REQUIREMENT FOR RIBOFLAVIN FOR EFFECTIVE SYMBIOSIS ON CLOVER BY AN AUXOTROPHIC MUTANT STRAIN OF *RHIZOBIUM TRIFOLII*

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Summary

A symbiotically ineffective auxotroph, T1/D-his^r-15, derived from an effective strain (T1) of *R. trifolii*, had a specific requirement for riboflavin in culture and produced an effective response on *Trifolium pratense* (red clover) only when riboflavin was added to the plant growth medium at *c.* 6 μg/ml. Addition of flavin mononucleotide to the plant medium produced a similar response but the requirement in symbiosis was only partly satisfied by flavin adenine dinucleotide. This biochemically induced change in symbiotic behaviour involved a transient restoration of effectiveness, distinct from genetic restoration by back mutation to prototrophy. Experiments involving the delayed addition of riboflavin after inoculation indicated that the critical time for riboflavin requirement in the symbiosis occurs within the period of about 4–8 days after inoculation.

The unaided auxotroph displayed different levels of effectiveness on some other clover hosts, including another cultivar of *T. pratense*, one of *T. repens*, and three of *T. subterraneum*. In all cases the addition of riboflavin enabled the mutant to attain a level of effective symbiosis approaching or equalling that of the effective T1 parent strain. The results suggest 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.

I. INTRODUCTION

Most of the available knowledge on the physiology or biochemistry of the *Rhizobium*–legume symbiotic relationship concerns mechanisms of nitrogen fixation, which occurs largely in an advanced stage of development of effective nodules. Comparatively less is known concerning critical bacterial–plant interactions at other stages of the symbiosis, from penetration of the root hair to development of the mature nodule and eventual senescence (Vincent 1967). Biochemical analyses of nodules produced on different hosts by one or more strains of rhizobia have yielded some useful information. For example, by using appropriate combinations of the symbionts it has been possible to study the fundamental question of whether the host or the bacterium governs the synthesis of leghaemoglobin (Dilworth 1969). Currently more emphasis is being placed on a search for meaningful differences in metabolism

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by the bacterial component *in vitro*—i.e. differences which appear to correlate with ability to nodulate or with effective (able to fix nitrogen) or ineffective symbiosis. Such studies may involve either available unrelated strains which differ in nodulation on closely related hosts (Hubbell and Elkan 1967) or closely related mutant rhizobia which differ in effectiveness (Schwinghamer 1967, 1968; Damery and Alexander 1969; Jordan, Yamamura, and McKague 1969) and in nutritional characteristics *in vitro* (Schwinghamer 1969). In the latter approach ineffective variants are derived from effective parent strains by isolation of mutants resistant to certain antimetabolites; some of these ineffective clones also have nutritional requirements which can be examined for possible involvement in ineffective symbiosis. Thus it was found that addition of adenine or adenine-containing compounds to the roots of pea seedlings would allow a non-nodulating mutant of *Rhizobium leguminosarum* to produce ineffective nodules (Schwinghamer 1967). This was the maximum response to biochemical amendment obtained, although it was possible to obtain genetic restoration to full effectiveness by isolation of prototrophic (nutritionally non-requiring) revertants.

The present paper describes the "biochemical restoration" of full effectiveness in a riboflavin-requiring auxotroph (mutant having nutritional requirement) of *R. trifolii* by supplying the *Rhizobium*–clover system with riboflavin or riboflavin-containing compounds.

II. Materials and Methods

(*a*) *Cultures*

T1, the strain from which the auxotrophic mutants were isolated, was obtained as strain 162P17 from the Nitragin Company, Milwaukee, Wisconsin. T1/D-his^r-15 was isolated from this parent as a D-histidine-resistant mutant. It was subsequently found to be ineffective on *Trifolium pratense*, cv. Kenland, and to have a strong requirement for a single growth factor, riboflavin (Schwinghamer 1969).

The temperate phage, ϕT10, is very specific for T1 or its derivatives and was used in the present work to identify these strains.

(*b*) *Media*

A glucose–salts–yeast extract agar medium (Schwinghamer 1960) was used for maintenance of all *Rhizobium* cultures. The synthetic agar medium of Bergersen (1961) was used for periodic growth requirement checks of T1/D-his^r-15 and for preliminary bioassay (T1/D-his^r-15 as test organism) of riboflavin in seeds or in the plant growth medium. The nitrogen-free "S_b" nutrient solution (Schwinghamer 1960) was used for plant growth and for preparation of cell suspensions used as inocula. Stock solutions of vitamins or other growth factors were sterilized by Millipore (pore size 0·45 μm) filtration.

(*c*) *Plant Nodulation Tests*

The following hosts were used for nodulation experiments: *T. pratense* L. (red clover), cv. Montgomeryshire and cv. Kenland; *T. subterraneum* L. (subterranean clover), cv. Mt. Barker, cv. Woogenellup, and cv. Tallarook; *T. repens* L. (white clover), cv. Grasslands Huia.

Clover seedlings were grown in sterile vermiculite in 8-oz glass jars covered with Cellophane-topped paper cylinders as described in earlier reports (Schwinghamer 1960, 1967). Approximately 15 surface-sterilized seeds were planted per jar and excess seedling stands thinned to four or five uniform plants of red clover and three or four plants of subterranean clover per jar. One replicate (jar) per treatment was used for preliminary experiments; three or more replicates were used for confirmatory experiments. Plants were inoculated (final bacterial concentration *c.* 5 $\times$ 10^6/ml of jar volume) on the eighth day after planting, and evaluation of effectiveness was usually made within 18–22 days after inoculation. Growth conditions in a temperature-controlled

cabinet were as follows: temperature 22°C (18 hr light period) and 19°C (6 hr dark period); light intensity *c.* 1000 f. c., from a mixture of daylight and warm white fluorescent lamps. The shoots were oven-dried for 36 hr at 70°C; following Kjeldahl digestion total nitrogen was determined by the colorimetric method of Williams and Twine (1967). The data were examined statistically by analysis of variance and values were transformed to common logarithms where the relationship between treatment means and error was linear. In preliminary experiments the treatment results were scored visually in three arbitrary classes (ineffective, partly effective, or effective) on the basis of nodule type and growth response (effective and ineffective growth responses shown in Fig. 1).

Sterile solutions (100 μg/ml) of growth factors were added to the plants immediately after inoculation or at various intervals after inoculation; the jars were inclined and rotated to assure uniform distribution of bacteria and chemicals in the vermiculite–nutrient solution substrate. The chemicals were added at one or more of three levels, corresponding to final concentrations of *c.* 0·5, 1·5, or 6·0 μg/ml of liquid in the jar.

III. Results

(*a*) *Comparative Response of Montgomeryshire Red Clover and Mt. Barker Subterranean Clover to Inoculation with a Variety of T1 Mutants*

Earlier studies on antimetabolite-resistant mutants of *Rhizobium* involving loss of effectiveness on Kenland red clover (Schwinghamer 1968, 1969) were extended to include another cultivar of red clover and several cultivars of subterranean clover. Cultivars from *T. pratense* and *T. subterraneum* are known to differ in level of effective response to many strains of *R. trifolii* (Vincent 1967). The parent strain T1, however, was fully effective on both host species, making it possible to note whether alteration of symbiotic effectiveness on one host was specific for that host or applied also to other hosts. Among 33 mutants isolated for resistance to antimetabolites and originally classified as ineffective or partly effective on Kenland, four retained the effectiveness of T1 on Montgomeryshire red clover or on Mt. Barker subterranean clover. The others were ineffective on all three clover cultivars. Only one of the four mutants, the riboflavin-requiring T1/D-his^{r}-15, differentiated fully between the two host groups in that it was ineffective on both red clover cultivars but almost fully effective on Mt. Barker.

(*b*) *Effect of Riboflavin and other Growth Factors on the Response of Red Clover Seedlings to Inoculation with some Ineffective Auxotrophs*

Attempts were made to promote an effective response on Montgomeryshire red clover inoculated with ineffective auxotrophs by adding to the plants immediately after inoculation the appropriate growth factors required in culture. The auxotrophs tested included T1/D-his^{r}-15 and four other D-histidine-resistant mutants which had more complex, partial requirements *in vitro*, mainly for combinations of thiamine, pantothenic acid, and inositol. There was no significant change in visual ratings of nodule type or plant growth when a mixture of these three vitamins was added to the plant growth medium at a concentration of 0·5 or 6 μg/ml. A positive change in both nodule type and plant growth was noted only for riboflavin, on plants inoculated with T1/D-his^{r}-15. Adding riboflavin at 0·5 μg/ml produced little change but at 6 μg/ml it induced almost full effectiveness. Flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), two flavin-containing coenzymes which also

stimulated growth in culture, likewise produced a change towards effectiveness at 6 μg/ml. The response to FAD, however, appeared to be only a partial one relative to that of riboflavin or FMN. Non-inoculated plants did not respond to the addition of riboflavin, FMN, or FAD at 6 μg/ml.

With regard to the four auxotrophs for which negative results were obtained on the plants it is recognized that a measurable reaction might possibly have been elicited under different experimental conditions involving different concentrations or proportions of the growth factors comprising the mixtures.

TABLE 1

PLANT DRY WEIGHT AND NITROGEN CONTENT OF MONTGOMERYSHIRE RED CLOVER SUPPLIED WITH RIBOFLAVIN AT DIFFERENT CONCENTRATIONS OR TIME INTERVALS FOLLOWING INOCULATION WITH T1/D-his^r-15

Inoculant Strain	Riboflavin or Flavin Compound Added to Plants	Approx. Concn. (μg/ml)	Days after Inoculation when Flavin Added	Mean Dry Weight of Shoots* (mg)	Mean Nitrogen Content of Shoots* (mg)
Non-inoculated	None	—	—	43	0·35 (0·54)†
Non-inoculated	Riboflavin	7·5	0	42	0·31 (0·49)
T1/D-his^r-15	None	—	—	46	0·46 (0·66)
T1/D-his^r-15 (cells washed)	None	—	—	45	0·46 (0·66)
T1/D-his^r-15	Riboflavin	0·4	0	37	0·40 (0·60)
T1/D-his^r-15		1·6	0	60	1·02 (1·01)
T1/D-his^r-15		7·5	0	109	2·71 (1·43)
T1/D-his^r-15		7·5	3	94	2·33 (1·37)
T1/D-his^r-15		7·5	6	67	1·70 (1·23)
T1/D-his^r-15		7·5	9	45	0·64 (0·81)
T1/D-his^r-15	FMN	9·0‡	0	103	2·41 (1·38)
T1/D-his^r-15	FAD	15·8‡	0	79	1·68 (1·23)
T1/D-his^r-15-p8	None	—	—	97	2·85 (1·46)
T1	None	—	—	115	3·35 (1·53)
L.S.D. (P=0·05)				20	(0·16)
L.S.D. (P=0·01)				26	(0·21)
L.S.D. (P=0·001)				35	(0·28)

* Shoots assayed 18 days after inoculation. Values given are means of four replicates (jars), four plants per replicate.

† Values in parentheses are logarithmically transformed values × 10.

‡ Concentration of FMN and FAD adjusted to provide approximately equal amounts of the riboflavin moiety.

(c) *Effect of Riboflavin Added at Different Concentrations or Different Times after Inoculation of Montgomeryshire Red Clover with T1/D-his^r-15*

The response produced by riboflavin, FMN, or FAD on Montgomeryshire inoculated with T1/D-his^r-15 was measured more accurately by plant dry weight and nitrogen determinations (Table 1). The time of riboflavin addition was varied to ascertain the approximate stage of nodule development where the vitamin appeared

to be most essential. A prototrophic derivative, T1/D-his^r-15-p8, and the parent T1 were included to compare the biochemically restored and the genetically (by back mutation) restored level of effectiveness with that of the wild type.

The results of the above experiment and related miscellaneous experiments are summarized as follows (see also Fig. 1):

(1) Both riboflavin and FMN, when added immediately after inoculation at the "high" concentration of 7·5 μg/ml, enabled the ineffective auxotroph to attain a level of effectiveness similar to that of the back mutant and nearly equal to that of the effective parent strain. All of these treaments differed clearly ($P<0·001$) from the non-inoculated or mutant-inoculated control treatments. Treatment with FAD also produced a highly significant increase over the controls in both plant weight and nitrogen content but the increase was significantly less ($P<0·05$) than for riboflavin or FMN. This intermediate reaction did not differ greatly from that of previous experiments even though the concentration of FAD (and FMN) was increased to allow comparison at approximately equivalent amounts of the riboflavin moiety.

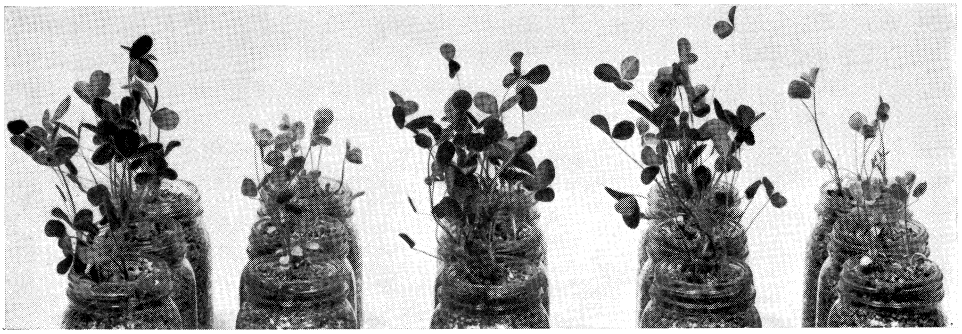

Fig. 1.—Growth response of seedlings of Montgomeryshire red clover to different inoculation treatments at 18 days after inoculation. Inoculants, left to right: T1, the effective parent strain of *R. trifolii*; T1/D-his^r-15, the riboflavin-requiring mutant derivative of T1; T1/D-his^r-15-p8, a prototrophic mutant from the above auxotroph; T1/D-his^r-15 plus riboflavin; non-inoculated control. The treatments illustrate sequentially the complete cycle of experimentally altered ability for symbiosis, involving loss of effectiveness and its restoration by either genetic or biochemical means.

(2) There was a decided concentration effect with riboflavin. At 0·4 μg/ml there was no measurable gain over the controls, while a fourfold increase in concentration produced a partial but significant gain ($P<0·01$) in nitrogen only. Another fivefold increase gave the maximum gain observed in this experiment for both plant weight and nitrogen. Higher concentrations of riboflavin have not been used but since a comparable growth response was obtained at about 6 μg/ml in the earlier experiment it appears that little gain could be expected above about 7 or 8 μg/ml.

(3) Delaying addition of riboflavin for 3 days produced only a slight decrease in growth (relative to the "day 0" treatment) which disappeared in a later harvest made at 22 days. A 6-day delay in providing riboflavin resulted in a significant decline in both dry matter and nitrogen. A delay of 9 days produced a further depression,

leaving only a slight, non-significant gain in nitrogen relative to the non-riboflavin controls. This difference became significant at the 22-day harvest. The prolonged period of nitrogen starvation in these plants precluded full recovery.

Limited observations were also made on nodule type at 7 days and 11 days after inoculation. T1/D-his^r-15 control plants had formed small, non-pigmented nodules by the seventh day. By the eleventh day there was only a trace of pigment visible and slight increase in nodule size; this stage represented the maximum development attained without riboflavin. Where riboflavin had been added at day 0 most of the nodules were already lightly pigmented by day 7 and subsequent development to the mature effective type did not differ noticeably from that of plants inoculated with T1. Delay of riboflavin addition for 3 days resulted in only a lag of about 1 day in nodule development; longer delay, notably with the 9-day interval, resulted in a correspondingly greater lag and incomplete development of nodules.

(4) The auxotroph, despite its very poor growth on minimal agar (growth in liquid medium not studied), was able to form ineffective nodules without the aid of any added riboflavin. The possibility that a significant amount of the vitamin might have been introduced to the root substrate through the inoculum (cells normally grown on yeast extract agar medium) was eliminated by the absence of any change in response when the bacteria were washed twice by centrifugation (Table 1). The plants still bore ineffective nodules and showed no gain in dry weight or nitrogen. Furthermore, a bioassay using the auxotroph as indicator (limit of detection about $0 \cdot 02$ μg) revealed no measurable riboflavin in the non-washed inoculum, even when the latter was concentrated to a cell density of about 10^9/ml. Some riboflavin is added to the substrate by seed coats which are shed following seed germination but this is below the demonstrated threshold level (at least 1 μg/ml) for influencing the symbiosis. This was confirmed in a separate experiment in which the seeds were germinated on water agar and the seedlings, minus seed coats, were transplanted to the vermiculite. The auxotroph was still able to produce ineffective nodules. The roots apparently provide enough of the vitamin for bacterial growth and limited development of the nodules up to the ineffective stage.

(5) The vitamin-induced modification to effectiveness was a transient biochemical change, not a change attributable to a low background of genetic variants in the bacterial population which might somehow be "enriched" selectively by the host plant plus riboflavin. Effective prototrophic mutants like T1/D-his^r-15-p8 can be isolated *in vitro* but the frequency of occurence is not high enough to allow their detection through an effective nodule on a small number of plants inoculated with the auxotroph. Evidence for the non-genetic nature of the change was obtained by positive identification of isolates from nodules harvested as follows: three effective nodules from the riboflavin treatment; two effective nodules each from the FMN and FAD treatments; two effective nodules from T1. All nine isolates were identified as T1 or T1 derivatives by the specific phage, ϕT10, and all except the two T1 isolates required riboflavin for growth in culture. When subsequently assayed on Montgomeryshire red clover all except the T1 isolates were still ineffective. Finally, the three isolates from the riboflavin treatment nodules again produced the anticipated effective reaction when the vitamin was added to the host at 6 μg/ml.

(*d*) *Assay of T1/D-his^r-15 on some other Clover Hosts*

In view of the riboflavin-modified symbiosis as described above for Montgomeryshire red clover, several additional clover hosts were tested to determine whether this type of repairable metabolic block in symbiosis is unique to one cultivar of red clover. Another cultivar of red clover and three of subterranean clover were assayed on the basis of shoot dry weight (Fig. 2). These results were further confirmed by observations on nodule type and plant appearance. As seen in Figure 2 the Kenland

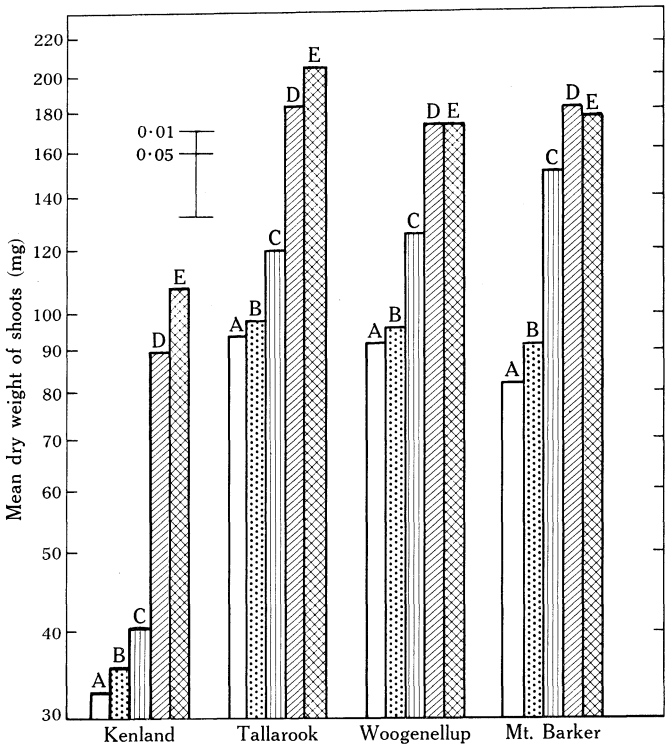

Fig. 2.—Plant growth response of four clover cultivars to riboflavin and inoculation treatments. *A*, non-inoculated, no riboflavin; *B*, as for *A*, plus riboflavin; *C*, inoculated with T1/D-his^r-15, no riboflavin; *D*, as for *C*, plus riboflavin; *E*, inoculated with T1, no riboflavin. Riboflavin concentration 7 μg/ml. Shoots harvested 20 days after inoculation. Means (log transformed data) represent three replicates of five plants each for Kenland red clover and four plants each for the three cultivars of subterranean clover. Least significant differences at the 5 and 1% levels are indicated.

cultivar of red clover was very similar to Montgomeryshire (Table 1) in its response to inoculation with this auxotroph or with the auxotroph plus riboflavin; the latter treatment approached the full effectiveness level of T1. With the three cultivars of subterranean clover riboflavin was also needed for an effective response. The unaided mutant, however, was not fully ineffective as on red clover, being instead partly effective on both Woogenellup and Tallarook, and almost fully effective on Mt. Barker.

A highly significant strain–cultivar interaction resulted mainly from the differences produced by inoculation with T1/D-his^r-15 without riboflavin. The mutant without riboflavin is able to distinguish between the clover cultivars at different levels of effectiveness, but in each instance the block in symbiosis can be largely corrected by riboflavin.

A limited test of a white clover (*T. repens*) cultivar, New Zealand White, indicated that the ineffectiveness of T1/D-his^r-15 on this host could also be altered by riboflavin. This was expected on the basis of the analogous nodulation pattern generally shown by red clover and white clover hosts to strains of *R. trifolii*.

Two additional miscellaneous trials from a separate experiment are relevant to the above studies on the host specificity of the auxotroph. In one trial two viomycin-resistant mutants selected from the auxotroph were found to be ineffective on Mt. Barker as well as on Montgomeryshire, thereby losing the ability to distinguish between these two hosts. Furthermore, neither mutant responded to riboflavin on Montgomeryshire, thus indicating that the block in symbiosis closely associated with resistance to viomycin (Schwinghamer 1967) is distinct from the block associated with riboflavin requirement. The second trial involved a single nodulation test, on Montgomeryshire, of five non-mutant *R. trifolii* strains (WU95, CC241, CC276, and two unnamed isolates from subterranean clover nodules) which were similar to the auxotroph only in their performance on these two hosts—i.e. ineffective on Montgomeryshire and effective on Mt. Barker. None of the five required the vitamin *in vitro* and none responded measurably to riboflavin on Montgomeryshire. As with the viomycin-resistant mutants, ineffectiveness in these strains is distinct from that of the auxotroph.

IV. Discussion

The experimental results demonstrate with reasonable certainty that the ineffective symbiosis of the mutant strain T1/D-his^r-15 with at least three species of clover is due to the mutant's inability to synthesize riboflavin or flavin-containing compounds. This provides a potentially valuable system for study of the nature of one specific block among the complex series of *Rhizobium*–legume interactions which normally lead to effective symbiosis. The mutant serves as a prototype for isolation of riboflavin auxotrophs in other rhizobia or of other types of ineffective auxotrophs having different specific requirements. By this approach it may eventually be possible to identify a series of biochemical deficiencies in the bacterium which contribute to defective symbiosis at different stages of nodule development. T1/D-his^r-15 and the adenine–thiamine auxotroph of *R. leguminosarum* described previously already point to blocks at two different stages. The former occurs at approximately the time of appearance of the nodules on red clover, and the latter appears on pea at an earlier stage, possibly in the root infection phase of nodule genesis. Both of these blocks can be fully corrected genetically by back mutation to prototrophy but that of the adenine–thiamine mutant could only be partially corrected biochemically, from non-nodulation to ineffective nodulation.

Information obtained thus far on the effect of riboflavin concentration and of the delayed addition of riboflavin is limited. The level of the vitamin added to the root substrate is considerably higher than that needed for good growth of the mutant

in vitro but nothing is known concerning uptake by the roots (including difference in uptake of FAD as compared to FMN or riboflavin) or inactivation within the roots. Adsorption on the vermiculite or inactivation in the plant nutrient solution did not measurably (bioassay) lower the concentration in the nutrient solution of the jar within 2 days after adding the vitamin but the concentration declined several-fold in 10 days. The bacteria apparently were able to infect the roots without the aid of riboflavin other than that presumably excreted by the roots, and to induce nodule development up to the ineffective stage. Within the tissue the host apparently provides nearly enough of the vitamin for about the first 3 days after infection. The following 4- or 5-day period, which includes the first macroscopic appearance of the nodules at about 7 days after inoculation, appeared to be the more critical time. During this period rapid growth and bacteroid formation occur in clover nodules following release of the rhizobia from the infection thread, and the associated higher energy demand could well be related to the added need for the flavin-containing coenzymes. It is therefore at this stage of nodule development where one may postulate a difference between the clover hosts, either in available riboflavin or in the bacterial demand for this growth factor. It is also the stage where structural changes like bacteroid or host cell breakdown may be meaningfully examined in relation to a specific metabolic defect like riboflavin requirement. Whatever the nature of the block it is still at least partly reversible by riboflavin even after the ineffective nodule has attained its maximum development; it is not known whether this revival involves continued functioning of existing bacteroid tissue or simply the production of new tissue by the nodule meristem. It also remains to be determined whether the block, once by-passed or corrected by riboflavin, would *remain* corrected in the absence of a continued external source of the vitamin.

Although direct evidence for involvement of a specific growth factor in effective symbiosis on legumes had hitherto been lacking, a possible role for vitamins has been examined by a number of investigators. Root nodules of legumes were found by Tuzimura (1950) to have a high content of riboflavin relative to other parts of the plant. Several vitamins, notably inositol, have been found to increase the speed of nodulation or the number of nodules on excised roots of clover (Weir 1960) or bean (Raggio, Raggio, and Burris 1959). Molina and Alexander (1967) studied the possible essentiality of various growth factors in nodulation by adding antimetabolites to the roots of intact birdsfoot trefoil or to excised bean roots. Some antimetabolites suppressed nodulation while others enhanced it, with two niacin antagonists (α-picolinic acid and pyridine-3-sulphonate) producing opposite effects. Damery and Alexander (1969) examined effective and kanamycin-resistant ineffective derivatives of *R. meliloti* and found that vitamin B_{12}, which is reported to be abundant in effective nodules, did not influence the symbiosis. In general the above studies differed from those described herein in the use of excised roots, unrelated non-mutant rhizobia, or more indirect approaches. In addition, the reports of stimulatory effects involved an increase in number of nodules per plant or percent of plants nodulated, rather than a distinct qualitative change in *type* of nodule or level of effectiveness as found for the riboflavin auxotroph. These reports do, however, suggest a possible essential role for some other growth factors (e.g. inositol or niacin) which should be amenable for study with appropriate auxotrophs of *Rhizobium*.

One may question whether nutritional requirements of the riboflavin or adenine–thiamine type occurring among laboratory-isolated spontaneous mutants also contribute to lowered effectiveness in naturally occurring rhizobia. The previously reported finding with auxotrophic, antimetabolite-resistant mutants (Schwinghamer 1969) suggested that partial auxotrophy involving more complex nutritional requirements (mainly vitamins) may increase the probability of ineffectiveness, but experimental evidence with ineffective strains from the field is as yet lacking. Field nodule isolates are most commonly cultured on yeast extract media and relatively infrequently tested for ability to grow well on a minimal medium. Moderate differences in growth on minimal agar have been noted among some geographically diverse strains of *R. trifolii* by the author but these differences have not been correlated with effectiveness on either red or subterranean clover.

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