THE ENZYMATIC PHOSPHORYLATION OF RIBOFLAVIN*

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While the existence and coenzymatic function of riboflavin-5-phosphate have been recognized for some time, the means by which this substance is synthesized from the free vitamin have not been satisfactorily demonstrated. Verzar and coworkers (3, 4) have reported extensive phosphorylation of riboflavin by means of inorganic phosphate in preparations of intestinal mucosa. The synthesis of FMN by such reversal of phosphatase action seemed an unlikely biological mechanism in view of the energetics of the reaction, but we none the less attempted to repeat this finding, closely duplicating their experimental conditions. Although these workers had reported as high as 50 per cent conversion of riboflavin to FMN, we were not able to demonstrate any FMN formation. Attempts to phosphorylate riboflavin with ATP in minces, homogenates, extracts, and acetone powders of hog and rat intestinal mucosa were likewise unsuccessful; added FMN was so rapidly split by phosphatase action, even in the presence of inhibitors such as fluoride or cysteine (5), that any formation of FMN could not have been detected.

It was possible, however, to demonstrate phosphorylation of riboflavin in suspensions of dried brewers’ yeast in which interfering enzymes could be inhibited by the addition of fluoride. The present paper describes the isolation and properties of the enzyme which catalyzes this transphosphorylation. On the basis of the specificity and stoichiometry of the reaction, the name “flavokinase” is suggested for the enzyme.

Materials and Methods

The yeast used in this work was a thoroughly washed, low temperature dried beer yeast, Lot D-422, obtained from Anheuser-Busch, Inc.2 Ribo-

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1 The following abbreviations are used in this paper: FMN, flavin mononucleotide (riboflavin-5-phosphate); FAD, flavin-adenine dinucleotide; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, adenosine-5-phosphate; ITP, inosinetriphosphate; THA tris(hydroxymethyl)aminomethane.

2 We are grateful to Mr. James D. Veron and Dr. J. E. McClary of Anheuser-Busch, Inc., for arranging the preparation of this yeast.
flavin, isoriboflavin, and pyridoxal, all three analytically pure compounds, were obtained from Merck and Company, Inc. Other sources were as follows: ribose, Nutritional Biochemicals Corporation; adenosine, B. L. Lemke; AMP, Ernst Bischoff Company, Inc. ATP was obtained either as the Ba salt (Sigma Chemical Company) or as the Na salt (Pabst Laboratories). For key experiments, pure ATP was separated by chromatography on Dowex-1 columns. ADP was prepared similarly from commercial preparations of ATP as well as by yeast hexokinase. ITP was prepared by the method of Kleinzeller and was ascertained to be free of ATP by spectrophotometric tests. FMN was obtained in the form of the monodiethanolamine salt, the Na salt, and the free acid, and was analytically pure by C, H, N, and P analysis, by potentiometric titration, absorption spectrum, fluorometry, microbiological assay, and by the cytochrome c reductase test. The molar extinction coefficient of these FMN samples at 450 nm was $\varepsilon = 11.8 \times 10^6$ sq. cm. mole$^{-1}$. Adenylic deaminase was prepared according to Kalckar, and d-amino acid oxidase according to Negelein and Bromel.

**Assay of Enzyme**—During the assay and subsequent analysis, solutions must be protected from light as much as possible. The enzyme is routinely assayed in low actinic test-tubes in a 5.0 ml reaction mixture containing 7.5 $\times$ $10^{-2}$ M THA buffer, pH 8.2, 1.0 $\times$ $10^{-4}$ M riboflavin, 1.0 $\times$ $10^{-3}$ M ATP, and 3.0 $\times$ $10^{-4}$ M MgSO$_4$. The final pH is 8.0 at 30$^\circ$. The mixture is incubated for 2 hours at 30$^\circ$ and the reaction is stopped by the addition of 2.0 ml. of 17.5 per cent trichloroacetic acid. After 10 minutes boiling to hydrolyze the small amount of FAD which may have been synthesized (see "Specificity"), the solution is cooled and filtered. A 5.0 ml. aliquot of the filtrate is neutralized with 1.25 ml. of 2.4 M K$_2$HPO$_4$. The FMN content of the solution is then determined by the following adaptation of the method of Burch et al. (12), based on the distribution coefficients of riboflavin and FMN between benzyl alcohol and aqueous solutions.

The total flavin content of the neutralized filtrate is determined by its light absorption at 450 nm in the Beckman spectrophotometer with the molar extinction coefficient, $\varepsilon = 12.2 \times 10^6$ sq. cm. mole$^{-1}$. 5 ml. of the neutralized filtrate are then extracted with 12.5 ml. of benzyl alcohol (saturated with water) by bubbling for 30 seconds in 20 $\times$ 150 mm. test-tubes. The two phases are separated by brief centrifugation and the benzyl alcohol layer is quantitatively removed. 5 ml. of chloroform (saturated with water) are added and the bubbling and centrifugation are re-

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3 We are indebted to Dr. W. Gibson and Dr. Karl Folkes of Merck and Company, Inc., for a gift of the last two compounds.

4 Kindly provided by Dr. S. H. Rubin, Hoffmann-La Roche, Inc.
peated. The top layer is removed directly to a cuvette and the light absorption at 450 μM is again determined.

From the light absorption of the unextracted sample, after suitable correction for dilution, the flavin content of the initial 5.0 ml. of reaction mixture is calculated as millimicromoles of total flavin per 5.0 ml. This is value A in the equation to follow. Value B is identically calculated from the light absorption of the extracted sample. The millimicromoles of FMN per 5.0 ml. of reaction mixture = 1.25 of value B-0.125 of value A. This value may be multiplied by the factor, total millimicromoles of riboflavin added per value A, to correct for the slight loss of flavin which occurs on deproteinization. The equation above is derived from accurate measurements of the distribution coefficients of riboflavin and FMN between benzyl alcohol and neutralized trichloroacetic acid solutions, as determined in our laboratory; these are 3.60 and 0.044, respectively. By this method, as little as 10 μM of FMN synthesized can be measured, and as much as 200 μM. Greater accuracy is obtained in the range of 50 to 150 μM. The over-all error is ±5 per cent.6 Fluorometric measurements may be substituted for spectrophotometric measurements for work on a smaller scale or with greater dilutions of flavin.

Protein Determinations—Protein concentrations were measured by the absorption at 280 μM in the Beckman spectrophotometer at pH 7; the relation between dry weight and D280 was determined at various stages of purification.

Results

Purification of Enzyme, Preparation A

Step 1. Autolysis—The conditions for autolysis are standardized for 500 gm. of dried yeast. The temperature and time relationship (Table I) are so chosen as to give the most satisfactory compromise between yield and purity of the enzyme, with relatively low concentrations of interfering enzymes on subsequent purification. The 500 gm. of yeast are allowed to autolyze in 1 liter of water at 35-36° with efficient stirring for 2 hours. At this time 500 ml. of water are added and the mixture is rapidly cooled to 30°. Stirring is continued for 15 minutes; the yeast is cooled to about 10° and centrifuged for 30 to 45 minutes at 4500 to 5000 r.p.m. in an angle type centrifuge. Activity ratio of supernatant = 0.2 to 0.5; total units = 40,000 to 45,000. A unit of activity has been arbitrarily defined as 1 μM of FMN synthesized in 2 hours at 30° per 5.0 ml. of re-

6 The use of the molar extinction coefficient of riboflavin in calculation of the A and B values does not introduce significant error, since the extinction coefficient of FMN is almost the same, and riboflavin is the main component.
action mixture, under the usual assay conditions (cf. "Methods"); activity ratio, units of activity per mg. of protein.

**Step 2. Ammonium Sulfate Precipitation**—The enzyme is precipitated from the supernatant at 0°, pH 6.0 (pH of the autolysate is usually 5.9 to 6.0), with solid (NH₄)₂SO₄ to give 0.40 saturation, as calculated from the figure 71.5 gm. per 100 ml. = 1.0 saturation. After 45 minutes stirring, the suspension is centrifuged for 45 to 60 minutes at 5000 r.p.m. and the opalescent supernatant is discarded. The precipitate is dissolved in about 30 to 40 ml. of water and dialyzed against running distilled water for 16 to 18 hours. The heavy precipitate after dialysis is centrifuged down at 10,000 r.p.m. for 30 minutes and discarded. Activity ratio of the clear supernatant = 23 to 27; total units = 30,000 to 40,000.

**Step 3. Precipitation at pH 5.0 and Adsorption on Aluminum Hydroxide Gel Cγ**—The dialyzed enzyme from Step 2 is diluted with water to a protein concentration of 8 to 10 mg. per ml. (D₂₅₀ = 13.2 for 1 per cent protein). The solution is brought to pH 5.0 at 0° by the addition of 0.2 to 0.3 ml. of 1 N acetic acid. Turbidity develops at this point, but the solution is not centrifuged. Aluminum hydroxide gel Cγ (13) is added in the ratio of 60 to 80 mg. of gel per gm. of protein, and after 15 minutes stirring the mixture is centrifuged for 30 minutes at 10,000 r.p.m. Enough gel should be added so that the amount of protein adsorbed, plus the precipitate which formed in adjustment to pH 5.0, is about 45 to 50 per cent of the initial protein. Since this step depends on the age of the gel and since the enzyme is not completely stable at pH 5.0, it is best to determine the amount of gel needed on a small aliquot prior to adjusting

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**TABLE I**

Effect of Time of Autolysis on Yield and Purity of Enzyme

<table>
<thead>
<tr>
<th>Autolysis hrs.</th>
<th>pH 6.0; 0.40 saturation*</th>
<th>pH 6.0; 0.45 saturation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total units</td>
<td>units per mg. protein</td>
<td>Total units</td>
</tr>
<tr>
<td>1.0</td>
<td>10,700</td>
<td>52</td>
</tr>
<tr>
<td>1.5</td>
<td>21,600</td>
<td>36</td>
</tr>
<tr>
<td>2.0</td>
<td>42,000</td>
<td>18</td>
</tr>
<tr>
<td>2.0</td>
<td>40,500</td>
<td>27</td>
</tr>
<tr>
<td>2.5</td>
<td>32,200</td>
<td>8</td>
</tr>
<tr>
<td>3.0</td>
<td>31,500</td>
<td>13</td>
</tr>
<tr>
<td>4.0</td>
<td>4,250</td>
<td>1</td>
</tr>
</tbody>
</table>

Autolysis conditions as in the text.

* Total units and units per mg. of protein are given for these preparations only after precipitation with (NH₄)₂SO₄ at the indicated concentrations and thorough dialysis.

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Autolysis-5
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the entire preparation to pH 5.0. The supernatant contains all of the enzyme at roughly twice the previous purity.

Step 4. Ammonium Sulfate Precipitation at pH 6.0 and 7.2—The supernatant from the previous step is brought to pH 6.0 at 0° by the addition of 0.4 to 0.5 ml. of 0.5 m \(K_2HPO_4\), and solid \((NH_4)_2SO_4\) is added to give 0.42 saturation. The suspension is stirred for 30 minutes and then centrifuged for 15 minutes at 10,000 r.p.m. The water-clear supernatant is discarded and the volume of the precipitate is estimated for a later calculation of the \((NH_4)_2SO_4\) concentration. The precipitate is dissolved in 0.015 m phosphate buffer, pH 7.2, in half of the volume at the end of Step 3, and the \((NH_4)_2SO_4\) concentration is calculated, considering the precipitate at pH 6.0 as 0.42 saturated \((NH_4)_2SO_4\). The solution is centrifuged if not clear, and the \((NH_4)_2SO_4\) concentration of the supernatant is raised, at 0°, to 0.42 saturation with solid \((NH_4)_2SO_4\). After 15 minutes stirring, the suspension is centrifuged for 10 minutes at 10,000 r.p.m. The precipitate is dissolved in a small volume of water and dialyzed against water at 0° for 2 to 3 hours. The dialyzed enzyme is centrifuged free of any precipitate which forms and is lyophilized to dryness.

The lyophilized precipitate is resuspended in 0.05 m succinate buffer, pH 6.0, and allowed to stand at 0° for 45 minutes. Much of the protein does not dissolve, and this is removed by 10 minutes centrifugation at 18,000 r.p.m. at 0°. The activity of the supernatant is measured in the presence of \(1 \times 10^{-3} \text{ M } MgSO_4\), since the usual \(Mg^{++}\) concentration of \(3 \times 10^{-4} \text{ M}\) is not optimal at this stage of purification (cf. "Metal requirements"). The activity ratio is 240:270, and the total units are 17,000 to 22,000. In the presence of \(Zn^{++}\), the activity ratio is as high as 350.

The enzyme may be lyophilized at any stage of purification and is stable in this form at −10° for at least several weeks. Aqueous solutions of the final preparation lose activity rapidly at 0°.

Preparation B

An alternate procedure for preparing the enzyme is outlined below. This method resulted in a preparation completely free of interfering enzymatic activities, with which certain key experiments could then be performed. It was later abandoned in favor of Preparation A, which yields a greater amount of enzyme and is more readily reproducible.

300 gm. of yeast were autolyzed in 600 ml. of water for 2.5 hours at 35-36°; 300 ml. of water were added and stirring was continued at 35-36° for 15 minutes more. The solids were centrifuged off and the supernatant was adjusted to pH 5.2 with 12 ml. of 2 n acetic acid. The enzyme was precipitated with solid \((NH_4)_2SO_4\) at 0.40 saturation, and the precipitate
was dissolved and dialyzed as for Preparation A. After dialysis, the sus-
pension was again centrifuged. Activity ratio of the supernatant = 5 to
6; total units = 11,000 to 20,000. The supernatant, at 2 per cent protein
concentration, was diluted with one-third volume of 0.2 M acetate buffer,
pH 5.0, and 95 per cent ethanol was added at 0° to −1° to a final con-
centration of 18 per cent by volume. The precipitate was discarded and
the ethanol concentration of the supernatant was raised to 35 per cent.
The precipitate from this fraction was dissolved in 0.05 M succinate buf-
fer, pH 6.0, and lyophilized to dryness. Activity ratio = 20 to 50; total
units = 3000 to 8000.

Interfering Enzymes—Crude preparations from brewers' yeast contain
certain enzymes which interfere with the quantitative estimation of FMN
synthesis, such as the FAD-synthesizing enzyme described by Schrecker
and Kornberg (14), nucleotide pyrophosphatase (15), a phosphatase which
hydrolyzes FMN, and probably also apyrases. All of these enzymes are
eliminated through Preparation B, and all but a small amount of the FAD
enzyme are removed by the end of Preparation A; this in no way interferes
with the routine assay, since FAD is hydrolyzed to FMN during the
deproteinization procedure. In the earlier stages of the preparation (Steps
1 and 2) 1 × 10⁻⁴ M KF and 2 × 10⁻⁵ M Na pyrophosphate are used to
inhibit the interfering enzymes.

Properties of Enzyme

Kinetics—Enzyme activity follows a zero order reaction for at least 2
hours and is directly proportional to enzyme concentration (Fig. 1). With
preparations having an activity ratio of 200 or greater, the reaction is
linear for 1 hour, but deviates slightly from linearity by 2 hours.

At pH 7.8 (THA buffer) the apparent temperature optimum is 38°
(Fig. 2, a). At 30°, the pH range of optimal activity is 7.8 to 8.5 (Fig.
2, b). THA is the preferred buffer for work with the enzyme; the activity
is somewhat lower in borate. Phosphate buffer in concentrations up to
7 × 10⁻² M has no inhibitory effect except at limiting metal concentrations.

Substrate Requirements—The enzyme is saturated at a concentration of
about 1 × 10⁻⁴ M riboflavin and half saturated at 1.0 × 10⁻⁶ M (Kₘ)
(Fig. 3). A source of high energy phosphate is obligatory for the phos-
phorylation of riboflavin in this system, and this requirement can be met
with either ATP or ADP. Inorganic phosphate does not enter the re-
action. Although the dissociation constants of the enzyme for ATP and
ADP are almost identical, 1.7 × 10⁻⁵ M and 1.6 × 10⁻⁵ M respectively,
ADP is only about half as effective as ATP as phosphate donor (Fig. 4).
This may in part be due to the fact that the product, AMP, acts as a
competitive inhibitor of the enzyme (Fig. 4). The dissociation constant
of the enzyme for this inhibitor ($K_I$) is calculated to be about $2.5 \times 10^{-5}$ M. ITP is inactive and non-inhibitory (Fig. 4). These data would suggest

![Fig. 1](image1.png)

**Fig. 1.** Proportionality of FMN formation with time and enzyme concentration. (a) Abscissa, time in hours; ordinate, millimicromoles of FMN synthesized per 5.0 ml. of reaction mixture. Standard conditions, except for the inclusion of $2 \times 10^{-3}$ M Na pyrophosphate and $1 \times 10^{-3}$ M MgSO$_4$. Enzyme, ammonium sulfate precipitate as in Preparation B, 13.75 mg. per tube, at an activity ratio of 7. (b) Abscissa, ml. of enzyme solution, 5.45 mg. per ml.; ordinate as in (a). Duration of experiment, 2 hours. Conditions and enzyme as in (a).

![Fig. 2](image2.png)

**Fig. 2**

**Fig. 3**

**Fig. 2.** The effect of temperature and pH. (a) Ordinate, millimicromoles of FMN per 5.0 ml.; abscissa, temperature in °C. Enzyme as in Fig. 1, 12 mg. per tube; conditions as in Fig. 1. (b) Ordinate, millimicromoles of FMN formed per 5.0 ml.; abscissa, pH of reaction mixture at 30°; ○, in $7.5 \times 10^{-2}$ M phosphate buffer, △, in $7.5 \times 10^{-2}$ M of THA buffer. Standard assay conditions; enzyme, alcohol-fractionated preparation, 3.9 mg. per tube at an activity ratio of 23.

**Fig. 3.** Affinity of enzyme for riboflavin (Lineweaver-Burk plot). Abscissa, $S$, average riboflavin concentration in micromoles per 5.0 ml. during the experimental period (20 minutes). Ordinate, $S/V$, where $V$ = millimicromoles of FMN synthesized per 5.0 ml. of reaction mixture. Enzyme, Preparation B, 4.4 mg. per tube at an activity ratio of 23.4. At the lowest substrate concentration, fluorometric measurement was used.

that the amino group in the 6 position of the purine ring is necessary for combination with the enzyme. A detailed study of this question is in progress.
It should be mentioned that, since ADP appeared to function as a phosphate donor in this system in contrast to other phosphokinase reactions, the enzyme preparation was tested for myokinase activity. The enzyme was incubated with ATP and AMP under the usual assay conditions, but without riboflavin; the proteins were precipitated with either perchloric acid or metaphosphoric acid, and the AMP content of the filtrates was determined by Schmidt's deaminase (10). No AMP disappearance could be detected, indicating the absence of myokinase activity. It may be added that the relative efficiencies of ATP and ADP in FMN synthesis are the same in the most highly purified preparation as in the alcohol-fractionated enzyme used here.

Metal Requirements—Without added bivalent cations, the activity of

<table>
<thead>
<tr>
<th>Metal</th>
<th>Optimal concentration</th>
<th>$V_{\text{max}}$</th>
<th>$K_M$</th>
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<tbody>
<tr>
<td>Mg$^{2+}$</td>
<td>$3 \times 10^{-4}$ M</td>
<td>100</td>
<td>$7.5 \times 10^{-5}$ M</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>$3 \times 10^{-4}$ &quot;</td>
<td>221</td>
<td>$2.9 \times 10^{-4}$ &quot;</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>$1 \times 10^{-4}$ &quot;</td>
<td>65</td>
<td>$2.2 \times 10^{-5}$ &quot;</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>$6 \times 10^{-4}$ &quot;</td>
<td>120</td>
<td>$9.0 \times 10^{-5}$ &quot;</td>
</tr>
</tbody>
</table>

Fe$^{3+}$, Al$^{3+}$, Ni$^{2+}$, inactive; Ca$^{2+}$, inhibitor.

Enzyme is an alcohol-fractionated preparation except in the experiments with Co$^{2+}$, in which the enzyme was made as for Preparation A, end of Step 5. Values for $K_M$ and $V_{\text{max}}$ are theoretical, derived from Lineweaver-Burk plots. The optimal concentration is that giving the best synthesis experimentally in the preparation used.

The enzyme is negligible from the end of Step 2 throughout purification. In Table II the activities of a number of metallic ions are compared. In preparations made by alcohol fractionation, or in the less purified stages of Preparation A, activation by Mg$^{2+}$ is optimal at a concentration of $3 \times 10^{-4}$ M; higher concentrations are somewhat inhibitory (Fig. 5, Curve 1). Preliminary evidence suggests that this inhibitory effect may be related to the binding of ATP by other proteins present. In highly purified preparations (end of Preparation A), both the optimal concentration of Mg$^{2+}$ and the $K_M$ value have changed (Fig. 5, Curve 2). The $K_M$, as calculated from the theoretical $V_{\text{max}}$ by the Lineweaver-Burk method, is $2.1 \times 10^{-4}$ M, as compared with $7.5 \times 10^{-5}$ M in the earlier preparations. The theoretical $V_{\text{max}}$ is more closely approached experimentally, however, in the crude preparations than in the material of higher purity, although no inhibition is apparent with the latter as the Mg$^{2+}$ concentration is raised (Curve 2, Fig. 5). Of the other metallic ions tested,
only Co++ acts similarly. This ion inhibits enzyme activity even in purified preparations at concentrations above the $K_M$ value. Mn++, with the lowest affinity constant, is not inhibitory but has a low $V_{max}$ which is closely approached experimentally.

Despite the changes in the apparent $K_M$ with purification, Mg++ is most consistent of these ions in activation of the enzyme, and has, there-

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** Affinity of enzyme for ATP, ADP, AMP, and ITP (Lineweaver-Burk plot). Abscissa, average substrate concentration during the experimental period (2 hours), in micromoles per 5.0 ml.; ordinate $S/V$ where $V$ is millimicromoles of FMN synthesized per 5.0 ml. of reaction mixture. $\bullet$, ADP; $\bigcirc$, ATP; $\triangle$, ATP plus $1 \times 10^{-4}$ M ITP; $\square$, ATP plus $9.4 \times 10^{-5}$ M AMP. Enzyme, Preparation B, 5.0 mg. per tube at an activity ratio of 21. $V_{max}$. for ATP, ATP + ITP, and ATP + AMP = 106 mmol per 5.0 ml.; $V_{max}$. for ADP = 41. These were used for the calculation of the $K_M$ values given in the text.

**Fig. 5.** Effect of Mg++ concentration. Abscissa, Mg++ concentration in moles per liter $\times 10^{-4}$; ordinate, millimicromoles of FMN formed per 5.0 ml. Standard assay conditions. Curve 1, alcohol-fractionated enzyme, 4 mg. per tube, at an activity ratio of 31. Curve 2, enzyme from Preparation A, final step, 0.84 mg. per tube, with an activity ratio of 110 at the standard Mg++ concentration of $3 \times 10^{-4}$ m. Activity of the two preparations related as equal at $3 \times 10^{-4}$ m MgSO₄, and the other points corrected from this activity for purposes of comparison.

fore, been used as a standard of reference throughout this work. This is in contrast to the effect of Zn++, which varies remarkably with the stage of purification. Thus, at the end of Step 2, the enzyme, though activated by Mg++, is only very slightly activated by Zn++. Alcohol preparations of similar purity are activated more by Zn++ than by Mg++ (Table II). Preparations carried through the entire procedure of Preparation A are again activated by Zn++, sometimes twice as much as by Mg++. Since the end of Step 2 is the only thoroughly dialyzed stage in the purification, it may be that other ions present in the other stages influence the acti-
vation by Zn++. Possibly not Zn++ itself, but a complex ion thereof is the activator. Similar variations in the effect of Zn on other enzymes have been reported by Johnson (16) and Smith (17). In view of these diverse observations on the metal requirements of this enzyme, a more complete study of this problem will be made.

Stoichiometry and Identification of Product—The stoichiometry of the reaction was determined by simultaneous analysis of the FMN synthesized and the riboflavin removed, and by the disappearance of acid-labile phosphate. The latter was determined by 7 minutes hydrolysis in 1 N H₂SO₄ at 100°; the phosphate of FMN is acid-stable. To obtain significant differences in acid-labile phosphate, a low concentration of ATP was used (3 X 10⁻⁵ M) at which the enzyme acted at about half its maximal rate, and the amount of enzyme was so chosen as to convert half of the ATP, or 25 per cent of the labile phosphate, to FMN. The results of this experiment are recorded in Table III. The balance of the reaction indicates that exactly 1 mole of acid-labile phosphate disappears for each mole of FMN synthesized, and the reaction may thus be formulated:

\[ \text{Riboflavin} + \text{ATP} \rightarrow \text{FMN + ADP} \]

or

\[ \text{Riboflavin} + \text{ADP} + \text{FMN} + \text{AMP} \]

That the reaction is essentially irreversible is to be expected from thermodynamic considerations, and is experimentally demonstrated by the quantitative conversion of riboflavin to FMN, in the presence of excess ATP. FMN and AMP remain unchanged in the presence of the enzyme.

The identity of the product as riboflavin-5-phosphate is indicated by its distribution coefficient between benzyl alcohol and water, and by its stability to acid. Further evidence of the identity of the product is its conversion, by the enzyme of Schrecker and Kornberg (14), to FAD as measured fluorometrically (12) and with the d-amino acid oxidase split protein. Finally the product is identified by its ability to activate the apoenzyme of cytochrome c reductase of yeast (9).
To demonstrate this, FMN was prepared by incubating 5 µM of ATP and a limiting amount of riboflavin (101.8 mM) with sufficient enzyme (250 units) to convert all of the riboflavin to the phosphorylated form. After incubation under the usual conditions, this solution was placed for 3 minutes in a boiling water bath, cooled, and filtered. A parallel experiment analyzed by the benzyl alcohol method established that the conversion of riboflavin to FMN was complete (106 mM of FMN). The flavin content of the filtrate was used as the basis of the calculated FMN content of the samples in subsequent assay with cytochrome c reductase apoenzyme. It is seen from Fig. 6 that FMN prepared by flavokinase replaces authentic riboflavin-5-phosphate as the prosthetic group of cytochrome c reductase.

**Specificity**—The specificity of flavokinase was tested at various stages of purification; namely, at the end of Step 2 and in the most highly purified fraction. The first of these contains phosphokinases for the phosphorylation of ribose (19), adenosine (19, 20), glucose, pyridoxal, and the FAD-synthesizing enzyme of Schrecker and Kornberg (14). The final preparation does not phosphorylate adenosine or ribose, although hexo-

Fig. 6. Activation of cytochrome c reductase apoenzyme. Ordinate, ΔD_{680} from 1 to 8 minutes after the addition of cytochrome; blank reduction without added FMN is subtracted. Abscissa, molar concentration of FMN × 10^{-7}. O, authentic FMN; A, enzymatically formed FMN. All constituents of the reductase system were prepared as specified by Haas et al. (9), except for cytochrome, which was a commercial preparation from Wyeth, Incorporated, and TPN, which was a 50 per cent pure substance prepared according to LePage and Mueller (18). The reductase apoenzyme and the FMN to be tested were incubated 15 minutes at 28°C to allow complete combination. FMN concentrations in the graph refer to this incubation mixture. 0.08 ml. aliquots were added to cells containing all other constituents except cytochrome, which was added immediately thereafter at zero time. Final concentrations in the cell were as follows: 5.16 × 10^{-8} M cytochrome c, 8.9 × 10^{-4} M TPN, 3.4 × 10^{-4} M glucose-6-phosphate, 2.8 × 10^{-2} M phosphate buffer, pH 7.1, 300 γ of Zwischenferment. The concentration of reductase was so chosen as to give about 50 per cent reduction of the added cytochrome in 8 minutes in the presence of excess FMN. The preparation of FMN from riboflavin is described in the text.
kinase and FAD-synthesizing activities are still present; the phosphokinase for pyridoxal is largely removed. The activity on ribose, adenosine, and glucose was measured by the disappearance of acid-labile phosphate, with Berenblum and Chain's micromethod for the determination of phosphorus (21). That flavokinase and hexokinase are different enzymes is indicated by the complete absence of flavokinase activity in highly purified bakers' yeast hexokinase and by the pronounced differences in the metal requirements of the two enzymes. Phosphorylation of pyridoxal was measured by activation of the tyrosine apodecarboxylase of Streptococcus faecalis (22). The enzyme which catalyzes this phosphorylation can be differentiated from flavokinase on the basis of its solubility in water and in alcohol, and its stability to the latter solvent. The enzyme of Schrecker and Kornberg is distinguished from flavokinase (a) by the almost complete absence of riboflavin phosphorylation in purified preparations of the former, (b) by the fact that ADP is inactive in FAD synthesis, and (c) by the complete absence of FAD formation from FMN and ATP in the alcohol-fractionated enzyme.

A close structural requirement by flavokinase for its substrate is suggested by experiments with isoriboflavin, which differs structurally from riboflavin only in the position of one methyl group (isoriboflavin, 5,6-dimethyl-9-(1-n-ribityl)isoalloxazine; riboflavin, 6,7-dimethyl-9-(1-n-ribityl)isoalloxazine). Isoriboflavin is not phosphorylated by the enzyme at any stage of purification. A systematic study of the phosphorylation of other riboflavin analogues and their possible inhibitory effects is in progress.

DISCUSSION

With the demonstration of this enzyme, it is possible to delineate the main features of the metabolism of riboflavin in yeast (Fig. 7). Starting with riboflavin (RF) and ATP or ADP, FMN is synthesized by means of flavokinase, an essentially irreversible reaction. FMN and ATP are condensed to FAD by the enzyme of Schrecker and Kornberg, with inorganic pyrophosphate (PP) as a side product; this is a reversible reaction. The breakdown of FAD is also catalyzed by nucleotide pyrophosphatase to yield FMN and AMP. FMN in turn is broken down to riboflavin and inorganic phosphate by the action of phosphatase. Thus far, the two key enzymes, flavokinase and the FAD-synthesizing enzyme, are known to exist only in yeast, while the other enzymes involved have been demon-

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6 Unpublished data by Mr. J. Hurwitz of this laboratory. We are grateful to Mr. Hurwitz for the determinations of pyridoxal phosphorylation.

7 Both enzymes are, however, activated by Zn++ as well as by Mg++ (Englard, S., and Kearney, E. B., unpublished data).
strated in animal tissues as well. FAD synthesis from riboflavin, however, has been shown to occur in animal tissues (23, 24), although the mechanism of the transformation is not known. It seems likely that this formation of FAD from riboflavin may proceed via the same cycle; i.e., with the initial phosphorylation of riboflavin. FMN may arise in three different ways, but ultimately all of these probably depend on flavokinase for the initial phosphorylation of riboflavin. Flavokinase seems ideally suited for the manufacture of a trace substance such as FMN in view of its low dissociation constants for its substrates and cofactors, and its ability to carry the reaction to completion. The flavokinase activity of brewers' yeast autolysate is of the same low order of magnitude as its content of the FAD-synthesizing enzyme (14). Both of these activities, however, appear to be sufficient to replenish rapidly the relatively low flavin nucleotide content of yeast cells (25). Once these enzymes are available in the pure state, it will be of interest to determine whether they are present in appreciable concentrations but have low turn-over numbers, or whether they are present in very low concentrations with high turn-over numbers.

It is a pleasure to thank Dr. Thomas P. Singer for his interest and help in the course of this study.

SUMMARY

1. An enzyme has been isolated from brewers' yeast which catalyzes the reaction:

\[ \text{Riboflavin} + \text{ATP} \rightarrow \text{riboflavin-5-phosphate} + \text{ADP} \]

2. The stoichiometry of the reaction has been demonstrated and the product of the reaction identified. In view of this reaction, the name "flavokinase" is proposed.

3. The activity of the enzyme follows zero order kinetics and is linear
with enzyme concentration. The pH range for optimal activity is 7.8 to 8.5 and the optimal temperature is 38°.

4. The dissociation constants of the enzyme for riboflavin, ATP, ADP, and AMP have been determined. ADP is about half as effective as ATP as phosphate donor in the reaction. ITP is inactive and non-inhibitory, and AMP is a competitive inhibitor.

5. A metallic ion is required for full activity, and Zn++, Mg++, Co++, and Mn++ are effective in this capacity. The relative affinities of these ions for the enzyme have been measured. Fe++, Al+++ and Ni++ are inactive; Ca++ is inhibitory.

6. The enzyme appears to be specific for riboflavin within the limits of the tests made thus far.

7. The role of the enzyme in the metabolism of riboflavin is discussed.

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THE ENZYMATIC PHOSPHORYLATION OF RIBOFLAVIN
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