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The Flavin Content of Clovers Relative to Symbiosis with a Riboflavin-requiring Mutant of *Rhizobium trifolii*[1]

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ABSTRACT

A riboflavin-requiring auxotroph of *Rhizobium trifolii* (T1/D-his^r-15) formed ineffective root nodules on red clover and on two cultivars of subterranean clover, but produced almost fully effective nodules on several other cultivars of subterranean clover. Fluorescence and bioassay measurements of the flavin content of the roots and shoots of these cultivars revealed no differences between cultivars which could be correlated with the differences in symbiotic response. The concentration of flavin in nodules formed by the auxotroph (in the absence of riboflavin), by the effective parent strain (T1), or by a partly effective mutant (penicillin-resistant) of T1 was roughly proportional to the effectiveness of the nodules. Effective nodules contained 20 times as much flavin, and ineffective nodules 3 to 4 times as much flavin as non-nodulated root tissue. Approximately 20 to 30% of the flavins in both root and nodule tissue was flavin adenine dinucleotide and 70 to 80% was riboflavin + flavin mononucleotide. Most of the flavin adenine dinucleotide in macerated nodules was associated with host cell fragments, and none was detected in a cell-free fraction. Bacteroids accounted for approximately 20% of the flavins in effective nodules and also contained more riboflavin + flavin mononucleotide than cultured rhizobial cells. The total flavin content of noninoculated roots increased from about 1.2 nmoles to 1.7 nmoles flavin/g of tissue after 3 days' exposure to 80 μM riboflavin. Exposure of only the upper or lower portion of preinoculated roots indicated negligible translocation, as effective nodulation occurred only on parts of the root in direct contact with riboflavin. Plants grown in a medium containing combined nitrogen (100 or 300 μM nitrogen added as $(NH_4)_2SO_4$), but no added riboflavin showed an increased root flavin content (about 2.1 nmoles flavin/g tissue) and a partly effective response when inoculated with the mutant. Nitrogen also promoted some upward translocation of exogenous riboflavin in the roots.

Requirement for riboflavin is known to be associated with loss of symbiotic effectiveness (ability to produce nodules capable of fixing nitrogen) in a mutant strain of *Rhizobium trifolii* (10, 11). Effectiveness on clover could readily be restored genetically in this mutant by back mutation to nonrequirement, but fully effective nodulation could also be accomplished by simply adding riboflavin or flavin nucleotides to clover plants inoculated with the auxotroph. The unaided auxotroph produced ineffective nodules on two cultivars each of red clover (*Trifolium pratense* L.) or white clover (*T. repens* L.) but produced almost fully effective or partly effective nodules on several cultivars of subterranean clover (*T. subterraneum* L.). On the basis of these results, it was suggested (11) that "the ability of the mutant to differentiate between different clover hosts may be related to content or availability of riboflavin or riboflavin-containing compounds in the host." Subsequent nodulation tests (unpublished data) have also shown the mutant to be fully ineffective on two cultivars of subterranean clover. The occurrence of contrasting nodulation responses in different cultivars of one species of clover (*T. subterraneum* L.) facilitated the present study, which examines the following: (*a*) the possibility of a direct relationship between flavin content of the host and degree of effectiveness of nodulation by the riboflavin auxotroph; (*b*) the distribution of flavins in nodule tissue; (*c*) the effect of the plant growth status, namely presence or absence of combined nitrogen, on the symbiosis and on flavin content; (*d*) the uptake and translocation (in the roots) of exogenous riboflavin.

MATERIALS AND METHODS

Cultures of *Rhizobium trifolii*. Cultures included the parent strain T1 and two mutants derived from T1, namely, T1/pen^r-4 (penicillin-resistant) and the riboflavin-requiring auxotroph T1/D-his^r-15 (D-histidine resistant) (9, 10). These were maintained on a glucose-salts-yeast extract agar medium (8).

Cultured rhizobia used for flavin analyses were grown in modified Bergersen's (1) broth (5 g mannitol, 0.5 g Na glutamate/l). Riboflavin was added to this minimal medium at 0.25 μM for growth of the auxotroph. Broth cultures were incubated on a shaker at 29 C for 48 hr. Centrifuged cells were weighed and sonicated for 5 min (Bronwill "Biosonik," Rochester, N. Y.), and flavins were extracted from sonicates by the methods described below.

Plant Culture. The following plant hosts were used in nodulation experiments for flavin assays: *Trifolium pratense* L. (red clover) cv. Kenland; *T. subterraneum* L. ssp. *subterraneum* Katzn. et Morley (subterranean clover) cv. Dwalganup, cv. Mt. Barker; *T. subterraneum* L. ssp. *brachycalycinum* Katzn. et Morley cv. Clare; and *T. subterraneum* L. ssp. *yanninicum* Katzn. et Morley cv. Yarloop. In experiments not concerning

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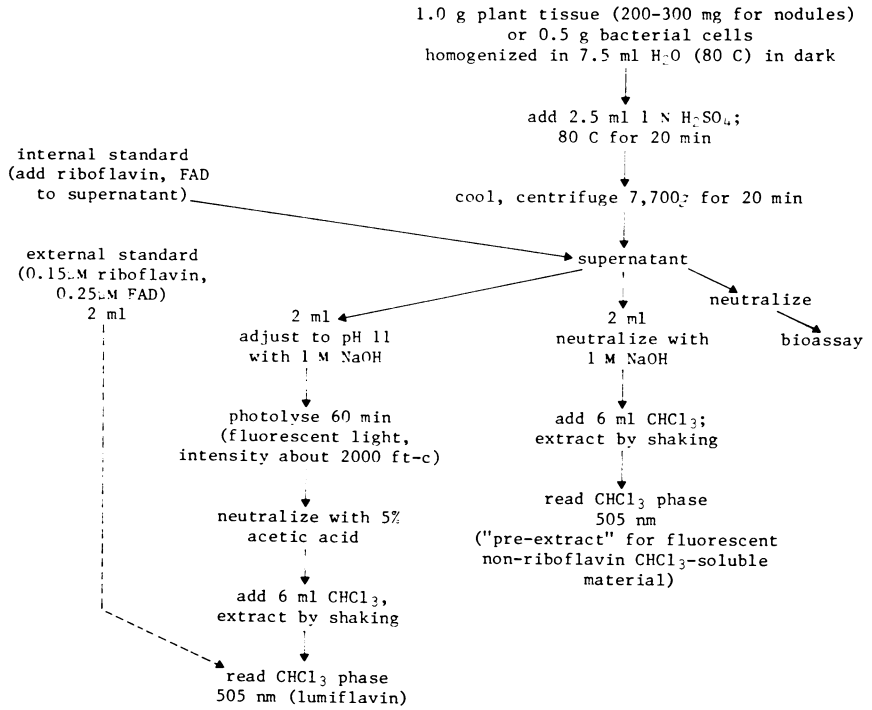

FIG. 1. Flow diagram of the modified lumiflavin fluorescence method for the determination of the total flavin content of plant tissues. The procedure for the external standard was the same as that for the lumiflavin sample.

translocation of flavin, seedlings were grown in sterile vermiculite in 140-ml glass jars as described previously (10). Seedlings were inoculated 8 days after germination with about 1×10^8 bacteria/ml of N-free plant solution in the jars. When required, riboflavin solution sterilized by Millipore membrane (0.45 μm pore size) filtration was added to the jars at the time of inoculation, to give an initial concentration of 20 μM.

For study of translocation of added riboflavin, a plant growth technique was developed in which the upper and lower root system of individual plants, grown aseptically, could be treated separately. Beakers (50 ml) wrapped in aluminum foil (to exclude light) and containing a separate small glass vial attached to the inside edge of the beaker were autoclaved. Six-day-old seedlings germinated in vermiculite were then transplanted to these containers (containing sterile N-free plant nutrient solution) by inserting the root through a slit in the aluminum foil covering the beaker. The seedling root was then placed so that the upper half of the root system was in the inner vial. In this way, the upper or the lower root system could be separately exposed to riboflavin or nitrogen as described under "Results."

In all experiments, the seedlings were maintained in a glasshouse under the following conditions: 22 C day (18-hr light period), 16 C night (6-hr dark period); light intensity about 1000 ft-c from a combination of warm white and daylight fluorescent tubes. Host plant response and type of nodules (designated as "level of effectiveness" in the tables) produced on the inoculated clover seedlings were rated visually as follows (9, 10): E (effective), E^- (slightly less than fully effective), PE (partly effective), I^+ (slightly better than ineffective), and I (ineffective).

Analysis of Flavins. The lumiflavin fluorescence method of Yagi (16) was used to estimate the total flavin content of plant tissues (shoots, roots, and nodules) or bacterial cells. This method employs the photoconversion of riboflavin and riboflavin-like compounds to lumiflavin and the extraction of lumiflavin with chloroform. Details of the procedure (including minor modifications) used in the experiments are outlined in Figure 1. Flavin adenine dinucleotide, as distinct from riboflavin plus flavin mononucleotide, was determined by the trichloroacetic acid extraction method of Bessey *et al.* (3). This method involves the trichloroacetic acid hydrolysis of FAD[3] to FMN and allows the determination of the total flavin content of a sample as well as FAD *versus* riboflavin + FMN content.

In all experiments, triplicate extracts were individually scanned over the range 580 to 500 nm in a fluorescence spectrophotometer using an excitation wavelength of 450 nm. Measurements were made at the 505 nm fluorescence peak for lumiflavin, and at 530 nm for trichloroacetic acid extracts. Solutions of 0.15 μM riboflavin and 0.25 μM FAD were used as standards for each experiment. The total flavin content of samples in the lumiflavin fluorescence method was calculated with reference to the fluorescence values (arbitrary units) obtained for standard riboflavin samples following photolysis to lumiflavin. In the trichloroacetic acid method, the total flavin content of extracts before and after hydrolysis (38 C, overnight) was first determined in terms of nmoles of riboflavin/g tissue. The FAD content was then obtained from the differences of these two values, corrected for fluorescence of FAD (equal to 15% of that of riboflavin at pH 6.8) in the unhydrolyzed sample (3).

Experiments involving estimation of the flavin content of root nodule fractions were done as follows. Nodules (400–500 mg) were gently crushed (mortar and pestle) in 4.5 ml of 25 mM Na-phosphate buffer, pH 7.0, containing 0.3 M sucrose and 2 mM $MgSO_4$. The crushed material was centrifuged at 480*g* for 5 min to remove the nodule debris (mostly plant cell fragments). The supernatant was then centrifuged at 12,000*g* for 20 min to sediment the bacteroids. All three fractions (nodule debris, cell-free fraction, bacteroids) were then extracted

[3] Abbreviations: FAD: flavin adenine dinucleotide; FMN: flavin mononucleotide.

Table I. *Total Flavin Content (Lumiflavin) of Shoots and Non-nodulated Roots of Five Clover Cultivars*

Symbols used to indicate levels of effectiveness are explained under "Materials and Methods." Mean values are based on three replicates, expressed as nmoles riboflavin/g tissue (fresh wt).

Cultivar	Level of Effectiveness with T1/D-his^r-15	Total Flavins			
		8-day-old seedlings[1]		14-day-old seedlings[2]	
		Shoots	Roots	Shoots	Roots
Dwalganup	E^-	2.26	1.06	2.36	1.09
Mt. Barker	E^-	2.20	1.01	2.60	1.14
Clare	I	2.44	0.91	2.58	0.93
Yarloop	I	2.02	0.98	2.20	1.17
Kenland	I^+	2.10	0.98	2.23	1.06

[1] LSD (0.05): 0.21.
[2] LSD (0.05): 0.31.

with either hot 0.25 N H_2SO_4 (for lumiflavin assay, Fig. 1) or cold 20% trichloroacetic acid (for trichloroacetic acid hydrolysis). Bacteroids were sonicated for 5 min prior to extraction.

Bioassays. Bioassay experiments using the T1/D-his^r-15 auxotroph as the test organism for the presence of riboflavin, FMN, and FAD were routinely done to supplement the fluorescence measurements, and to check for possible activity of nonfluorescing flavin precursors in extracts. The extracts were neutralized, sterilized by exposure to chloroform for 30 min, and added to wells cut into minimal agar which had been surface-layered with T1/D-his^r-15. The diameter of zones of growth of the auxotroph (grown in the dark) were measured and the effective flavin concentration of test samples was estimated from similar assays of a series of concentrations of a riboflavin standard.

Acetylene Reduction by Nodulated Root Systems. The acetylene reduction technique, involving the reduction of acetylene to ethylene, was used to assay nitrogenase activity in the nodules (5). Nodulated roots from three plants of a given treatment were placed in glass vials (13.5-ml capacity) sealed with air-tight rubber stoppers. The vials were evacuated and filled with a gas mixture of O_2-C_2H_2-Ar (1:1:3), then incubated at 22 C for 30 to 60 min. Analysis of C_2H_2 and C_2H_4 was done with a Philips P4000 gas chromatograph (2).

RESULTS

Total Flavin Content of Noninoculated Seedlings of Different Clover Cultivars. To examine the possibility that a relationship may exist between the flavin content of different clover cultivars and the effectiveness of nodules formed on them by the auxotroph, the flavin content of non-nodulated shoot and root tissue was determined.

Fluorescence measurements showed significant but small differences between the five clover cultivars examined in the total flavin content of shoot tissue, but not root tissue (Table I). Shoot tissue of all cultivars contained about 2.1 times more extractable flavin than the root tissue while the concentration (nmole/g tissue) of both tissues increased slightly over an 8- to 14-day period of growth. Further analysis of the flavins of roots (14 days old) showed no significant differences between cultivars in relative amounts of FAD and riboflavin + FMN. The mean values for all cultivars with respect to these two flavin fractions were 0.36 nmole and 0.83 nmole of flavin/g tissue (fresh weight).

Although the roots of cultivars like Clare and Kenland contained as much total flavin as roots of Dwalganup, it had earlier (11) been shown that addition of riboflavin to roots was a prequisite for effective nodulation of Clare and Kenland by the auxotroph. The possibility that roots of these cultures absorbed and concentrated exogenous riboflavin to levels much higher than those normally found in root tissue was therefore examined with plants grown in vermiculite in the presence of several levels of exogenous riboflavin. Riboflavin was added to the roots of 11-day-old seedlings at concentrations of 1.5, 20, and 80 μM, and uptake by the roots was determined 3 days later. This period of exposure (11–14 days after planting) was chosen because it corresponds to the time when nodules normally appear on seedlings inoculated 8 days after planting, and because this is the most critical period of flavin requirement for effective symbiosis by T1/D-his^r-15 (6). The results of one of these experiments (Table II) show that there was only a small increase in riboflavin content in the roots of all three cultivars. All were similar in their maximum increase (per cent increase, relative to controls) of tissue flavin in the 80 μM riboflavin treatment. However, the relative uptake by roots of Dwalganup differed significantly from that of Clare and Kenland at the lowest (1.5 μM) concentration of added riboflavin. Since the auxotroph produces effective type nodules on Dwalganup in the absence of exogenous flavin, this difference in uptake could indicate greater root tissue permeability or more uniform distribution in the root cells of this cultivar. The general low level of uptake was not due to lability of the compound in the substrate as analysis showed that 70 to 80% of the flavin was still present in the substrate at the end of the 3-day interval.

To confirm the fluorescence measurements, the flavin content of neutralized tissue extracts of the cultivars was also estimated by bioassay. The bioassays were also made to check for the possible occurrence of nonfluorescent riboflavin precursor compounds utilizable by the auxotroph.

The values obtained for total flavin in extracts by this method were somewhat lower (about 20%) than corresponding values obtained by the fluorescence method but the relative amounts of total flavin in the shoots versus roots were the same (shoot to root ratio of 2.1:1). As found in the fluorescence assays (Table I), there were no differences between cultivars in root flavin content and only minor differences in shoot flavins. In addition, the results minimize the possibility that cultivars on which the auxotroph is effective could contain appreciable amounts of nonfluorescent riboflavin precursors.

Table II. *Uptake of Externally Added Riboflavin by the Roots of Three Clover Cultivars*

Mean values are based on three replicates, expressed as nmoles riboflavin/g tissue (fresh wt).

Treatment	Total Flavin Content of Roots[1]					
	Dwalganup		Clare		Kenland	
	Total flavin	Increase	Total flavin	Increase	Total flavin	Increase
		%		%		%
Control roots	1.17		1.14		1.30	
Riboflavin added (concn in substrate)						
1.5 μM	1.49	27.3	1.11	0.0	1.38	6.1
20.0 μM	1.62	38.4	1.51	32.5	1.48	13.8
80.0 μM	1.64	40.2	1.60	40.3	1.80	38.5

[1] LSD (0.05): 0.29; LSD (0.01): 0.41.

In a companion experiment, hot (80 C) *versus* cold (ice) extracts and acid (0.25 N H_2SO_4) *versus* nonacid extracts of 8- and 14-day-old root tissue of the different cultivars were tested by bioassay for possible differences in the release of tissue-bound (protein-bound) flavins. Comparable growth stimulation of the auxotroph was obtained for all cultivars in all treatments. There was thus no evidence for incomplete extraction in the fluorescence assays to account for the observed differences in growth of the auxotroph in roots of the various cultivars.

In another series of experiments, surface-sterilized (chloroform-treated) segments of roots were placed on the surface of minimal agar plates seeded with the auxotroph, to test for possible differences between cultivars in the amount of flavin diffusing from the intact or cut portions of the root. All root segments stimulated the growth of the auxotroph in a 2 to 4 mm zone adjoining their intact or cut surfaces, but no differences were found between cultivars. A similar result was obtained when seedlings of the various cultivars were aseptically germinated and grown on the surface of agar plates seeded with the auxotroph.

Total Flavin Content of Nodules Produced by *Rhizobium* Strains T1, T1/D-his^r-15, and T1/pen^r-4 on Three Clover Cultivars. Following the failure to demonstrate any significant difference between cultivars in total flavin content of non-nodulated root tissue, the flavin content was determined for root nodules produced on three cultivars by the parent strain T1, the auxotroph T1/D-his^r-15 (in the presence or absence of riboflavin), and by a penicillin-resistant mutant of T1, T1/pen^r-4. T1/pen^r-4, not auxotrophic, formed partly effective nodules on all three cultivars and was included here to represent an intermediate defective nodule type where the block in the symbiosis was due to factors other than flavin availability. The aim of these comparisons was to ascertain whether the observed differences in the cultivar response to the auxotroph were confined to the nodules, and to compare the "natural" concentration of flavins in different types of nodules with concentrations attained in nodules when riboflavin was added to the root medium. Nodules of two ages were examined (10-day-old and 16-day-old) to allow for possible changes in flavin content with age of tissue.

It was found that the total flavin concentration in the nodules corresponded roughly to their level of symbiotic effectiveness, irrespective of cultivar (Table III). Effective nodules produced on all three cultivars by T1 or by the auxotroph in the presence of riboflavin contained similar concentrations of flavin, nearly 20 times greater than that found in non-nodulated roots. Ineffective nodules produced by the unaided auxotroph contained the lowest level of flavin, although this level still exceeded that of non-nodulated root tissue by a factor of almost three. Partly effective nodules produced by T1/pen^r-4 were intermediate in flavin content. There was no significant difference in the flavin concentration of nodule tissue in young (10-day) or mature (16-day) nodules of any treatment, and only results for the 10-day-old nodules are show in Table III.

Distribution of Flavins within Nodules Formed by T1 and by T1/D-his^r-15 plus Riboflavin on Kenland Red Clover. A further comparison was made of flavin distribution in the bacterial (bacteroid) *versus* plant cell components of effective nodules produced on Kenland red clover by the parent strain (T1) and by the auxotroph (T1/D-his^r-15) plus added riboflavin. Ineffective nodules produced by the auxotroph on this host in the absence of riboflavin were not examined as they are largely devoid of bacteroids (6).

Three fractions (host cell debris or fragments, cell-free fraction, and bacteroids) were examined for comparative content of total flavins (Table IV). Almost half the total flavin in T1 nodules was associated with the nodule debris, while in T1/D-his^r-15 nodules formed in the presence of riboflavin more flavin was present in the cell-free fraction (Table IV). The bacteroids accounted for about 20% of the total flavin in both types of nodules.

The relative proportions of FAD and riboflavin + FMN in the different nodule fractions are also given in Table IV. Whole nodule flavin consisted of about 20% FAD and 80% riboflavin + FMN. There was no measurable FAD present in the cell-free fraction. Most of the FAD was associated with the nodule debris (*i.e.* apparently bound to plant cell components), whereas most of the riboflavin + FMN was found in the cell-free fraction of nodules produced by either strain. Nodules formed by T1 and T1/D-his^r-15 did not differ significantly on a whole nodule basis in total flavins or in the ratio of the two flavin components. They did, however, differ significantly ($P < 0.01$) in the distribution of the flavins in the nodule debris *versus* cell-free fractions; nodules formed by the auxotroph contained less FAD in the debris fraction but more riboflavin + FMN in the cell-free fraction. Bacteroids of the two strains did not differ measurably in either total flavins or relative amounts of the flavin components.

Table III. *Total Flavin (Lumiflavin) Content of Nodules Formed on Seedlings of Three Clover Cultivars by Rhizobium Strains T1, T1/D-his^r-15, and T1/pen^r-4*

Symbols used to indicate levels of effectiveness are explained under "Materials and Methods." Mean values are based on three replicates, expressed as nmoles riboflavin/g tissue (fresh wt). Nodules were assayed when 10 days old.

Rhizobium Strain or Treatment	Dwalganup		Clare		Kenland	
	Level of effectiveness	Nodule total flavin[1]	Level of effectiveness	Nodule total flavin[1]	Level of effectiveness	Nodule total flavin[1]
T1 (parent)	E	18.3	E	19.6	E	22.3
T1/D-his^r-15	E$^-$	12.7	I	2.3	I$^+$	6.1
T1/D-his^r-15 + ribo.	E	19.6	E	21.2	E	21.8
T1/pen^r-4	PE	8.2	PE	8.2	PE	9.0
Noninoculated roots		1.01		0.90		0.87

[1] LSD (0.05): 2.7; LSD (0.01): 3.8.

Table IV. *Distribution of Total Flavin and of the Component Flavins (Riboflavin plus FMN and FAD) in Fractions of Nodules formed by T1 and by T1/D-his^r-15 + Riboflavin on Kenland Red Clover*

Mean values are based on three replicates, expressed as nmoles flavin/g tissue (fresh wt).

Nodule Fraction	T1			T1/D-his^r-15 + Riboflavin		
	Total flavin (lumiflavin)[1]	Component flavins of the trichloroacetic acid hydrolysis[2]		Total flavin (lumiflavin)[1]	Component flavins of the trichloroacetic acid hydrolysis[2]	
		Riboflavin + FMN	FAD		Riboflavin + FMN	FAD
Nodule debris	9.3	2.8	4.2	8.7	2.6	2.4
Cell-free	5.8	7.2	0	9.8	10.9	0
Bacteroids	5.0	2.2	1.1	4.7	2.4	1.3
Whole nodules (control)	22.3	16.7	4.7	24.7	17.3	4.3

[1] LSD (0.05): 2.3; LSD (0.01): 3.6.
[2] LSD (0.05): 0.8; LSD (0.01): 1.4.

Comparison of Total Flavin in Cultured Rhizobia and in Bacteroids from Nodules on Kenland Red Clover. In view of previous evidence (6, 11) obtained for a high demand for riboflavin (or other flavin compounds) during the time of bacteroid formation in nodules, a comparison was made of the total flavin, FAD, and riboflavin + FMN content of cultured cells and of bacteroids of T1 and T1/D-his^r-15 (Table V). On a wet weight basis, the bacteroids of both strains were found to contain significantly ($P < 0.01$) more total flavin and significantly more riboflavin + FMN than the cultured cells. When the total flavin content was compared on a per cell basis, individual bacteroids, which are much larger than cultured cells, were approximately 22 times richer in flavins than cultured cells. On a per gram, dry weight basis, T1 cultured cells had a total flavin concentration of 46 nmoles/g, while T1/D-his^r-15 cells contained 55 nmoles/g.

Modification of the Symbiosis and of Nodule Flavin Content by Combined Nitrogen. In all of the aforementioned experiments, the plants were grown in a nitrogen-free medium, which is standard procedure for experiments involving plant response measurements of nitrogen-fixing ability of rhizobia. It was therefore considered possible that the ineffectiveness of the riboflavin auxotroph on some clover cultivars could result from "unavailability" of flavin due to nitrogen stress conditions of seedling growth. It was known from previous work (6, 11) that the ameliorative effect of riboflavin on ineffective symbiosis by T1/D-his^r-15 could not be attributed to the nitrogen content of the vitamin, since the addition of an equivalent amount of combined nitrogen (5 μM of N, added either as $(NH_4)_2SO_4$ or as NH_4Cl) had no observable effect on the nodulation. However, in subsequent preliminary experiments the addition of N at considerably higher concentrations (100 or 300 μM of N, added as $(NH_4)_2SO_4$) to Kenland or Clare seedlings allowed the auxotroph to produce partly effective nodules on these plants without the aid of exogenous riboflavin. These results were confirmed in the experiments described below, involving evaluation of nodule appearance, nitrogenase activity, and nodule flavin content.

The effect of combined nitrogen on the number and pigmentation of nodules formed by T1 (300 μM N) and T1/D-his^r-15 (100 and 300 μM N) in the presence or absence of riboflavin on Kenland red clover is shown in Table VI. The effect on C_2H_2-reducing activity is shown in Figure 2. Similar results (not presented here) were obtained for Clare subterranean clover.

Both levels of combined nitrogen allowed the auxotroph to form the full number of nodules (*i.e.* similar to the number observed in the nitrogen + riboflavin treatment), although this

Table V. *Flavin Content of Cultured Cells and of Bacteroids Isolated from Nodules Formed on Kenland Red Clover by Rhizobium Strains T1 and T1/D-his^r-15*

Mean values are based on three replicates, and expressed as nmoles flavin/g cells (fresh wt) or nmoles flavin/cell. Counts of cultured cells and bacteroids were made from cell suspensions with a Petroff-Hauser counting chamber. Each value given is the mean of 20 individual counts.

Determinations	T1		T1/D-his^r-15 + Riboflavin	
	Cultured cells	Bacteroids	Cultured cells	Bacteroids
Total flavin (lumiflavin method)[1]	9.1	12.6	9.9	13.2
Total riboflavin + FMN[2]	4.6	7.4	5.2	7.0
FAD[2]	3.9	4.2	4.1	4.9
No. cells or bacteroids per mg (wet wt)	1.06×10^8	6.61×10^6	1.23×10^8	7.42×10^6
Total flavin/cell	8.5×10^{-11}	190.6×10^{-11}	8.0×10^{-11}	177.9×10^{-11}
Total riboflavin + FMN/cell	4.3×10^{-11}	112.0×10^{-11}	4.2×10^{-11}	94.3×10^{-11}
Total FAD/cell	3.7×10^{-11}	63.5×10^{-11}	3.3×10^{-11}	66.0×10^{-11}

[1] LSD (0.05): 1.5; LSD (0.01): 2.3.
[2] LSD (0.05): 0.7; LSD (0.01): 1.1.

Table VI. *Effect of Combined Nitrogen on the Number and Pigmentation of Nodules Formed by T1 and T1/D-his^r-15 on Kenland Red Clover*

Nodule numbers given represent the mean from eight plants. The nitrogen and riboflavin were added to the plants at the time of inoculation.

Time after Inoculation	Nodules[1]						Nodules Pigmented					
	T1		T1/D-his^r-15				T1		T1/D-his^r-15			
	No N	N (300 μM)	No N	N (100 μM)	N (300 μM)	N (300 μM) riboflavin (20 μM)	No N	N (300 μM)	No N	N (100 μM)	N (300 μM)	N (300 μM) riboflavin (20 μM)
days	*No.*						%					
8	22	8	9	6	9	11	98	95	0	0	8	75
10	27	13	7	9	10	13	98	97	10	8	15	94
12	25	11	9	20	19	20	100	100	19	24	16	94
14	30	22	13	25	17	27	92	96	18	33	48	96
16	34	36	15	20	23	24	100	91	22	41	60	90
18	36	34	17	30	35	33	100	100	30	46	79	83

[1] LSD (0.05): 1.4; LSD (0.01): 3.1.

increase was not evident until about 12 days after inoculation (Table VI). Absence of an increase before this time can be attributed largely to a depressive effect (4, 15) of combined nitrogen on infection or nodule initiation, as shown in the nitrogen + riboflavin treatment with the auxotroph and in the nitrogen + T1 treatment. There was, however, very little depression of nodule pigmentation (an indicator of effective nodule development). Pigmentation of nodules produced by the auxotroph in the presence of nitrogen commenced slowly, relative to the nitrogen + riboflavin treatment, then increased rapidly after day 12.

The effect of combined nitrogen on nitrogenase activity in the nodules (Fig. 2) corresponded, in general, to the effects described above for nodule pigmentation. In particular, a significant dose effect of added nitrogen was evident for plants inoculated with the auxotroph. The nitrogen also produced a moderate depressive effect on the activity of T1 nodules prior to day 14 after inoculation.

Combined nitrogen also induced a significant increase in total flavin in the nodules produced by T1 and T1/D-his^r-15 on the cultivars Dwalganup, Clare, and Kenland (Table VII). This increase was proportionately greater in nodules produced on Kenland and Clare than on Dwalganup.

The total flavin content of noninoculated roots of the three clover cultivars grown in the presence of combined nitrogen (300 μM N) was also found to be significantly greater ($P < 0.05$) than that of roots of seedlings grown in a nitrogen-free substrate (Table VII). This increase in total flavins was slightly greater than when the roots of these seedlings were exposed to riboflavin (80 μM) for 3 days (Table II) and was similar for all cultivars irrespective of their nodulation response to the unaided auxotroph.

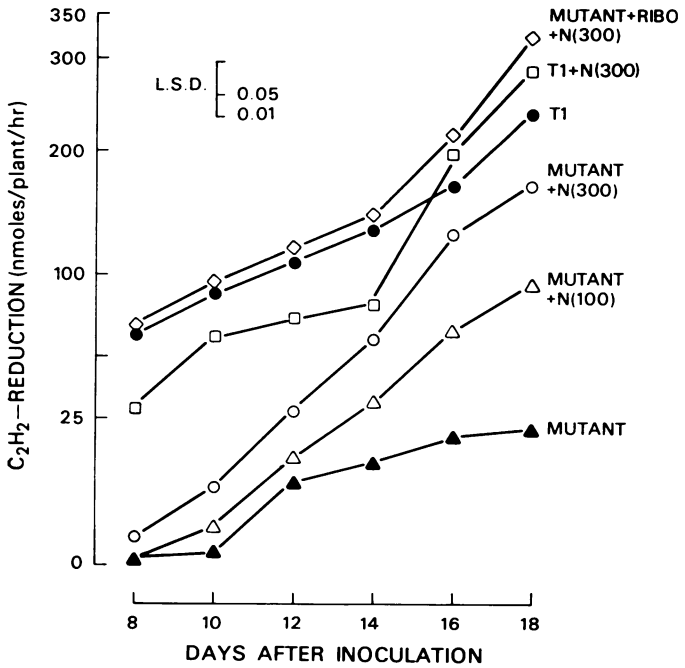

FIG. 2. Effect of combined nitrogen on the C_2H_2-reducing activity (values shown on square-root scale) of Kenland red clover plants inoculated with T1, T1/D-his^r-15 (mutant), and T1/D-his^r-15 + riboflavin (20 μM). Numbers in parentheses indicate the concentration (μM) of nitrogen (N), added as $(NH_4)_2SO_4$, present in the root substrate. Nitrogen and riboflavin were added with the inoculum. Each point is the mean of four assays, three nodulated root systems per assay. Least significant differences were obtained by square-root transformation of individual assay values.

Table VII. *Effect of Combined Nitrogen on the Flavin Content of Nodules Formed by Rhizobium Strains T1 and T1/D-his^r-15 on Three Clover Cultivars*

Symbols used for levels of effectiveness are explained under "Materials and Methods." These are given for the non-nitrogen treatments only (growth stimulation by added nitrogen precludes similar rating of plant response in other inoculation treatments) for comparison of flavin level with level of effectiveness. The mean values given are based on three replicates, expressed as nmoles riboflavin/g tissue (fresh wt). Nodules were assayed when 10 days old.

Rhizobium Strain or Treatment	Dwalganup		Clare		Kenland	
	Level of effectiveness	Nodule total flavin[1]	Level of effectiveness	Nodule total flavin[1]	Level of effectiveness	Nodule total flavin[1]
T1 (parent)	E	22.8	E	25.2	E	22.3
T1 + N (300 μM)		26.0		20.2		25.2
T1/D-his^r-15	E$^-$	12.2	I	2.9	I$^+$	5.8
+ N (100 μM)		15.1		6.6		10.3
+ N (300 μM)		17.8		7.9		10.9
T1/D-his^r-15 + riboflavin (20 μM) + N (300 μM)		32.4		26.8		29.5
Noninoculated roots		1.01		0.90		0.87
Noninoculated roots + N (300 μM)		2.00		2.26		1.96

[1] LSD (0.05): 1.0; LSD (0.01): 1.7.

Limited Translocation of Added Riboflavin by Clover Roots. Since adding riboflavin to the roots produced only a minor increase in total root flavin content, in contrast to its major effect on symbiosis by T1/D-his^r-15, the question of mobility or translocation (affecting distribution within root tissue) of flavins in roots was examined by application of riboflavin to only a part of the root system (see "Materials and Methods"). The experiments were confined to the cultivars Kenland and Clare, on which the unaided auxotroph is ineffective, as the criterion used for translocation of riboflavin was the formation of effective nodules on untreated parts of the roots. Nodules developed effectively (Table VIII) only on those parts of the root system exposed directly to riboflavin, suggesting that not enough flavin was translocated in either an upward or downward direction to alleviate the defect in nodule development. There was, however, evidence for some upward movement of flavin as indicated by an increase in nodule number in the upper half of the roots when the lower half was exposed (Table VIII). This interpretation was based on previous information (6) that a low concentration of added riboflavin satisfied the requirement for nodule initiation (*i.e.* release of the early defect which restricts the formation of approximately half the full number of nodules), but did not suffice to overcome the later "major" defect or block in bacteroid formation.

The same partial root technique was used to test for a possible effect of added combined nitrogen on movement of riboflavin in the roots. When the upper root system of both cultivars (Kenland and Clare) was exposed to combined nitrogen at a concentration of 300 μM of N (added as $(NH_4)_2SO_4$) and the lower root system was exposed to riboflavin (20 μM), nodules formed by the auxotroph on the upper root system were almost all effective. This was in contrast to only a slight increase in effectiveness achieved when the whole root system was exposed to the nitrogen and suggested that nitrogen may favor the upward translocation of riboflavin. When the nitrogen and riboflavin treatments were reversed (upper roots in riboflavin, lower roots in nitrogen) there was no significant change in the effectiveness of the nodules formed on the lower roots, thus

Table VIII. *Effect of Partial Root Application of Riboflavin on the Number and Pigmentation of Nodules Formed by T1/D-his^r-15 on Kenland Red Clover and Clare Subterranean Clover*

Both cultivars are nodulated ineffectively by the auxotroph in the absence of added flavin. Nodule numbers (16 days after inoculation) represent the mean from five plants. Riboflavin was added where indicated with the inoculum to give an initial concentration of 20 μM in the plant nutrient solution.

Rhizobium Strain	Riboflavin Added	Kenland				Clare			
		Upper roots		Lower roots		Upper roots		Lower roots	
		Nodules[1]	Nodules pigmented	Nodules[1]	Nodules pigmented	Nodules[1]	Nodules pigmented	Nodules[1]	Nodules pigmented
		No.	%	*No.*	%	*No.*	%	*No.*	%
T1 (parent)	None	16	100	19	100	23	100	32	100
T1/D-his^r-15	None	4	16	10	11	11	7	19	9
	Upper roots	19	97	13	8	48	83	10	0
	Lower roots	15	20	27	100	20	4	29	100
	Upper and lower roots	14	100	16	100	26	97	34	90

[1] Nodule numbers: LSD (0.05): 1.6; LSD (0.01): 2.9.

providing no evidence for downward translocation of riboflavin.

DISCUSSION

A riboflavin-requiring mutant of *R. trifolii* formed ineffective root nodules on red clover and on two cultivars of subterranean clover and almost fully effective nodules on several other cultivars of subterranean clover. This pattern of symbiosis was shown to be unrelated to the total flavin content of the roots and shoots of these clovers. This finding was unexpected in view of the fact that a fully effective symbiosis could be achieved by adding riboflavin externally to the roots of the inoculated seedlings (11) or by back mutation of the auxotroph to riboflavin nonrequirement (10).

Bioassays provided no evidence that the two cultivars on which the auxotroph produced effective nodules were rich in nonfluorescent riboflavin precursor compounds usable by the auxotroph, nor did they provide any evidence for the presence of inhibitors of bacterial growth in the three cultivars on which ineffective nodules were produced. Instead, root segments of all cultivars excreted enough flavin to stimulate growth of the bacteria in a volume of agar much greater than the tissue volume. Thus the flavin content in the roots appears more than adequate for vegetative growth of the auxotroph *in vitro*. The auxotroph is, in fact, able to infect the root and develop in the "vegetative" form within infection threads up to the bacteroid formation stage, but at a suboptimum level in the case of clover cultivars like Clare, Yarloop, or Kenland (nodules ineffective on these hosts). Addition of riboflavin at a concentration as high or slightly higher than that found in the over-all root tissue (about 1.0 nmole/g tissue) is necessary for the maximum number of nodules to appear on these clovers. Thus even for the infection phase of nodule development the amount of flavin present in the roots, although similar for all clovers examined, is not sufficiently "available" in some cultivars.

During the bacteroid formation stage of nodule growth when the flavin requirement is much higher (6), the amount of available flavin in the host tissue is clearly inadequate, and the bacteria must be able to either synthesize these substances or concentrate host-produced flavins to a level of about 18.0 to 23.5 nmoles/g tissue, for effective symbiosis. Nonauxotrophic strains like the effective T1 parent strain or the partly effective T1/pen^r-4 (partial block in symbiosis not attributable to flavins with this mutant) presumably synthesize the required flavin rather than concentrate host-produced flavin to such high levels, although this has not been experimentally confirmed. The riboflavin auxotroph, barring some unlikely host inductive mechanism which would allow synthesis by the bacteria in Dwalganup or Mt. Barker roots (effective nodules on these hosts), would have to be able to concentrate host-produced flavins to the required level. This apparently occurs on Dwalganup roots, where a final concentration of about 12.0 nmoles/g tissue is attained, but not on roots of Clare, Yarloop, or Kenland. As there was no evidence for appreciable uptake (on a whole root basis) or translocation of exogenous riboflavin by the roots of Clare or Kenland (Yarloop not examined), it is noteworthy that the concentration of riboflavin (about 20.0 nmoles/ml of substrate) that had to be added to promote fully effective nodulation by the auxotroph was similar to the flavin concentration of effective nodules. This suggested that much of the added vitamin was absorbed directly through the broken, more permeable surface of the young nodules rather than indirectly via absorption and translocation by the non-nodulated portions of the roots. The occurrence of specialized transfer cells in the pericycle tissue surrounding vascular strands in clover nodules (7) may facilitate such an influx of riboflavin from the external medium across the nodule cortex to the developing bacteroids.

Evidence that total flavins occur at high concentrations in nodulated relative to non-nodulated soybean tissue has also been reported by Tuzimura (14) who used an effective strain of *R. japonicum*. Shemakhanova and Bunk'o (12) found that effective bean nodules contained nearly twice as much riboflavin as ineffective nodules. Shemakhanova and Sidorenko (13) also compared the vitamin-synthesizing capacity of various rhizobia having different levels of symbiotic effectiveness and concluded that effectiveness was correlated with ability to synthesize high levels of riboflavin as well as several other vitamins. These results support the evidence, previously reported (6) and presented in this paper, for a critical role of riboflavin in effective symbiosis, but there appears to be no further relevant information on differences between legume plants in the synthesis or availability of flavins.

The stimulative effect of combined nitrogen on root flavin content, on upward movement of absorbed riboflavin, and on the effectiveness of the symbiosis (in the absence of added flavin) suggests that ineffectiveness of the auxotroph on cultivars of clovers like Kenland and Clare is partly attributable to nitrogen stress in the seedlings. Some increase in flavin content

was noted even in the effective type nodules produced by the auxotroph on Dwalganup. However, the acetylene-reducing activity of the nodules formed in the presence of nitrogen, although much greater than the activity of nodules on control plants, was still significantly below the activity of nodules produced by T1 or by the auxotroph plus riboflavin. The requirement for riboflavin in effective symbiosis was thus clearly not dependent solely on the nitrogen status of the plant. Since the nitrogen treatment induced a somewhat higher root flavin content than the riboflavin treatment but induced only partial effectiveness, it would appear that the ameliorative effect of nitrogen may be related more to translocation or uniform distribution of flavin in root cells than to enhanced synthesis by the plant. More definitive information concerning this question and other questions relating to the flavin-associated differences between clover cultivars will require the application of appropriately labeled flavins and flavin precursor compounds.

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