standing at room temperature over night, the crystalline mass produced was collected and washed thoroughly with benzene and then a small amount of water. Recrystallization of the crude crystalline mass gave 0.7 g. of pale yellow prisms.

Preparation of Methyl 2-Cyano-3-(5-nitro-2-furyl)acrylate—The following general procedure was used for preparation of the 7 esters listed in Table II. A solution of 1.0 g. (0.005 mole) of 2-cyano-3-(5-nitro-2-furyl)acryloyl chloride in 30 cc. of MeOH containing a few drops of pyridine was heated on a water bath for 1 hr. and then allowed to cool. The reaction mixture was diluted with about 50 cc. of water, the crude crystalline mass that separated was collected, and washed well with water. The crude crystals were recrystallized from EtOH.

Summary

Thirty-one new 2-cyano-3-(5-nitro-2-furyl)acrylamides and esters were synthesized by condensation of 2-cyano-3-(5-nitro-2-furyl)acryloyl chloride and various amines and alcohols. Unexpectedly, these derivatives did not show the desired antibacterial activity *in vitro*. It seemed that this might be due to a small solubility of these derivatives in water.

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84. Jun Okuda and Kunio Yagi : Metabolism of Flavin Nucleotides. IV. Properties of the Enzyme for Dephosphorylation of Flavin Mononucleotide in the Small Intestine.

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In previous papers of this series,[1,2] the decomposition of flavin nucleotides in the digestive canal was reported. Dephosphorylation of flavin mononucleotide (FMN) was demonstrated chiefly in the cavity of the small intestine. The homogenate of the mucosa of the small intestine was found to dephosphorylate FMN very rapidly. Then, the histochemical study[3] showed that the enzyme for dephosphorylation of FMN was localized mainly in the epithelial and endothelial cells of the mucosa. Furthermore, the distribution of this enzyme in the subcellular fraction of the mucosa was examined and its highest activity was found in the cytoplasm fraction of jejunum and duodenum.[4]

To find the physiological action of this enzyme present in the mucosa attempt was made to extract and purify this enzyme and to examine its enzymatic properties, results of which are reported in this paper.

Materials

FMN—Chemically synthesized and supplied as its monosodium salt.

Buffer solutions—A mixture of 1 cc. each of 0.1*M* citrate, 0.1*M* veronal, and 0.1*M* borate was adjusted to pH 2~13 by addition of 0.5*N* HCl or 0.5*N* NaOH, and made up to 10 cc. with distilled water.

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1) J. Okuda : This Bulletin, **6**, 662(1958).
2) J. *Idem.* : *Ibid.*, **6**, 665(1958).
3) K. Yamada, J. Okuda : 16th Meeting of the Japanese Society of Anatomy (Chubu Local Section), 1957.
4) J. Okuda : This Bulletin, **7**, 295(1959).

Methods

Incubation—The reaction mixture (total, 0.6 cc.) consisted of 40 μg. of enzyme powder, FMN (final concentration, $1.7\times10^{-3}M$), and $MgCl_2$ (final concentration, $1.7\times10^{-3}M$) in $0.1M$ monoethanolamine hydrochloride buffer (pH 9.4). Incubation was carried out at 37° for 10 min. in a dark room to prevent photodecomposition of flavins.

Measurement of Enzyme Activity—Two methods were used for measurement of enzyme activity. One is to measure the amount of riboflavin produced by dephosphorylation of FMN, and the other is to estimate the inorganic phosphate liberated from monophosphoric esters by this enzyme as will be described below.

1) **Estimation of Riboflavin produced from FMN** : After incubation, 0.01 cc. of the reaction mixture was placed on a paper strip and flavins were separated by paper chromatography using the upper layer of a mixture of *n*-BuOH•AcOH•H_2O (4:1:5, v/v) as the mobile phase. After drying the paper in a dark room, the fluorescent zone of flavins on paper strip was estimated by the aid of the attachment[5] of a microphotofluorometer.[6] The molar ratio of flavins was then calculated as described in the previous paper.[7]

2) **Estimation of Inorganic Phosphate** : After incubation, 0.1 cc. of the reaction mixture was pipetted into 1.0 cc. of $3N$ H_2SO_4 to stop the enzyme action and followed by estimation of the liberated inorganic phosphate by the method of Fiske-Subbarow.[8]

Definition of Unit of Enzyme Activity and Specific Activity—One unit of enzyme activity is defined as μM of substrate attacked during 10 min. under the above condition. The specific activity is expressed as the enzyme activity per mg. of nitrogen.

Results and Discussion

1) Purification of the Enzyme for Dephosphorylation of FMN

It had been demonstrated by histochemical studies[3] that the enzyme for dephosphorylation of FMN was localized chiefly in the epithelial cells and a little in the endothelial cells of the mucosa. It means that the enzyme existed chiefly in the surface of the mucosa. Therefore, the mucosa for the extraction of the enzyme was collected thinly from the surface of the small intestine.

As the study with subcellular fraction of the mucosa showed that the supernatant of the homogenate obtained by centrifugation (11,000*g* at 0°, 10 min.) contained 80% of the total enzyme activity in the cell,[4] it was supposed that the decomposition of the cells by homogenization is sufficient to extract the enzyme from the mucosa.

In preliminary experiments using the homogenate of the mucosa of the small intestine, it was observed that the enzyme for dephosphorylation of FMN in the homogenate was rather stable to heating or aging in ice box. This property of the enzyme was mainly considered in the purification procedure.

Step 1 : Dog's small intestine was collected from the slaughter house immediately after killing and the mesenteric membranes were removed. The mucosa was then obtained by rinsing the cavity with tap water rapidly and scraping thinly the mucosa of the small intestine with the edge of a plastic spatula. From six dogs, 175 g. (wet weight) of the mucosa of the small intestine was obtained. The mucosa was cooled with ice and dispersed in two volumes of distilled water. It was homogenized in a glass homogenizer at 0° and 420 cc. of homogenate was obtained.

Step 2 : The homogenate was heated at 58° for 15 min., cooled rapidly, and the supernatant was collected by centrifugation (9,000*g* at 0°, 20 min.). The precipitate was suspended in its volume of distilled water and the supernatant obtained by centrifugation as described above was mixed with the former extract. Supernatant (I) was 320 cc. and the precipitate was discarded.

5) K. Yagi, T. Tabata : J. Japan. Biochem. Soc., **27**, 55(1956).
6) K. Yagi, T. Arakawa : Vitamins (Japan), **6**, 523(1953).
7) K. Yagi, J. Okuda : This Bulletin, **6**, 659(1958).
8) C. H. Fiske, Y. Subbarow : J. Biol. Chem., **81**, 629(1929).

Step 3 : The supernatant (I) was frozen and stored for 1 day in a refrigerator. After melting, the resultant precipitate was removed by centrifugation (20,000*g* at 0°, 20 min.). The supernatant was frozen again and the same centrifugations were repeated until a clear supernatant (II) was obtained.

Step 4 : The supernatant (II) was saturated with $(NH_4)_2SO_4$ and the precipitate was collected by centrifugation (900*g* at 0°, 10 min.). The precipitate was suspended in 23 cc. of distilled water and dialyzed at 0° to 5° against distilled water to remove $(NH_4)_2SO_4$ completely. The resultant precipitate was removed by centrifugation (20,000*g* at 0°, 20 min.) and 20 cc. of a clear supernatant (III) was obtained.

Step 5 : The supernatant (III) was lyophilized to dryness and about 0.2 g. of a slightly yellowish white powder was obtained. This powder was suspended in 3.0 cc. of glass-distilled water and insoluble matter was removed by centrifugation (20,000*g* at 0°, 20 min.). Clear and colorless supernatant (IV) so obtained was lyophilized to dryness. The activity of this sample for dephosphorylation of FMN per its dry weight was 200 times higher than that of the original homogenate.

This purification procedure is summarized in Chart 1.

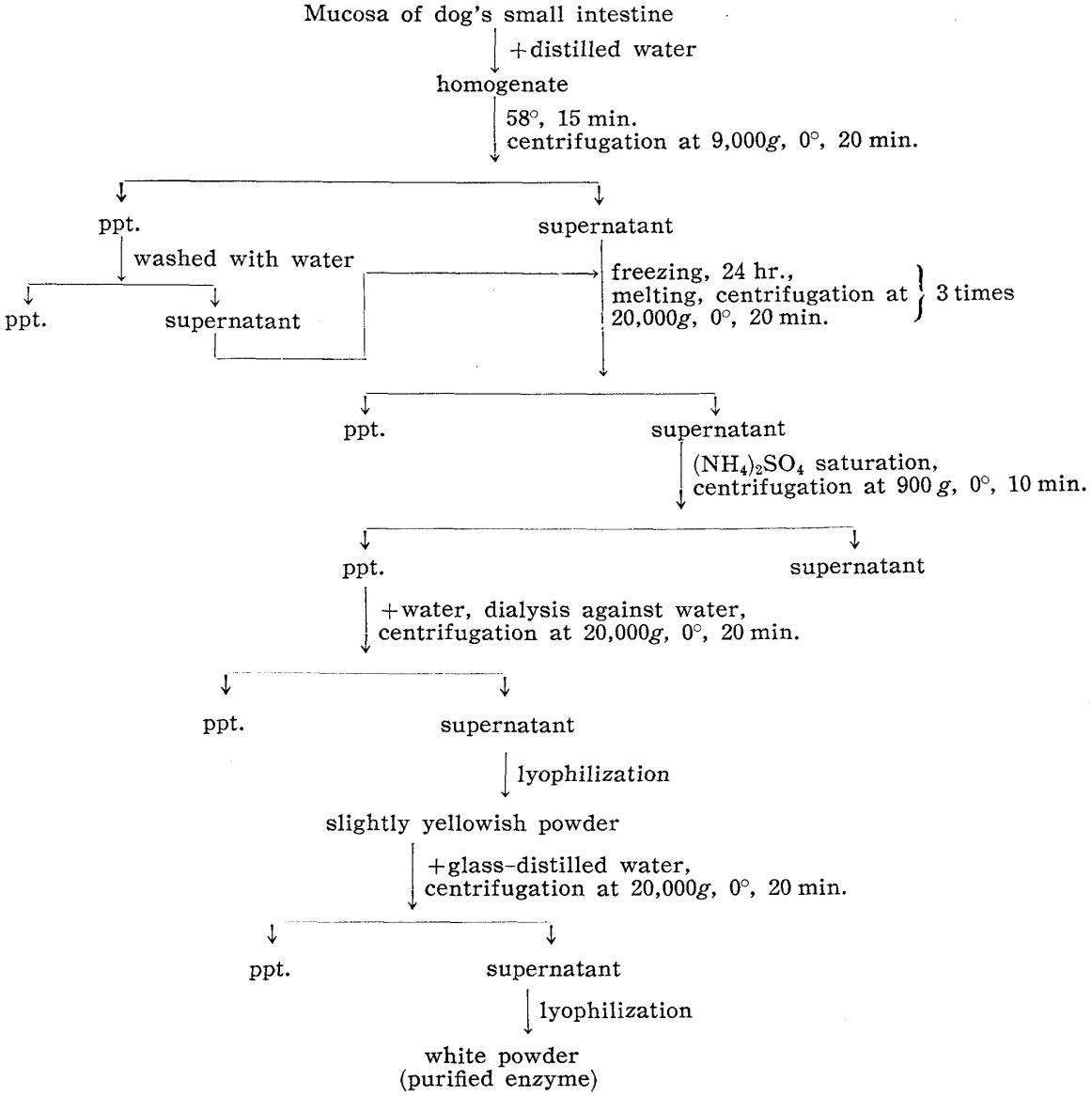

Chart 1. Purification of the Enzyme for Dephosphorylation of FMN

2) Properties of the Enzyme for Dephosphorylation of FMN

Using the sample prepared by the method mentioned above, the properties of this enzyme was examined.

Optimum pH : As shown in Fig. 1, optimum pH of this enzyme was found in the range between pH 9 and 10. From this result, this enzyme can be distinguished from nucleotidase.

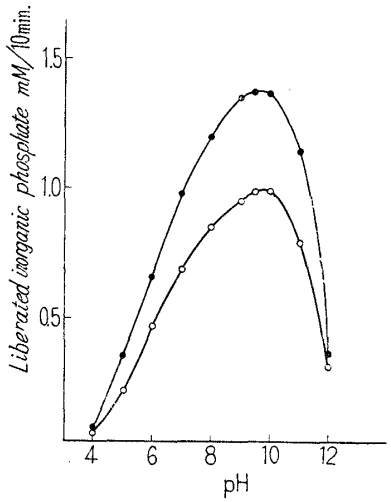

Fig. 1. pH-Activity Curve of the Enzyme for Dephosphorylation of FMN and β-Glycerophosphate

•——• β-glycerophosphate
○——○ FMN

The reaction mixture (total, 0.6 cc.) contained FMN or β-glycerophosphate (final concentration, $1.7\times10^{-3}M$), $MgCl_2$ (final concentration, $1.7\times10^{-3}M$), and 40 μg. of purified enzyme in various pH buffers. Incubation was at 37° for 10 min.

Optimum Temperature : As shown in Fig. 2, the optimum temperature was found to be 45° for this enzyme.

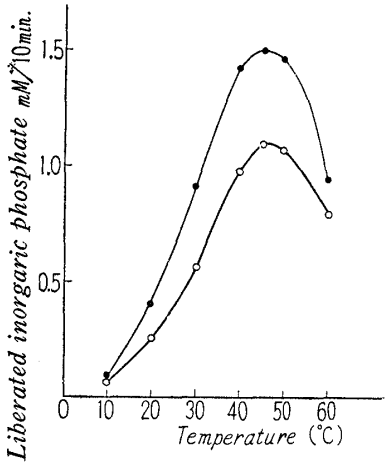

Fig. 2. Temperature-Activity Curve of the Enzyme for Dephosphorylation of FMN and β-Glycerophosphate

•——• β-glycerophosphate
○——○ FMN

The reaction mixture (total, 0.6 cc.) contained FMN or β-glycerophosphate (final concentration, $1.7\times10^{-3}M$), $MgCl_2$ (final concentration, $1.7\times10^{-3}M$), and 40 μg. of purified enzyme at various temperatures as indicated. Incubation was at pH 9.4 for 10 min.

Substrate Specificity : Other phosphomonoesters were examined to see whether they can be attacked by this enzyme preparation. β-Glycerophosphate, phenyl phosphate, glucose 1-phosphate, glucose 6-phosphate, and adenosine monophosphate were also dephosphorylated. The dissociation constants of the complex of these substrates were obtained by the method of Lineweaver-Burk and the results are shown in Table I. The value obtained with β-glycerophosphate was smaller than that obtained by Roche and Sarles.[9]

Table I. Dissociation Constant of the Complex of Enzyme Protein with Substrate

Substrate	Dissociation Constant
FMN	$7.0\times10^{-3}M$
β-Glycerophosphate	1.5 〃
5′-Adenylic acid	7.8 〃
Phenyl phosphate	3.7 〃
Glucose 1-phosphate	1.5 〃
Glucose 6-phosphate	1.6 〃

9) J. Roche, H. Sarles : Biochim. Biophys. Acta, **5**, 275(1950)

This enzyme did not act on adenosine di- and triphosphate.

Inhibition : In an earlier report[2] of this series, it was shown that FMN dephosphorylation in the homogenate of the mucosa was inhibited by ethylenediaminetetraacetic acid (EDTA), pyrophosphate, or orthophosphate. The same inhibitors were tested for dephosphorylation of FMN by this enzyme and the inhibition was demonstrated. The concentration of inhibitors for 50% inhibition is shown in Table II.

Table II. Concentration of Inhibitor for 50% Inhibition of the Enzyme for Dephosphorylation of FMN, β-Glycerophosphate, or Phenyl Phosphate

Inhibitor	Substrate		
	FMN (*M*)	β-Glycerophosphate (*M*)	Phenyl phosphate (*M*)
EDTA	1.5×10^{-3}	1.1×10^{-3}	1.2×10^{-3}
Pyrophosphate	8.0×10^{-3}	—	9.3×10^{-3}
Orthophosphate	1.2×10^{-2}	—	1.3×10^{-2}

3) Identification of the Enzyme with Alkaline Phosphomonoesterase

As described before, optimum pH and substrate specificity of this enzyme are very similar to those of alkaline phosphomonoesterase and it was supposed that the enzyme may be identical with general alkaline phosphomonoesterase. To examine the identity of these two enzymes, dephosphorylation of both FMN was compared with that of β-glycerophosphate in each step of the purification procedure and the results are summarized in Table III. As shown in the table, the effect of each step on elevation of specific activity, i.e. rate of purification, was the same in these two substrates.

Table III. Specific Activity and Rate of Purification of the Enzyme for Dephosphorylation of FMN and β-Glycerophosphate

Step No.	1	2	3	4	5
Total volume (cc.)	420	320	250	20	2.5
Dry weight (mg./cc.)	69.0	19.5	3.2	9.4	6.5
Nitrogen (mg./cc.)	9.8	3.0	0.5	1.5	1.0
Yield (as the amount of nitrogen)	100.0	23.2	3.03	0.70	0.06
Specific activity[a] (FMN)	0.82	2.7	9.4	32.0	142.0
Rate of purification[b]	1.0	3.3	11.4	39.0	173.2
Recovery (%)	100.0	76.2	35.0	28.5	10.5
Specific activity[a] (β-glycerophosphate)	1.10	3.5	12.0	41.0	204.0
Rate of purification[b]	1.0	3.2	10.9	37.3	185.5
Recovery %	100.0	74.0	33.1	27.1	11.5

a) Value represents the enzyme activity per mg. of N. The enzyme activity is defined as μM of substrate attacked during 10 min. in the reaction mixture.

b) Ratio of the specific activity to that of the original sample.

Activity curve of pH or of temperature of this enzyme was estimated for dephosphorylation of FMN and of β-glycerophosphate, and the results agreed with both substrates as shown in Figs. 1 and 2.

The inhibitory action of inhibitors was examined and the concentration of inhibitor for 50% inhibition was also found to be of the same order, in either FMN or other phosphomonoesters, as shown in Table II.

From these results, the enzyme for dephosphorylation of FMN was considered to be identical with general alkaline phosphomonoesterase.

Summary

The enzyme for dephosphorylation of FMN was purified from the mucosa of dog's small intestine. From the results obtained on optimum pH, optimum temperature, and substrate specificity of the enzyme, and on the rate of purification of the enzyme for dephosphorylation of both FMN and β-glycerophosphate, it is considered that the enzyme is identical with intestinal alkaline phosphomonoesterase.

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