JOURNAL OF
THE
INDIAN INSTITUTE OF SCIENCE

VOLUME 43 JANUARY 1961 NUMBER 1

STUDIES ON THE OCCURRENCE, CHANGES DURING GERMINATION AND DISTRIBUTION OF THE FLAVIN NUCLEOTIDE COENZYMES IN SEEDS

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Received on September 12, 1960

Abstract

Although the occurrence of riboflavin in plants is well known, there is no information on the forms in which it occurs in plants. The present paper deals with the riboflavin nucleotide contents of some resting seeds and the changes in their quantitative make up during germination. The effect of light on the synthesis of flavin nucleotides has been reported and discussed.

The flavin content is largely made up of FAD. There is very little free riboflavin in resting seeds. The radicle of the germinating seedlings contain almost all the FAD and the major portion of the flavins in the seedlings, while the plumule contains FMN as the major flavin.

It is well known that the vitamins of the B-complex group occur widely distributed in higher plants (Bonner, 1942). A good summarised description of the behaviour of B-vitamins during germination is given by Bonner and Bonner (1948). Burkholder and McVeigh (1945) have reported an appreciable increase in the riboflavin content of a variety of plant material on germination. Simpson, Chou and Soh (1953) have studied the changes and distribution of riboflavin and thiamine in the germinating seedlings of mung bean (*Phaseolus aureus*). Cheldelein and Lane (1943), Burkholder and McVeigh (1945) and Naik and Narayana (1954) have studied the effect of light on the riboflavin content of the germinating seedlings. All these workers used either the micro-biological assay method described by Snell and Strong (1939) or the

fluorimetric method after acid hydrolysis. These methods are incapable of distinguishing between the free vitamin and its nucleotides.

No information is available on the quantitative make up of the riboflavin nucleotide content of plants. Bessey, Lowry and Love (1949) and Burch, Lowry, Pedilla and Combs (1956) have shown that riboflavin occurs in animal tissues as its nucleotides with little or no riboflavin.

In this paper are presented the riboflavin nucleotide contents of eight pulse seeds, the changes during germination of these in two plants and the distribution of these nucleotides in the various parts of the seedlings of *Phaseolus radiatus*.

EXPERIMENTAL

Riboflavin and FMN were Hoffman La Roche products; FAD was was obtained as a gift from Messrs. Essai Ltd., Japan. The flavins were chromatographically purified before use. The seeds were purchased from the local market. All the solvents were redistilled before use.

Estimation of flavin nucleotides in plants:—The method employed was essentially that of Bessey *et. al.* (1949) with modifications to suit the estimation of the nucleotides in plant material and is briefly outlined. The plant material was thoroughly ground with ten times its volume of ice-cold water in a pestle and mortar, chilled in an ice-salt mixture. It was found that extraction with 10 times the volume is sufficient, since there was no increase in the flavin content by extraction with larger volumes (25 to 50 times) recommended by Bessey *et. al.* The cold suspension was immediately deproteinised by the addition of an equal volume of 20% *w/v* trichloracetic acid (TCA). After 15 minutes the extract was centrifuged at 2,000 g. for 10 minutes. An aliquot of the supernatant liquid was immediately neutralised with one-fourth its volume of 4 M. K_2HPO_4. During these processes, extreme care was taken to maintain the temperature between 0° and 4° to prevent the hydrolysis of FAD. A second aliquot of the supernatant was stored in the dark at 37° for 18 hours to hydrolyse FAD. After hydrolysis it was neutralised with K_2HPO_4 as described earlier. Care was taken to prevent undue exposure to light during these procedures as the flavins are extremely photolabile at these high salt concentrations. The fluorescence of both the neutralised extracts was measured in a Klett fluorimeter using primary B_2 and orange filters (Lamp: Corning 5113 + 3389 and photocell, Corning 3486). The blanks were obtained by quenching the fluorescence of the extracts with 0·1 ml. of 10% sodium dithionate in 5% sodium bicarbonate. The apparent riboflavin was calculated by reference to the standard curve obtained by using increasing concentratrations of riboflavin as internal standards in the same aliquot. FAD (calculated as riboflavin) has a fluorescence equal to 15% of riboflavin, whereas riboflavin and FMN have equal fluorescence, under these high salt concentrations. Therefore, if the

apparent riboflavin of the initial unhydrolysed sample is R_i and that of the latter, hydrolysed sample is R_t,

$$\text{FAD} = (R_t - R_i)/0.85 \qquad [1]$$

The balance is composed of FMN and free riboflavin, *i.e.*,

$$\text{Total riboflavin } (R_t) = \text{FAD} + \text{non FAD riboflavin.} \qquad [2]$$

Free riboflavin was determined as follows. An aliquot of the initial unhydrolysed sample was neutralised and thoroughly shaken with an equal volume of water saturated benzyl alcohol. The partition coefficients for riboflavin FMN and FAD between water saturated benzyl alcohol and 10% TCA neutralised with K_2HPO_4, are 4.1 ; 0.032 and 0.020, respectively. Since FAD has a fluorescence approximately 60% of that of riboflavin in a mixture of benzyl alcohol and 45% ethanol, the apparent riboflavin in benzyl alcohol extract $(R_{BZ}) = 4.1/5.1$ free riboflavin + $0.032/1.032$ FMN + $0.02/1.02 \times 0.6$ FAD [3]
Since, $R_{\text{non FAD}}$ = FMN + free riboflavin,

$$\text{Free riboflavin} = 1.30\ R_{BZ} - 0.040\ R_{\text{non FAD}} - 0.015\ \text{FAD} \qquad [4]$$

To avoid the interfering flourescence of benzyl alcohol, the benzyl alcohol layer was re-extracted with 15 volumes of toluene and 1 volume of a solution which is 0.05 M with respect to both acetic acid and sodium acetate. All the riboflavin was quantitatively extracted into the aqueous layer. The fluorescence of the water extract was measured as before. Since the fluorescence of FAD is lesser in aqueous solutions, a correction in the above equation [4], changing 0.015 to 0·003 FAD was applied. Since very little free riboflavin was present, non-FAD riboflavin = FMN. Total flavins were determined by the microbiological method using *Lactobacillus casei* 7584 Snell and Strong (1939) and the values obtained were in agreement with those obtained by the method described above.

Recovery of added flavins:—Riboflavin, FMN and FAD were added to the cold homogenates before extraction with TCA at three different concentrations *viz.*, 10 μg, 20 μg and 30 μg per gram of seeds. The recovery of the added flavins averaged 90 to 95%.

Results and Discussions

Occurrence of flavin nucleotides in seeds:—The seeds were homogenised in cold at 0 to 5° and the homogenate was added to the split D-amino acid oxidase prepared as described by Huennekens and Felton (1957). The D-amino acid oxidase activity was restored by the addition of seed homogenates. This test indicated the presence of FAD in the seed homogenates.

The seed homogenates were deproteinised with an equal volume of 20% *w/v* TCA. The TCA extracts were neutralised with 4M. K_2HPO_4 and ammonium sulphate was added to saturation. This extract was centrifuged in the cold at

2000 g. for 15 minutes. The supernatant (100 ml.) was extracted with phenol (20 ml.). The phenol layer was partitioned between ether (200 ml.) and water (5 ml.). The acqueous layer was concentrated *in vacuo* over phosphorus pentoxide in the cold. The concentrated extract was subjected to preparative circular paper chromatography (Giri, 1954, 1955) using Butanol-Acetic acid-Water 4 : 1 : 5 *v/v* as the solvent system. The flavins with R_f values 0.40 and 0.25 were located and marked out under an U.V. lamp. The bands were cut and eluted into glass distilled water. Eluate of band with R_f value 0.40 was subjected to chromatography with an authentic sample of FMN in three different solvent systems, *viz.*, *n*-butanol-acetic acid-water 4:1:5 *v/v*; *n*-butanol-*n*-propanol-water 2:2:1 *v/v* and *n*-butanol-formic acid-water 77:10:13. The eluate moved as a single band in all the three solvents, and had absorption maxima at 263, 373 and 445 $m\mu$. On concentration and acid hydrolysis of the eluate with NH_2SO_4 at 100° for 4 hours riboflavin was the only flavin detected. Phosphate was detected in the extract by the method of Fiske and Subbarow (1925). Eluate of the band with R_f value 0.25 had absorption maxima at 263, 375 and 450 $m\mu$. On cochromatography with an authentic sample of FAD, in the three different solvents mentioned above it moved as a single spot. On acid hydrolysis with 6 N. HCl for 15 minutes at 120° in an autoclave, neutralisation and concentration gave a positive test for 4-amino-5-imidazole carboxamidine identified by spraying the chromatograms with the Follins reagent. This indicated the presence of the adenine moiety in the flavin. The eluate was hydrolysed by crude extracts of *Phaseolus radiatus* and FMN and riboflavin were detected on the chromatograms. These tests conclusively prove the occurrence of FMN and FAD in the seed extracts.

Flavin nucleotide content of the resting seeds:—Eight pulses listed in Table I were examined for their flavin nucleotide content. The major flavin in the

TABLE I
Flavin Nucleotide Content of Seeds
(Average of 10 samples)

Plant	FMN *	FAD *	Total *
	Microgram per gram weight seeds dry		
Cicer arietinum (Bengal gram)	0.41	0.57	0.98
Dolichos biflorus (Horse gram)	0.51	0.76	1.27
Dolichos lablab (Field bean)	0.58	1.43	2.01
Phaseolus radiatus (Green gram)	0.10	3.30	3.40
Phaseolus mungo (Black gram)	0.12	1.90	2.02
Phaseolus vulgaris (French bean)	1.11	2.01	3.12
Pisum sativum (Peas)	1.20	0.90	2.10
Vigna catiang (Cow pea)	0.20	2.40	2.60

* Calculated as riboflavin.

seeds was FAD with varying amounts of FMN. No free riboflavin could be detected in the resting seeds of the plant sources examined.

Effect of germination on the flavin nucleotide content of Phaseolus radiatus *and* Vigna catiang :—Equal amounts of seeds were allowed to germinate in direct sunlight, diffused day light (inside the laboratory) and in total darkness. The seeds were placed on wet cotton covered with a filter paper cut to the size of the petri dishes. Water was frequently sprinkled to keep the filter paper moist. At the end of definite intervals of time, they were taken out and the flavin content determined as described earlier. The changes in the total flavin content of the two plant sources are presented in Fig. I. There is almost a three-fold increase in the

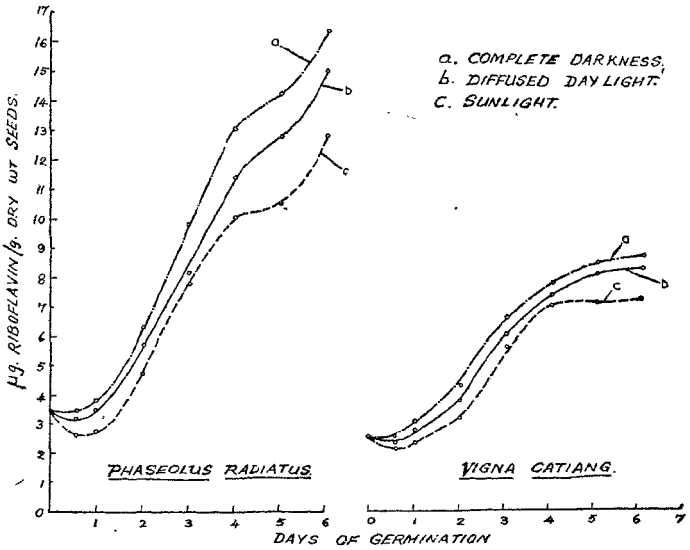

FIG. I
Effect of Light on Total Flavin Content during Germination of *Phaseolus Radiatus and Vigna Catiang*

total flavin content during germination for six days. In the very early stages of germination (upto about 12 hours) there is a small decrease in the flavin content which is possibly due to the utilisation of the stored flavin for metabolic purposes and no synthesis occurs at this stage. After about 24 hours of germi-

nation, a rapid increase in the flavin content is observed till the fifth day. Both FMN and FAD increase simultaneously (Fig. II and III). The increase is

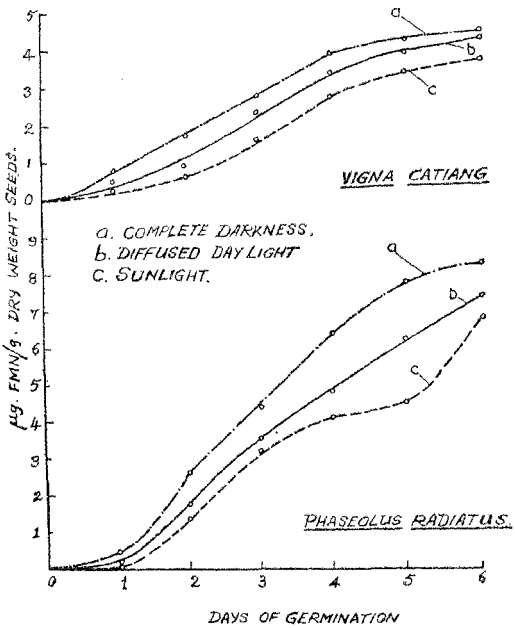

FIG. II

Effect of Light on FMN Content during Germination of *Vigna Catiang and Phaseolus Radiatus*

very rapid upto fifth day. After the fifth day the increase is not as rapid as before. At this stage a small quantity of riboflavin was detected, but it was not sufficient for accurate quantitative evalution. Our findings that free riboflavin is absent in resting seeds and during the early stages of germination suggest that during this period of high metabolic activity, the riboflavin synthesised by the plant is being converted immediately into coenzymatic forms. Giri, Appaji Rao, Cama and Kumar (1959) have demonstrated the occurrence of

enzymes synthesising FMN and FAD in plants. They have shown that there is a positive correlation between FMN and FAD synthesising activities and the total flavin content during germination.

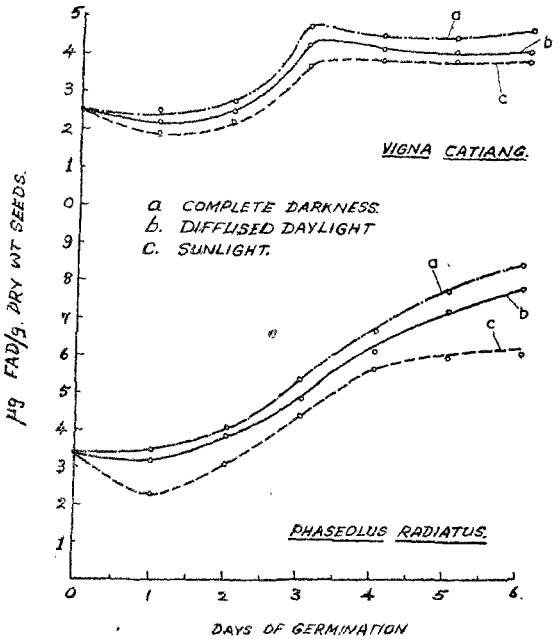

FIGURE III

Effect of Light on FAD Content during Germination of *Vigna Catiang and Phaseolus radiatus*

Maximum synthesis occurs when the seeds are grown in the dark. Sunlight seems to have an inhibitory effect. Gustafson (1948) studied the effect of light on the riboflavin content of chlorophyll-free albino corn plants and found that plants grown in total darkness had more riboflavin than those grown in light. Similar observations have been made by Galston (1949, 1950), Galston and Baker (1951) and Naik and Narayana (1954). This might be either due to decreased synthesis or photo-decomposition of flavins.

The distribution of the flavin nucleotides was studied in diffused day light in the seedlings of *Phaseolus radiatus*. The plumule, radicle and cotyledons were dissected out and their nucleotide content determined. The results are presented in Table II. The major flavin in the plumule is FMN and in the

TABLE II

Distribution of flavin nucleotides in germinating seedlings of *Phaseolus radiatus* (Average of 4 readings)

Days	Plumule		Radicle		Cotyledons		Total	
	FMN*	FAD*	FMN*	FAD*	FMN*	FAD*	FMN*	FAD*
	Microgram per gram dry weight of seeds							
1	.....	.....	0.00	0.93	0.80	1.10	0.80	2.03
2	0.95	0.00	0.00	3.80	1.66	1.20	2.61	5.00
3	1.90	0.00	0.28	4.50	2.13	0.72	4.31	5.19
4	2.75	0.80	0.68	4.68	1.10	1.08	4.56	6.60
5	2.75	0.83	2.18	5.46	0.95	0.00	5.88	6.30
6	4.50	1.66	1.86	6.66	.....	.....	6.72	8.33

* Calculated as riboflavin.

radicle, FAD. During germination there is an enormous increase in the flavin contents of the embryo. Thus after five days of germination, the plumule and the radicle contain 29.4 % and 62.76 %, respectively of the total flavins of the seedlings. The radicle contains 86.68 % of the total FAD and 37.08 % of the total FMN, while the plumule contains 46.77 % of the total FMN and 13.17 % of the total FAD.

Simpson, Chou and Soh (1953) have shown by photagraphic examination of the seedlings of *Phaseolus aureus*, higher concentrations of riboflavin in the cells of the radicle than in the cotyledons. Wai, Bishop, Mack and Cotton (1947) have reported that in three day-old soybean seedlings the hypocotyl portion accounted for 20 % of the total riboflavin content of the seedlings. Our observation that 60 % of the flavins present in the seedlings are concentrated in the radicle and 30 % in the plumule, supports the fact that growing parts synthesise the flavins. Bonner (1941) has reported the synthesis of riboflavin by isolated root cultures of tomato, clover and alfalfa. Gustafson (1954, 1955) has also shown that excised roots of white lupine seedlings can synthesise riboflavin, when grown in a synthetic medium. Naik and Narayana (1954) have reported that on removal of the radicle, the plants ceased to synthesise the flavins. Therefore, it is very probable that the radicle is the site of synthesis of the flavins.

However, although the mechanism of the biosynthesis of riboflavin is not yet clear, with the demonstration of the enzymes synthesising the flavin nucleotides, and the occurrence of flavin nucleotides it is reasonable to suggest that this has a profound influence on the intermediary metabolism of the plant by its effect on enzymes.

ACKNOWLEDGEMENT

The authors wish to acknowledge the financial aid received from the Council of Scientific and Industrial Research (India) and thank Dr. C. S. Vaidyanathan for his helpful suggestions.

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