TABLE II

H..........O (hydrogen bond)

R—N—CON(R')C—R''

No.	R	R'	R''	Concn.[a] moles/liter	Absorption band, cm.$^{-1}$			
1	C_6H_5	C_6H_5	OC_2H_5	1.056[b]	4843			
2	C_6H_5	C_6H_5	OC_2H_5	0.3520[c]	4843			
3	C_6H_5	C_6H_5	OC_2H_5	.278[b]	4843			
4	C_6H_5	C_6H_5	OC_2H_5	.1408[c]	4843			
5	C_6H_5	C_6H_5	OC_2H_5	.0704[c]	4843			
6	C_6H_5	p-$CH_3OC_6H_5$	OC_2H_5	.1592[c]	4831			
7	C_6H_5	p-ClC_6H_5	OC_2H_5	.3140[c]	4836			
8	C_6H_5	C_2H_5	$N(C_2H_5)_2$	.3800[c]	4877			
9	C_2H_5	C_2H_5	$N(C_2H_5)_2$	.0807[c]	4877			
10	C_6H_5	C_6H_5	NHC_6H_5	.1620[b]	6711	5276	4943	4870
11	C_2H_5	C_2H_5	NHC_2H_5	.1605[c]	6725	5000	4920	4825

[a] All spectra were determined at 26 ± 0.5° and carried out in 10.00- and 25.00-mm. quartz cells. [b] Carbon tetrachloride was used as the solvent. [c] Benzene was used as the solvent.

prepared according to the procedure of Kogon.[11] 1-Ethyl-3-phenylurea was prepared according to the procedure of Thiele and Pickard,[12] m.p. 96–97°. 1-Methyl-1,3-diphenylurea was prepared according to the procedure of Gebhardt[13] m.p. 104°. 1,1,3-Triethylurea was prepared according to the procedure of Hofmann,[14] m.p. 62°. 1,3,5-Triphenylbiuret, m.p. 150–151°, and 1,1,3-triethyl-5-phenylbiuret, m.p. 113–113.5°, were kindly supplied by E. J. Goldberg.[15] 1,3,5-Triethylbiuret[16] (*Anal.* Calcd. for $C_8H_{17}N_3O_2$: N, 22.4. Found: N, 22.6) and 1,1,3,5-tetraethylbiuret (*Anal.* Calcd. for $C_{10}H_{21}N_3O_2$: N, 19.4. Found: N, 19.6) were prepared according to the procedure of Baker and Holdsworth.[17] A boiling point for these compounds was not obtained due to their ease of decomposition.

Preparation of Solutions.—The solutions were carefully made up by weighing the compound directly into a 50-ml. or 100-ml. volumetric flask and diluting with sufficient carbon tetrachloride or benzene to give 50 or 100 ml. of solution. The solution was then added directly to a 100.0- or 25.0-mm. quartz cell. Prior to each run the cells were washed once with acetone, followed by three to five washings with reagent grade carbon tetrachloride, then dried *in vacuo*. The cells were rinsed twice with 2 ml. of the solution to be examined and then filled.

Spectrophotometric Method.—Measurements were made in a 100.0- or 25.0-mm. quartz cell using the Cary spectrophotometer model No. 14. The cell holder was held at a temperature of 26 ± 0.5°. The samples were run at a scan speed of 50 Å./sec., chart speed of 5''/min., and a slit control of 0.15 mm.

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(15) E. J. Goldberg, Elastomer Chemicals Dept., E. I. du Pont de Nemours, Wilmington, Del.
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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON]

Paper Chromatography of Flavins and Flavin Nucleotides[1,2]

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Methods and solvent systems are described for the paper chromatographic separation and identification of flavins and flavin nucleotides.

Numerous investigations concerned with flavins and flavin nucleotides have been facilitated greatly by the application of chromatographic methods to these problems. Following the work of Crammer,[4] who first separated Rb,[5] FMN and FAD by paper chromatography, this technique has been utilized further in such studies as: the isolation[6–8] and chemical synthesis[9–11] of FAD and FMN, the identification of riboflavinyl glucoside,[12] the identification of Rb analogs,[13] the failure of P^{32}-labeled orthophosphate to be incorporated into FAD by respiring tissue homogenates[14] and the identifica-

(1) Paper IV in the series "Flavin Nucleotides and Flavoproteins"; for paper III, see THIS JOURNAL, **77**, 6716 (1955).
(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) J. L. Crammer, *Nature*, **161**, 349 (1948).
(5) The following abbreviations will be used: Rb, riboflavin; Lx, lyxoflavin; RbG, riboflavinyl glucoside; GalF, galactoflavin; IsoRb, isoriboflavin; LF, lumiflavin; LC, lumichrome; FMN, riboflavin 5'-phosphate (flavin mononucleotide); cyc-FMN, riboflavin-4',5'-phosphate (cyclic); RbPP, riboflavin 5'-pyrophosphate; Rb-diP, riboflavin 4',5'-diphosphate; FAD, flavin-adenine dinucleotide; FAD-X, cyclic analog of FAD.
(6) E. Dimant, D. R. Sanadi and F. M. Huennekens, THIS JOURNAL, **74**, 5440 (1952).
(7) L. G. Whitby, *Biochem. J.*, **54**, 437 (1953); *Biochim. Biophys. Acta*, **15**, 148 (1954).
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(9) S. Christie, G. W. Kenner and A. R. Todd, *Nature*, **170**, 924 (1952); *J. Chem. Soc.*, 46 (1954).
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(13) W. Forter and P. Karrer, *Helv. Chim. Acta*, **36**, 1530 (1953).
(14) J. C. Rabinowitz, G. Jacobs, L. J. Teply, V. H. Cheldelin, E. Dimant, H. R. Mahler and F. M. Huennekens, *Biochim. Biophys. Acta*, **13**, 413 (1954).

tion of the prosthetic group of various flavoproteins.

The present communication describes methods of paper chromatography which are applicable for the separation and identification of known flavins, and which provide information concerning the structure of unknown flavins of natural or synthetic origin.

Experimental

Materials.—Rb and FMN were commercial products from Merck and Co. and Hoffmann-La Roche Inc., respectively. FAD[6] and FAD-X[15] were isolated by methods previously described. LF and LC were prepared by ultraviolet irradiation of neutral and basic solutions of Rb, with subsequent isolation of the products by chloroform extraction of the acidified aqueous phase. Rb-4',5'-diphosphate was obtained as a by-product in the synthesis of Rb-5'-pyrophosphate; details of these preparations are described in a subsequent communication.[16]

Intestinal phosphatase was purchased from Armour & Co. and nucleotide pyrophosphatase was isolated from potatoes.[17]

The following materials were generously provided for this investigation: Lx by Drs. K. Folkers and E. Snell; IsoRb and GalF by Dr. Folkers; RbG by Dr. L. G. Whitby; and cyc-FMN by Drs. A. R. Todd and G. Kenner.

All solvents were reagent grade commercial products and were used without further purification. Phenol of the required high purity was Merck reagent grade.

Methods.—Paper chromatography was carried out on 28 × 42 cm. sheets of Whatman #1 paper. Spots of approximately 4 mm. diameter were applied at the starting line, and the papers developed either ascending in 15 × 45 cm. cylindrical jars or descending in a Chromatocab. These operations were carried out in very dim light in order to minimize photodegradation of the flavins to LF or LC. An equilibration period of 3 hours was required for all organic systems, but not for aqueous systems.

As little as 0.01 μg. of flavin can be detected by means of its fluorescence under the Mineralight ultraviolet lamp, Model RB-50. When it was desired to locate, in addition, other nucleotides by their dark blue "quenching" spots, the shorter wave length Mineralight lamp, Model V41, was employed. Larger amounts of the dinucleotides, such as FAD, must be used in comparison to Rb or FMN, since the molar fluorescence of FAD is only 10–15% of that of the other two compounds at neutral *p*H.[18] In addition, considerable quenching of the fluorescence of all the flavins is caused by the presence of many organic compounds and metal ions.[19]

Due to the great sensitivity of detection by means of fluorescence, the usual spray reagents are largely unnecessary, and, in fact, are somewhat interfered with by the yellow color of the flavins. Thus, the phosphate reagent of Bandurski and Axelrod[20] yields spots of a greenish shade rather than blue. It is possible by spraying with an alkaline hydrosulfite reagent to bleach temporarily the yellow color of flavins, which are then re-oxidized by air. This serves to distinguish flavins from many other colored materials.

A semi-quantitative estimate of the amount of each flavin on a paper chromatogram may be obtained by photographing the flavins by their own fluorescence, and subsequently analyzing the negative with a Photovolt Model 501A densitometer.[21] The photography is accomplished by illuminating the paper evenly with the ultraviolet light source and using a red (*e.g.*, Wratten A) filter at the camera with Contrast Process Panchromatic Sheet film (Eastman). The picture also provides a permanent record of the chromatogram. An alternate method for obtaining quantitative data consists of treating the paper strip with a light Mineral oil, passing it through a Beckman Model DU spectrophotometer equipped with a paper-strip scanner,[22] Model 2001, and taking optical density readings at 450 mμ.

Results and Discussion

R_f Values and Solvent Systems.—Table I lists a series of acidic, basic and neutral solvents which have been found to be most suitable for the separation and identification of flavins. It is seen that essentially all of the systems readily separate the four *classes* of flavins: (a) degradation products, LC and LF; (b) nucleosides, Rb, Lx, GalF, IsoRb and RbG; (c) mononucleotides, FMN and cyc-FMN; and (d) dinucleotides, FAD and FAD-X. Within a given class, the individual members are usually separable, with the exception of Rb and Lx for which no separation has been devised.[23]

TABLE I

PAPER CHROMATOGRAPHY OF FLAVINS

Substance	R_f Values × 100 in various solvent systems[a] 1	2	3	4	5	6	7	8	9
Dinucleotides									
FAD	5	36	38	20	17	8	4	24	14
FAD-X	16	37	38	41	30	28	20	29	30
Mononucleotides									
FMN	10	33	50	17	4	17	4	17	14
cyc-FMN	13	27	36	49	15	29	12	29	23
Nucleosides									
Rb	31	58	28	79	69	37	41	33	39
Lx	32	55	30	77	64	34	40	32	38
IsoRb	33	55	27	80	74	45	46	35	42
GalF	30	51	41	73	57	37	41	32	39
RbG	23	42	43	59	50	34	35	31	36
Degradation Products									
LF	47	67	18	94	68	34	43	34	44
LC	68	75	7	88	72	61	73	53	58

[a] Composition of solvent systems: 1, *n*-butanol:acetic acid:H_2O (40:10:50)—use organic phase; 2, isobutyric acid:NH_4OH:H_2O (66:1:33); 3, $Na_2HPO_4{\cdot}7H_2O$ (5% in H_2O); 4, phenol:*n*-butanol:H_2O—160 g. of phenol is dissolved with warming in 30 ml. of *n*-butanol. The resulting solution is cooled to room temperature, shaken with 100 ml. of water and the organic phase used. 5, collidine—saturate with H_2O and use organic phase; 6, *t*-butyl alcohol:H_2O (60:40); 7, *t*-butyl alcohol:pyridine:H_2O (60:15:25); 8, *t*-butyl alcohol:NH_4OH:H_2O (60:5:35); 9, *n*-propanol:NH_4OH:H_2O (60:30:10). After being run in solvent system 4 and 5, the dried papers must be washed repeatedly in $CHCl_3$ before the fluorescent spots can be seen.

Solvent system 3, and similar ones employing aqueous buffered solutions, are susceptible to extreme variation in R_f values depending upon both the *p*H and the buffer concentration. The R_f values (× 100) for Rb, FMN and FAD vary with the molar concentration of 1.0, 0.1, 0.01 and 0.001 *M* phosphate buffer, *p*H 8.0, as follows: Rb—19, 32, 34, 32; FMN—40, 75, 76, 89; and FAD—24, 62, 74, 79. The relative order of the compounds does not change in spite of wide variations in their relative separation. This latter finding is not the case, however, when the *p*H is varied using different buffers, but with the buffer concentration held at 0.1 *M*. The R_f of Rb is not greatly influenced from *p*H 2 to 10, while FAD precedes FMN at *p*H values below 5.5, and follows FMN at *p*H values above that. Maximum separation may be ob-

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tained for a mixture of all three components in the *p*H 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

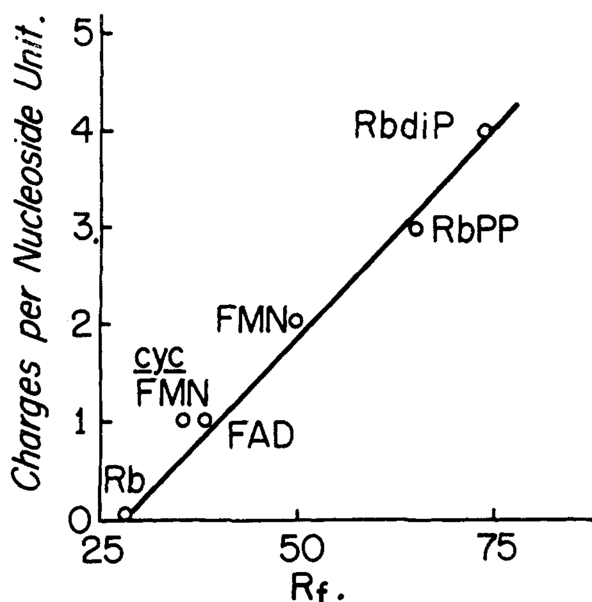

Fig. 1.—R_f value as a function of charge per nucleoside.

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 *p*H 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.

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

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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.

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