in the strength of intermolecular linkage of FAD between flavin and adenine rings in its molecule in the two cases.[12]


## Summary

Fluorescence spectra of flavins on paper strip were determined and the peak of the fluorescence spectra of free riboflavin, FMN, and FAD was identically recorded at 530 mμ. The relation between the quantity and fluorescence energy on paper strip was linear over a range of the quantities of flavin from $2.66\times10^{-10}$ moles to $0.53\times10^{-11}$ moles. The relative fluorescence energies of equimolar quantities of free riboflavin, FMN, and FAD on a paper strip were determined under experimental conditions reported herein.

The molar quantity of flavins on a paper strip can be determined directly by fluorometry using a standard concentration of free riboflavin placed on the paper strip as a scale.


(Received June 18, 1958)

12) G. Weber: Biochem. J., **47**, 114 (1950).

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*UDC 577.164.12*

## 132. Jun Okuda: Metabolism of Flavin Nucleotides. I. Decomposition of Flavin Nucleotides in Digestive Juice.

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It has already been shown that many coenzymes are derivatives of vitamins and are divided into two classes, i.e. monophosphoric and pyrophosphoric esters.

Vitamin $B_2$, riboflavin, has both of them, namely, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).[1]

It has been shown that these esters could be synthesized from free riboflavin in living body[2~4] and the enzymes for the phosphorylation of free riboflavin and FMN could be purified.[5,6]

In the case of a rat, it was found that administered free riboflavin was phosphorylated to FMN in the mucous membrane of the small intestines and then it was changed to FAD in the liver and kidney,[7] and the enzymes responsible for these reactions were demonstrated *in vitro* using acetone-dried powder of these organs.[8,9]

However, investigations on the decomposition of these phosphoric esters of riboflavin in the digestive organs have not been reported yet. Results on these subjects might be quite important in understanding the absorption mechanism of flavin nucleotides, which is closely related to nutritional and pharmaceutical fields. They will also give some presumption on the decomposition of other phosphoric esters of vitamins and nucleotides in the digestive organs. The present paper deals with the decomposition of flavin nucleotides in saliva, gastric juice, bile, and pancreatic juice.

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1) Hereafter following abbreviations will be used in this report: Flavin mononucleotide=FMN, flavin adenine dinucleotide=FAD.
2) H. Rudy: Naturwiss., **23**, 286 (1935).
3) H. Hübner, F. Verzár: Helv. Chim. Acta, **21**, 1006 (1938).
4) J. R. Klein, I. H. Kohn: J. Biol. Chem., **136**, 177 (1940).
5) E. B. Kearney: *Ibid.*, **194**, 747 (1952).
6) A. W. Schrecker, A. Kornberg: *Ibid.*, **182**, 795 (1950).
7) K. Yagi: J. Biochem. (Tokyo), **41**, 757 (1954).
8) K. Yagi: Igaku-to-Seibutsugaku, **19**, 233 (1951).
9) K. Yagi: *Ibid.*, **19**, 305 (1951).

## Materials

**Free Riboflavin**—Synthesized chemically and recrystallized from water.

**FMN**—Prepared from riboflavin with $POCl_3$ and derived to its sodium salt. The purity of this sample was determined by fluorometry and spectrophotometry to be over 99%.

**FAD**—Obtained from *Eremothecium ashbyii* by the method of Yagi, *et al.*[10] The purity of this sample was estimated to be over 92% by fluorometry and spectrophotometry. Other flavins, nucleic acids, and metals were not detected in the impurity.

**Saliva**—Collected from a healthy human mouth after washing the oral cavity several times: pH of the saliva was 6.6.

**Gastric Juice**—Collected from a healthy human stomach by means of the Levin's tube. pH of the gastric juice was 1.5.

**Simulated Gastric Juice**—It consisted of 1.0 cc. of HCl (35%) and 0.5 g. of saccharated pepsin, and made up to 100 cc. with distilled water as directed in the Japanese Pharmacopoeia.[11]

**Bile**—Collected from dog's gall bladder by means of an injection syringe directly after laparatomy. pH of the bile was 7.0.

**Pancreatic Juice**—Collected directly through a rubber tubing attached to the pancreatic duct of a dog after laparatomy. pH of the pancreatic juice was 8.0.

## Methods

Free riboflavin, FMN, or FAD was incubated at 37° for 1 hr. in saliva, 3 hrs. in gastric juice, or 2 hrs. in bile and pancreatic juice. The incubation was carried out in a dark room. The final concentration of flavin in the reaction mixture was adjusted to $6.7\times10^{-4}$ *M*.

After incubation, the total quantity of flavin was estimated by the lumiflavin-fluorescence method.[12] To estimate the molar ratio of each flavin in the reaction mixture, paper chromatography was used. A small amount of the reaction mixture was placed on paper strips (Toyo Roshi No. 51, 2×30 cm.) and *n*-BuOH-AcOH-$H_2O$ mixture (4 : 1 : 5, v/v, upper layer) was used as the mobile phase. The Rf values of FAD, FMN, and free riboflavin were 0.03, 0.09 and 0.3, respectively.[13] After drying in a dark room, a part of paper strip containing each flavin was cut out from the paper strips. As reported before,[14] the fluorescence intensities of equimoles of FAD, FMN, and free riboflavin on the paper strip were 0.46, 1, and 1, respectively. The molar ratio of each flavin was estimated directly on the paper strip by the aid of an attachment[15] of a microphotofluorometer.[16] pH was measured by a glass electrode pH meter.

## Results

**In Saliva**—Free riboflavin, FMN, and FAD were not decomposed in saliva during 1 hr. of incubation at 37°.

**In Gastric Juice**—Free riboflavin and FMN were not decomposed in gastric juice during 3hrs. of incubation at 37°. A slight decomposition of FAD to FMN was observed in gastric juice as shown in Fig. 1. In this experiment, as the decomposing factor was supposed to be HCl in gastric juice, examination in simulated gastric juice was made, in which FAD is decomposed to FMN in the same way as in the case of the normal gastric juice. The result is shown in Fig. 1. The curve of decomposition was similar to the

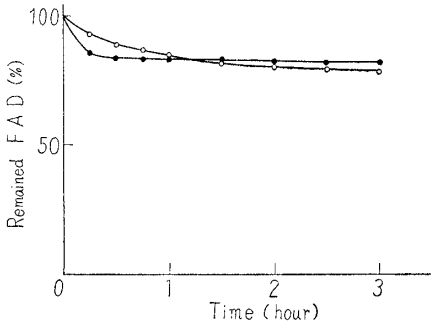


Fig. 1. Decomposition of FAD in Gastric Juice and Simulated Gastric Juice
Final concn. of FAD, $6.7\times10^{-4}$ *M*
○——○ Gastric juice (pH 1.5)
●——● Simulated gastric juice (pH 1.5)

10) K. Yagi, Y. Matsuoka, S. Kuyama, M. Tada: J. Biochem. (Tokyo), **43**, 93 (1956).
11) Japanese Pharmacopeia, VI Ed., 628 (1951).
12) K. Yagi: J. Biochem. (Tokyo), **43**, 635 (1956).
13) J. L. Crammer: Nature, **161**, 349 (1948).
14) K. Yagi, J. Okuda: This Bulletin., **6**, 659(1958).
15) K. Yagi, T. Tabata: J. Japan. Biochem. Soc., **27**, 779 (1956).
16) K. Yagi, T. Arakawa: Vitamins (Kyoto), **6**, 523 (1953).

above, though in simulated juice, FAD was decomposed to FMN rather rapidly at first and then gradually. On the contrary, FAD was decomposed in grastic juice, at the same speed during incubation.

The heated human gastric juice decomposed FAD to FMN in almost the same speed. Aqueous HCl solution (0.35%), corresponding to its concentration in simulated gastric juice, also decomposed FAD to FMN in the same speed as in the case of simulated gastric juice.

**In Bile**—Free riboflavin was not decomposed in bile. FMN was decomposed slightly during 2 hrs. of incubation at 37°, as shown in Fig. 2, but heated bile of over 80° for 5 mins. did not decompose FMN to free rivoflavin. FAD was not decomposed to either FMN or free riboflavin directly in bile.

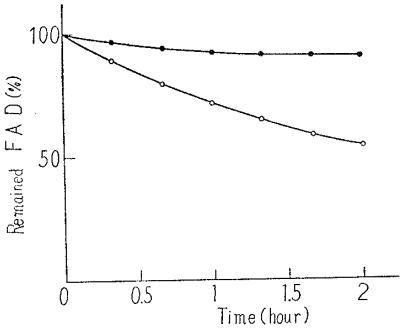


Fig. 2. Decomposition of FMN in Bile and Pancreatic Juice
Final concn. of FMN, $6.7 \times 10^{-4}$ *M*
●——● Bile (pH 7.0)
○——○ Pancreatic juice (pH 8.0)

**In Pancreatic Juice**—Free riboflavin was not decomposed in pancreatic juice. FMN was decomposed to free riboflavin in fresh pancreatic juice as shown in Fig. 2, about 45% of it being dephosphorylated to free riboflavin during 2 hrs. of incubation at 37°. FAD was not decomposed. A heated pancreatic juice could not decompose FMN during the same incubation period. The pancreatic juice stored at 0° for 1 day lost one-half of its activity compared with the fresh one.

## Discussion

Free riboflavin, FMN, and FAD were not decomposed in saliva. Some amount of acid phosphatase and a small amount of alkaline phosphatase were found in human saliva by Chauncey, *et al.*[17] However, FMN and FAD were not decomposed in saliva. This means that both acid and alkaline phosphatase hardly act on flavin nucleotides at around pH 6.6 of the saliva.

In gastric juice, free riboflavin and FMN were not decomposed, but 20% of FAD was decomposed to FMN during 3 hours of incubation and it was shown that this decomposition was due to hydrochloric acid in gastric juice. The difference in decomposition of FAD between the human and simulated gastric juice will be due to the presence of some amounts of protein, for instance, mucin in human gastric juice, which resulted in disturbance of the progress of decomposition of FAD.

It will be possible to assume by analogy that other biochemically important pyrophosphoric esters, such as diphosphopyridine nucleotide, triphosphopyridine nucleotide, coenzyme-A, etc., will be decomposed to their mononucleotides in gastric juice.

In bile and pancreatic juice, only FMN was decomposed to free riboflavin and this decomposing factor will be a kind of phosphatase, as the heated bile and pancreatic juice did not decompose FMN at all.

In bile and pancreatic juice, FAD was not decomposed, and therefore pyrophosphatase may not be present in them. Although the true state of digestive juice in the digestive tracts will be much more complicated, it was found from these experiments that FMN can be administered orally as far as the pyrolus without dephosphorylation, but FAD will not be able to reach there without decomposition when administered orally.

In these experiments, decomposition of flavin nucleotides was discussed by using pure digestive juice. In the digestion process, the digestive juices will be mixed together and with many kinds of food, and their pH will be changing continuously during their movement, so that the decomposition of flavin nucleotides should progress much faster in the digestive organs than that reported here.

17) H. Chauncey, F. Lionetti, R. A. Winer, V. F. Lisanti: J. Dental Research, **33**, 321 (1954).

The author expresses his deep gratitude to Prof. Dr. T. Takahashi of the University of Kyoto and Dr. K. Yagi of the University of Nagoya for their kind guidances and encouragements throughout this work.

**Summary**

Decomposition of flavin nucleotides in digestive juices was studied.

1. Free riboflavin, FMN, and FAD were not decomposed in saliva.
2. In gastric juice, about 20% of FAD was decomposed to FMN by the hydrochloric acid in gastric juice during 3 hours' incubation at 37° while free riboflavin and FMN were not decomposed.
3. In bile, 10% of FMN was decomposed to free riboflavin during 2 hours' incubation, free riboflavin and FAD were not decomposed.
4. In pancreatic juice, 45% of FMN was decomposed to free riboflavin during 2 hours' incubation, but free riboflavin and FAD were not decomposed.

(Received June 23, 1958)

*UDC 577.164.12*

## 133. Jun Okuda: Metabolism of Flavin Nucleotides. II.[1] Decomposition of Flavin Nucleotides in the Small Intestine.

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As reported in the first paper of this series,[1] free riboflavin was not decomposed in the digestive juice, and dephosphorylation of FMN[2] was not observed in saliva and gastric juice, though a slight dephosphorylation was observed in bile and pancreatic juice. FAD[2] was not decomposed in saliva, bile, or pancreatic juice, but did so in gastric juice, about 20% of FAD being decomposed to FMN during 3 hours of incubation.

In the small intestine, a quite strong dephosphorylation of FMN was observed and it would be probably due to the presence of phosphomonoesterase in the mucous membrane of the small intestine. As in the case of FMN, FAD was decomposed to free riboflavin through FMN rapidly and it will be attributed to the existences of nucleotide pyrophosphatase and phosphomonoesterase of the small intestine. This paper deals with the decomposition of flavin nucleotides in the small intestine.

### Materials

**Free riboflavin, FMN, and FAD**—The same samples as described before[1] were used.

**Simulated Intestinal Fluid**[3]—$NaHCO_3$ (1.5 g.) and pancreatin (0.28 g.) were dissolved and made up to 100 cc. with distilled water.

**Homogenate of the Mucosa of the Small Intestine**—Small intestine of a dog was collected from a slaughter house immediately after killing and mesenteric membranes were removed. The mucosa was then obtained by rinsing the cavity with tap water and scraping the mucosa with the edge of a plastic spatula. The mucosa was cooled to 0° in an ice bath and homogenized with 4 volumes of cold physiological saline solution.

**Buffer Solution of Various pH**—To determine the optimal pH for dephosphorylation by the homogenate, the following buffer solutions were prepared: Each buffer solution contained 1 cc. each of 0.1*M*

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1) J. Okuda: This Bulletin, **6**, 662(1958).
2) Hereafter following abbreviations will be used in this report:
FR=free riboflavin, FMN=flavin mononucleotide, FAD=flavin adenine dinucleotide.
3) Japanese Pharmacopoeia VI Ed. (Supplement), p. 77 (1953).