UDC 547.866.4-118.5 : 577.157

85. Kunio Yagi and Jun Okuda : Metabolism of Flavin Nucleotides. V. Phosphorylation of Riboflavin by Transferase Action.

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When riboflavin was administered to a rat, a large amount of riboflavin was concentrated in the mucosa of the small intestine and an increase of flavin mononucleotide (FMN) also occurred.[1,2] This result suggested that the riboflavin administered might be phosphorylated to FMN in the mucosa of the small intestine.

As a supposed mechanism of phosphorylation of riboflavin in this tissue, the flavokinase action[3] was first assumed and it was demonstrated using the acetone-dried powder of the mucosa of the small intestine.[4] In this case, about 5% of the substrate riboflavin was synthesized to FMN in the presence of a large excess of adenosine triphosphate.

On the other hand, it was reported by Axelrod[5] and Nishibori[6] that transphosphorylase action is catalyzed by acid phosphatase, and it was also demonstrated by Green and Meyerhof,[7] and by Morton[8] that the transfer could be catalyzed by alkaline phosphomonoesterase. Therefore, possibility of a transphosphorylation from phosphomonoester compound to riboflavin was imagined.

During the investigation on dephosphorylation of FMN in the small intestine, FMN dephosphorylating enzyme was purified and this was considered to be identical with intestinal alkaline phosphomonoesterase.[9] Considering the specificity of this enzyme, phosphorylation of riboflavin by transferase action of this alkaline phosphomonoesterase preparation was studied and the results are described in this paper, which is a detailed report of the preliminary note.[10]

Materials

Riboflavin—Special care was taken in the purification of riboflavin. Crystals of synthesized product were recrystallized from distilled water and further purified by cellulose column chromatography to separate a trace of other fluorescent substances.

Phosphomonoesterase—This was purified from the mucosa of dog's small intestine by the method described in detail in the preceding paper.[9] The activity per dry weight of this enzyme was 200 times higher than that of the original homogenate.

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

Methods

Phosphatase Action—To estimate phosphatase action, inorganic phosphate liberated from phosphomonoester was estimated. After the incubation, 0.1 cc. of the reaction mixture was pipetted into 1.0 cc. of $3N$ H_2SO_4 to stop the reaction and the liberated inorganic phosphate was estimated by the method of Fiske-Subbarow.[11]

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Transferase Action—The reaction mixture (total volume, 1.3 cc.) consisted of riboflavin (final concn., $1.5\times10^{-4}M$), $MgCl_2$ (final concn., $1.0\times10^{-3}M$), phosphomonoesterase (0.1 mg.), and $0.05M$ veronal-HCl buffer (pH 9.4). The incubation was at 37° for 15 min.

For the estimation of synthesized FMN, a small amount of the reaction mixture was placed on a paper strip (5×30 cm.), dried rapidly, and then newly synthesized FMN was separated by paper chromatography using the upper layer of the mixture of *n*-BuOH:AcOH:H_2O (4:1:5, v/v) as a mobile phase. The parts of paper strip containing flavins were cut out and they were eluted separately with water of 80°. After cool, the eluate was diluted with phosphate buffer (pH 7.0) and the fluorescence intensity was estimated by a microphotofluorometer designed by Yagi, *et al.*,[12] and the molar ratio of FMN to the total flavin was calculated.

For the identification of the newly synthesized flavin with FMN, paper chromatography was adopted using several solvents.[13]

Throughout the procedures of the incubation of the reaction mixture and the following separatory estimation of flavins, operations were carried out under orange-colored light by which flavins are not decomposed.[14]

Results

Relationship between Phosphorylation of Riboflavin and the Concentration of β-Glycerophosphate—The reaction mixture contained riboflavin, phosphomonoesterase, Mg^{2+}, and graduated concentrations of β-glycerophosphate from 1.5×10^{-2} to $3.8\times10^{-2}M$ in veronal buffer (pH 9.4). After the mixture was incubated at 37° for 15 min., the presence of FMN was demonstrated. The amount of newly synthesized FMN was found to be in parallel with that of β-glycerophosphate added as shown in Fig. 1.

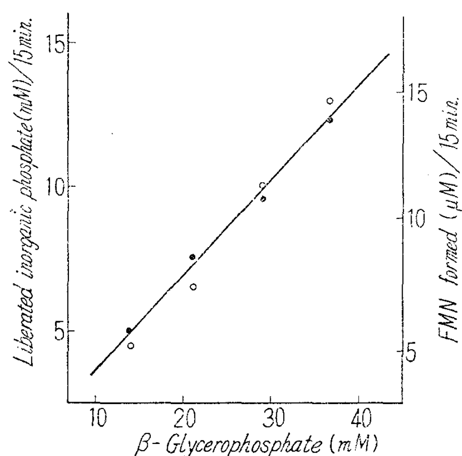

Fig. 1. Relationship between Concentration of β-Glycerophosphate, FMN formed, and Liberated Inorgnaic Phosphate

○ FMN formed
• Liberated inorganic phosphate

Reaction mixture (1.3 cc., pH 9.4) contained riboflavin (final concentration, $1.5\times10^{-4}M$), $MgCl_2$ (final concentration, $1.0\times10^{-3}M$), enzyme powder (0.1 mg.), and β-glycerophosphate as indicated. Incubation: 37°, 15 min.

It was also noted that the amount of inorganic phosphate liberated by phosphomonoesterase from β-glycerophosphate was parallel to the concentration of FMN formed. It was calculated that about $^1/_{1000}$ of the liberated inorganic phosphate was transferred to riboflavin, the phosphate acceptor.

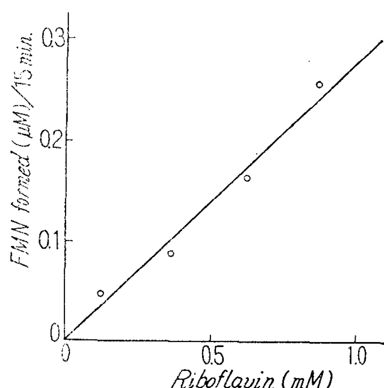

Fig. 2. Relationship between Concentration of Riboflavin and FMN formed

Reaction mixture (1.3 cc., pH 9.4) contained β-glycerophosphate (final concentration, $3.3\times10^{-2}M$), $MgCl_2$ (final concentration, $1.0\times10^{-3}M$), enzyme powder (0.1 mg.), and riboflavin as indicated. Incubation: 37°, 15 min.

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Relationship between Phosphorylation of Riboflavin and the Initial Concentration of Riboflavin—The reaction mixture contained phosphomonoesterase, Mg^{2+}, $3.3 \times 10^{-2}M$ of β-glycerophosphate, and graduated concentrations of riboflavin from 1.2×10^{-4} to $8.8 \times 10^{-4}M$. After incubation at 37° for 15 min., the formation of FMN was found to be in parallel with the initial concentration of riboflavin, the phosphate acceptor, in the incubation mixture as shown in Fig. 2.

Optimum pH for Transferase Action—By using buffer solutions of various pH, the optimum pH for transphosphorylation of riboflavin from β-glycerophosphate by this phosphomonoesterase preparation was examined and it was found to be in the range of pH 9~10 as shown in Fig. 3.

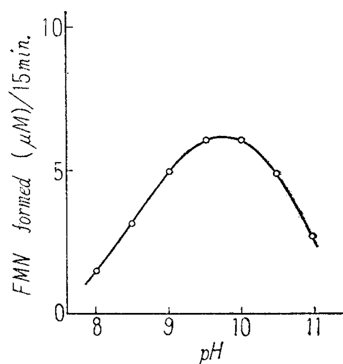

Fig. 3. pH-Activity Curve of Transferase Action

Reaction mixture (1.3 cc.) contained riboflavin (final concentration, $1.5 \times 10^{-4}M$), β-glycerophosphate (final concentration, $2.0 \times 10^{-2}M$), $MgCl_2$ (final concentration, $1.0 \times 10^{-3}M$) and enzyme powder (0.1 mg.). Incubation: 37°, 15 min.

Optimum Temperature for Transferase Action—The optimum temperature for transphosphorylation of riboflavin from β-glycerophosphate by this phosphomonoesterase preparation was examined and it was found to be 45° as shown in Fig. 4.

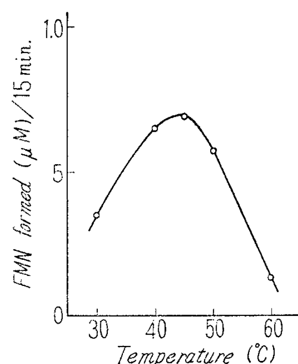

Fig. 4. Temperature-Activity Curve of Transferase Action

Reaction mixture (1.3 cc.) contained riboflavin (final concentration, $1.5 \times 10^{-4}M$), β-glycerophosphate (final concentration, $2.0 \times 10^{-2} M$), $MgCl_2$ (final concentration, $1.0 \times 10^{-3}M$), and enzyme powder (0.1 mg.). Incubation: pH 9.4, 15 min.

Other Phosphate Donors—The transferase action was examined by substituting β-glycerophosphate with other supposed phosphate donors.

Using phenyl phosphate as a phosphate donor, rate of phosphorylation of riboflavin similar to that with β-glycerophosphate was demonstrated. Adenosine monophosphate (AMP) could be a phosphate donor. In the case of AMP, lower rate of phosphorylation of riboflavin than that using β-glycerophosphate or phenyl phosphate was observed by the experiment in their equimolar concentrations. When orthophosphate was used as a phosphate donor, phosphorylation of riboflavin was zero. Adenosine di- and triphosphate could not be a phosphate donor in these experiments.

Discussion

In the reaction mixture used in this work, phosphorylation of riboflavin occurred in parallel with dephosphorylation of β-glycerophosphate by the catalysis of the enzyme preparation obtained for this experiment, which is considered to be alkaline phosphomonoesterase.

Optimal pH and temperature for the FMN synthesis agreed with those of phosphatase action of the enzyme preparation. This result suggests that phosphorylation may be catalysed by the same enzyme as that for phosphatase action.

On the other hand, the fact that orthophosphate could not serve as a phosphate donor showed that this enzyme reaction is not the reverse reaction of dephosphorylation,

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and not the action of phosphorylase. The fact that adenosine di- or triphosphate did not serve as a phosphate donor indicates that this enzyme is not a flavokinase.

It is also observed that other phosphomonoesters which can be dephosphorylated by this enzyme could be a phosphate donor, i.e., the phosphate donor for the transferase action is the same as the substrate for phosphatase. Therefore, this reaction may be considered as a transphosphorylation catalyzed by alkaline phosphomonoesterase.

The difference in the rate of phosphorylation of riboflavin with different phosphate donors in equimolar amount may be attributed to the difference in their dissociation constants of their complex with the enzyme protein.

Brawerman and Chargaff[15] reported that their enzyme for the phosphorylation of nucleosides by transferase action is specific to nucleosides, the phosphate acceptor, and was thus named nucleoside phosphotransferase. The specificity of phosphate acceptor of the present enzyme will be studied in the future.

The possibility of separating a transferase from the phosphomonoesterase cannot be denied in the present preparation until it is purified completely. In this direction, studies will also be continued.

From these results, it is considered that riboflavin could be converted by the present enzyme to FMN with a high concentration of riboflavin. Such a high concentration of riboflavin in the intestinal canal can often be observed when riboflavin is administered orally, even in the case of injection.[1] Therefore, this type of phosphorylation of riboflavin may be expected to be of physiological significance.

Summary

1) The phosphorylation of riboflavin to FMN was demonstrated by intestinal phosphomonoesterase using β-glycerophosphate as a phosphate donor.

2) Phenyl phosphate and adenosine monophosphate could be used as a phosphate donor, but not adenosine di- and triphosphate, and orthophosphate.

3) Optimal pH and temperature for the phosphorylation of riboflavin agreed with those of phosphatase action.

From these results, the phosphorylation of riboflavin to FMN may be attributed to the transferase action of the alkaline phosphomonoesterase.

4) Physiological significance of this reaction was discussed.

(Received November 26, 1958)

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