THE OCCURRENCE OF A PREVIOUSLY UNOBSERVED POLYSACCHARIDE IN IMMATURE INFECTED CELLS OF ROOT NODULES OF *TRIFOLIUM AMBIGUUM* M. BIEB. AND OTHER MEMBERS OF THE TRIFOLIEAE

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Summary

A type of ineffective nodulation is described in which the bacteroids in the nodule cells fail to mature, and in which the infected host cells accumulate water-soluble polysaccharide between protoplast and cell wall. Ineffectiveness of this kind is characteristic of nodules on *Trifolium ambiguum* M. Bieb. produced by unadapted strains of bacteria, or produced by adapted strains on a small proportion of plants. It is also found when strains effective with *T. ambiguum* nodulate subterranean or white clover. The polysaccharide in the peripheries of infected cells is readily seen by phase-contrast observation, provided the sections are not hydrated after removal of wax, but it is not visible by ordinary staining procedures.

Examination of a number of effective nodules from various members of the Trifolieae revealed that the above type of polysaccharide accumulation always occurs as a transitory deposit in a narrow layer of cells across the immature region of the bacteroid-containing tissue. The possible significance of this deposit is discussed.

I. INTRODUCTION

A study of the symbiosis of *Trifolium ambiguum* M. Bieb. in progress in this Laboratory has revealed very extensive variation in the host, which particularly affects nodulating capacity (i.e. susceptibility to infection), and the symbiotic effectiveness of the nodules produced by adapted and unadapted strains of nodule bacteria. The extreme expression of variation found in this species is thought to be related to its cross-fertilizing habit (Hely 1956).

With adapted strains of bacteria (i.e. those obtained from the region of natural occurrence of *T. ambiguum*) a largely effective symbiosis was established, only a small proportion of the plants remaining nitrogen deficient. In some of the latter the nodules were found to be too few and too small to be able to effect any detectable nitrogen fixation, but in others the number of nodules produced was larger and an appreciable amount of potentially fixing tissue was formed. Most Australian isolates of *Rhizobium trifolii* either failed to nodulate *T. ambiguum* or did so in a very sparse and ineffective manner.

Ineffective nodules of these kinds have been examined by various cytological and cytochemical procedures, and their structure compared with that of effective nodules on *T. ambiguum* and nodules produced by the same strains of bacteria on other *Trifolium* species, as well as with nodules produced by known effective strains of bacteria on *Trifolium* and *Medicago* species.

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II. Materials and Methods

(a) *Plant and Bacterial Material*

Nodules were examined from plants grown in test-tube culture after the method of Thornton (1930), and from inoculated plants grown in pots. For the most part the hexaploid, 6*n*(3), strain of *T. ambiguum* (Hely 1956), and commercial samples of *T. subterraneum* L. (var. Mt. Barker), *T. repens* L. (New Zealand certified mother seed), and *Medicago tribuloides* Desr. were used. Nodules from *M. hispida* J. Gaertn. grown in plots at Temora, N.S.W., were also compared with the others. The various

Table 1

PLANT AND BACTERIAL MATERIALS USED AND TYPE OF SYMBIOSIS ESTABLISHED

Host	Bacterial Strains	Type of Nodules Examined
T. ambiguum (6*n*(3))*	A*	Effective and ineffective
	B*	Effective and ineffective
	C*	Effective and ineffective
	D*	Effective and ineffective
	Australian isolates	Ineffective
T. subterraneum (var. Mt. Barker)	A	Ineffective
	B	Ineffective
	C	Ineffective
	D	Ineffective
	SU297	Effective
T. repens (New Zealand certified mother seed)	A	Ineffective
	B	Ineffective
	C	Ineffective
	D	Ineffective
	C1F†	Effective
M. tribuloides	CC146	Effective
	CC157	Effective
M. hispida	Nodules from the field, Temora, N.S.W.	Effective

* See Hely (1956). † Rothamsted culture collection.

host–bacterial strain combinations examined are set out in Table 1. Cultures were maintained on yeast–water–mannitol–agar, and plants were inoculated immediately after germination. Potted plants were grown from plants initially cultured in test tubes where their symbiotic response had been determined.

(b) *Microscopic Methods*

Nodules were fixed in Flemming's solution, dehydrated in graded ethanol, and embedded in paraffin wax. For unstained preparations the following procedure was employed. Sections 3–5 μ in thickness were cut and mounted on slides, the wax was removed by two changes of xylol, and the sections mounted under thin

coverslips in "Xam" (Gurr). These were examined with a "Wild Varicolor" phase-contrast system using monochromatic green light, the exact wavelength (between 500 and 530 mμ) being selected for each preparation to give minimal refractility of cell wall material and maximal contrast in the various cytological features being examined. Such changes in wavelength as were necessary were effected by tilting the interference filter in the light source of the equipment. The principles of this method are discussed by Dufour and Locquin (1951).

Normal bright-field illumination was used for stained preparations and sections were cut with a thickness of 5–7 μ. Rose bengal–light green (Brenchely and Thornton 1925) was used as a general anatomical stain and polysaccharide was stained by the Hotchkiss method (Glick 1949).

Bacteroids from nodules were examined as previously described (Bergersen 1955) using phase-contrast observation and staining with tetrazolium blue or neotetrazolium chloride (Synthetical Laboratories, Chicago, U.S.A.) in M/15 phosphate buffer

Photomicrographs were obtained with a "Mikas" camera attachment using Ilford "Micro-neg Pan" film and processing for maximum gradation.

III. Results

(*a*) *Nodule Structure in* T. ambiguum

Stained sections showed that there were no gross structural abnormalities in the ineffective nodules of *T. ambiguum*. The decay of the bacteroid-containing tissue took place somewhat earlier in ineffective than in effective modules, but the bacteroid-containing tissue persisted for some time in the former. The degree of persistence of the bacteroid-containing tissue was independent of the kind of ineffectiveness produced, whether associated with adapted or unadapted strains of bacteria.

Phase-contrast methods, however, revealed marked histological differences between effective and ineffective nodules, particularly with regard to carbohydrate accumulation. In all ineffective nodules excessive accumulation of starch occurred: this appeared as very bright grains. It was also observed that the boundary of the protoplast of the nodule cells containing ineffective bacteroids was brightly refractile compared with the same structures in effective nodules. Closer examination showed that this refractility was due to deposition of material between protoplast and cell wall of the infected cells (Plate 1, Figs. 2, 3, and 4). The amount of deposit paralleled starch accumulation which occurred in adjacent, uninfected cells; the material appeared first as refractile granules at the periphery of the young infected cells into which the bacteria had been recently released, had multiplied, and were undergoing the first stages of bacteroid formation. These deposits of refractile material coalesced as development of the host cell proceeded, and eventually formed a more or less complete layer around the bacteroid-filled cells. Deposition was much greater at peripheries adjacent to uninfected cells than between infected cells (Plate 1, Figs. 2, 3, and 5).

Because of the similar refractile nature of the starch grains and the deposited material it was thought that this material was probably also polysaccharide in

nature. Preparations, stained by the Hotchkiss method, were made using 70 per cent. (v/v) alcoholic solutions to prevent possible loss of the deposited material during the staining procedure due to solubility in water. These tests confirmed the polysaccharide nature of the material (Plate 1, Fig. 5). Comparison of sections stained by the rose bengal–light green method and unstained sections, seen by the phase-contrast method, showed that some of the deposited carbohydrate was removed during staining in aqueous solutions, and that which remained was unstained. It is doubtless for this reason that this material has not previously been reported. Experiments were then performed which showed that the polysaccharide was almost entirely removed from sections by immersion for 1 hr in water at 30°C: a treatment which did not alter the appearance of the starch in these sections. In sections mounted in Lugol's or Gram's iodine the starch was stained blue and the peripheral polysaccharide brownish blue. From these observations it was concluded that the polysaccharide deposited in the periphery of infected cells of ineffective nodules from *T. ambiguum* was in a fairly highly polymerized form, possibly a dextrin.

(*b*) *Structure of Nodules Produced by Strains A, B, C, and D on Subterranean and White Clover*

Observations were next extended to an examination of ineffective nodulation produced by the *T. ambiguum* bacteria strains A, B, C, and D on subterranean and white clovers. Sections of such nodules revealed the same persistence of bacteroid-containing tissue as occurred with the ineffective *T. ambiguum* nodules. This tissue was similar in appearance in the three clovers, and also accumulated carbohydrate at the periphery of infected cells. This phenomenon would therefore appear to be general in ineffective *Trifolium* nodules produced by these strains of *Rhizobium*.

(*c*) *The Transitory Accumulation of Carbohydrate in Effective Nodules in the Trifolieae*

Closer examination of effective nodules on *T. ambiguum* by the phase-contrast method showed that a limited amount of peripheral carbohydrate also occurred in actively nitrogen-fixing nodules. It was, however, limited to a narrow zone of cells a short distance behind the nodule meristem and within which bacteroid formation was not yet complete. In section, the cells containing the peripheral carbohydrate appeared as a narrow band transverse to the main axis of the nodule and were one or two cells in thickness (Fig. 1).

An examination of effective nodules on subterranean and white clovers was then undertaken together with *Medicago tribuloides* and *M. hispida* nodules. In all these species a band of cells was again found which contained immature bacteroids and a deposit of peripheral polysaccharide (Plate 1, Fig. 6).

(*d*) *Peripheral Carbohydrate Accumulation and Immaturity of Bacteroids*

The association of a layer of carbohydrate with a certain stage of bacteroid development suggested that the uniform distribution of peripheral carbohydrate throughout the ineffective nodules on *T. ambiguum* may be associated with a persistence of a juvenile stage of bacteroid formation.

Examination of *T. ambiguum* showed that the bacteroids from ineffective nodules were, in fact, identical with bacteroids from very young effective nodules: morphologically in that they were much thinner and metabolically in possessing fewer oxidatively active granules than mature effective bacteroids (Fig. 2), as shown by staining with tetrazolium compounds in the presence of a substrate such as glucose or succinate. Phase-contrast observation suggested that the perinuclear areas found in effective subterranean clover bacteroids (Bergersen 1955) were also absent from the immature bacteroids. These findings suggested that the bacteroid-containing tissue found in ineffective nodules of *T. ambiguum* is unable to fix nitrogen because the bacteroid development is incomplete.

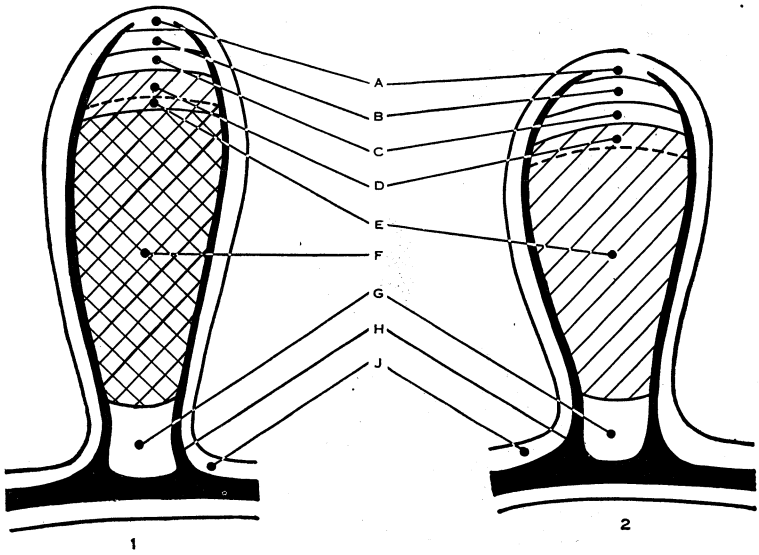

Fig. 1.—Diagrammatic representation of the arrangement of tissues in effective (*1*) and ineffective (*2*) nodules in *T. ambiguum*. *A*, meristem; *B*, region of infection thread penetration; *C*, release of bacteria from threads and their intracellular multiplication; *D*, region of bacteroid formation; *E*, region of cells containing immature bacteroids and peripheral polysaccharide; *F*, region of active, mature, bacteroid-containing tissue; *G*, decay of old tissue; *H*, vascular system of nodule; *J*, cortex of nodule and root.

IV. Discussion

The existence of a zone of cells in which polysaccharide accumulates between cell wall and protoplast is a hitherto undescribed feature in the development of effective nodule tissue, and, together with the persistence of these cells in ineffective nodules of the *T. ambiguum* type, invites consideration of its relation to the nitrogen-fixing processes. In ineffective nodules absence of nitrogen fixation appears in all cases to be correlated with the failure of the bacteroids to complete their development. This failure may be caused by inadequacy of the host plant or of the bacteria since some strains of bacteria gave both effective and ineffective nodules on *T. ambiguum* and entirely ineffective nodules on two other host species. From

this and previous work (Bergersen 1955, Bergersen and Nutman 1957) it has become clear that successful symbiotic nitrogen fixation depends on the completion of a series of steps, each of which involves interactions between host cells and bacteria. The failure or blockage of any of these steps prevents fixation. This concept has already been stated in a preliminary report (Bergersen 1956). In the present case evidence points to the failure of a fairly late step in which some essential change in the bacteria has not taken place.

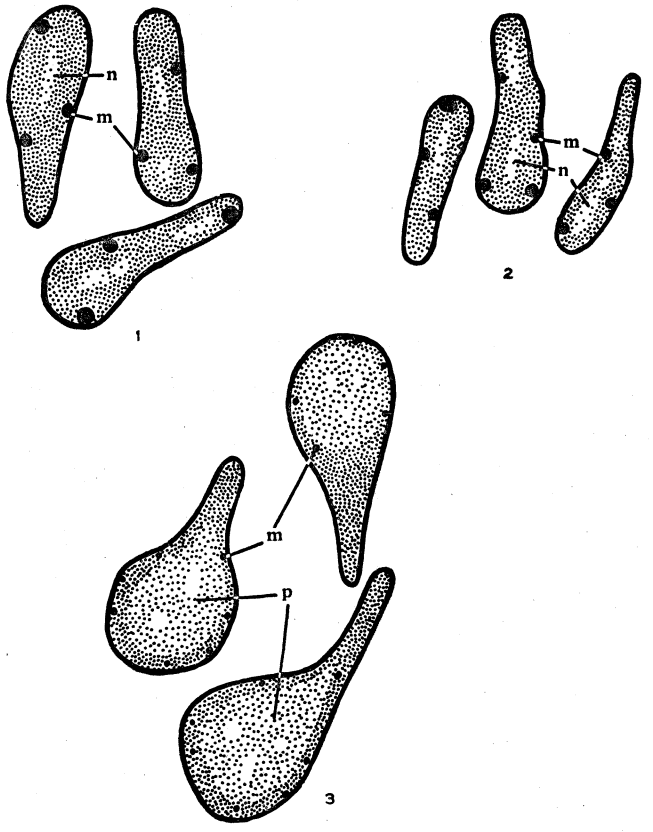

Fig. 2.—Drawings of bacteroids seen by phase-contrast microscopy. *1*, Bacteroids from a very young effective *T. ambiguum* nodule. *2*, Bacteroids from an ineffective *T. ambiguum* nodule. *3*, Bacteroids from mature tissue of an effective *T. ambiguum* nodule. *n*, Site of nuclear material; *m*, metabolically active mitochondria-like granules; *p*, perinuclear region of mature bacteroids.

The occurrence of carbohydrate as a transitory deposit in the periphery of infected cells of effective nodules on a number of host plants suggests the following hypothesis.

Host tissues provide appropriate intermediate substances which are used as substrates for the metabolism of the bacteroids inside infected cells. Some of these substances are transformed into amino acids etc. by combination with the primary compound of nitrogen fixation. The intermediate substances appear to be

provided before the bacteroids are fully matured and are synthesized into a temporary reserve material until fixation commences within the cells. The arrangement of the polysaccharide supports this hypothesis, since it is most abundant adjacent to uninfected cells and least abundant, or even absent, between infected cells: i.e. it occurs in regions in which one would expect to find accumulation if its utilization cannot take place.

The bacteroid-containing tissue consists of cells which have twice the chromosome number of uninfected tissue (Wipf and Cooper 1940). Differences in osmotic relations and permeability to non-electrolytes have been found between diploid and tetraploid plant tissues (Kostov and Nikolov 1941; Chen and Tang 1945; Chandler and Barton 1955): such differences may be a necessary condition for the passage of chemical substances into bacteroid-containing cells, and the carbohydrate accumulation may be a function of the relative ploidy of adjacent infected and uninfected cells. Further work on the nature of substances supplied by the host plant to bacteroid-containing cells may prove of value in elucidating more of the details of symbiotic nitrogen fixation in root nodules on leguminous plants.

The methods described have been used for many years with the exception of the phase-contrast technique; this has several noteworthy advantages:

(i) It renders visible nodule structures which required at least two different staining methods to demonstrate.

(ii) By eliminating the aqueous staining stages, loss of water-soluble material is minimized, although care should be taken in the interpretation of the appearance of precipitated water soluble substances.

(iii) It proves very useful as a screening method for the examination of large numbers of preparations, since it eliminates hydration, staining, and dehydration of the sections. The general aspect of tissues at first seems unfamiliar, but with practice, all histological features can be readily recognized.

V. Acknowledgments

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Explanation of Plate 1

Figures 1, 2, 3, 4, and 6 are 'Varicolor' photomicrographs of unstained nodule sections, 3–5 μ in thickness, and photographed in light of wavelength 500–530 mμ. All magnifications $\times 1400$. *b*, Bacteroid-filled host cytoplasm; *p*, peripheral polysaccharide; *s*, starch

Fig. 1.—Effective *T. ambiguum* nodule—bacterial strain A: bacteroid-filled cells.

Fig. 2.—Ineffective *T. ambiguum* nodule—bacterial strain A. Showing deposition of refractile polysaccharide in bacteroid-filled cells.

Fig. 3.—Ineffective *T. repens* nodule—bacterial strain A: as for Plate 1, Figure 2.

Fig. 4.—Ineffective *T. subterraneum* nodule—bacterial strain A: as for Plate 1, Figure 2.

Fig. 5.—Ineffective *T. ambiguum* nodule: Hotchkiss strain of bacteroid-containing tissue. Starch and peripheral polysaccharide stained dark red.

Fig. 6.—Effective *M. tribuloides* nodule—bacterial strain CC146: polysaccharide deposition in the periphery of a layer of cells, one cell deep, across the nodule tip. The meristem is out of the picture to the left and mature bacteroid-containing tissue is to the right.

POLYSACCHARIDE IN ROOT NODULES