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.

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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).

glycine, 0.1*M* AcONa, 0.1*M* monoethanolamine, and pH was adjusted by addition of 0.5*M* HCl or 0.5 *M* NaOH. Then each buffer solution (pH 2~13) was made up to 10 cc. with distilled water.

0.1*M* Monoethanolamine Hydrochloride Buffer (pH 9.5)

Methods

Reaction Mixture for Incubation—Free riboflavin, FMN, or FAD was incubated in the homogenate. The final concentration of flavins in the mixture was 6.7×10^{-4} *M*. The reaction mixture was prepared as follows:

Test tube No.	1	2
Free riboflavin, FMN, or FAD (2.7×10^{-3} *M*) (cc.)	0.5	0.5
Homogenate of the mucosa of the small intestine (cc.)	0.5	0.5
Monoethanolamine-HCl buffer (pH 9.5) (cc.)	1.0	0.5
Inhibitor dissolved in monoethanolamine-HCl buffer (cc.)	—	0.5

Incubation was carried out at 37° for 4 mins. in a dark room in order to prevent photodecomposition of flavins.

Determination of Flavins—Total quantity of flavins was determined by the lumiflavin-fluorescence method,[4] and the separatory determination of flavin compounds, by the measurement of flavins on paper strips as described in a previous paper.[5]

Results

1. Decomposition of Flavin Nucleotides in the Small Intestinal Cavity—Prior to the homogenate experiments, the decomposing action of dog's small intestine on free riboflavin, FMN, and FAD was examined. After laparatomy, the small intestine was ligated at two places with an interval of 10 cm. The intestinal cavity was washed with physiological saline solution and free riboflavin, FMN, or FAD (2.7×10^{-6} moles) dissolved in physiological saline solution (1 cc.) was injected into the intestinal cavity.

After 20 mins., flavins in the intestinal cavity were washed out with physiological saline solution and decomposition of flavin nucleotides was examined by paper chromatography.

Of the three flavins, free riboflavin was not changed to other decomposition products, such as lumiflavin or lumichrome, during the incubation of 20 mins. in the small intestinal cavity. However, FMN or FAD injected in the small intestinal cavity was decomposed completely to free riboflavin.

2. Decomposition of Flavin Nucleotides by Homogenate of the Small Intestine—Using the homogenate of the small intestine, the same results as the above was demonstrated. As it was not clear whether the decomposition of flavin nucleotides was caused by the enzyme or not, simulated intestinal fluid was used to examine the decomposition of FMN or FAD in it, but the results were negative. Then the homogenate was heated at 90° for 5 mins., but the heated homogenate did not affect them under the same conditions as in the case of normal homogenate. From these experiments, it was

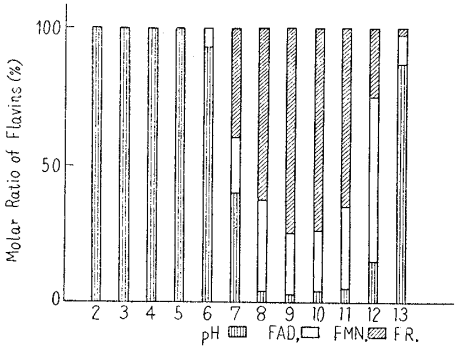

Fig. 1. Decomposition of FAD by homogenate of the mucosa of the small intestine at various pH. The incubation mixture contained 0.5 cc. of aqueous FAD solution (2.7×10^{-3} *M*), 0.5 cc. of homogenate, and 1.0 cc. of buffer solution. Incubation was at 37° for 4 mins.

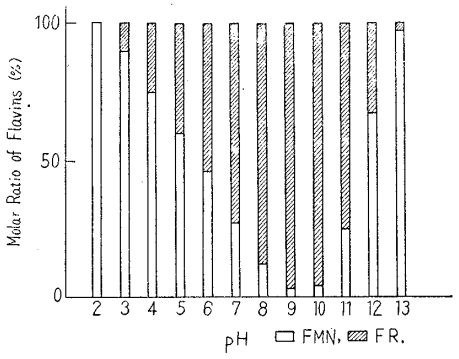

Fig. 2. Dephosphorylation of FMN by homogenate of the mucosa of the small intestine at various pH. The incubation mixture contained 0.5 cc. of aqueous FMN solution (2.7×10^{-3} *M*), 0.5 cc. of homogenate, and 1.0 cc. of buffer solution. Incubation was at 37° for 4 mins.

4) K. Yagi: J. Biochem. (Tokyo), **43**, 635 (1956).
5) K. Yagi, J. Okuda: This Bulletin, **6**, 659(1958).

assumed that the decomposition of flavin nucleotides is an enzymatic action. It was also supposed that FMN and FAD were decomposed on the surface of the mucosa of the small intestine or by the small intestinal juice.

Decomposition of flavin nucleotides in the homogenate of the mucosa of the small intestine was examined as a preliminary step and the strong decomposition of FMN or FAD was found.

Optimal pH—Optimal pH for the decomposition of flavin nucleotides in the homogenate of the small intestine was tested by 4-min. incubation at 37° using buffer solutions of various pH (pH 2~13). The optimal pH was found at pH 9 for decomposition of both FMN and FAD, as shown in Figs. 1 and 2.

In Fig. 1, it is shown that FAD was not decomposed below pH 6.0. At pH 7, free riboflavin, FMN, and FAD were present in the reaction mixture.

The greatest decomposition was found at pH 9.0 Above pH 9.0, molar ratio of free riboflavin and FMN in the reaction mixture decreased and that of FAD increased with the increasing pH for 4 mins. of incubation at 37°.

Decomposition Processes—As shown in the above-mentioned results, the decomposition of FAD may be effected by two enzymes. The process of this decomposition was studied to prove the two-step decomposition. The decomposition products were analyzed during incubation of 7 mins., when FAD was decomposed completely to free riboflavin as shown in Fig. 3. The dephosphorylation of FMN reached its maximum earlier than that of FAD.

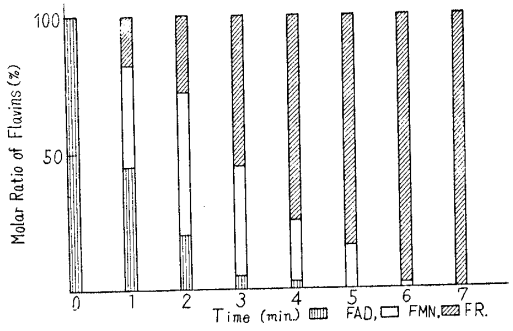

Fig. 3. Decomposition of FAD by the homogenate of the mucosa of the small intestine. The incubation mixture contained 0.5 cc. of aqueous FAD solution ($2.7\times10^{-3}M$), 0.5 cc. of homogenate, and 1.0 cc. of $0.1M$ monoethanolamine-HCl buffer (pH 9.5). Incubation was at 37° for period indicated.

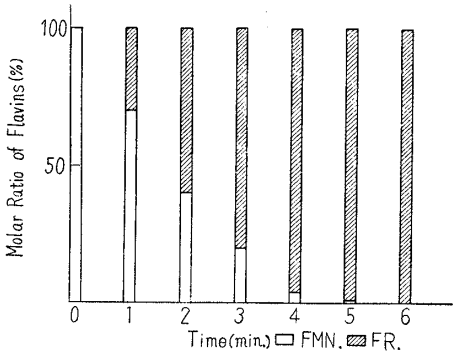

Fig. 4. Dephosphorylation of FMN by the homogenate of the mucosa of the small intestine. The incubation mixture contained 0.5 cc. of aqueous FMN solution ($2.7\times10^{-3}M$), 0.5 cc. of homogenate, and 1.0 cc. of $0.1M$ monoethanolamine-HCl buffer (pH 9.5). Incubation was at 37° for period indicated.

Some Inhibition Experiments—Among the inhibitors tested, pyrophosphate, orthophosphate, and ethylenediaminetetraacetic acid (EDTA) were found to be strong inhibitors of the decomposition of FAD and dephosphorylation of FMN. The minimum concentration of the inhibitor for 50% or 100% inhibition of the above reactions is shown in Table I. EDTA was the strongest inhibitor for FMN and FAD decompositions.

TABLE I. Inhibition Rate of Inhibitors

	100%*	50%*
Substrate—FMN		
Pyrophosphate	$7\times10^{-2}M$	$1\times10^{-2}M$
Orthophosphate	$2\times10^{-1}M$	$2\times10^{-2}M$
EDTA	$1\times10^{-2}M$	$3\times10^{-3}M$
Substrate—FAD		
Pyrophosphate	$1\times10^{-1}M$	$2\times10^{-2}M$
Orthophosphate	$2\times10^{-1}M$	$2\times10^{-2}M$
EDTA	$1\times10^{-2}M$	$3\times10^{-3}M$

* Minimum concentration of inhibitors which shows 100% or 50% inhibition.

Although the inhibitory action of other inorganic substances was examined, carbonate, molybdate, fluoride, Mg ions, and Ca ions did not show any inhibitory action against decomposition of flavin nucleotides in $0.05M$ (final concentration). Lower concentrations (final concentration, $0.001M$) of Mg or Ca ions did not show any enhancement of this decomposition.

Discussion

In the experimental data described in the preceding[1] and present papers, it was found that free riboflavin was not decomposed in the digestive juice and the small intestinal cavity, in which free riboflavin decomposing-enzyme, like riboflavinase,[6] which was found in the stool, may not be present.

FMN was not decomposed in the saliva and gastric juice but it was decomposed slowly in the bile and pancreatic juice, as reported before. In the small intestinal cavity, decomposition of FMN was very rapid, due to the presence of phosphatase in the mucosa of the small intestine. From the pH-activity curve for dephosphorylation of FMN, it is supposed that the existence of acid phosphatase or 5′-nucleotidase in the mucosa of the small intestine is rather small, even if present in the mucosa. The activity can probably be attributed to specific dephosphorylating enzyme of FMN or alkaline phosphomonoesterase in the mucosa. The purification of the enzyme will answer that.

In the case of FAD, as shown in the preceding paper,[1] only about 20% of it was decomposed to FMN in gastric juice during 3 hours of incubation at 37°, but no decomposition of it was observed in saliva, bile, or pancreatic juice.

In the present series of experiments, strong decomposition of FAD to free riboflavin through FMN in the homogenate of the mucosa of the small intestine was demonstrated.

All these data suggest that the mucosa of the small intestine will contain at least two kinds of phosphatase, FAD was supposed to be decomposed first to FMN by nucleotide pyrophosphatase, and then to free riboflavin by phosphomonoesterase as follows:

$$\mathrm{FAD} \xrightarrow{(1)} \mathrm{FMN} \xrightarrow{(2)} \mathrm{FR}$$

(1) Nucleotide pyrophosphatase
(2) Phosphomonoesterase

It has been shown, on the one hand, that the dephosphorylation of FMN took place in the experiment of the ligated and washed cavity, and a similar dephosphorylation process of FMN was found by the experiment using homogenate of the mucosa of the small intestine. On the other hand, the presence of FMN-dephosphorylating enzyme was proved by the present author and others[7] in the epithelial cells of the mucosa of the small intestine from the point of histochemistry.

These data suggest that FMN dephosphorylation in the cavity will occur by phosphatase present extensively in the exterior cells of the mucosa. These problems may be connected with the absorption mechanism of flavins in the small intestine.

However, the possibility of the action of phosphatase in intestinal juice which comes from the Brünner's gland of the duodenum and the Liberkühn's gland of the small intestine could not be completely denied, for the pure intestinal juice was hardly collected from the small intestine.

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 guidances and encouragements throughout the study.

Summary

Decomposition of flavin nucleotides in the small intestine was studied. Free riboflavin was not decomposed at all in the homogenate of the mucosa of the small intestine. FMN was decomposed to free riboflavin very rapidly in the homogenate and FAD was decomposed to free riboflavin through FMN by two steps in the homogenate of the mucosa of the small intestine.

6) K. Hotta, K. Yagi: Vitamins (Kyoto), **6**, 454 (1953).
7) K. Yamada, J. Okuda: 16th Japanese Anatomical Society (Chubu Local Section), 1957.

Inhibitors for the decomposition of these flavin nucleotides were studied. EDTA, pyrophosphate, and orthophosphate were found to inhibit decomposition of both FMN and FAD in the homogenate. The minimum concentrations of these inhibitors for 50% or 100% inhibition were obtained.

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134. Tsutomu Momose, Yo Ueda, Tatsuo Shoji, and Hiroshige Yano: Organic Analysis. XII.[5] Infrared Spectra of Phenylsulfonyl Derivatives. (2). SO_2-Stretching Frequencies of Benzenesulfonamide Derivatives and CO-Stretching Frequencies of N-Acetylsulfonamide Groups.

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A few infrared spectral studies on benzenesulfonamide derivatives were reported by Adams, *et al.*,[1] Schreiber,[2] Bellamy,[3] and Baxter, *et al.*,[4] but effect of substitution on the SO_2-stretching frequencies is hardly known.

In this work, infrared spectra of 48 benzenesulfonamide derivatives were measured and the effect of a substituent on the SO_2-stretching frequency is discussed. The CO-stretching frequency of the N-acetylsulfonamide group is also discussed.

Results and Discussion

Nature of the Spectra of SO_2-Stretching Vibrations

Since most of benzenesulfonamide derivatives were sparingly soluble in organic solvents except alcohols, a Nujol mull method was used for all samples in the measurement.

All compounds exhibited very strong absorption bands of an asymmetric (ν_{as}) and symmetric (ν_s) stretching mode of SO_2 group. Both absorption bands appeared as one or two bands, but in general, ν_{as} was more complex than ν_s. Their frequencies are tabulated in Table I.

Similarly as in the case of phenyl alkyl sulfone derivatives[5] all maximum bands, listed in bold-face type in the table, are used in this discussion. The ν_{as} and ν_s of benzenesulfonamide derivatives were in the ranges of 1358~1303 cm^{-1} (7.37~7.68 μ) and of 1173~1130 cm^{-1} (8.53~8.85 μ), respectively, although those of phenyl alkyl sulfone derivatives were in the ranges of 1339~1279 cm^{-1} and 1172~1136 cm^{-1}, respectively. Therefore, the SO_2-frequencies, especially ν_{as}, of benzenesulfonamide derivatives existed in a shorter wavelength region than that of phenyl alkyl sulfone derivatives, as shown in Table II. This shift is reverse of that of CO-frequencies between carbonamides and carbonyl compounds.

The NH_2 group has both mesomeric and inductive effect. In carbonamides +M effect is larger than -I effect and the binding of CO group will be weakened by a resonance form

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1) R. Adams, J. J. Tjepkema: J. Am. Chem. Soc., **70**, 4204(1948).
2) Kurt C. Schreiber: Anal. Chem., **21**, 1168(1949).
3) L. J. Bellamy: "The Infrared Spectra of Complex Molecules," Methuen, 300(1954).
4) J. N. Baxter, J. Cymerman-Craig, J. B. Willis: J. Chem. Soc., **1955**, 669.
5) Part XI: This Bulletin, **6**, 415(1958).