Nucleotides. Part XXV. A Synthesis of Flavin–Adenine Dinucleotide.*

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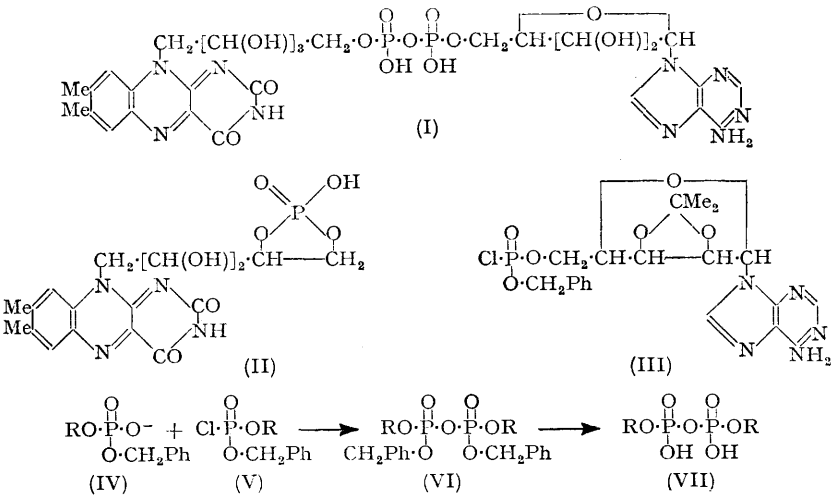
Condensation of salts of riboflavin-5′ phosphate with 2′ : 3′-*O*-*iso*propylideneadenosine-5′ benzyl phosphorochloridate in warm phenol, followed by removal of the *iso*propylidene residue, yields P^1-adenosine-5′ P^2-riboflavin-5′ pyrophosphate identical with the natural coenzyme flavin–adenine dinucleotide (FAD).

Flavin–adenine dinucleotide (usually abbreviated to FAD) is of considerable importance since it occurs as the prosthetic group or coenzyme of a variety of flavoproteins active in hydrogen transport in biological systems. It was isolated as the coenzyme of D-amino-acid oxidase from liver, kidney, and yeast by Warburg and Christian (*Naturwiss.*, 1938, **26**, 235; *Biochem. Z.*, 1938, **298**, 150) in the form of its barium salt. On the basis of its analytical composition, its cleavage to adenosine-5′ phosphate and riboflavin-5′ phosphate (Abraham, *Biochem. J.*, 1939, **33**, 543), and its failure to yield formaldehyde with periodic acid (Karrer and Frank, *Helv. Chim. Acta*, 1940, **23**, 948), the originally postulated structure (I) for FAD has been widely accepted. Nevertheless, it seems at least doubtful whether the pure coenzyme has been isolated as such or as one of its salts since the original work of Warburg and Christian (*loc. cit.*); this has no doubt been due to the difficulty of isolation from natural sources, its lability to both acid and alkali, and to the fact that relatively crude preparations of the coenzyme suffice for most biochemical purposes. According to (I), FAD is an unsymmetrical diester of pyrophosphoric acid (P^1-adenosine-5′ P^2-riboflavin-5′ pyrophosphate) and is a member of a group of analogously constituted coenzymes (*e.g.*, coenzymes I and II, uridine-diphosphate-glucose or UDPG, and coenzyme A) usually described as nucleotide coenzymes since one of the esterifying groups in each case is a natural nucleoside or one of its simple derivatives. The synthesis of coenzymes of this group has been one of the major aims of investigations in the nucleotide field carried out in this laboratory during recent years; no member of the group has hitherto been synthesised by other than enzymic methods. Several preliminary attempts to synthesise FAD have been described in Part XI of this series (Forrest, Mason, and Todd, *J.*, 1952, 2530) and some of the difficulties inherent in any method involving exchange reactions were there discussed. After the work described in Part XI, new methods of pyrophosphate synthesis suitable for application in the nucleotide field were devised. These new methods opened the way to a successful synthesis of flavin–adenine dinucleotide identical with the natural coenzyme. A brief announcement of this synthesis has already been published (Christie, Kenner, and Todd, *Nature*, 1952, **170**, 924); the present paper records full details of the synthesis and associated experiments.

The key to successful synthesis lay in the discovery of a relatively easy preparation of 2′ : 3′-*O*-*iso*propylideneadenosine-5′ benzyl phosphorochloridate (III) (Corby, Kenner, and

* Part XXIV, preceding paper.

Todd, *J.*, 1952, 3669; Kenner, Todd, and Weymouth, *J.*, 1952, 3675). P^1P^2-dialkyl pyrophosphates (VII) can be prepared from alkyl benzyl phosphorochloridates (V) by reaction with a salt of an alkyl benzyl phosphate (IV) and subsequent anionic debenzylation (Clark and Todd, *J.*, 1950, 2031) of the initially formed P^1P^2-dialkyl P^1P^2-didenzyl pyrophosphate. A synthesis of the symmetrical P^1P^2-diuridine-5′ pyrophosphate (VII; R = uridine-5′ residue) by this route has been described in Part XXII (Christie, Elmore, Kenner, Todd, and Weymouth, *J.*, 1953, 2947). This method seemed capable of extension to the preparation of unsymmetrical P^1P^2-diesters of pyrophosphoric acid, although complications were expected in view of the ease with which unsymmetrical fully esterified pyrophosphates undergo hydrolysis and disproportionation reactions. In the specific case of flavin–adenine dinucleotide (I) synthesis a derivative of riboflavin which appeared suitable for reaction with (III), *viz.*, 2′ : 3′ : 4′-triacetylriboflavin-5′ benzyl phosphate, had been prepared with some difficulty by Dr. H. S. Forrest in this laboratory. This substance, in the form of its triethylammonium salt, was therefore brought into reaction with (III) in acetonitrile solution. Condensation appeared to occur, but we were unable to detect FAD in the product obtained after various treatments designed to remove all the protecting groups. This result was not wholly surprising, since FAD itself is readily degraded by mild alkaline treatment to riboflavin-4′ : 5′ phosphate (II) (Forrest and Todd, *J.*, 1950, 3295) and it was to be expected that the initial product of condensation—a fully esterified pyrophosphate—would be even more labile. The same difficulty had balked many of the attempted syntheses described in Part XI (*loc. cit.*) and in many of them, too, (II) had been detected in the products of reaction.



In these circumstances it seemed worth while to investigate the reaction between (III) and a salt of the readily available unprotected riboflavin-5′ phosphate. Such a reaction, if successful, might produce several compounds in addition to a derivative of FAD but it was unlikely to lead to any ambiguity, and, if rather less elegant than the route explored above, it would have the advantage of involving a smaller number of steps. The unprotected riboflavin-5′ phosphate is freely soluble only in phenols, but it is known that pyrophosphates can be prepared from phosphorochloridates and silver salts in phenolic media (Baddiley, Michelson, and Todd, *J.*, 1949, 582). During a re-investigation of this earlier work, Dr. A. S. Curry made the important discovery that at temperatures of 50° or above benzyl pyrophosphates are rapidly debenzylated by phenol with production of nuclear benzylated phenols. Further information about this interesting reaction will be published in due course, but it will suffice for present purposes to mention that it is acid-catalysed, whereas anionic debenzylation occurs under neutral or alkaline conditions; it is therefore an alternative to hydrogenolysis, which is sometimes unsatisfactory (cf. *e.g.*, Anand, Clark, Hall, and Todd, *J.*, 1952, 3665). The main relevance of this discovery to

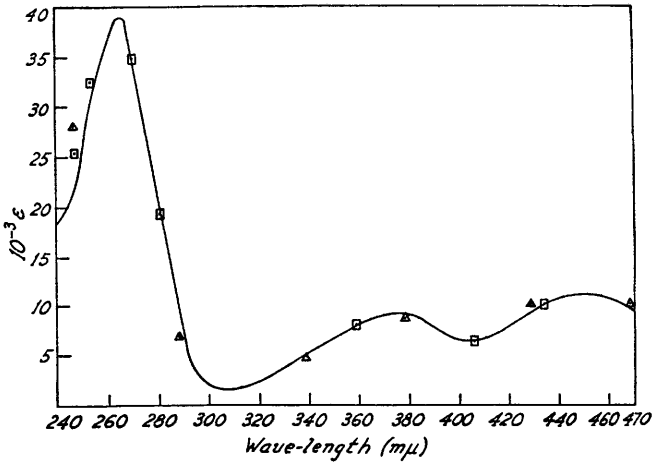
the synthesis under discussion is that, since the debenzylating action of phenol is much more marked with pyrophosphates than phosphates, it is possible to carry out pyrophosphate syntheses in phenol with benzylated intermediates and to debenzylate (and thereby stabilise) the product almost as soon as it is formed. This paper is therefore concerned with successful syntheses of this simplified type, *i.e.*, by reaction between (III) and a salt of riboflavin-5′ phosphate in phenolic solution.

In preliminary experiments, small amounts of FAD were detected by paper chromatography (Crammer, *Nature*, 1948, **161**, 349) in the acid-treated product of reaction of (III) and a suspension of the disilver salt of riboflavin-5′ phosphate in a mixture of phenol and acetonitrile (5 : 1). By means of the chromatographic technique described below it was possible to estimate the overall yields of FAD in a considerable number of runs under varying conditions. In this way it was established that the optimum conversion of riboflavin-5′ phosphate salt into FAD was obtained by using three mols. of (III), which suffers considerable decomposition in warm phenol. The monothallous salt gave rather higher yields than the other metal salts tried. It was also advantageous to add one equivalent of triethylamine to the salt, but larger quantities caused a drastic reduction in the yield, probably by causing breakdown of the pyrophosphate intermediate to the cyclic phosphate (II). It was better to carry out the reaction in warm phenol (up to 80°) rather than in phenol at room temperature; under these conditions complete debenzylation evidently occurred, since subsequent hydrogenation with palladium oxide had no effect (other than the reversible reduction of the flavin system). Removal of the *iso*propylidene residue from the product to give FAD presented considerable difficulty and no fully satisfactory method was found. Model experiments on 2′ : 3′-*O*-*iso*propylideneadenosine showed that when 0·1N-mineral acid was used removal of the acetone residue was incomplete at room temperature even after 24 hours. During such prolonged treatment of the reaction product considerable breakdown of the pyrophosphate occurred. This could be minimised by interrupting the hydrolysis after 7 hours, separating the FAD already produced, and repeating the hydrolysis on the residue. An alternative method of hydrolysis was to boil a solution of the product in acetic acid–ethanol–water (2 : 9 : 9 by volume) for 10 minutes. These acid treatments were, however, unsatisfactory and even under the most carefully controlled conditions substantial fission of the pyrophosphate linkage occurred with a consequent heavy fall in the yield of FAD obtained.

Under the best conditions found the yield of FAD in the final product corresponded to *ca.* 6%, calculated on the riboflavin-5′ phosphate employed. Separation of the FAD from the complex reaction product by the method used by Warburg and Christian (*loc. cit.*) for the natural coenzyme (*i.e.*, precipitation of silver and barium salts) was impracticable. Preliminary experiments indicated that counter-current distribution between phenol–chloroform and water would give a separation from the large amounts of riboflavin-5′ phosphate present, but a much more convenient method was found in chromatography on a cellulose column with *n*-butanol–acetic acid–water. This was carried out in an unusual way which avoided the common difficulty of poor separation due to " channelling " in the column. The mixture was adsorbed on a little cellulose powder which was then dry-packed at the foot of the column. The solvent mixture was then allowed to rise through the column, largely under capillary tension; after development the slowest band, which contained the FAD, was cut out and eluted with water. The dry powder obtained on evaporation of the eluate was almost free from riboflavin-5′ phosphate and adenylic acids, as shown by enzymic assay and light-absorption measurements. Prof. D. Keilin and Dr. E. F. Hartree, to whom we are greatly indebted, determined the activity of the synthetic material obtained in this way as coenzyme of D-amino-acid oxidase and found that it showed full activity, the FAD content corresponding to *ca.* 90% of the riboflavin derivatives present as measured by the intensity of light absorption at 450 mμ. The presence of small amounts of adenylic acids could be detected by comparing the optical density at 260 mμ with that at 450 mμ. According to Warburg and Christian (*loc. cit.*) and Whitby (*Biochem. J.*, 1953, **54**, 437) the ratio of these values should be 3·28 for pure FAD, although other workers have reported values in the region of 3·8 (Dimant, Sanadi, and Huennekens, *J. Amer. Chem. Soc.*, 1952, **74**, 5440). In various preparations obtained by

us as described above the ratio varied between 3·6 and 4·2. The solid material obtained from the cellulose column usually contained only about 30% of FAD, the remainder being largely phosphorus-free material derived from the cellulose. We attempted to remove the impurities by fractional dissolution and precipitation of the barium salt of FAD according to Warburg and Christian (*loc. cit.*) but, in our hands, there was always some concurrent cleavage of the pyrophosphate linkage and the salt was not obtained chromatographically homogeneous. Attempts to obtain crystalline salts with several organic bases failed and we therefore had recourse to the red, virtually insoluble, silver salt which we were able to obtain in a pure condition by precipitation. This salt gave analytical values for a hydrated trisilver salt of P^1-adenosine-5′ P^2-riboflavin-5′ pyrophosphate, in accord with the fact that riboflavin itself forms a red monosilver salt (Kuhn, Rudy, and Wagner-Jauregg, *Ber.*, 1933, **66**, 1954). The free P^1-adenosine-5′ P^2-riboflavin-5′ pyrophosphate regenerated from this synthetic silver salt was identical in its chemical and chromatographic behaviour and in its absorption characteristics (see Fig.) (including the optical density ratio 260 mμ/450 mμ = 3·28) with natural flavin–adenine dinucleotide.

The synthesis described above gives modest but reproducible yields of FAD from



Absorption spectrum of synthetic FAD (——). *Values obtained by Warburg and Christian* (loc. cit.) △, *and by Whitby* (loc. cit.) ⊡, *are recorded for comparison.*

fairly easily accessible starting materials, and it provides confirmation of structure (I) earlier allotted to the coenzyme. On the other hand, there is much scope for improvement in the yield and work aimed at such improvement is being continued. Perhaps the main importance of the synthesis as it stands is that it demonstrates that the difficulties which beset the synthesis of such complex and unstable molecules can be overcome; other related pyrophosphate coenzymes should be capable of synthesis by analogous methods.

Experimental

Salts of Riboflavin-5′ Phosphate.—The riboflavin-5′ phosphate used was prepared from its bisethanolamine salt and purified by chromatography on Dowex-2 ion-exchange resin.

(a) *Monothallous salt.* Riboflavin-5′ phosphate (1·27 g.) was suspended in water (150 c.c.) and aqueous ammonia (N) was added to pH 7; dissolution of the flavin was then complete. Aqueous thallous hydroxide (31·2 c.c. of 0·079N; 1 mol.) was added and the mixture was evaporated to dryness under reduced pressure. The residue was dissolved in water (100 c.c.), and the *monothallous* salt (1·49 g.) precipitated with ethanol; this decomposed at 235° (Found, in material dried at 20°: C, 30·2; H, 3·0; N, 8·4; P, 4·8. $C_{17}H_{20}O_9N_4PTl$ requires C, 30·9; H, 3·1; N, 8·5; P, 4·7%).

(b) *Disilver salt.* A mixture of riboflavin-5′ phosphate (0·996 g.), silver carbonate (1·605 g.), and water (20 c.c.) was shaken for 30 min. and then centrifuged. The supernatant liquor and aqueous washings were combined and freeze-dried, to give the *disilver* salt as a deep red powder (Found, in material dried at 20°: C, 29·4; H, 3·4; N, 8·4. $C_{17}H_{19}O_9N_4PAg_2,H_2O$ requires C, 29·7; H, 3·1; N, 8·1%). An equimolar mixture of this salt with riboflavin-5′ phosphate,

prepared by freeze-drying an aqueous solution, was used in several experiments as the " monosilver salt."

2′ : 3′ : 4′-*Tri*-O-*acetylriboflavin*-5′ *Phosphate* (Dr. H. S. Forrest).—Riboflavin-5′ phosphate (0·3 g.) was suspended in acetic anhydride (3 c.c.) and perchloric acid (72%; 3—5 drops) was added until all the phosphate had dissolved to a clear solution. The product was isolated from this solution either by direct precipitation with ether or by cautious addition of ethanol to the cooled solution, followed by concentration to small bulk and precipitation with ether. The *triacetyl* derivative (390 mg.) was obtained as a yellow water-soluble microcrystalline powder; on paper chromatography it ran as a single homogeneous spot and on treatment with ethanolic ammonia at 0° for 16 hr. it reverted to riboflavin-5′ phosphate (Found: C, 45·0; H, 4·8; N, 9·2. $C_{23}H_{27}O_{12}N_4P,2H_2O$ requires C, 44·7; H, 5·0; N, 9·1%). The same product, in lower yield, was obtained by treating riboflavin-5′ phosphate with acetic anhydride in pyridine at 40°.

2′ : 3′ : 4′-*Tri*-O-*acetylriboflavin*-5′ *Benzyl Phosphate* (Dr. H. S. Forrest).—Phenyldiazomethane (from 2·25 g. of benzaldehyde hydrazone) was added to a solution of 2′ : 3′ : 4′-tri-*O*-acetylriboflavin-5′ phosphate (100 mg.) in dry dioxan (10 c.c.), and the mixture set aside for 1 hr. Most of the dioxan was removed under reduced pressure and excess of ether was added to the residual solution. The flocculent yellow precipitate (100 mg.) was collected and washed by centrifugation. It appeared to be mainly the expected dibenzyl ester since it could not be extracted from chloroform solution with sodium hydrogen carbonate, and with alcoholic solvents on paper chromatograms it moved rapidly and mainly as a single spot.

The ester obtained in this way (35 mg.) was heated in a hot saturated solution of lithium chloride in 2-ethoxyethanol (0·2 c.c.) for 3 hr. at 100—120°. Solvent was then removed under reduced pressure and the residue dissolved in water and extracted with chloroform. The chloroform extract was discarded, the acidified aqueous layer was again extracted with chloroform, and the extract was dried (Na_2SO_4) and evaporated. The residue was dissolved in a minimum of ethanol and the *monobenzyl* ester was precipitated as a yellow powder (23 mg.) by means of ether. It was chromatographically homogeneous, could be extracted from its chloroform solution with sodium hydrogen carbonate, and when treated with ethanolic ammonia at 0° for 16 hr. yielded a substance assumed to be riboflavin-5′ benzyl phosphate since it was identical chromatographically with the acidic ester obtained by treating riboflavin-5′ phosphate with phenyldiazomethane in dimethylformamide (Found: C, 51·0; H, 4·9; N, 8·4. $C_{30}H_{33}O_{12}N_4P,2H_2O$ requires C, 50·9; H, 5·2; N, 7·9%).

Reaction of 2′ : 3′-O-iso*Propylideneadenosine*-5′ *Benzyl Phosphorochloridate with* 2′ : 3′ : 4′-*Tri*-O-*acetylriboflavin*-5′ *Benzyl Phosphate*.—2′ : 3′-*O-iso*Propylideneadenosine-5′ benzyl phosphite (0·19 g.), dissolved in a mixture of benzene (3 c.c.) and methyl cyanide (1 c.c.), was chlorinated by treatment with *N*-chlorosuccinimide (0·054 g.) for 2 hr. at room temperature, and 2′ : 3′ : 4′-tri-*O*-acetylriboflavin-5′ benzyl phosphate (0·227 g.), dissolved in methyl cyanide (3 c.c.), was added. Triethylamine (0·047 c.c., 1 mol.) in benzene (5 c.c.) was added with stirring during 5 min. and the mixture was set aside for 2 hr. Solvents were removed under reduced pressure, a saturated solution of lithium chloride in 2-ethoxyethanol (5 c.c.) was added, and the mixture heated at 100° for 2 hr. before concentration to small bulk (1 c.c.) under reduced pressure. The resultant solution was poured into dry ether (50 c.c.) and the precipitated resin was collected, redissolved in methanol (1 c.c.), and again precipitated with ether.

Paper chromatography of this resin indicated (*a*) that some condensation had occurred, since the main flavin-containing spot had R_F 0·78 and the starting material had R_F 0·56 [*n*-butanol–acetic acid–water (5 : 2 : 3)], and (*b*) that treatment with lithium chloride left about 70% of the initial reaction product unchanged. Hydrogenation (uptake 15 c.c.) of the resin (0·386 g.) in aqueous methanol with palladium oxide–palladised charcoal, followed by treatment with methanolic hydrogen chloride (0·5M) for 1 hr. and then with barium methoxide in methanol (0·5M), caused considerable decomposition and no FAD could be detected among the products.

P^1-*Adenosine*-5′ P^2-*Riboflavin*-5′ *Pyrophosphate* (*Flavin–Adenine Dinucleotide*).—(*a*) A solution of 2′ : 3′-*O-iso*propylideneadenosine-5′ benzyl phosphite (1·05 g.) and N-chlorosuccinimide (0·253 g.) in methyl cyanide (10 c.c.) was kept at room temperature for 2 hr. before being added to a solution of monothallous riboflavin-5′ phosphate (0·5 g.) and triethylamine (0·11 c.c., 1 mol.) in phenol (15 c.c.) at 70°. All reagents were dry and moisture was rigidly excluded. Methyl cyanide was now removed under reduced pressure at 70° and the phenolic solution kept at this temperature for 40 min. in all. Dry ether (3 c.c.) was then added and the precipitated thallous chloride removed in a centrifuge. Water (20 c.c.) was added and the phenol was removed from the mixture by extraction with ether (3 × 100 c.c.); a little precipitated resin was taken up in phenol and re-partitioned between ether and water. The combined

aqueous solutions were brought to 0·1N-acidity by means of 3N-hydrochloric acid and were kept at 20° for 7 hr. They were then neutralised with ammonia, saturated with ammonium sulphate, and extracted with phenol (3 × 5 c.c.). The mixed flavins were driven out of the phenol extract into water (10 c.c.) by ether (3 × 100 c.c.) and then adsorbed on powdered cellulose (7 g.; Whatman Standard Grade). The adsorbate was dried *in vacuo* over sulphuric acid and placed as a layer on a sintered-glass disc (4 cm. diam.) in a short tube (5 cm.). A long chromatogram tube was now fitted on to the short tube by a ground-glass joint and filled to a height of 30 cm. with tamped-down cellulose powder. When a mixture of *n*-butanol–acetic acid–water (5 : 2 : 3 by vol.) was allowed to flow through the disc up the column by capillary tension, the product moved with an R_F *ca.* 0·2 and was therefore easily separated from riboflavin-5′ phosphate (R_F *ca.* 0·4) and other unidentified faster-moving flavin derivatives. Development of the column was complete in 5 hr.

The two portions of the column were separated and the required slower-moving yellow band, which had just passed the ground joint, was removed, washed with acetone (2 × 100 c.c.), and packed into a short column (3 × 10 cm.); percolation of this with water (61 c.c.) eluted the product. The faster-moving flavins in the main column were also eluted and their aqueous solution made 0·1N with respect to hydrochloric acid and kept at 20° for 17 hr. Repetition of the above process of extraction and chromatography with this solution yielded a further quantity of product. The intensity of light absorption at 450 mμ of the two solutions so obtained corresponded to the presence of 39 and 11 μmoles of FAD, a total yield of 6·6%. The combined solutions were freeze-dried to a yellow solid, which had an optical density ratio 260/450 mμ = 4·2, and contained 29% by weight of FAD.

(*b*) A considerable number of experiments of the same general type as (*a*) were carried out. In some of these the red trisilver salt of FAD described in (*c*) (below) was precipitated from the de-salted aqueous solution [which in (*a*) was adsorbed directly on cellulose] by acidification to pH 3 with nitric acid and addition of 20% silver nitrate solution. The salt was suspended in water and dissolved by adding 20% aqueous potassium chloride. Hydrochloric acid (0·5N) was added, silver chloride was removed in a centrifuge, and the clear solution neutralised with potassium hydroxide, before adsorption on cellulose and chromatography. The product finally obtained by this means was not noticeably purer than that obtained in (*a*) and the recovery was lower.

The consistency of the yields obtained in repeating individual experiments was surprising and the following general conclusions were drawn. *p*-Chlorophenol may be used in place of phenol without marked effect. The technique of adding the phosphorochloridate solution to the warm phenolic solution as in (*a*) is superior to that of allowing condensation to take place at a lower temperature (*e.g.*, 20° for 3 days) and then debenzylating in a separate operation (*e.g.*, at 60° for 30 min.) after removal of the methyl cyanide. The monothallous salt is the most convenient since it is anhydrous and dissolves readily in phenol. Yields about half as great or somewhat better are given by the monosilver, disodium, and triethylammonium salts. In the last case, a series of runs with increasing quantities of triethylamine showed two molar proportions to be the optimum amount; with three, yields were negligible, but small yields could be obtained with only one or even with none added to the riboflavin-5′ phosphate. When no triethylamine was added to the monothallous salt the yield by method (*a*) fell to 4%.

(*c*) Although the product obtained in (*a*) above showed the full biological activity of FAD, and was free from inhibitory substances, it was desirable to obtain a chemically pure specimen of FAD or one of its salts from it.

The freeze-dried synthetic product was dissolved in water (2 c.c.) and shaken with a small quantity of IR-120 ion-exchange resin in the hydrogen form. The resin was removed at a centrifuge, and silver nitrate solution (0·5 c.c. of 20% w/v) was added to the supernatant liquid. The flocculent red precipitate of the *trisilver* salt of P^1-adenosine-5′ P^2-riboflavin-5′ pyrophosphate was collected, washed with water (2 × 1 c.c.) and then acetone (2 × 2 c.c.), and dried *in vacuo* at room temperature. The deep red silver salt was evidently hydrated, but drying at high temperature was impracticable owing to decomposition; it appeared to be stable when stored for prolonged periods in the dark at 0° (Found, in material dried at 20° : C, 26·6; H, 3·5; N, 10·1; P, 5·8; Ag, 27·3. $C_{27}H_{30}O_{15}N_9P_2Ag_3,5H_2O$ requires C, 27·1; H, 3·4; N, 10·5; P, 5·2; Ag, 27·1%).

The synthetic product regenerated from the trisilver salt had the same R_F as natural flavin-adenine dinucleotide when submitted to paper chromatography in (1) 5% disodium hydrogen phosphate saturated with *iso*amyl alcohol and (2) *n*-butanol–acetic acid–water (5 : 2 : 3), and it migrated as a single spot on paper electrophoresis in 10% acetic acid. On hydrolysis with

sulphuric acid (0·1N) at 100° for 4 hr. it yielded, like the natural coenzyme, adenine and riboflavin-5′ phosphate, identified by paper chromatography. The absorption spectrum was determined in M/15-phosphate buffer at pH 7 (see Fig.). Molecular extinction coefficients at 260 mμ and 375 mμ, as well as the optical density ratio 260/450 mμ are compared below with the recorded literature values given by (1) Warburg and Christian (*loc. cit.*) and (2) Whitby (*loc. cit.*), FAD concentrations being in each case based on intensity of absorption of solutions at 450 mμ.

	$10^{-3}\varepsilon$, 260 mμ	$10^{-3}\varepsilon$, 375 mμ	Ratio 260/450 mμ
Synthetic FAD	37·0	9·21	3·28
Natural FAD	(1) 37·0, (2) 37·0	(1) 9·0, (2) 9·3	(1) 3·28, (2) 3·28

Bioassay of Synthetic FAD.—The ability of the synthetic material to reconstitute D-amino-acid oxidase when combined with the flavin-free enzyme protein was tested, following Keilin and Hartree's method (*Nature*, 1946, **157**, 801) in which the velocity of oxidation of DL-alanine is measured by the uptake of oxygen in presence of catalase at 39° and pH 8·3. An aqueous solution of material similar to that obtained in (*a*) (0·565 mg. in 4 c.c.) had optical density 0·620 at 450 mμ and 0·025 c.c. was equivalent in coenzyme activity to 0·0041 c.c. of natural FAD ($3{\cdot}07 \times 10^{-4}$M). Hence the solution of synthetic material was $0{\cdot}504 \times 10^{-4}$M and 28% of the dry solid and 91% of the flavin in the solution were coenzyme.

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