Studies on Plant Flavokinase

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As riboflavin is a component of several enzymes concerned with respiration, cells must be capable of synthesizing the active forms of the vitamin, namely both flavin mononucleotide and flavin-adenine dinucleotide. The earlier observations (Hubner & Verzar, 1939; Pulner & Verzar, 1939), that phosphorylation of riboflavin could take place by means of inorganic phosphate in preparations of intestinal mucosa, seemed unlikely in view of the energetics of the reaction. A conclusive demonstration of the phosphorylation of riboflavin by adenosine triphosphate was made by Kearney & Englard (1951), using a purified enzyme preparation from yeast. The only reports in the literature pertain to its isolation and purification from different yeasts (Kearney & Englard, 1951; Giri & Krishnaswamy, 1956). Though the occurrence of riboflavin in plants is well known, little is known about the mechanism of synthesis of flavin nucleotides in plants. It was observed that synthesis of flavin mononucleotide took place in plant extracts when they were incubated under the same conditions as those used with yeasts (Giri, Krishnaswamy & Appaji Rao, 1957). The properties of this plant enzyme have been investigated and are described in this paper.

MATERIALS AND METHODS

Substrates and reagents. Riboflavin and flavin mononucleotide (FMN) were products of Hoffman La-Roche and Nutrition Biochemicals Corp. respectively. Adenosine

Table 1. *Progress of purification of flavokinase from* Phaseolus radiatus

For details of stages of purification see text. Reaction mixtures were as follows: 0·2 ml. of riboflavin, 0·1 mM; 0·1 ml. of ATP, mM; 0·1 ml. of NaF, 0·1M; 0·7 ml. of 0·1M-veronal–HCl buffer (pH 8·6); 0·8 ml. of enzyme preparation from the various stages and 0·1 ml. of $MgSO_4$, 0·3 mM. Total volume 2 ml. Incubated at 55° for 1 hr.

Stage of purification	Volume (ml.)	Protein (mg.)	Total activity (units)	Specific activity (units/mg. of protein)
I	165	15 820	6198	0·3908
II	180	4 842	8246	1·7030
III	60	820	6726	8·2040
IV	130	416	5981	14·5100
V	40	105	3144	29·9300

triphosphate (ATP) used included preparations made according to LePage (1949), of purity 60–65%, and one purchased from California Foundation for Biochemical Research of purity 95%. Some differences in reactions were noted with samples of ATP of different purity but these differences were only quantitative in nature. All other chemicals used were of a reagent grade and the purest currently available.

Preparation and purification of enzyme. Finely powdered green gram (*Phaseolus radiatus*) was used as the source of the enzyme, and various procedures were tried to obtain extracts with maximum activity. Extraction of the powder at a temperature of 0–5° with water was found to be more efficient than extraction with buffer solutions or with water at 25° and 37°. The procedure finally adopted, which was very similar to that used by Kearney & Englard (1951) for yeast flavokinase, was as follows.

Finely powdered material was extracted with water (100 g./300 ml.) for 60 min. at 0–5° by periodic shaking. The extract obtained by centrifuging at 1000 *g* (165 ml., stage I, Table 1) was dialysed in the cold against water for 18–20 hr. and the precipitate which appeared was centrifuged at 2500 *g*. To the opalescent supernatant (180 ml., stage II) ammonium sulphate to 0·40 saturation was added and precipitate obtained by centrifuging at 5000 *g* was dissolved in 50 ml. of water and dialysed for 18 hr. against water at 0–5° (60 ml., stage III). An equal volume of alumina-C_γ gel (15 mg./ml.) was added to the dialysed solution and kept for 20 min. in the cold, and centrifuged at 1000 *g*. This procedure removed much inactive protein; the precipitate was eluted three times with 10 ml. portions of 0·02M-sodium phosphate buffer (pH 7·2) and recombined with the supernatant (130 ml., stage IV). This was again fractionated with ammonium sulphate to 0·40 saturation; the precipitate obtained by centrifuging at 5000 *g* was dissolved in 30 ml. of 0·01M-veronal–HCl buffer (pH 8·6) and the solution dialysed for 18 hr. against water in the cold (40 ml., stage V).

A 75-fold purification of the enzyme was achieved with a recovery of about 38%. It is likely that crude extracts contain certain inhibitory factors which are eliminated on dialysis, since the activity increased on dialysis.

Enzyme assay. During the assay and analysis, tubes (10 cm. × 1 cm.) in which reactions were carried out were protected from light. The reaction mixtures, unless otherwise stated, consisted of 0·2 ml. of riboflavin solution, 0·1 mM; 0·1 ml. of ATP solution, mM; 0·1 ml. of NaF solution, 0·1M; 0·1 ml. of $MgSO_4$ solution, 0·3 mM; 0·7 ml. of 0·1M-veronal–HCl buffer (pH 8·6) and 0·8 ml. of enzyme preparation; total volume, 2 ml. They were incubated at 55° and at specified intervals, usually after 60 min., the reaction was arrested by the addition of 0·8 ml. of 17·5% (w/v) trichloroacetic acid. The tubes were kept in a boiling-water bath for 5 min. to hydrolyse traces of flavin-adenine dinucleotide (FAD) formed. The solutions were cooled, centrifuged and suitable portions of the supernatant were analysed for FMN formed by the circular-paper-chromatographic method of Giri & Krishnaswamy (1956), with butanol–acetic acid–water (4:1:5, v/v) as the solvent system. The procedure briefly consisted in locating FMN on the chromatograms under u.v. light, eluting the paper strips in glass-distilled water and measuring the fluorescence in a Klett fluorimeter. Appropriate blanks and controls were always run. For preparative purposes, the technique of Giri (1954, 1955) was used. Absorption spectra were determined with a Beckman Spectrophotometer, Model DU.

Riboflavin concentrations in germinating seedlings were determined by the method of Snell & Strong (1939). Protein was determined by the Biuret method. One unit of enzyme activity is defined as the amount that synthesizes 1 μm-mole of FMN at pH 8·6, temperature 55°, in 60 min.

RESULTS

Identification of flavin mononucleotide formed by enzymic synthesis. A reaction mixture (40 ml.), containing the substrates, buffer, etc., as described was incubated for 18 hr. at 37°. The reaction was stopped with 16 ml. of 17·5% (w/v) trichloroacetic acid and the mixture was kept in a boiling-water bath for 5 min. The supernatant was subjected to preparative circular-paper chromatography (Giri, 1954, 1955). The isolated product showed absorption maxima at 264–266, 372–374 and 446 mμ and was chromatographically identical with an authentic sample of FMN. On enzymic hydrolysis by phosphatases [0·2 ml. of 0·1 mM-FMN; 0·8 ml. of 0·1M-veronal–HCl buffer (pH 8·6) and 1 ml. of enzyme preparation from green gram], as well as acid hydrolysis with N-H_2SO_4 at 100° for 1 hr., phosphate and riboflavin were detected as degradation products.

Reverse reaction. FMN (0·2 ml., 0·1 mM); 0·1 ml. of $MgSO_4$, 0·3 mM; 0·8 ml. veronal–HCl buffer (pH 8·6); and 0·9 ml. of the purest enzyme preparation (total volume 2 ml.) were incubated at

37° for 2 hr. Reaction was arrested by the addition of 0·8 ml. of 17·5% (w/v) trichloroacetic acid. Portions of supernatant were analysed chromatographically with butanol–acetic acid–water (4:1:5, v/v) as the solvent system. No riboflavin could be detected on the chromatograms. Kearney & Englard (1951) have reported that the synthesis of FMN from riboflavin and ATP was virtually an irreversible reaction.

Optimum temperature for activity of enzyme. In preliminary experiments reactions were carried out at 37°. However, it was found later that the enzyme showed maximum activity at 55° (Fig. 1).

The plant flavokinase was different from the yeast flavokinase in that it had a much higher temperature for optimum activity. Whereas the enzyme was totally inactivated when incubated alone at 60°, much activity (90%) was retained when it was incubated with its substrates (riboflavin 0·1 mM, ATP 1·0 mM). This would explain to a certain extent the high temperature of optimum activity.

Progress curve of the reaction. This is shown in Fig. 2; 2 ml. reaction mixtures were set up under standard conditions as already stated. At different intervals, they were analysed for FMN. Separate sets of tubes were used for each time interval.

pH–*Activity curve.* This is shown in Fig. 3. Variation of the initial rate of reaction as a function of pH was determined by carrying out experiments,

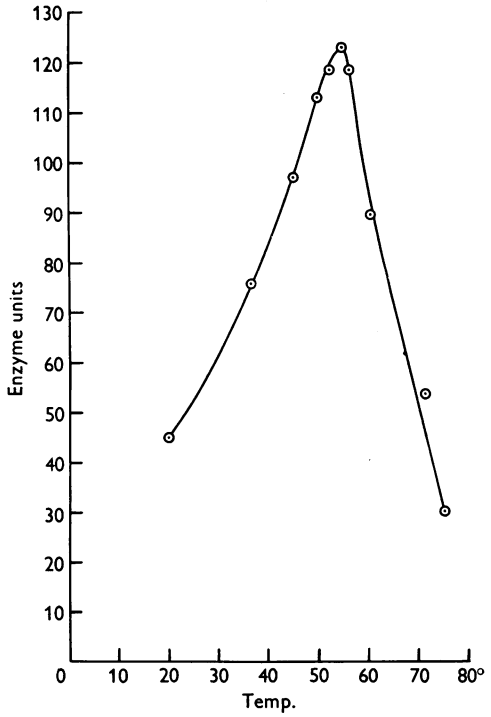

Fig. 1. Optimum temperature for activity of enzyme. Reaction mixtures contained: 0·2 ml. of riboflavin, 0·1 mM; 0·1 ml. of ATP, mM; 0·1 ml. of NaF, 0·1 M; 0·1 ml. of magnesium sulphate, 0·3 mM; 0·7 ml. of 0·1 M-veronal–HCl buffer (pH 8·6); 0·8 ml. of enzyme; total volume 2 ml. Incubated for 1 hr. at various temperatures.

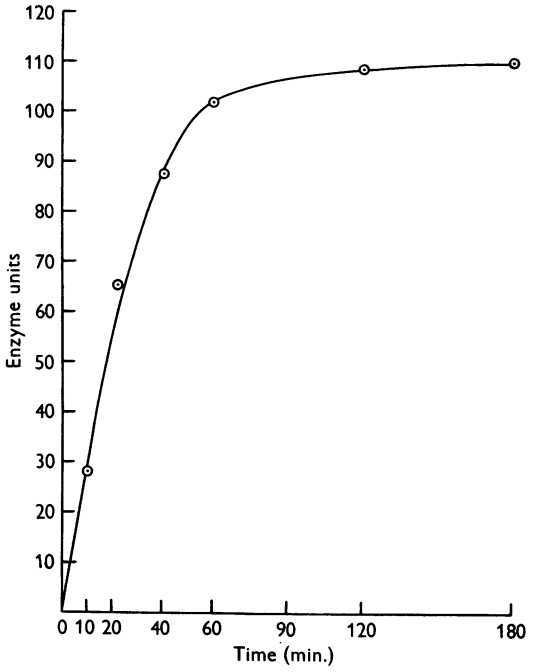

Fig. 2. Progress curve. Reaction mixtures were as in Fig. 1 and were incubated for 1 hr. at 55° for the times indicated.

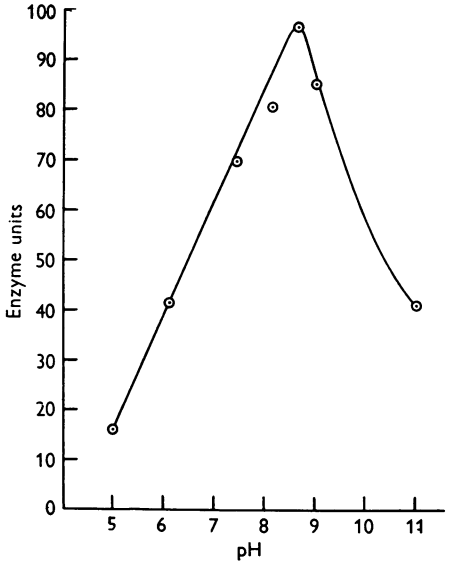

Fig. 3. pH–Activity curve. Reaction mixtures were as in Fig. 1 but with 0·7 ml. of various buffers (pH 5·0–11·0); total volume 2 ml. They were incubated for 1 hr. at 55°.

under conditions already specified, in different buffers of known pH [0·1 M-potassium hydrogen phthalate–NaOH (pH 4·0 and 5·0), 0·1 M-veronal–HCl (pH 6·0–9·0), 0·1 M-boric acid–NaOH (pH 10·0) and 0·1 M-potassium dihydrogen phosphate–NaOH (pH 11·0)].

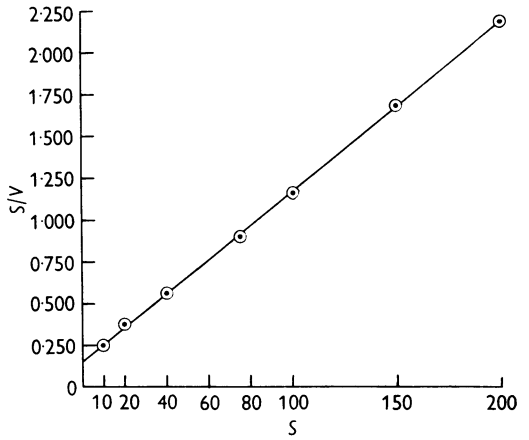

Fig. 4. Lineweaver–Burk plots. Reaction mixtures contained 0·2 ml. of riboflavin, of concentrations indicated in the graph; 0·1 ml. of ATP, mM; 0·1 ml. of NaF, 0·1M; 0·1 ml. of magnesium sulphate, 0·3 mM; 0·7 ml. of veronal–HCl buffer (pH 8·6); and 0·8 ml. of enzyme; total volume 2 ml. The mixtures were incubated for 1 hr. at 55°. *S*, Concentration of riboflavin in reaction mixtures in μmoles; *V*, μm-moles of FMN synthesized/2 ml./hr. at 55° and pH 8·6.

Table 2. *Effect of various ions on flavokinase activity*

Reaction mixtures were as follows: 0·2 ml. of riboflavin, 0·1 mM; 0·1 ml. of ATP, mM; 0·1 ml. of NaF, 0·1 M; 0·7 ml. of 0·1 M-veronal–HCl buffer (pH 8·6); 0·8 ml. of enzyme preparation from stage III and the various ions as shown in Table 2. Total volume 2 ml. Incubated at 55° for 1 hr. +, Activation; −, inhibition.

	Percentage inhibition or activation of flavokinase activity at different concentrations of ions added		
	0·1 mM	mM	10 mM
Calcium chloride	−5·1	+5·2	+5·2
Magnesium sulphate	+36·8	+26·2	+28·9
Zinc sulphate	+15·8	−2·5	−75·5
Potassium cyanide	−74·9	−74·9	−87·5
Lithium chloride	+10·5	+3·5	+0·7
Manganese sulphate	+31·5	+5·3	−26·4
Ferrous sulphate	+5·2	−31·6	−52·7
Potassium permanganate	0	−47·6	−100·0
Copper sulphate	0	+5·2	−47·6
Nickel chloride	+10·0	+5·2	+5·2
Cadmium sulphate	0	+5·2	−26·2
Cobalt acetate	0	−21·0	−42·1
Mercuric chloride	−51·9	−49·3	−63·2
	0·01 mM	0·1 mM	mM

Various buffers such as veronal–HCl, borate–boric acid, tris, phosphate and glycine were tried at pH 8·5. Maximum amount of FMN was synthesized with veronal–HCl buffer and this buffer has been used for enzyme assay.

Substrate affinity of enzyme. Lineweaver–Burk plots are shown in Fig. 4. At a constant ATP concentration, mM, different concentrations of riboflavin were used in the reaction system.

The enzyme functions at the maximum rate at a riboflavin concentration of 0·1 mM. It was half saturated with riboflavin at a concentration of 0·015 mM. At a constant riboflavin concentration of 0·1 mM, varying concentrations of ATP were used and it was observed that the enzyme functions at a maximum rate at an ATP concentration of 2 mM.

Effect of various ions. A number of ions were tested for their effect on flavokinase activity. They were added to the system in different molar concentrations. In these experiments an enzyme preparation from stage III was used. The results are summarized in Table 2.

Of the ions used, Mg^{2+} activated the enzyme at all concentrations tested, Mn^{2+} and Zn^{2+} ions activated at low concentrations, but inhibited at higher concentrations. For routine assay of the enzyme Mg^{2+} ions were used as the activator. Kearney & Englard (1951) used Mg^{2+} ions as the standard activator for yeast flavokinase since they observed that it was the most consistent in activating the enzyme, Zn^{2+} ions having a slight effect at some stages of purification and a much greater effect at other stages of purification. As suggested by Englard (1953), Zn^{2+} ions may affect the level of ATP in the system, thus resulting in a lowered synthesis of FMN.

CN^-, Hg^{2+}, Fe^{2+}, MnO_4^- and Cu^{2+} ions were found to be inhibitory, whereas Ca^{2+}, Ni^{2+}, Li^+ and Cd^{2+} ions were without effect. Co^{2+} ions were inhibitory at concentrations higher than mM. Hydroxylamine was also found to be an inhibitor.

Distribution of flavokinase activity in plants. Examination of a number of plants indicated that flavokinase activity was present in all of those plant materials examined (Table 3).

Germination and flavokinase activity. Riboflavin is one of the vitamins which not only occurs in fairly large amounts in plants but also increases considerably during germination (Bonner, 1942). Flavokinase activity during germination of *Phaseolus radiatus* was followed and riboflavin concentrations were also determined at the various stages (Fig. 5). Seeds were allowed to germinate in the dark, in Petri dishes. They were placed on moistened filter papers cut to the same size. Water was frequently sprinkled to keep the filter paper moist. At the end of definite intervals, they were taken

out, homogenized in water (1:3, w/v), centrifuged and the supernatant obtained after dialysis of the crude extract was used as the enzyme. Riboflavin concentrations were determined on a parallel set of samples (Snell & Strong, 1939). Increase in activity of the enzyme paralleled an increase in concentration of riboflavin.

Table 3. *Occurrence of flavokinase activity in different plants*

Reaction mixtures were as follows: 0·2 ml. of riboflavin, 0·1 mM; 0·1 ml. of ATP, mM; 0·1 ml. of NaF, 0·1 M; 0·1 ml. of $MgSO_4$, 0·3 mM; 0·7 ml. of 0·1 M-veronal–HCl buffer (pH 8·4); and 0·8 ml. of enzyme from stage II. Total volume 2 ml. Incubated at 37° for 5 hr.

Plant source*	Activity†
1. *Cicer arietinum* (Bengal gram)	99·7
2. *Ricinus communis* (castor seed)	85·9
3. *Brassica campesteris* (mustard)	82·8
4. *Phaseolus mungo* (black gram)	76·7
5. *Phaseolus radiatus* (green gram)	79·7
6. *Dolichos lablab* (field bean)	148·8
7. *Dolichos biflorus* (horse gram)	99·7
8. *Canavalia gladiata* (sword bean)	118·1
9. *Pisum sativum* (peas)	99·7
10. *Sesamum indicum* (sesame)	99·7
11. *Cajanus indicus* (red gram)	99·7
12. *Solanum tuberosum* (potato)	73·6
13. *Ipomea batatas* (sweet potato)	70·0

* 1–11 were resting seeds.

† FMN synthesized at 37° and pH 8·4 (μm-moles/2 ml. of reaction mixture/5 hr.).

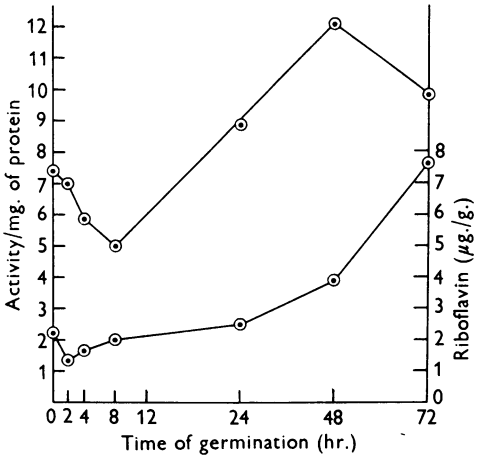

Fig. 5. Effect of germination on flavokinase activity. Flavokinase activity (top curve) and riboflavin content of green gram (*Phaseolus radiatus*) (bottom curve). Reaction mixtures were as in Fig. 1, and contained 0·8 ml. of enzyme in a total volume of 2 ml. and were incubated for 1 hr. at 55°.

DISCUSSION

The widespread occurrence of flavokinase activity in plants, as in yeasts, suggests that the main features of riboflavin metabolism are the same in plants as in yeast. Starting with riboflavin, flavin mononucleotide is synthesized by flavokinase by means of a reaction with adenosine triphosphate. The synthesis of flavin-adenine dinucleotide from riboflavin proceeds through the initial phosphorylation of riboflavin and subsequent interaction of flavin mononucleotide and adenosine triphosphate to form flavin-adenine dinucleotide by another enzymic step (Schrecker & Kornberg, 1950). During the course of these investigations synthesis of flavin-adenine dinucleotide could be demonstrated in plant extracts. Though the occurrence of riboflavin in plants is well known (Bonner, 1942; Bonner & Bonner, 1948; Watson & Nogelle, 1947), information about the enzymes concerned in its metabolism is scanty. Riboflavin is a component of many flavoprotein enzymes present in plants. Hence a study of these two enzymes which synthesize the biologically active coenzyme forms of riboflavin from plant sources may help to delineate their role in the physiology of plants.

SUMMARY

1. Flavokinase activity has been detected for the first time in plants, and its properties were studied by the application of circular-paper chromatography. The plant investigated was *Phaseolus radiatus*.

2. Optimum pH for flavokinase activity was found to be 8·6 and temperature 55°; conversion into flavin mononucleotide was nearly 60%. The Michaelis constant for riboflavin was found to be 0·015 mM.

3. Mg^{2+}, Zn^{2+} and Mn^{2+} ions showed an activating effect on flavokinase; Zn^{2+} and Mn^{2+} ions, however, were inhibitory at higher concentrations. Ca^{2+}, Ni^{2+}, Li^{+} and Cd^{2+} ions were without effect. CN^{-}, Hg^{2+}, Fe^{2+}, Cu^{2+}, MnO_4^{-} ions and hydroxylamine were inhibitory.

4. A 75-fold purification was obtained by fractionation with ammonium sulphate and adsorption with alumina-C_γ. There was a parallel increase in riboflavin levels and flavokinase activity in germinating seedlings of *Phaseolus radiatus*.

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