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Studies on Flavinadenine Dinucleotide-Synthesizing Enzyme in Plants

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There is practically no information on the biosynthesis of flavinadenine dinucleotide in plants. The available evidence relating to this process in animal tissues or micro-organisms is meagre, although the function of flavin nucleotides as components of respiratory enzymes has long been known. Trufanov (1941) suggested that flavinadenine dinucleotide is synthesized in rat-tissue slices by a condensation of riboflavin and adenosine diphosphate and that maintenance of cell structure is essential for the reaction. Schrecker & Kornberg (1950) demonstrated synthesis of flavinadenine dinucleotide from flavin mononucleotide and adenosine triphosphate in yeast. A similar pathway for synthesis of flavinadenine dinucleotide was reported in a riboflavin-secreting mutant yeast (Giri & Krishnaswamy, 1956). Recently Deluca & Kaplan (1958) showed that synthesis of flavinadenine dinucleotide occurs in rat-liver supernatants.

The occurrence of riboflavin in plants has long been known (Bonner, 1942; Bonner & Bonner, 1948; Watson & Nogelle, 1947), but there is no information on the concentration of flavin nucleotide coenzymes and the enzymes synthesizing these.

After the observation on the widespread occurrence of the flavin nucleotides (Giri, Appaji Rao, Cama & Kumar, 1959) and on the biosynthesis of flavin mononucleotide in plants by phosphorylation of riboflavin by adenosine triphosphate (Giri, Krishnaswamy & Appaji Rao, 1958), the mechanism of biosynthesis of flavinadenine dinucleotide was studied, and the results are described and discussed in this paper.

* Deceased.

MATERIALS AND METHODS

Substrates and reagents. Flavin mononucleotide (FMN) was a Hoffman–La Roche and Co. product obtained as a gift from Voltas Ltd., Bombay, and was purified before use. Flavinadenine dinucleotide (FAD; 60% pure) was obtained as a gift from Esai Ltd., Japan, and was purified by preparative circular-paper chromatography before use (Giri, 1954, 1955). Adenosine triphosphate (ATP) used included preparations made according to LePage (1949) of 65–70% purity and another (95% purity) obtained from California Foundation for Biochemical Research. The purity of ATP was determined by the method described by LePage (1949). All other chemicals used were of reagent grade.

Preparation of alumina Cγ-gel. Ammonium sulphate (22 g.) was dissolved in 600 ml. of water. Ammonia soln. (100 ml. of 10%; previously standardized by titration) was added to it and the mixture was warmed to 58°. A solution containing 76·7 g. of aluminium ammonium sulphate dodecahydrate in 150 ml. of water was also warmed to 58° and was added all at once to the basic ammonium sulphate solution. This mixture was warmed to 60° and stirred for 15 min. The contents were transferred to a tall jar with 5 l. of water and the precipitate was washed three times with 3 l. of water. To the fourth wash was added 80 ml. of 20% ammonia soln. to decompose the basic sulphate and the gel was washed repeatedly with water. After twenty washings the water remained opalescent and the gel was washed twice more with water. A suspension containing 18 mg. (dry wt.)/ml. was prepared. It was aged at 4° for at least 2 months before use (Willstätter & Kraut, 1923).

Preparation and purification of the enzyme. Finely powdered green gram (*Phaseolus radiatus*) was used as the source of the enzyme. An extract with maximum specific activity was obtained by the following procedure.

Freshly powdered green-gram seeds (100 g./300 ml.) were extracted with 0·1 M-sodium bicarbonate soln. with constant stirring at 0–5° for 2 hr. The extract was centrifuged

at 1000 *g* for 15 min. and the supernatant (stage I, Table 1) was dialysed against glass-distilled water for 18 hr. at 0–5°. The diffusate was centrifuged at 2000 *g* for 15 min. (stage II), the supernatant was brought to 0·45 saturation with ammonium sulphate by the addition of recrystallized solid ammonium sulphate and the precipitate obtained on centrifuging at 5000 *g* was rejected. The supernatant was raised to 0·55 saturation with ammonium sulphate, the precipitate obtained on centrifuging at 5000 *g* for 30 min. was dissolved in glass-distilled water and was dialysed free from ammonium sulphate (stage III). To the dialysed extract (40 ml.) was added 4 ml. of ethanol at 0° to −5° and the precipitate obtained on centrifuging was rejected. To the supernatant 5·5 ml. of ethanol was added and the precipitate obtained on centrifuging at 5000 *g* for 20 min. was dissolved in 35 ml. of glass-distilled water (stage IV). An equal volume of alumina C*γ*-gel (18 mg./ml.) was added and the enzyme–gel mixture was kept cold for 20 min. with constant stirring, centrifuged at 1000 *g* for 5 min. and the supernatant was rejected. The gel was extracted three times with 10 ml. volumes of 0·02 M-sodium phosphate buffer (pH 7·8) and the eluates were combined. The combined eluates were precipitated with ethanol, to 8% concentration, centrifuged at 5000 *g* for 45 min. and the supernatant was rejected. The precipitate was dissolved in 20 ml. of glass-distilled water and used as the enzyme (stage V). An 83-fold purification with 26% recovery was achieved.

Enzyme assay. During assay and analysis, tubes (1 cm. × 10 cm.) in which the reactions were carried out were protected from light. The reaction mixture contained: FMN, 1 mM; ATP, 20 mM; NaF, 0·1 M; $MgSO_4$, 0·1 mM; veronal–HCl buffer (pH 7·4), 0·1 M; 0·8 ml. of the enzyme preparation; total volume, 2 ml. Reaction was stopped by heating in boiling water for 20 min. The tubes were centrifuged at 3000 *g* for 20 min. and suitable portions (usually 0·3 ml.) were analysed for FAD by the circular-paper-chromatographic technique (Giri & Krishnaswamy, 1956), butanol–acetic acid–water (4:1:5, by vol.) being used as the solvent system. The FAD band was located under u.v. light and marked with a pencil. The band was eluted into 5 ml. of glass-distilled water for 6 hr. and fluorescence of the eluates measured in a Klett fluorimeter with filters (*a*) at the lamp source, Corning 5113 + 3389 (4358 Å), and (*b*) at the photocell, Corning 3486 (5200 Å). The necessary blanks and controls were always run.

Protein was determined by the biuret method of Robinson & Hogden (1940). One unit of enzyme activity is defined as the amount that synthesizes 1 μm-mole of FAD at pH 7·4, temperature 37°, in 60 min.

For preparative purposes the technique of Giri (1954, 1955) was used. Absorption spectra were determined in a Beckman spectrophotometer model DU.

Determination of flavinadenine dinucleotide in seedlings. FAD in germinating seedlings was determined by the method of Bessey, Lowry & Love (1949). The seeds were homogenized and the homogenates deproteinized with trichloroacetic acid. The deproteinized extracts were neutralized and fluorescence was measured in a Klett fluorimeter with appropriate filters. Another portion of the deproteinized extract was hydrolysed by incubating at 37° for 18 hr., and it was neutralized and the fluorescence was measured in a Klett fluorimeter. With the equation suggested by Bessey *et al.* (1949), the FAD content of the portions was determined.

RESULTS

Identification of enzymically synthesized flavinadenine dinucleotide. A 20 ml. reaction mixture was incubated at 37° for 18 hr. and the reaction stopped as indicated above. The concentrated extract was chromatographed and the flavin band (R_F 0·18) was eluted into glass-distilled water. The eluate showed absorption maxima at 263, 375 and 450 mμ and the spectrum was identical in all respects with that of an authentic sample of FAD. The eluate, on chromatography in four different solvent systems [(i) butan-1-ol–acetic acid–water (4:1:5, by vol.); (ii) butan-1-ol–propanol–water (2:2:1, by vol.); (iii) butan-1-ol–formic acid–water (77:10:13, by vol.); (iv) *iso*amyl alcohol saturated with water], was identical with an authentic sample of FAD. Cochromatography in all these solvent systems was carried out and the authentic sample moved as a single band with enzymically synthesized FAD. The eluate was evaporated to dryness and the dry residue hydrolysed with 1 ml. of 6 N-hydrochloric acid for 2 hr. at 120°, in a sealed tube. Hydrochloric acid was removed *in vacuo* and the neutral extract was subjected to circular-paper chromatography with butanol–acetic acid–water (4:1:5, by vol.); the air-dried chromatograms were sprayed with Folin's reagent and the characteristic pink band of 4-amino-5-imidazole carboxamidine was identified with reference to a standard. Since 4-amino-5-imidazole carboxamidine is a degradation product of adenine and adenine-containing compounds this test proved the presence of an adenine moiety in the flavin in the eluate. The reaction product was hydrolysed by the crude

Table 1. *Progress of purification of flavinadenine dinucleotide-synthesizing enzyme from* Phaseolus radiatus

For details of purification, see text.

Stage of purification	Volume (ml.)	Total activity (units)	Total protein (mg.)	Specific activity (units/mg. of protein)
I	165	4062	14 930	0·272
II	180	3868	4 493	0·861
III	40	3499	483	7·232
IV	35	1928	114	14·426
V	22	1069	47	22·580

extracts of *P. radiatus* and FMN detected on chromatograms. The FAD synthesized was further identified by the D-amino acid-oxidase test (Huennekens & Felton, 1957). The substrate used was DL-alanine. There was no uptake of oxygen by the apoenzyme and the uptake of oxygen was linear with graded amounts of enzymically synthesized FAD and also with standard FAD. These tests conclusively proved the identity of the reaction product to be FAD.

Stoicheiometry of the reaction. The stoicheiometry of the FAD synthesis by the plant enzyme was established by estimating FMN utilized and FAD synthesized at different time intervals.

From Table 2 it is evident that a stoicheiometric relationship exists between FMN used and FAD synthesized.

Progress curve of the reaction. The activity at different intervals of time was determined by incubating separate sets of reaction mixtures for the time intervals mentioned in Fig. 1. There is a linearity between FAD synthesized and time up to 60 min.

pH–*Activity curves.* The effect of pH on FAD synthesis was determined by carrying out the reactions in phthalate–HCl buffer (pH 4·0–6·0; 0·1 M), veronal–HCl buffer (pH 6·0–9·0; 0·1 M) and glycine–NaOH buffer (pH 9·0–10·0; 0·1 M). The results are presented in Fig. 2.

The enzyme has a pH optimum at 7·5 in 0·1 M-veronal–HCl buffer. The following buffers were tried at the optimum pH for the effect on FAD

Table 2. *Stoicheiometry of synthesis of flavinadenine dinucleotide*

Reaction mixtures (see text) were incubated, for the time intervals mentioned, at 37°.

Time (min.)	FMN used (μm-moles/ml.)	FAD synthesized (μm-moles/ml.)
10	16·58	16·26
20	39·23	38·50
30	54·01	52·17
60	84·10	83·21
180	92·34	91·82

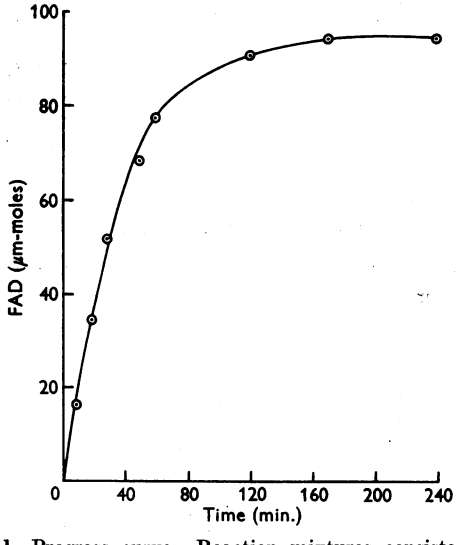


Fig. 1. Progress curve. Reaction mixtures consisted of FMN, 1 mM; ATP, 20 mM; NaF, 0·1 M; $MgSO_4$, 0·1 mM; veronal–HCl buffer (pH 7·4), 0·1 M; 0·8 ml. of enzyme preparation; total volume, 2 ml. Incubation was at 37° for the time intervals mentioned in the figure.

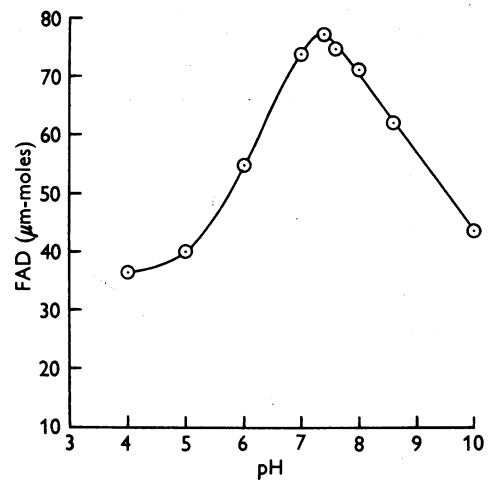


Fig. 2. pH–Activity curve. Reaction mixtures were the same as for Fig. 1 except that various buffers were used. Incubation was at 37° for 60 min.

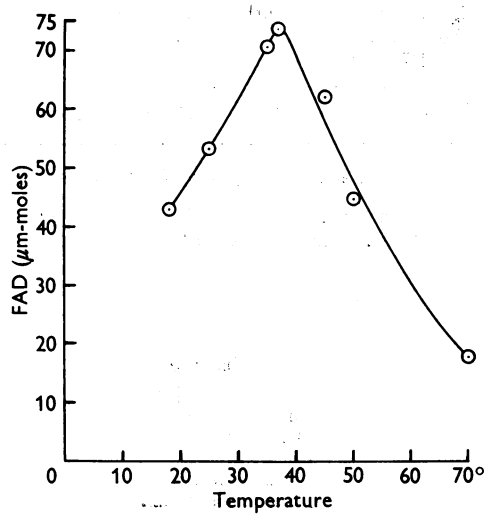


Fig. 3. Optimum temperature for activity. Reaction mixtures were the same as for Fig. 1, incubated at different temperatures for 60 min.

synthesis: 2-amino-2-hydroxymethylpropane-1:3-diol, sodium phosphate and veronal–HCl, and it was found that veronal–HCl buffer was the best.

Optimum temperature for activity. Variation of the initial rate of reaction as a function of temperature was determined by incubating the reaction mixtures at different temperatures (Fig. 3). The enzyme functions optimally at a temperature of 37°.

Substrate affinity of the enzyme. The Lineweaver–Burk plots for FMN and ATP are shown in Fig. 4. At a constant ATP concentration of 20 mM, varying concentrations of FMN were used in the reaction mixture. The enzyme functions optimally at an FMN concentration of 0·15 mM and the K_m calculated from Fig. 4 is 0·043 mM. At a constant FMN concentration of 0·20 mM varying concentrations of ATP were used in the reaction mixture. Maximum FAD synthesis was observed at an ATP concentration of 2·25 mM and K_m calculated to be 0·75 mM.

Substrate specificity. At the same molar concentration, ADP and adenosine monophosphate could not replace ATP. Riboflavin, lumiflavin or lumichrome could not replace FMN, at the same molar concentration.

Effect of ions. The various ions listed in Table 3 were tried for their effect on the FAD-synthesizing enzyme. Of the ions used, Mg^{2+} and Zn^{2+} activated at all of the three concentrations, whereas Mn^{2+} ions activated at the lowest but had no effect at mM concentration and inhibited at the highest concentration. Ni^{2+}, Cu^{2+}, Hg^{2+} and CN^- ions in-

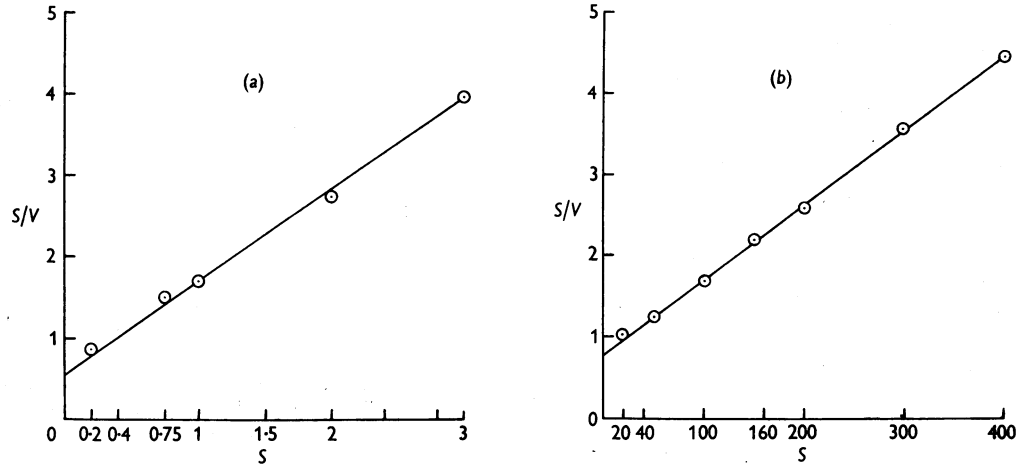


Fig. 4. Lineweaver–Burk plots. (*a*) Reaction mixtures contained 0·2 ml. of FMN, at concentrations indicated; other constituents were as given for Fig. 1; total volume, 2 ml. Incubation was at 37° for 60 min. *S*, Concn. of FMN (μM); *V*, μm-moles of FAD synthesized. (*b*) Reaction mixtures contained 0·1 ml. of ATP, at concentrations indicated; other constituents were as given for Fig. 1; total volume, 2 ml. Incubation was at 37° for 60 min. *S*, Concn. of ATP (mM); *V*, μm-moles of FAD synthesized.

Table 3. *Effect of ions on the flavinadenine dinucleotide-synthesizing enzyme*

Reaction mixtures were as follows: FMN, 1 mM; ATP, 20 mM; NaF, 0·1 M; ions at the concentrations indicated; veronal–HCl buffer (pH 7·4), 0·1 M; 0·8 ml. of enzyme; total volume, 2 ml. Incubation was at 37° for 60 min.

	Percentage inhibition (−) or activation (+) at different concentrations of ions added		
Substance	Concn. 0·1 mM	Concn. 1·0 mM	Concn. 10·0 mM
Magnesium sulphate	+12·5	+29·2	+49·2
Zinc sulphate	+4·2	+20·8	+29·4
Copper sulphate	−20·8	−16·7	−4·2
Cobalt acetate	−4·2	−4·1	−4·1
Nickel chloride	−20·8	−20·8	−12·5
Manganous sulphate	+12·5	+4·1	−20·8
Ferric sulphate	−7·5	−4·1	−4·2
Mercuric chloride	−29·1	−39·2	−40·7
Cadmium sulphate	+4·2	+4·6	+4·7
Potassium cyanide	−45·8	−50·8	−60·2

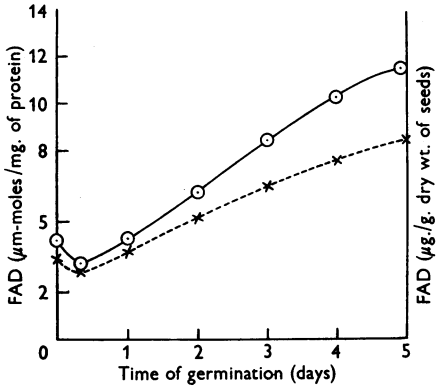


Fig. 5. Effect of germination on FAD-synthesizing activity (top curve) and FAD content of green gram (*Phaseolus radiatus*) (bottom curve). Reaction mixtures were the same as those given for Fig. 1, and were incubated at 37° for 60 min.

Table 4. *Occurrence of flavinadenine dinucleotide-synthesizing activity in plants*

Reaction mixtures were as follows: 0·2 ml. of FMN, 1 mM; 0·1 ml. of ATP, 20 mM; 0·1 ml. of NaF, 0·1 M; 0·1 ml. of $MgSO_4$, 0·1 mM; 0·7 ml. of veronal–HCl buffer (pH 7·4), 0·1 M; 0·8 ml. of enzyme from stage II; total volume, 2 ml. Incubation was at 37° for 5 hr. Sources 1–12 were resting seeds.

Source	Activity (μm-moles of FAD synthesized/ml. at 37° for 5 hr.)
1. *Cicer arietinum* (Bengal gram)	60·3
2. *Ricinus communis* (castor)	72·1
3. *Brassica campesteris* (mustard)	72·1
4. *Phaseolus mungo* (black gram)	76·7
5. *Phaseolus radiatus* (green gram)	92·1
6. *Dolichos biflorus* (horse gram)	82·1
7. *Dolichos lablab* (field bean)	93·8
8. *Cannavalia gladiata* (sword bean)	76·2
9. *Pisum sativum* (peas)	82·1
10. *Sesamum indicum* (sesame)	82·1
11. *Cajanus indicus* (red gram)	76·1
12. *Arachis hypogea* (groundnut)	60·3
13. *Solanum tuberosum* (potato)	58·1
14. *Ipomea batatus* (sweet potato)	52·7

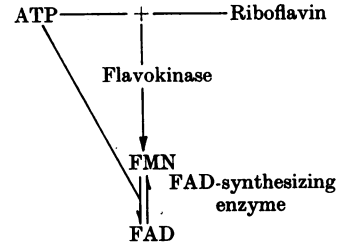
hibited at all the concentrations, whereas Co^{2+}, Fe^{3+} and Cd^{2+} ions had no effect.

Effect of germination on flavinadenine dinucleotide-synthesizing enzyme activity. In view of the observation that riboflavin content of the seedlings increases many-fold during germination (Burkholder & McViegh, 1942; Simpson, Chow & Soh, 1953; Giri *et al.* 1958), seeds were allowed to germinate in the dark, in Petri dishes. They were placed on moist cotton covered with filter-paper disks cut to the size of the Petri dishes. At definite intervals of time they were taken out, homogenized and the supernatants obtained on centrifuging at 1000 *g* for 10 min. were dialysed for 18 hr. in the cold. The supernatant obtained on centrifuging the dialysed extract at 3000 *g* for 5 min. was used as the enzyme. FAD content was determined on a parallel set of seedlings. The increase in FAD concentration paralleled the enzyme activity during germination for 6 days (Fig. 5).

Distribution of flavinadenine dinucleotide-synthesizing enzyme in plants. Fourteen plant materials were examined for this enzyme. All the plant sources investigated showed the presence of this enzyme activity (Table 4).

DISCUSSION

In the light of the experimental evidence presented in this paper the following scheme for the biosynthesis of FAD in plants appears probable:



Starting with riboflavin, FMN is synthesized by means of an irreversible reaction with ATP (Giri *et al.* 1958). The FMN so synthesized is converted into FAD by a reversible reaction with ATP.

SUMMARY

1. The occurrence of flavinadenine dinucleotide-synthesizing enzyme has been demonstrated in plants and its properties have been studied by the application of circular-paper chromatography. Fourteen plants were examined and the detailed study was confined to one of them, *Phaseolus radiatus*.

2. Under the experimental conditions described, maximum synthesis of flavinadenine dinucleotide was observed at pH 7·4. The Michaelis constant for flavin mononucleotide was 0·043 mM and adenosine triphosphate was 0·75 mM.

3. Mg^{2+} and Zn^{2+} ions activated at all the concentrations studied, whereas Mn^{2+} ions activated at the lowest concentration, but had no effect at mM and inhibited at the highest concentration. Ni^{2+}, Cu^{2+}, Hg^{2+} and CN^- ions inhibited at all the concentrations, whereas Co^{2+}, Fe^{3+} and Cd^{2+} ions had no effect.

4. An 83-fold purification of the enzyme was achieved by dialysis, fractionation with ammonium sulphate, adsorption and elution from alumina $C\gamma$-gel, and precipitation with ethanol.

5. There was a parallel increase in flavinadenine dinucleotide content and synthesizing activity for this compound with the progress of germination.

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