tained for a mixture of all three components in the pH range of 7.0–8.0.

It is possible also in aqueous solvent systems, such as No. 3, to observe a linear relationship, as shown in Fig. 1, between the R_f value of a flavin

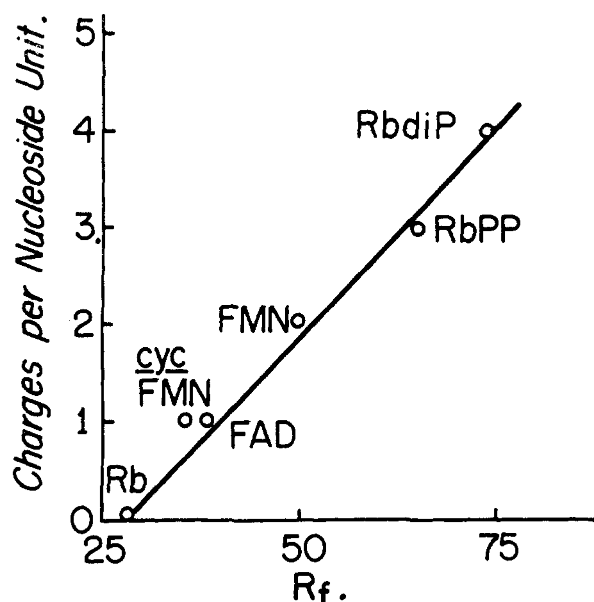

Fig. 1.—R_f value as a function of charge per nucleoside.

and its "charge per nucleoside" ratio. This latter quantity is obtained by dividing the number of negative charges, due to ionization of the phosphate groups, by the total number of nucleoside residues in the molecule. At pH 8, these values are 0, 1 and 2, respectively, for Rb, FAD and FMN.

The acidic solvents in Table I have been chosen partially because of the relative stability of sensitive dinucleotides, such as FAD, in these systems. The basic solvents, especially those containing ammonia, all have shown a tendency to cause cyclization of the flavin nucleotides.

Identification of Unknown Flavin Nucleotides.—Considerable information concerning the structure of a flavin prosthetic group or synthetic flavin may be obtained on small amounts of material by the above techniques. All flavoproteins, with the exception of succinic dehydrogenase,[24] have their prosthetic groups readily detached by heat denaturation or treatment in the cold with 10% trichloroacetic or perchloric acid. Heat is preferable both because it minimizes breakdown and because it avoids the addition of extraneous materials. Excess of the two precipitating agents should be removed, however, by ether extraction or neutralization with KOH, respectively. Prior to examination by paper chromatography, the flavins may be concentrated by methods described previously, or by adaptation of paper chromatographic methods, wherein the flavins are run as streaks and eluted with minimum volumes of water. It should be noted that acids, bases, light and some organic solvents, (*e.g.*, acetone and ethyl alcohol), tend to degrade flavins and should be avoided during the concentration procedures.

In addition to obtaining information from chromatography of the intact flavin, it is possible to degrade the material by means of treatment with acid, base or enzymes, and to compare the breakdown products with known standards. For example, after spotting the flavin at the origin, the paper may be exposed to HCl or NH_3 vapors for 1 hour in the dark, and then aerated in a hood for a few minutes before being placed in the solvent. Similarly, the spot may be overlaid with a concentrated solution of intestinal phosphatase or nucleotide pyrophosphatase and the paper suspended in a moist tank for an hour prior to running the chromatogram. Such techniques have been employed previously for establishing the structure of FAD-X.[15]

Acknowledgment.—The authors are indebted to Mrs. Enid Vercamer for her assistance in this problem.

(24) E. B. Kearney and T. P. Singer, *Biochim. Biophys. Acta*, **17**, 596 (1955).

SEATTLE 5, WASHINGTON

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON]

Chemical Synthesis of Riboflavin 5′-Pyrophosphate[1,2]

BY G. L. KILGOUR[3] AND F. M. HUENNEKENS

RECEIVED SEPTEMBER 19, 1956

The chemical synthesis of riboflavin 5′-pyrophosphate (RbPP) from readily available riboflavin 5′-phosphate (FMN) has been investigated, using several phosphorylating agents, the most suitable being phosphoric acid in the presence of di-*p*-tolyl carbodiimide (DPTC). By means of these reactions, and subsequent isolation of the product by large-scale, preparative paper chromatography, RbPP has been obtained and characterized.

The chemical synthesis of flavin–adenine dinucleotide (FAD[4]) was first achieved by A. R. Todd and his colleagues[5] through a route utilizing the condensation of the mono-silver salt of FMN with 2′,3′-isopropylidene adenosine-5′-benzyl phos-

(1) Paper V in the series "Flavin Nucleotides and Flavoproteins"; for paper IV see G. L. Kilgour, S. P. Felton and F. M. Huennekens, THIS JOURNAL, **79**, 2254 (1957).

(2) Supported by research grants from Eli Lilly and Co. and Initiative 171, State of Washington.

(3) A portion of this material is taken from the Dissertation of Gordon L. Kilgour offered in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(4) The following abbreviations will be used: LC, lumichrome; LF, lumiflavin; Rb riboflavin; FMN, riboflavin-5′-phosphate; cyc-FMN, riboflavin-4′,5′-(cyclic)-phosphate; FAD, flavin–adenine dinucleotide; RbPP, riboflavin-5′-pyrophosphate; RbdiP, riboflavin diphosphate; RbI, 5′-deoxy-5′-iodo-riboflavin; AdI, 5′-deoxy-5′-iodo-adenosine; DCC, dicyclohexyl carbodiimide; DPTC, di *p*-tolyl carbodiimide.

(5) S. Christie, G. W. Kenner and A. R. Todd, *Nature*, **170**, 924 (1952); *J. Chem. Soc.*, 46 (1954).

phorochloridate, followed by removal of the protective groups. Subsequently, an alternate procedure was devised in this Laboratory, wherein FMN and AMP are condensed directly using di-*p*-tolyl carbodiimide as the dehydrating agent.[6] Other projected pathways to FAD, not requiring protective groups, would envision either (a) the condensation of RbI with mono-silver ADP, or (b) mono-silver RbPP with AdI.[7] The present communication describes briefly the attempted preparation of RbI and, in more detail, the successful synthesis of RbPP.

Synthesis of RbI would be expected to proceed through replacement of the primary tosyl group, at the 5'-position of riboflavin, with iodine.[8] Preliminary experiments, however, confirmed the finding of Forrest, *et al.*,[9] that neither the 5'-tosyl nor the 5'-mesyl ester of riboflavin could be obtained, even with wide variation in reaction conditions. Attempts to halogenate the 5'-position directly with ICl were likewise unproductive; accordingly the first route was not explored further.

The preparation of RbPP was approached in several ways. It was decided first to attempt the condensation of the silver salt of FMN with $POCl_3$ at temperatures below 0° in anhydrous dimethylformamide. Upon chromatographing an aliquot of the resulting solution in 5% Na_2HPO_4 and determining the amount of each component by quantitative densitometry,[1] three products were found in addition to unreacted FMN (R_f = 0.57, 18% yield) and large quantities of cyc-FMN (R_f = 0.36, 63% yield). Following purification by means of large-scale paper chromatography, one product (R_f = 0.66, 6% yield) was identified as RbPP. It was found to exhibit a P_7:P_{tot}:flavin ratio[10] of 1:2:1, and upon treatment with dilute H^+, NH_3 or Ba^{++}, was hydrolyzed to FMN (with NH_3, some cyc-FMN was formed also). The second flavin product (R_f = 0.73, 8% yield) is believed to be riboflavin-4',5'diphosphate by the P_{tot}:flavin ratio of 2:1, the absence of any acid-labile phosphate, and the positive periodate reaction.[11] This second compound has been detected also in small amounts (*ca.* 3–6%) by paper chromatography of commercial FMN. The third flavin (R_f = 0.60, 5% yield) has been designated tentatively as Rb-4',5'-cyc-diphosphate because of its chromatographic behavior, ease of hydrolysis in dilute acids to yield Rb-4',5'-diphosphate, and positive reaction to the periodate reagent.

The yield of RbPP could not be increased, nor could the amount of unwanted cyc-FMN be decreased, by substituting monochlorophosphoric acid[12] or diphenylphosphoryl chloride for $POCl_3$.

The synthesis of ATP and ADP from AMP and H_3PO_4 using dicyclohexyl carbodiimide[13] suggested the possibility of RbPP synthesis from FMN and H_3PO_4 by this method. However, DCC reacted rapidly and completely with FMN to give only cyc-FMN. When the less reactive DPTC was substituted for the dicyclohexyl derivative, the reduced reaction rate permitted the hydroxyl group of the entering phosphate to compete with the sterically preferred 4'-hydroxyl of FMN. In addition to FMN and cyc-FMN, the products included one compound chromatographically and chemically indistinguishable from the RbPP prepared previously by condensation of Ag_2FMN and $POCl_3$. Several other flavins obtained in lower yield appeared to be, on the basis of R_f values and acid hydrolysis to FMN, higher polyphosphates of riboflavin, Rb(P)x, analogous to the ATP and adenosine tetraphosphate obtained by Khorana[13] in the synthesis of ADP.

Experimental

Materials and Methods.—Commercial chemicals used in this investigation were obtained from the following sources: monosodium FMN, Hoffman-LaRoche, Inc.; Rb, Nutritional Biochemical Co.; DCC, Dajac Laboratories; Florisil, Floridin Company; ICl, Eastman Kodak Co. DPTC was prepared by the method of Khorana.[14] Cyc-FMN was prepared by the method of Forrest and Todd.[15]

Paper chromatography was carried out as described previously.[1]

Preparation of Disilver FMN.—NaFMN (1.0 g., 2.1 mmoles) was dissolved in 10 ml. of water and a solution of 1.0 g. (6.0 mmoles) of $AgNO_3$ in 10 ml. of water added. To the resultant bright red solution was added 10 ml. of absolute methanol. At this point a flocculent red precipitate formed and was centrifuged readily from the pale yellow supernatant solution. The precipitate was washed twice with absolute methanol, twice with dry ether, and dried under vacuum at room temperature to yield 1.080 g. of a fine, red powder. Found: Ag = 33.17%; Calcd. for AgFMN, Ag = 19.04%; for Ag_2 FMN, Ag = 31.73%; for Ag_3FMN, Ag = 41.70%.

Synthesis of RbPP Using H_3PO_4 and Carbodiimide.—Ten mg. of NaFMN was dissolved in 0.1 ml. of water and 0.5 ml. of pyridine was added. To this was added a solution of 500 mg. of DPTC in 0.5 ml. of pyridine. Finally, 0.25 ml. of 85% H_3PO_4 was added with shaking, and the final mixture was shaken mechanically in the dark at room temperature for 24 hours. Addition of water (*ca.* 2 ml.) to the mixture caused precipitation of unreacted DPTC and the by-product, di-*p*-tolylurea. These materials were removed by filtration and the resulting clear solution analyzed by paper chromatography in order to ascertain the amount of RbPP present. Quantitative densitometry of the paper chromatograms revealed that 17% of the total flavin was RbPP. Decreasing the amount of H_3PO_4 or water, increasing the amount of pyridine (to 3.0 ml.), or substitution of FMN (free acid) for NaFMN, greatly diminished the yield of RbPP, while increasing the time beyond 24 hours appeared to have no significant effect. Shorter times were found to result in increased amounts of unreacted FMN.

(6) F. M. Huennekens and G. L. Kilgour, THIS JOURNAL, **77**, 6716 (1955).

(7) Since the difficulties of preparing AdI by the conventional route through the condensation of 2',3'-isopropylidene-5'-tosyladenosine with NaI are well recognized (J. Baddiley, *J. Chem. Soc.*, 1349 (1951); V. M. Clark, A. R. Todd and V. Zussman, *ibid.*, 2952 (1951)), this pathway would be useful only if an alternate route to AdI were available. The analog, flavin–inosine dinucleotide, previously synthesized by another route,[6] could be prepared, however, in this manner, since the synthesis of inosine-5'-iodide is reported to proceed smoothly by NaI treatment of the tosyl intermediate.

(8) J. W. H. Oldham and J. K. Rutherford, THIS JOURNAL, **54**, 366 (1932); P. A. Levene and A. L. Raymond, *J. Biol. Chem.*, **102**, 317 (1933).

(9) H. S. Forrest, H. S. Mason and A. R. Todd, *J. Chem. Soc.*, 2530 (1952).

(10) P_7 = moles phosphate released in 7 minutes at 100° in 1 *N* acid/mole flavin; P_{tot} = total moles phosphate present/mole flavin.

(11) J. G. Buchanan, C. A. Dekker and A. G. Long, *J. Chem. Soc.*, 3162 (1950).

(12) L. A. Flexser and W. G. Farkas, 12th International Congress of Pure and Applied Chemistry, New York, Sept. 1951, Abstracts, p. 71.

(13) H. G. Khorana, THIS JOURNAL, **76**, 3517 (1954).

(14) H. G. Khorana, *Can. J. Chem.*, **32**, 230 (1954).

(15) H. S. Forrest and A. R. Todd, *J. Chem. Soc.*, 3295 (1950).

RbPP was isolated from the above mixture of flavins by the following procedure. The solution was diluted to 50 ml. with water and was shaken three times with four volumes of $CHCl_3$ in order to remove most of the pyridine. Removal of inorganic impurities was carried out by adsorption of the flavins on a Florisil column. The solution was acidified to *p*H 5 with acetic acid and passed through a washed column of Florisil (*ca.* 10 × 30 cm.). The column was washed with acetic acid, water and pyridine, as described previously,[16] and the flavins removed by elution with 5% pyridine. Pyridine was removed by shaking with $CHCl_3$, giving a clear yellow solution of the flavins.

The solution was concentrated to a small volume by lyophilization. Bulk separation of the flavins was achieved by applying the concentrated flavin solution to the starting line of Munktell's Cremer–Tiselius paper[6] (*ca.* 50 mg. per sheet) and developing the chromatogram in 5% Na_2HPO_4. The separate bands were located by inspection under a Mineralite, cut out, and eluted with water. Inorganic phosphate was removed from each fraction as follows. First, the solutions were stored at 0–5° for several days in order to crystallize out excess phosphate. Silver nitrate was then added in slight excess, with stirring, and the solution was adjusted with dilute acetic acid until the flavin went back into solution as a red complex. The residual silver phosphate was removed by centrifugation and the red supernatant solution passed through a short, chilled column of IR-120(H^+) cation exchange resin to remove silver. The effluent was collected in a tube immersed in a bath of acetone and Dry Ice and was thus frozen immediately. The solution was later thawed and adjusted immediately to *p*H 7 with dilute NaOH. Large-scale paper chromatography was repeated as above but employing *t*-BuOH:water (60:40) as the solvent system.[6] The pure components thus obtained, with the exception of FMN and cyc-FMN, were present in amounts too small to handle conveniently in the dry state, and the analyses and characterization were carried out using concentrated solutions. At this point, all of the flavins including RbPP behaved chromatographically as single components having the R_f values given previously. RbPP was obtained after purification in *ca.* 5% over-all yield, based upon the original NaFMN. The P_7: $P_{tot.}$: flavin ratio was 1.13:2.07:1 and 1.09:2.17:1 in duplicate analyses, and upon gentle acid hydrolysis there was quantitative conversion to FMN.

(16) E. Dimant, D. R. Sanadi and F. M. Huennekens, THIS JOURNAL, **74**, 5440 (1952).

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON, AND THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF TEXAS]

Lyxoflavin Nucleotides[1,2]

BY F. M. HUENNEKENS, S. P. FELTON AND ESMOND E. SNELL

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L. lactis cells, grown on lyxoflavin, incorporate the vitamin into lyxoflavin mononucleotide (LMN) and dinucleotide (LAD), which can be isolated by means of chromatography on Florisil and paper columns. LMN and LAD can be distinguished from their riboflavin analogs by means of a differential microbiological assay using *L. lactis* and *L. casei*. They cannot be distinguished, however, by means of paper chromatography. In TPNH cytochrome c apo-reductase from yeast FMN is utilized preferentially to LMN, although the latter exhibits appreciable activity. In a D-amino acid apo-oxidase from pig kidney, FAD is utilized preferentially to LAD.

In 1949 Pallares and Garza[3] reported the isolation of L-lyxoflavin from human cardiac muscle. The proof of its structure, and its non-identity with D-riboflavin, rested principally upon the preparation of the tetraacetyl derivative, whose melting point agreed with that of Lx,[4] but not Rb. Synthetic Lx was first prepared by these authors, using a modification of the Karrer procedure[5] and, subsequently, by other independent routes.

The failure of other workers[6,7] to find Lx in a variety of animal tissues has cast doubt as to the natural occurrence of this substance. In addition, Gardner, *et al.*,[7] have pointed out the difficulty in distinguishing Lx and Rb on the basis of a single derivative.

In spite of the doubtful natural occurrence of Lx, other investigations have been carried out with the synthetic material. The addition of Lx to experimental rations increased the growth of rats,[8,9] chicks[9,10] and pigs.[11] Snell, *et al.*,[6] have shown that Lx does not support growth of *Lactobacillus casei* in the absence of Rb, but that low concentrations of Lx increase the growth response to limiting amounts of Rb. Under the latter conditions it was shown that Lx was not deposited in cells of *L. casei*, and that it actually decreased the concentration of Rb deposited in such cells. In contrast, the similar organism, *Lactobacillus lactis*, grew in the absence of Rb when Lx was supplied to the medium.[6,12] In this case, cells grown with Lx contain no Rb, but do contain Lx; *i.e.*, Lx apparently can fulfill the essential metabolic roles normally played by Rb. The growth requirements of these two organisms made it possible to determine Lx and Rb by means of a differential microbiological assay.[6]

Since it had been established that *L. lactis* utilized Lx directly for growth, it seemed possible that the Lx was being incorporated into the cellular flavin nucleotides. In the present study, the

(1) Paper VI in the series "Flavin Nucleotides and Flavoproteins." For paper V see G. L. Kilgour and F. M. Huennekens, THIS JOURNAL, **79**, 2256 (1956).

(2) Supported by grants from Eli Lilly and Co. and Initiative 171, State of Washington.

(3) E. S. Pallares and H. M. Garza, *Arch. Biochem.*, **22**, 63 (1949).

(4) The following abbreviations will be used: Lx, lyxoflavin; Rb, riboflavin; LMN and FMN, lyxoflavin and riboflavin mononucleotides; LAD and FAD, lyxoflavin- and riboflavin-adenine dinucleotides.

(5) P. Karrer, H. Salmon, K. Schopp, F. Benz and B. Becker, *Helv. Chim. Acta*, **18**, 910 (1935).

(6) E. E. Snell, O. A. Klatt, H. W. Bruins and W. W. Cravens, *Proc. Soc. Exp. Biol. Med.*, **82**, 583 (1953).

(7) T. S. Gardner, E. Wenis and J. Lee, *Arch. Biochem. Biophys.*, **34**, 98 (1951).

(8) G. A. Emerson and K. Folkers, THIS JOURNAL, **73**, 2398, 5383 (1951).

(9) J. M. Cooperman, W. L. Marsuick, J. Scheiner, L. Drekter, E. DeRitter and S. H. Rubin, *Proc. Soc. Exp. Biol. Med.*, **81**, 57 (1952).

(10) H. W. Bruins, M. L. Sunde, W. W. Cravens and E. E. Snell, *ibid.*, **78**, 535 (1951).

(11) R. C. Wahlstrom and B. C. Johnson, *ibid.*, **79**, 636 (1952).

(12) M. S. Shorb, *ibid.*, **79**, 611 (1952).