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75. Tyunosin Ukita and Masachika Irie: Organic Phosphates. IX.[1] An Effective Separation of Flavin Nucleotides and Adenine Nucleotide from Hydrolysates of Flavin Adenine Dinucleotide.

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Since Abraham[2] reported the enzymatic detection of adenine nucleotide and FMP[*2] in mild acid or alkaline hydrolysate of FAD and suggested that the hydrolysis of FAD resulted in formation of these two coenzymes, several works on more precise chemical identification of the products were published. Masuda, *et al.*[3] detected FR, FMP, AMP, and adenine in the acid hydrolysate of FAD by paper chromatography. Todd and his collaborator[4] showed the occurrence of cyFMP by decomposition of FAD with aqueous ammonia, and since cyFMP is convertible to FMP by further acid or alkaline treatment, they proposed a mechanism of the decomposition reaction of FAD as schematically given in Chart 1.

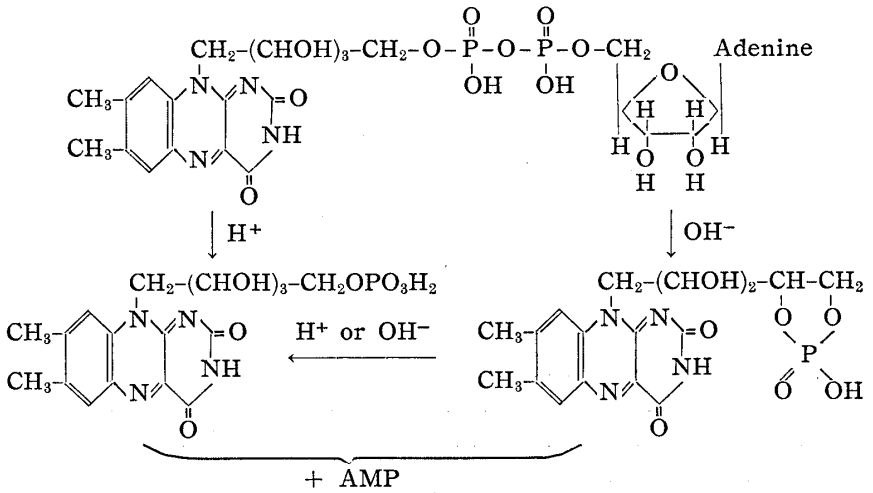

Chart 1.

However, in the above cited works, detections of the products were made qualitatively by the use of a celullose column or paper chromatography and they were not necessarily satisfactory for their isolation or quantitative estimation.

The present paper is concerned with fractionation of the mild hydrolysis products of FAD by ion exchanger chromatography which furnished further advantages in connection with above-mentioned problems.

Experimental

Materials—FAD: The commercial product (Takeda Pharm. Ind., 75% purity) was used. This material contained 5% of FMP detectable by the ion exchange column chromatography using Dowex-

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*2 Following abbreviations are used: FR=riboflavin, FMP=riboflavin 5'-phosphate, cyFMP=riboflavin 4',5'-cyclic phosphate, FAD=flavin adenine dinucleotide, AMP=adenosine 5'-phosphate, FDP=riboflavin diphosphate.

1) Part Ⅷ. T. Ukita, K. Nagasawa: This Bulletin, **7**, 465(1959).
2) E. P. Abraham: Biochem. J., **33**, 543(1939).
3) T. Masuda, Y. Sawa, M. Asai, S. Kuwada: Yakugaku Zasshi, **75**, 799(1955).
4) H. S. Forrest, A. R. Todd: J. Chem. Soc., **1950**, 3295.

1 (Cl^-) as described below (Fig. 1-d). The FMP values found in the fraction obtained from the hydrolysate were corrected by subtracting the FMP values found in control experiment (Table II).

FMP: The commercial product (Takeda Pharm. Ind.) was purified by paper electrophoresis to remove a small amount of FDP present.

cyFMP: cyFMP was prepared by the treatment of FMP with dicyclohexylcarbodiimide according to the procedure reported by Tanaka.[5]

Ion Exchanger Column Chromatography—Ca. 10~12 mg. of a sample was dissolved in 50 cc. of water and placed on top of a column (10×110 mm.) of Dowex-1 (×8) (Cl^-, 200~400 mesh). The column was eluted with the solvent systems given in Fig. 1 and 8 cc. each of the effluent was collected. Elution rate was 0.8 cc./min. and each fraction was submitted to measurement of absorbance at 260 mμ.

For quantitative analysis, the standard samples of adenine, adenosine, FMP, cyFMP, and FAD were applied to above column chromatography and the total absorbance of each compound eluted was divided by respective molecular absorbance of the compound*3 given by Whitby[6] to calculate the recovery rate (Table I). The total absorbance measured for each hydrolysis product of FAD separated by fractional chromatography was divided by the corresponding recovery rate to give the yield of each product (Table II).

Paper Chromatography—The solvent systems used for paper chromatography were as follows: Solvent 1: BuOH:H_2O:AcOH (4:5:1); Solvent 2: *tert*-BuOH:NH_4OH:H_2O (60:5:35); Solvent 3: 5% solution of $Na_2HPO_4 \cdot 7H_2O$.

The effluent containing flavin derivatives was saturated with $(NH_4)_2SO_4$ and shaken with phenol. Et_2O was added to the phenol layer, after repeated washing with water. The Et_2O-phenol mixture was then extracted with water. The separated aqueous layer was again washed with Et_2O and concentrated under a diminished pressure. The concentrated solutions of flavin derivatives thus obtained were submitted to paper chromatography.

Results and Discussion

Not many reports have hitherto been encountered on the separation of flavin derivatives by ion exchanger chromatography. Yagi and co-workers[7] reported the separation of FR, FMP, and FAD using Dowex-1 (borate) with $Na_2B_4O_7$ and NaCl solutions as elution solvents or Dowex-1 (Cl^-) with a solvent system composed of NH_4Cl, HCl, NaCl, and NH_4OH. Siliprandi, *et al.*[8] reported the isolation of FAD from natural source by combined electrophoresis and ion exchanger chromatography with Amberlite IRC-50.

To avoid drastic conditions in the isolation of fractionated hydrolysis products, the solvent system consisting of NH_4Cl, HCl, and NH_4OH was selected for elution from adsorbed Dowex-1 (Cl^-). According to Yagi, *et al.*[7] FR and FMP are fractionated successively from the resin with the solvent system of 0.01*N* NH_4Cl+0.01*N* NH_4OH and 0.05*N* NH_4Cl solutions. Duplication of this experiment, however, showed that neither of these two flavin derivatives could be fractionated by the use of these solvents.

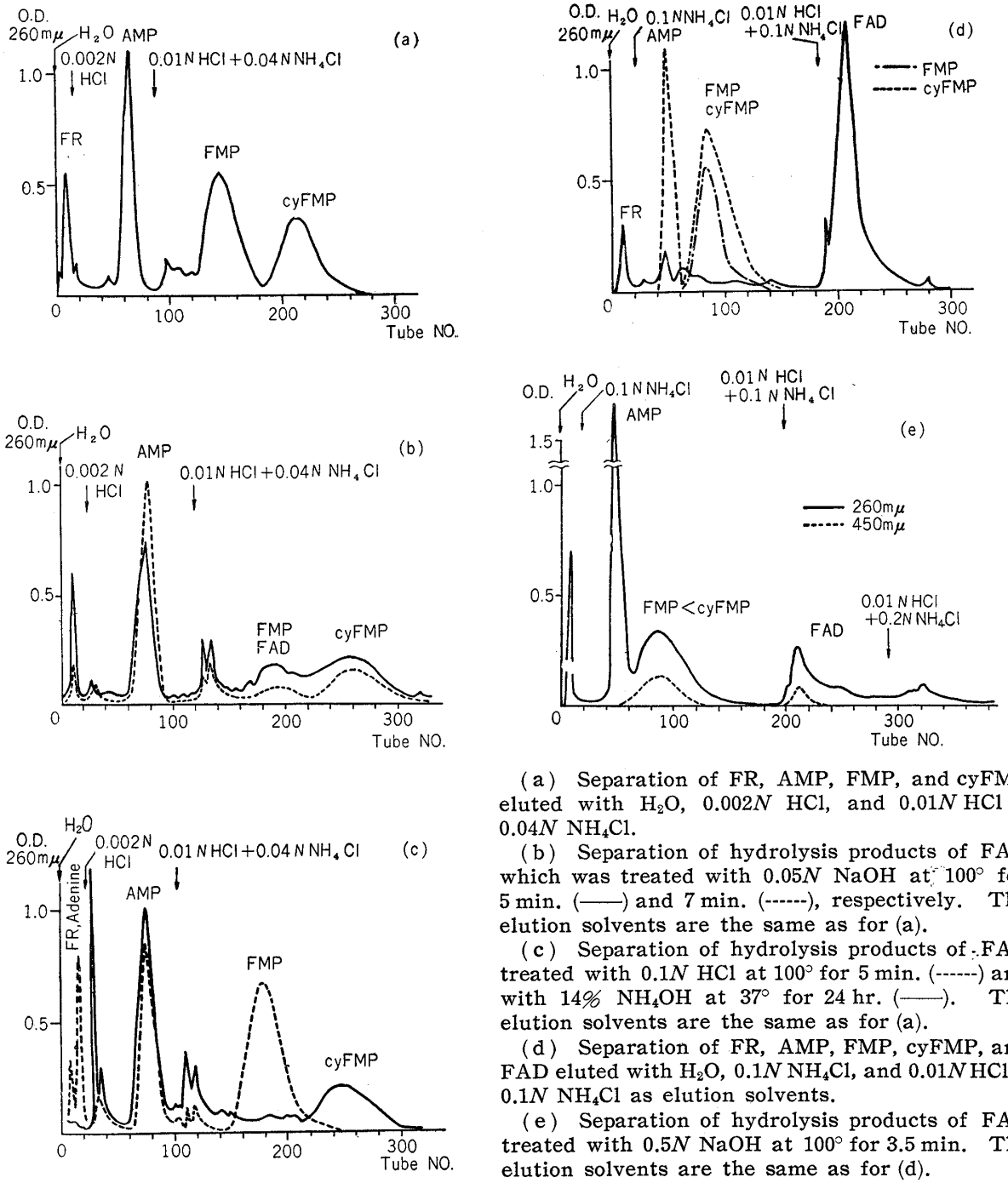
In the present experiment, a mixture of standard FR, FMP, cyFMP, and AMP adsorbed on Dowex-1 (Cl^-) was effectively fractionated by stepwise elutions with 0.002*N* HCl and 0.01*N* HCl+0.04*N* NH_4Cl after washing the adsorbed resin with water.

FR, adenine, adenosine, and lumiflavin were eluted with water, AMP was found in the eluate of 0.002*N* HCl, and both FMP and cyFMP in this order, were fractionated in the eluate with 0.01*N* HCl+0.04*N* NH_4Cl. The results are given in Fig. 1-a.

By this analysis system, FAD was found in the fraction eluted with 0.01*N* HCl+0.04*N* NH_4Cl and this fraction overlapped those containing FMP. Thus this system is not suitable for the purification of FAD or quantitative estimation of FMP from a mixture containing FAD. From FAD-free mixture of FR, FMP, AMP, and cyFMP, this system

*3 AMP=15.9, FMP=27.1, FR=27.7, (L, mole, cm.×10^{-3}) at 260 mμ.

5) T. Tanaka: Yakugaku Zasshi, **78**, 627(1958).
6) L. G. Whitby: Biochem. J., **50**, 435(1952).
7) K. Yagi, J. Okuda, Y. Matsuoka: Nature, **175**, 555(1955).
8) P. Cerletti, N. Siliprandi: Arch. Biochem. Biophys., **76**, 214(1958).



(a) Separation of FR, AMP, FMP, and cyFMP eluted with H_2O, 0.002*N* HCl, and 0.01*N* HCl + 0.04*N* NH_4Cl.

(b) Separation of hydrolysis products of FAD which was treated with 0.05*N* NaOH at 100° for 5 min. (——) and 7 min. (------), respectively. The elution solvents are the same as for (a).

(c) Separation of hydrolysis products of FAD treated with 0.1*N* HCl at 100° for 5 min. (------) and with 14% NH_4OH at 37° for 24 hr. (——). The elution solvents are the same as for (a).

(d) Separation of FR, AMP, FMP, cyFMP, and FAD eluted with H_2O, 0.1*N* NH_4Cl, and 0.01*N* HCl+ 0.1*N* NH_4Cl as elution solvents.

(e) Separation of hydrolysis products of FAD treated with 0.5*N* NaOH at 100° for 3.5 min. The elution solvents are the same as for (d).

Fig. 1. Stepwise Elution of Hydrolysis Products of FAD adsorbed on Dowex-1 (Cl^-)

Table I. Recovery Rate of Standard Samples

Sample	Recovery (%)	Sample	Recovery (%)
FR	78.0	FMP	86.0, 84.2
AMP	90.5, 90.0	cyFMP	83.0

gives fractions containing each component in pure state with excellent constant recovery rates, the results of which are given in Table I.

The above-described system was applied to hydrolysates of FAD obtained with several different hydrolysis conditions.

FAD was hydrolysed under several conditions, such as (1) with 0.05*N* NaOH at 100°

for 5~7 min. which seemed sufficiently strong for the cleavage of pyrophosphate linkage of FAD but milder than that used by Masuda,[3] (2) with 0.1*N* HCl at 100° for 5 min., and (3) with 14% NH_4OH at room temperature for 24 hr., the condition used by Todd.[4]

Of the four kinds of hydrolysate obtained by these conditions, only the one treated with 0.05*N* NaOH at 100° for 5 min. contained a detectable amount of FAD as checked by paper chromatography.

The results of fractionation are shown in Fig. 1-b and -c, and as expected, not cyFMP but FMP was found in the acid hydrolysate, while both cyFMP and FMP were found in NaOH hydrolysate although in the latter case the total yield of flavin derivatives was less than 40% of the theoretical amount.

In the case of hydrolysis with NH_4OH, the yield of total flavin nucleotides was greatest with 14% concentration of the reagent and in this case, as reported by Todd,[4] the main constituent of flavin nucleotide was cyFMP. Further, this concentration of NH_4OH was found to be the most suitable to yield the theoretical amount of AMP.

The yield of AMP decreased in acid hydrolysis and this should be due to the instability of AMP in acid medium. In this case a large amount of adenine besides FR was detected in the effluent eluted with water. The results of these fractionations are given in Table II.

Table II. Hydrolysis Conditions of FAD and Yield of Products

Hydrolysis condition	Yield (%)* AMP (%)	Flavin nucleotide (%) FMP	cyFMP
i) 0.5*N* NaOH, 100°, 8 min.	70.5	22.8	23.0
ii) 〃 〃 7 〃	88.0	11.0	25.4
iii) 〃 〃 5 〃	65.0	—	38.0
iv) 14% NH_4OH, 30°, 24 hr.	100.0	13.5	45.0
v) 0.1*N* HCl, 100°, 5 min.	63.0	74.0	0

* Percentage to the respective theoretical amount of AMP and total flavin nucleotide produced each one to one mole of FAD and calculated by the following equations :

for AMP and cyFMP $\left(a\times\frac{100}{b}\right)\times 100/c$

for FMP $\left(a\times\frac{100}{b}-d\right)\times 100/c$

where a : Amount of the product (in mg.) calculated from moles obtained by dividing the total absorbance for the product by corresponding molecular absorbance.
b : Recovery rate found for standard material (Table I).
c : Theoretical amount of the product in mg.
d : Recovery of FMP (in mg.) contained in original FAD after its treatment under the hydrolysis conditions given in i) to v). The respective recovery rate, 1.3, 3.0, 6.0, 59.6, and 100%, of the FMP for hydrolysis condition of i, ii, iii, iv, and v, were obtained by blank tests with standard FMP.

Although these solvent systems were found to effect separation of the hydrolysis products of FAD, some disadvantages remained i.e. because of the acidic reaction of the effluents, it was difficult to isolate cyFMP from the fractionated medium which converted cyFMP to FMP and as described above, to estimate the amount of FMP from the hydrolysate containing the remaining FAD.

Efforts were made to find another solvent system to overcome these disadvantages. Solvents such as water, 0.1*N* NH_4Cl solution, and 0.1*N* NH_4Cl+0.01*N* HCl, were successively used. Of the five standard compounds, FR, AMP, FMP, cyFMP, and FAD, FR was eluted out primarily with water and on subsequent elution with 0.1*N* NH_4Cl, AMP appeared as the second fraction, and FMP was fractionated together with cyFMP.

In the final effluent with the mixed solvent of 0.1*N* NH_4Cl+0.01*N* HCl, only FAD was eluted.

The fraction containing FAD was checked on its purity by paper chromatography using Solvents 1, 2, and 3, and it was found to contain practically no impurities. Thus this solvent system was found to be useful for the purification of FAD as well as for the quantitative estimation of non-degraded FAD in the hydrolysate.

Fig. 1-e shows the fractionation of FAD after treatment with 0.5*N* NaOH at 100° for 3.5 min., a hydrolytic condition by which some amount of FAD still remained intact. In this case, on further analysis of the fraction containing both FMP and cyFMP by paper chromatography using three different solvents (Solvent 1, 2, and 3), this fraction contained ca. 80% of cyFMP.

Some of the hydrolysis products of FAD separated by above two systems of ion exchanger chromatography contained traces of contamination which gave a fluorescent spot when checked by paper chromatography. However, these impurities showed practically no effect on the quantitative analysis of the products. Thus, the combined use of these two fractionation procedures was found useful for the isolation and quantitative detection of the main products contained in mild acid or alkaline hydrolysate of FAD.

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Summary

For the purpose of isolation and quantitative estimation of the hydrolysis products of FAD, ion exchange column chromatography was used. The hydrolysates were fractionated stepwise from Dowex-1 (Cl^-) with two systems of combined solvents : (1) Water, 0.002*N* hydrochloric acid, and 0.01*N* hydrochloric acid+0.04*N* ammonium chloride, and (2) 0.1*N* ammonium chloride and 0.1*N* ammonium chloride+0.01*N* hydrochloric acid.

The former solvent system could effectively separate FR, AMP, FMP with FAD, and cyFMP, and the latter AMP, FMP with cyFMP, and FAD. The combined use of these two solvent systems was shown to effect clear-cut separation of the flavin nucleotides and adenine nucleotide contained in the hydrolysate of FAD.

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