479. *Nucleotides. Part XI.* Some Preliminary Experiments on a Projected Synthesis of Flavin-adenine Dinucleotide. New Methods for the Preparation of Riboflavin-4′ : 5′ Phosphate.*

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

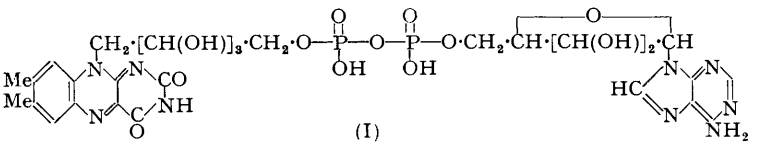
The possibility of using 4′ : 5′-anhydroriboflavin as an intermediate for a synthesis of flavin-adenine dinucleotide (FAD) has been explored and abandoned. Reaction of riboflavin-5′ phosphate with 2′ : 3′-*iso*propylidene-adenosine-5′ benzyl diphenyl pyrophosphate yielded the cyclic riboflavin-4′ : 5′ phosphate, probably by breakdown of the initially formed FAD derivative under the conditions of experiment. Riboflavin-4′ : 5′ phosphate is also produced from riboflavin-5′ phosphate with tetraphenyl or tetrabenzyl pyrophosphate, and reaction of trifluoroacetic anhydride with riboflavin-5′ phosphate followed by treatment with ammonia is a convenient method for its preparation.

Flavin-adenine dinucleotide (commonly abbreviated to FAD) was first isolated as the coenzyme of D-amino-acid oxidase (Warburg and Christian, *Naturwiss.*, 1938, **26**, 235; *Biochem. Z.*, 1938, **298**, 150) and it was later shown to be the prosthetic group of a number of other flavoproteins involved in oxidation–reduction processes in living organisms. According to the generally accepted structure (I), FAD can be regarded as an unsymmetrical

* Part X, *J.*, 1952, 52.

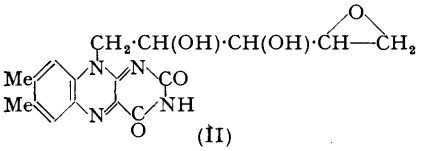
diester of pyrophosphoric acid and so belongs to a class of compounds no representative of which has so far been prepared synthetically other than by the use of enzymes. Indeed the only compounds which are known with some degree of certainty to belong to this type are, in addition to FAD, the natural coenzymes I and II (DPN and TPN) and the coenzyme (UDPG) of the system which converts galactose into glucose. Theoretically the problem of FAD synthesis could be approached in several ways, *e.g.*, (*a*) by linking together riboflavin-5′ phosphate and adenosine-5′ phosphate, (*b*) by linking adenosine-5′ pyrophosphate to riboflavin, or (*c*) by joining together riboflavin-5′ pyrophosphate and adenosine. The present paper deals with some exploratory studies on this problem using approaches (*a*) and (*b*) which, although they did not lead to a successful solution, have yielded results which not only indicate directions for further work but also provide information relevant to several other lines of investigation currently in progress in this laboratory.

Previous work on the addition of phosphoric acid and its diesters to ethylene oxide derivatives (Atherton, Openshaw, and Todd, *J.*, 1945, 382; Harvey, Michalski, and Todd, *J.*, 1951, 2271) suggested that a very simple synthesis of (I) might be achieved by addition



(I)

of adenosine-5′ pyrophosphate (or its dibenzyl ester) to 4′ : 5′-anhydroriboflavin (II), and accordingly attempts were made to prepare the latter substance. An obvious starting material for (II) would be 5′-toluene-*p*-sulphonylriboflavin but as already reported (Forrest and Todd, *J.*, 1950, 3295) we were unable to toluene-*p*-sulphonylate 2′ : 3′ : 4′-triacetylriboflavin. We next tried to prepare the appropriate toluene-*p*-sulphonyl derivative of an intermediate in one of the known total syntheses of riboflavin and to obtain the desired riboflavin derivative by completing the synthesis of the flavin ring system; a method of this type was used successfully for riboflavin-5′ phosphate by Karrer, Frei, and Meerwein (*Helv. Chim. Acta*, 1937, **20**, 79). Treatment of 3 : 4-dimethyl-*N*-D-ribitylaniline (III; R = H) with toluene-*p*-sulphonyl chloride gave an excellent yield of a product which, however, was evidently an *N*-toluene-*p*-sulphonyl derivative since it yielded a stable tetra-acetyl derivative and, on periodate titration, consumed 3 mols. of oxidant. Efforts to prepare a toluene-*p*-sulphonyl derivative of the azo-compound (III; R = Ph·N : N-) were unsuccessful, and 3 : 4-dimethyl-D-ribonanilide gave in poor yield a product which was sulphur-free and gave analytical figures corresponding to the starting material minus the



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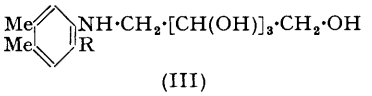

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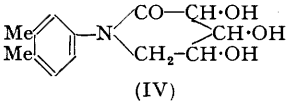
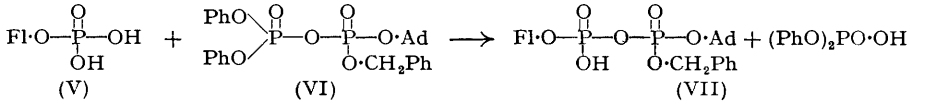


(IV)



(V) (VI) (VII)

(Fl = flavin residue; Ad = adenine residue.)

elements of water. Since it gives a triacetyl derivative it is possible that this product may be (IV) which could be produced from an initially formed toluene-*p*-sulphonyl derivative by intramolecular alkylation. In view of these results experiments along these lines were abandoned.

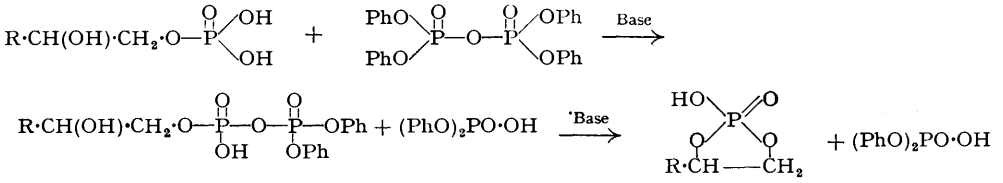
When it was discovered that riboflavin is moderately soluble in dimethylformamide it was decided to examine again the direct reaction of riboflavin with toluene-*p*-sulphonyl chloride in this solvent although earlier experiments in other media had been unsuccessful. The results were rather unsatisfactory although a large number of experiments were performed under varying conditions, the products being examined by paper chromatography with a *tert.*-butanol–pyridine–water system. When equimolecular quantities of the reactants were used some reaction occurred, giving a yellow material very similar to riboflavin but with a slightly higher R_F value. The amount of this material increased with increasing proportions of toluene-*p*-sulphonyl chloride up to 4—6 mols. but with larger amounts considerable darkening and decomposition seemed to occur. It was finally found that fairly rapid addition of the halide (6 mols.) in pyridine solution to riboflavin in dimethylformamide at room temperature yielded an almost homogeneous product. This product was sulphur-free and gave analytical figures similar to those of riboflavin itself but on periodate titration took up one mol. of oxidant rapidly (1 hour) and then there was a lag of 4 hours before a further slow uptake set in. This behaviour is what would be expected of 4′ : 5′-anhydroriboflavin and the production of this substance under the reaction conditions employed would not be unreasonable: the same product appeared to result when methanesulphonyl chloride was substituted for toluene-*p*-sulphonyl chloride. Nevertheless we were unable to obtain confirmatory evidence for this structure; attempts to prepare riboflavin-5′ phosphate or its ester by reaction of the supposed anhydro-compound with phosphoric acid, dibenzyl hydrogen phosphate, or their salts either left the substance unchanged or brought about conversion into riboflavin which was identified chromatographically and/or by isolation in substance. While the exact nature of this product remained in doubt it was, at any rate, clear that the likelihood of a successful synthesis of flavin-adenine dinucleotide along these lines was small indeed.

We therefore turned our attention to possible routes to FAD which involve the condensation of riboflavin phosphate with a phosphorylated adenosine derivative. At about this time the observation was made that tetrabenzyl pyrophosphate could be prepared in high yield by allowing tetraphenyl pyrophosphate to react with dibenzyl hydrogen phosphate at room temperature in presence of a base (Mason and Todd, *J.*, 1951, 2267). It was clear that this reaction might be made the basis of a process leading to unsymmetrical pyrophosphates and investigations were undertaken on model compounds to clarify and broaden the scope of this exchange reaction of pyrophosphates and of mixed anhydrides of phosphoric acid with other strong acids; the results of these investigations have been already reported (Corby, Kenner, and Todd, *J.*, 1952, 1234). Simultaneously, however, experiments were carried out in which direct application to FAD synthesis was attempted by endeavouring to bring about an exchange reaction between riboflavin-5′ phosphate (V; Fl = riboflavin) and adenosine-5′ benzyl diphenyl pyrophosphate (VI; Ad = adenosine), to yield the monobenzyl ester (VII) of FAD, from which the benzyl group might subsequently be removed.

The choice of (VI) as a reactant rested on the assumption that diphenyl hydrogen phosphate would be a stronger acid than adenosine-5′ monobenzyl phosphate, so that reaction would proceed in the direction indicated (cf. Corby, Kenner, and Todd, *loc. cit.*). It was, of course, realised that the reaction might proceed more readily if riboflavin-5′ monobenzyl phosphate were employed in place of the dibasic acid riboflavin-5′ phosphate, but it seemed probable that the validity of the method might well be tested initially without recourse to the tedious preparation of the former compound. It may be noted in passing that the enzymic synthesis due to Shrecker and Kornberg (*J. Biol. Chem.*, 1950, **182**, 795) in which riboflavin-5′ phosphate and adenosine triphosphate react in presence of an enzyme to give FAD and pyrophosphate is similar in principle to the purely chemical synthesis here envisaged; the probable significance of this has been discussed by one of us elsewhere (Todd, Harvey Lecture, New York, 1951).

When (V) and a crude sample of the 2′ : 3′-*iso*propylidene derivative of (VI) (prepared by treating the silver salt of 2′ : 3′-*iso*propylideneadenosine-5′ benzyl phosphate with diphenyl chlorophosphonate) were brought into reaction in dimethylformamide solution alone or in presence of bases and the products examined by paper chromatography it was evident that

some reaction had occurred, giving a riboflavin derivative which travelled on paper chromatograms at approximately the sane speed as natural FAD in 5% aqueous disodium hydrogen phosphate. This product, which could be obtained chromatographically homogeneous by the chromatopile technique, proved to be the cyclic riboflavin-4′ : 5′ phosphate of Forrest and Todd (*loc. cit.*) and it was also produced in almost quantitative yield by treating riboflavin-5′ phosphate with tetraphenyl or tetrabenzyl pyrophosphate in presence of bases. A large number of experiments along these lines were made in which conditions of reaction were varied but riboflavin-4′ : 5′ phosphate was the only product obtainable. It may be reasonably assumed that in these reactions the desired exchange did in fact occur and that the initially produced pyrophosphate of riboflavin then behaved, in presence of base, as a phosphorylating agent towards the adjacent 4′-hydroxyl of the riboflavin residue. The reaction between riboflavin-5′ phosphate and tetraphenyl pyrophosphate would thus be formulated :



This is in accord with the known behaviour of pyrophosphates as phosphorylating agents (cf. Atherton and Todd, *J.*, 1947, 674) and recalls the production of riboflavin-4′ : 5′ phosphate from FAD on treatment with ammonia (Forrest and Todd, *loc. cit.*), although the cyclic phosphate does not appear to be produced from natural FAD by triethylamine, the base commonly used in our exchange reactions. On this basis it is not improbable that, in the attempted FAD synthesis mentioned above, the benzyl ester (VII) of FAD (or its 2′ : 3′-*iso*propylidene derivative) was actually produced but then reacted further to yield the cyclic riboflavin phosphate.

The results of these experiments, although disappointing, were not wholly unexpected, for it had been realised that a major difficulty in any synthesis of FAD, in which the acidic hydroxyl groups in the pyrophosphate linkage were blocked by protecting ester groups, would lie in the very great lability of the penultimate product and its tendency to act as a powerful phosphorylating agent, although we had not at first realised how readily the cyclic riboflavin phosphate would be formed. It was evident that one of the most promising lines of advance from the point now reached would be to substitute for riboflavin-5′ phosphate, in the reactions described, a fully acylated derivative of this substance so that cyclic phosphate formation would be impossible, provided a derivative could be prepared in which the acyl groups would be sufficiently labile to permit their removal from the product of the exchange reaction without rupture of the pyrophosphate linkage. Further studies along these lines will be described in a later communication, but it is appropriate to mention at this point our attempt to meet the requirement by preparing a 2′ : 3′ : 4′-tristrifluoroacetylriboflavin-5′ phosphate. Trifluoroacetic anhydride reacted readily with riboflavin-5′ phosphate, to give a substance of the expected composition, but on treatment with alcoholic ammonia the product yielded riboflavin-4′ : 5′ phosphate; heating with dilute acid gave riboflavin-5′ phosphate. It seems clear that this product was a hydrated tristrifluoroacetyl derivative of the cyclic riboflavin-4′ : 5′ phosphate and owed its formation to reaction of the trifluoroacetic anhydride with riboflavin-5′ phosphate to give a mixed anhydride which then underwent fission with formation of the cyclic phosphate, the latter being subsequently trifluoroacetylated (presumably in the 3-, 2′-, and 3′-positions). Whatever be the precise orientation of this trifluoroacetyl derivative it is undoubtedly true that reaction of trifluoroacetic anhydride with riboflavin-5′ phosphate followed by treatment with alcoholic ammonia provides an excellent method for preparing riboflavin-4′ : 5′ phosphate, and trifluoroacetic anhydride could presumably be applied successfully as a general reagent for the preparation of cyclic phosphates from monoesters of phosphoric acid in which the esterifying alcohol bears a *cis*-hydroxyl group adjacent to the point of attachment of the

phosphoryl group. Preparative methods for such cyclic phosphates have recently assumed considerable importance in connexion with the mechanism of hydrolytic breakdown of ribonucleic acids and the interconversion of the isomeric *a* and *b* ribonucleotides (Brown and Todd, *J.*, 1952, 44, 52).

Experimental

*Action of Toluene-*p*-sulphonyl Chloride on* 3 : 4-*Dimethyl-*N-D-*ribitylaniline.*—A solution of 3 : 4-dimethyl-*N*-D-ribitylaniline (1·92 g.) in dry pyridine (18 c.c.) was cooled to 0° and toluene-*p*-sulphonyl chloride (1·44 g.) in pyridine (18 c.c.) containing a little toluene-*p*-sulphonic acid (0·06 g.) was added during 10 minutes. The mixture was stirred at 0° for 6 hours, then left overnight at room temperature. Pyridine was removed under reduced pressure and the residue stirred with water. The solid product (2·45 g.) was recrystallised from ethyl acetate, 3 : 4-*dimethyl-*N-D-*ribityl-*N-*toluene-*p*-*sulphonylaniline* (as III; R = H) being obtained as colourless needles, m. p. 110° (Found: C, 58·2; H, 6·6; N, 3·4. $C_{20}H_{27}O_6NS$ requires C, 58·7; H, 6·6; N, 3·4%). The substance consumed 3 mols. of oxidant per mol. when titrated with sodium metaperiodate in aqueous dioxan (80%), no further uptake occurring in 48 hours. Treatment with acetic anhydride in pyridine gave a *tetra-acetyl* derivative which separated from benzene–light petroleum in colourless needles, m. p. 92° (Found: C, 58·0; H, 6·0; N, 2·4. $C_{28}H_{35}O_{10}NS$ requires C, 58·2; H, 6·0; N, 2·4%).

*Action of Toluene-*p*-sulphonyl Chloride on* 3 : 4-*Dimethyl-*D-*ribonanilide.*—A solution of the ribonanilide (8·13 g.) in dry pyridine (65 c.c.) was cooled in an ice–salt bath, and toluene-*p*-sulphonyl chloride (5·73 g.) in pyridine (35 c.c.) containing toluene-*p*-sulphonic acid (0·25 g.) was added during 10 minutes. The cold solution was stirred for 6 hours, then allowed to come to room temperature and set aside overnight. Pyridine was removed under reduced pressure, the residue dissolved as far as possible in chloroform, and the solution washed with dilute hydrochloric acid and water. Evaporation of the dried chloroform solution and recrystallisation of the residue from methanol gave the *product* (IV?) (2·4 g.) as colourless felted needles, m. p. 167° (Found: C, 62·3; H, 6·5; N, 5·6. $C_{13}H_{17}O_4N$ requires C, 62·2; H, 6·8; N, 5·6%). The substance contained no sulphur and with acetic anhydride in pyridine solution gave a *triacetyl* derivative crystallising from benzene–light petroleum in colourless plates, m. p. 96° (Found: C, 60·5; H, 6·6; N, 4·2. $C_{19}H_{27}O_7N$ requires C, 60·4; H, 6·1; N, 3·7%).

*Action of Toluene-*p*-sulphonyl Chloride on Riboflavin.*—Many experiments (*ca.* 40) were carried out with varying quantities of reagents, reaction medium, and conditions of reaction. The working up and separation of reaction products was rendered very difficult by the extreme insolubility of riboflavin and its derivatives in suitable solvents. The following is an example of a comparatively large-scale experiment in which the product was isolated in substance for further study.

Riboflavin (500 mg.) in dimethylformamide (60 c.c.) was treated with toluene-*p*-sulphonyl chloride (1·52 g.) in pyridine added during 5 minutes at room temperature. After 20 minutes the dark yellow solution was diluted with half its volume of water and concentrated to small bulk under reduced pressure. Further addition of water precipitated the product as a yellow solid (450 mg.) which was collected, washed with water, and dried. Recrystallised from water, it was obtained as a microcrystalline yellow powder, m. p. 250° (the riboflavin used has m. p. 280°), which contained no sulphur [Found: C, 53·6; H, 6·0; N, 14·3. Calc. for $C_{17}H_{20}O_6N_4$ (riboflavin): C, 54·1; H, 5·4; N, 14·9%]. The product was apparently not riboflavin although it yielded the latter when heated with acids or with alkali; on paper chromatography in *tert.*-butanol–pyridine–water (12 : 3 : 5) it gave one main spot just ahead of a trace of riboflavin which it contained as an impurity. In view of this and of the results of periodate titrations mentioned in the introductory part of this paper it is possible that this product may be 4′ : 5′-anhydroriboflavin but some 25 experiments in which opening of the oxide ring to yield riboflavin-5′ phosphate was attempted under a variety of conditions failed; when reaction occurred the sole product was riboflavin, identified by m. p., mixed m. p., and paper chromatography.

Attempted Exchange Reactions using Riboflavin-5′ Phosphate.—The following examples typify the experiments carried out.

(a) *With* 2′ : 3′-iso*propylideneadenosine-5′ benzyl diphenyl pyrophosphate* (VI: Ad = adenosine). The crude product obtained by treating silver 2′ : 3′-*iso*propylideneadenosine-5′ benzyl phosphate (100 mg.) with diphenyl chlorophosphonate (70 mg.) in dry benzene was dissolved together with riboflavin-5′ phosphate (50 mg.) in dimethylformamide (10 c.c.) containing triethylamine (0·2 c.c.). After 24 hours the reaction product was isolated, by precipitation with ether, as a yellow solid. Paper chromatography with aqueous disodium hydrogen phos-

phate (5%) showed that it contained in addition to unchanged riboflavin-5′ phosphate a second component travelling at the same speed as natural flavin-adenine dinucleotide (R_F 0·42). This second material (7 mg.) was isolated in a pure state by the chromatopile technique (cf. Forrest and Todd, *loc. cit.*) and was shown to be riboflavin-4′ : 5′ phosphate by paper-chromatographic comparison in several solvent systems, by periodate titration (1·03 mols. uptake), and by conversion into riboflavin-5′ phosphate with acids. No improvement in yield or change in the nature of product could be obtained despite considerable variation in the solvent used or the conditions of reaction.

(b) *With tetraphenyl pyrophosphate.* An experiment similar to the above was carried out with riboflavin-5′ phosphate (250 mg.), tetraphenyl pyrophosphate (600 mg.) in dimethylformamide (10 c.c.), and triethylamine (0·3 c.c.). The reaction was carried out at room temperature during 2 hours. Worked up as before, the product (230 mg.) isolated was shown to be riboflavin-4′ : 5′ phosphate. Chromatopile purification was unnecessary.

(c) *With tetrabenzyl pyrophosphate.* Under the same conditions as in (b) riboflavin-5′ phosphate (60 mg.) and tetrabenzyl pyrophosphate (300 mg.) gave again riboflavin-4′ : 5′ phosphate (50 mg.). Under the same conditions, but with riboflavin instead of riboflavin-5′ phosphate, no phosphorylation occurred.

Riboflavin-5′ Phosphate and Trifluoroacetic Anhydride.—Riboflavin-5′ phosphate (100 mg.) was set aside with trifluoroacetic anhydride (0·2 c.c.) for 12 hours, during which it gradually passed into solution. Dry ether (40 c.c.) was added giving a yellow precipitate (120 mg.) of a trifluoroacetylated compound (probably 3 : 2′ : 3′-*tristrifluoroacetylriboflavin*-4′ : 5′ *phosphate*), purified by dissolution in ethanol and reprecipitation with ether (Found : C, 36·7; H, 2·8; N, 7·5. $C_{23}H_{16}O_{11}N_4F_9P,H_2O$ requires C, 37·1; H, 2·4; N, 7·6%). When set aside with saturated ethanolic ammonia this product was converted almost quantitatively into riboflavin-4′ : 5′ phosphate, identified by direct comparison with an authentic specimen.

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