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86. Tyunosin Ukita and Kinzo Nagasawa: Organic Phosphates. VIII.[1] A Novel Synthesis of Riboflavin 5'-Phosphate.

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It was reported in the preceding paper[1] that on the alcoholysis of catechol cyclic phosphate (CCP[*2]) (I) with polyols containing three vicinal hydroxyls, the reaction proceeded to give polyol cyclic phosphate (IV) with simultaneous liberation of catechol, and on further hydrolysis (IV) was converted into the monophosphate (V) of the polyol used. Thus, as shown in Chart 1, DL-erythritol 1-phosphate (Va) or D-mannitol 1-phosphate (Vb) was obtained from erythritol or D-mannitol and CCP.

This paper describes the phosphorylation of riboflavin by a similar reaction and isolation of a product which afforded riboflavin 5'-phosphate (FMP) (Vc) in a high yield.

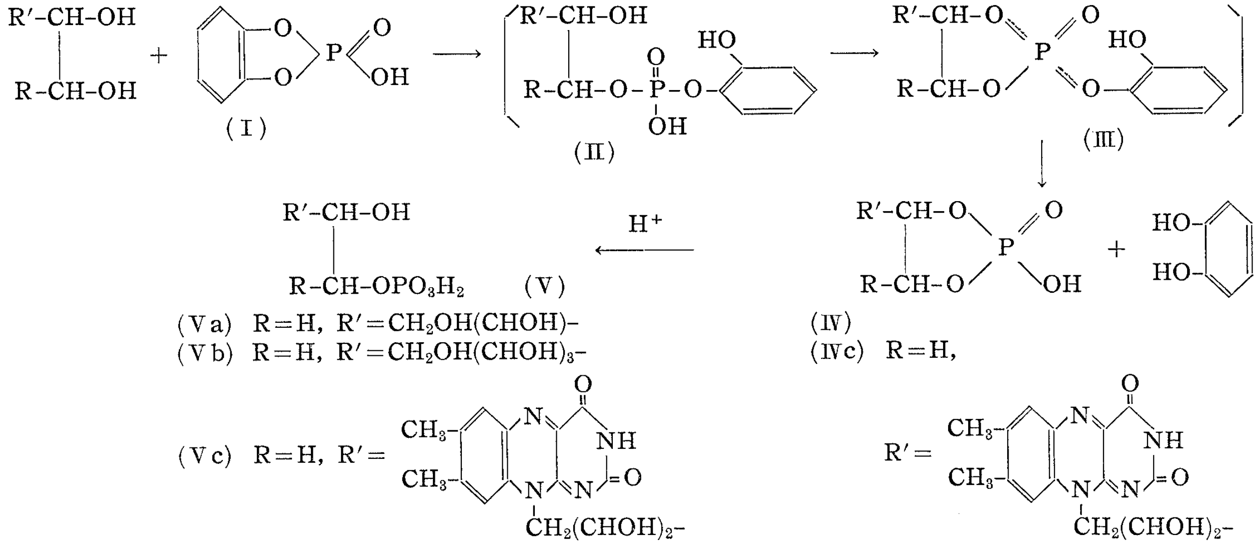

Chart 1.

Several reports on the synthesis of riboflavin 5'-phosphate (FMP) have been published. Thus, Kuhn and Rudy,[2,3] Forrest and Todd,[4] and Flexer and Farkas[5] obtained this compound by phosphorylation of riboflavin or its acetyl derivative with phosphoryl chloride. However, in all of these cases the product, FMP, contained several by-products which were separated from the desired FMP only by tedious operations.

Riboflavin and CCP were reacted in pyridine under various conditions and the results were compared with that reported in the literature, which are summarized in Table I.

As shown in Table I the yield of the total phosphorus product was 50% and 75%, respectively, by the method reported by Forrest and Todd (No. 7), and by Flexer, Farkas (No. 8) and that of FMP were 42% and 66.5%, respectively. Among the results of the

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[*1] Hongo, Tokyo (浮田忠之進, 長沢金蔵).

[*2] The following abbreviations are used: CCP, catechol cyclic phosphate; RF, riboflavin; FMP, riboflavin 5'-phosphate; FDP, riboflavin diphosphate; cyclic-FMP, riboflavin 4',5'-cyclic phosphate; *o*-HPP, *o*-hydroxyphenyl phosphate.

1) Part VII: T. Ukita, K. Nagasawa: This Bulletin, **7**, 401(1959).
2) R. Kuhn, H. Rudy: Ber., **68**, 383(1935).
3) R. Kuhn, H. Rudy, F. Weygand: *Ibid.*, **69**, 1543(1936).
4) H.S. Forrest, A.R. Todd: J. Chem. Soc., **1950**, 3295.
5) L.A. Flexer, W.G. Farkas: Abstracts of papers, XIIth International Congress of Pure and Applied Chemistry (New York, 1951), p. 71.

Table I.

React. No.	React. conditions: Reactants RF (mg.)	CCP (mg.)		Solvent Pyridine (cc.)	React. temp. (°C)	React. time (hr.)	React. product Yield of RF	cyclic-FMP FMP	FDP (%)
1	500 (1 eq.)	1150 (5 eq.)		200	120\~140	2	15.7	71.7	12.6
2	200 (〃)	370 (4 eq.)		70	110\~115	3	4.94	77.6	17.3
3	200 (〃)	180 (2 eq.)		70	110	3	62.9	34.8	2.3
4	200 (〃)	180 (2 eq.)		70	130\~140	3	32.6	61.8	5.8
5	50 (〃)	50 (2.2 eq.)		70	130\~140	3	44.6	51.8	3.48
6	500 (〃)	1500 (6.6 eq.)		110	110\~120	4	7.4	50.0	42.3
	RF	$POCl_3$	H_2O						
7	190 (mg.)	0.09 cc.	0.009 cc.	100	room temp.	1	47.9	41.9	10.1
8	752 (mg.)	9.13 g.	0.72 g.		room temp.	6	24.6	66.5	8.3

experiments No. 1 to No. 6, the starting compound was phosphorylated to the extent of 95% in the case of No. 2 and the product contained 78% of FMP.

These data show that the new method is a desirable one for the practical preparation of FMP provided that some effective procedure in separating the main product is found.

The method of separation hitherto used was applied in the present case with extraction of the crude phosphorus product with water and followed by precipitation of the desired phosphate with acetone or ethanol. By this procedure, however, unavoidable loss of the product resulted and the final yield of the pure FMP was 34\~45%.

For the separation of FMP from the mixture of riboflavin and its phosphorylated products, a zone electrophoresis on a block of cellulose powder was used. A concentrated aqueous solution of the crude product, which was pretreated with cation exchange resin,[*3] was subjected to electrophoresis. The separation was effected to give distinct zones of each of riboflavin, riboflavin 5′-monophosphate, and riboflavin diphosphate. The result of the separation is illustrated in Fig. 1.

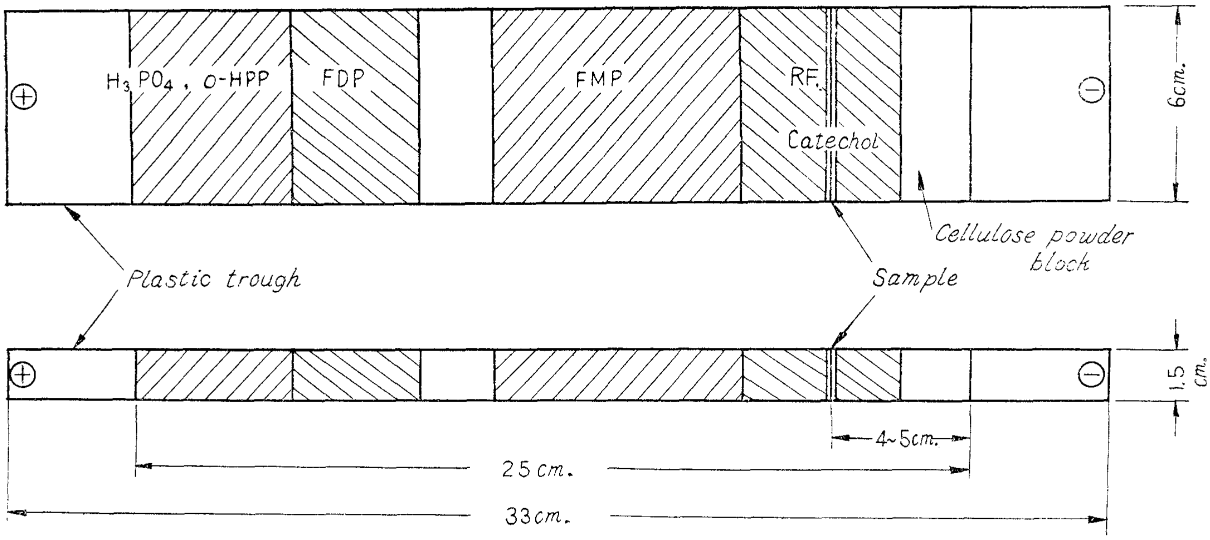

Fig. 1.

The zone of the cellulose powder which contained riboflavin 5′-phosphate was extracted with water to obtain the desired phosphate in a yield of 75\~78%.

The combination of these procedures of phosphorylation of riboflavin and separation

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[*3] Riboflavin 4′,5′-cyclic phosphate (IVc) contained in the crude product from phosphorylation was hydrolyzed to riboflavin 5′-phosphate (Vc) (see experimental part).

of the products by zone electrophoresis is a novel and effective method for the preparation of riboflavin 5′-monophosphate.

The authors are indebted to Misses R. Ohta and S. Ohno for carrying out microanalyses.

Experimental

Detections of Products from Alcoholysis of CCP with Riboflavin by Paper Chromatography and Paper Electrophoresis—Ascending paper chromatography was performed with Toyo Roshi No. 53 filter paper. The solvent systems used were (1) $Na_2HPO_4 \cdot 7H_2O$ (5% in water) and (2) *tert*-BuOH: conc. $NH_4OH:H_2O$ (60:5:35).

For paper electrophoresis, Toyo Roshi No. 53 and No. 27 papers were used. After spotting the sample, the paper was moistened with a buffer solution of pH 6.0, consisting of *n*-BuOH (15 cc.): AcOH (2 cc.): pyridine (10 cc.): H_2O (500 cc.), and subjected to a potential of 20~30 V/cm. for 50~120 min.

Spots on chromatograms were detected by their fluorescence under ultraviolet lamp and by detection of P according to the method of Bandurski and Axelrod.[6]

Quantitative Estimation of Products—An aliquot (ca. 1 mg. as riboflavin) of reaction mixture was withdrawn and streaked on Toyo Roshi No. 27 filter paper (10 cm. width). The paper was subjected to electrophoresis at 30 V/cm. for 90 min. as described above. The separated bands were detected by their fluorescence and cut out. Each cutting was completely eluted with water to make a constant volume and determined by reading optical density at 450 mμ.[7]

Isolation of the Alcoholysis Products of CCP with Riboflavin: (a) Riboflavin 5′-Phosphate (FMP) (Vc)—A mixture of 500 mg. of riboflavin, 650 mg. of CCP, and 200 cc. of dehyd. pyridine was heated at 130~140° under vigorous stirring. After 30 min., 500 mg. of CCP was added to the mixture, the heating was continued for additional 1.5 hr. (reaction No. 2 shown in Table I). The solvent was evaporated *in vacuo* to dryness in a dim light and the syrup obtained was suspended in 15 cc. of water. The suspension was passed through a column of Amberlite IR-120 (H^+) and the acid effluent and washings were combined (20 cc., 0.3*M* solution), and heated at 95° for 15 min. to hydrolyze cyclic-FMP to FMP. 2 cc. of the hydrolysate thus obtained was absorbed on 4 sheets of Toyo Roshi No. 27 filter paper (6×1.5 cm.) and dried. A cellulose powder block was prepared with 120 g. of Toyo Roshi cellulose powder (100~120 mesh) and buffer solution of pH 6.0, consisting of *n*-BuOH (20 cc.): AcOH (1 cc.): pyridine (5 cc.): water (500 cc.). The papers were inserted in the block set on a plastic trough (see Fig. 1). Zone electrophoretic separation was performed at 10 V/cm. for 15 hr. The orange-yellow band second from the starting line was taken and extracted with a minimum volume of water. The extract was evaporated to ca. 10 cc. *in vacuo* and filtered to remove the insoluble material. The filtrate was lyophilized to an orange colored powder, which consisted of monopyridinium salt of FMP with a trace of contaminated FDP; yield, 49.2 mg. (69%).

85 mg. of monopyridinium FMP thus obtained was dissolved in 3 cc. of hot *N* HCl and insoluble impurity was immediately removed by centrifugation. On keeping the supernatant in a refrigerator overnight, microcrystals separated. After washing twice with 0.5 cc. each of dehyd. EtOH, the sample was dried over P_2O_5 *in vacuo* for 5 hr.; yield, 40 mg. (44.0%). *Anal.* Calcd. for $C_{17}H_{21}O_9N_4P \cdot \frac{1}{2}H_2O$ (Riboflavin 5′-phosphate): C, 43.84; H, 4.76; N, 12.01; P, 6.66. Found: C, 43.90; H, 5.14; N, 11.82; P, 6.99.

On periodate oxidation the product consumed 1.92 mol. equiv. of the reagent.

(b) Riboflavin Diphosphate (FDP)—A mixture of 200 mg. of riboflavin, 370 mg. of CCP, and 70 cc. of dehyd. pyridine was heated at 110~115° under vigorous stirring for 3 hr. (run No. 3 in Table I). After concentration, decationization of the reaction mixture, and hydrolysis of the product as described in (a), the hydrolysate and washings were concentrated *in vacuo* to a small volume (ca. 4 cc.) and added with 30 cc. of dehyd. acetone. The yellow precipitate obtained was reprecipitated once more to complete the removal of both *o*-HPP and inorganic phosphate, which are hardly separated from FDP by subsequent zone electrophoresis. The precipitate thus obtained was dissolved in 10 cc. of water and 2 cc. of the solution was absorbed on 4 sheets of Toyo Roshi No. 27 filter paper (6×1.5 cm.). These papers were submitted to zone electrophoresis under the same conditions as described in the previous experiment (a). The yellow zone third from the starting line (shown in Fig. 1) was taken and eluted with 50 cc. of water. The yellow-fluorescent effluent was concentrated *in vacuo* to a small volume (ca. 2 cc.) in a dim light and centrifuged to remove some impurity. On evaporation of the supernatant, a resinous material was obtained (10.1 mg.).

Crude FDP (55.8 mg.) thus obtained was dissolved in 1 cc. of water and added with 40 mg. of anhydr. NaOAc and 5 cc. of dehyd. EtOH, and warmed on a water bath for several minutes. Some

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6) R.S. Bandurski, B. Axelrod: J. Biol. Chem., **193**, 405(1951).

7) G.L. Kilgour, S.P. Felton, F.M. Huennekens: J. Am. Chem. Soc., **79**, 2254(1957).

flocculent impurity that appeared was removed by centrifugation and the supernatant was kept in a refrigerator overnight to separate yellow microcrystals; yield, 12 mg. The sample for analysis was dried over P_2O_5 *in vacuo* for 5 hr. *Anal.* Calcd. for $C_{17}H_{20}O_{12}N_4Na_2P_2 \cdot 4H_2O$ (Sodium riboflavin diphosphate): N, 8.58; P, 9.51. Found: N, 8.32; P, 9.75.

Summary

The alcoholysis reaction of catechol cyclic phosphate (CCP) (I) with polyols reported in the preceding paper was applied to phosphorylation of riboflavin. The latter was phosphorylated to give a mixture of riboflavin 5′-phosphate (FMP) (Vc) and riboflavin 4′,5′-cyclic phosphate in a yield of 75～78%. After acid hydrolysis the pure riboflavin 5′-phosphate was obtained by zone electrophoresis without any loss of the product during isolation.

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87. **Minoru Sekiya and Toshio Oishi**: Reactions of Amide Homologs. I. Reaction between Azomethines and Amides.

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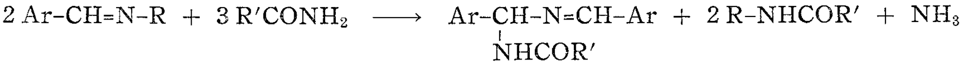
In an earlier paper[1)] dealing with the reaction between azomethines and formamide, it was shown that a reductive cleavage occurred in azomethines by reaction with formamide to afford N-alkylformamide.

$$R\text{-}CH{=}N\text{-}R' + 3\,HCONH_2 + H_2O \longrightarrow R\text{-}CH_2\text{-}NHCHO + R'\text{-}NHCHO + CO_2 + NH_3$$

(Compound $(R\text{-}CH_2)_2N\text{-}CHO$ was also produced in a side reaction)

In this reaction a portion of formamide served as a reducing agent, so that the reaction temperature is high. When the same reagents, azomethine and formamide, were allowed to react at a low temperature (about 95°) at which formamide could not act as a reducing agent, a new reaction was found to occur. This reaction which may be represented by the following equation will be discussed below.



$$2\,Ar\text{-}CH{=}N\text{-}R + 3\,R'CONH_2 \longrightarrow Ar\text{-}\underset{\displaystyle NHCOR'}{\underset{|}{CH}}\text{-}N{=}CH\text{-}Ar + 2\,R\text{-}NHCOR' + NH_3$$

The main product of this reaction was a compound of a new type, which may be termed N-arylmethylene-1-acylamino-1-arylmethylamine.

Several reactions were carried out using azomethines such as N-arylmethylenealkylamines, i.e. N-benzylidenemethylamine, N-benzylideneëthylamine, N-(*p*-chlorobenzylidene)methylamine, and N-anisylidenemethylamine.[*2] In the reaction not only formamide but acetamide could also be used as a reactant, but was less reactive.

The reaction between N-benzylidenemethylamine (I) and formamide is first discussed below from the viewpoints both of confirmation of structure of the main product and of the nature of the reaction. A mixture of (I) and formamide (molar ratio, 1:3) was heated on a boiling water bath with occasional shaking. From the evaporation residue, colorless prisms of m.p. 124～125° (II) were obtained, which were analysed to give the molecular formula of $C_{15}H_{14}ON_2$. When (II) was hydrolysed with 10% phosphoric acid, 2 moles of benzaldehyde proved as its phenylhydrazone, 2 moles of ammonia, and some formic acid,

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[*] Oshika, Shizuoka (関屋　実, 大石俊夫).

1) M. Sekiya, T. Fujita: Yakugaku Zasshi, **71**, 942(1951).

[*2] Anisyl = *p*-methoxybenzyl; anisylidene = *p*-methoxybenzylidene