

Role of Lectins in Plant-Microorganism Interactions

I. BINDING OF SOYBEAN LECTIN TO RHIZOBIA¹

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ABSTRACT

Highly purified soybean lectin (SBL) was labeled with fluorescein isothiocyanate (FITC-SBL) or tritium (³H-SBL) and repurified by affinity chromatography. FITC-SBL was found to bind to living cells of 15 of the 22 *Rhizobium japonicum* strains tested. The lectin did not bind to cells of the other seven *R. japonicum* strains, or to cells of any of the nine *Rhizobium* strains tested which do not nodulate soybean. The binding of the lectin to the SBL-positive strains of *R. japonicum* was shown to be specific and reversible by hapten inhibition with D-galactose or N-acetyl-D-galactosamine.

The lectin-binding properties of the SBL-positive *R. japonicum* strains were found to change substantially with culture age. The percentage of cells in a population exhibiting fluorescence after exposure to FITC-SBL varied between 0 and 70%. The average number of SBL molecules bound per cell varied between 0 and 2×10^6 . While most strains had their highest percentage of SBL-positive cells and maximum number of SBL-binding sites per cell in the early and midlog phases of growth, one strain had a distinctly different pattern. The SBL-negative strains did not bind lectin at any stage of growth.

Quantitative binding studies with ³H-SBL indicated that the affinity constant for binding of SBL to its receptor sites on *R. japonicum* is approximately 4×10^7 M⁻¹. Many of the binding curves were biphasic. An inhibitor of SBL binding was found to be present in *R. japonicum* culture filtrates.

Intimate and specific symbiotic associations between leguminous plants and bacteria of the genus *Rhizobium* provide most of the biologically fixed nitrogen available for agriculture. The rhizobia enter root hairs of the host plant in a structure called the infection thread (4). The infection thread, which is believed to be a tubular, inward growing invagination of the host cell wall, carries the bacterial symbiont into the cortex of the root (11). There the bacteria enter the cytoplasm of host cell, surrounded by an envelope of host cell plasma membrane (12). Both the bacteria and cortical cells of the host proliferate to form a root nodule where nitrogen fixation takes place.

The specificity of the *Rhizobium*-legume symbiosis is manifested by the failure of soil microorganisms other than rhizobia to gain effective entry into the plant by induction of infection thread structures, and also by host range specificity between members of the genus *Rhizobium* and the family Leguminosae. Rhizobia that infect and nodulate soybean, for example, cannot nodulate garden bean or white clover and vice versa. Such host

range specificity is the operational basis of species differentiation and cross-inoculation grouping within the genus *Rhizobium*. Conversely, and perhaps more accurately, such specificity may be viewed as the ability of a particular type of legume to recognize only certain types of rhizobia as potential symbionts.

Several investigators have recently examined the possibility that the lectins of host legumes may serve as determinants of host range specificity through binding of the lectins to characteristic carbohydrate structures on the cell surfaces of symbiotic rhizobia. In a pioneering work, Bohlool and Schmidt (2) tested the ability of FITC³-labeled soybean seed lectin to bind to 25 strains of *Rhizobium japonicum*, the symbiotic species for soybean, and to 23 other strains representative of several *Rhizobium* species which do not nodulate soybean. They reported that the SBL bound to 22 to the 25 strains of the soybean symbiont, *R. japonicum*, and that the lectin did not bind to any of the 23 nonsymbiotic strains.

Dazzo and Hubbell (6), in a study of the symbiotic association between *Rhizobium trifolii* and white clover, have found that clover roots and *R. trifolii* possess a common antigen. This common antigen was shown to be present on the cell surfaces of infective strains of *R. trifolii*, but absent, inaccessible, or present in reduced quantities on noninfective strains. These authors isolated capsular polysaccharide material from infective *R. trifolii* which possessed cross-reactive antigenicity. They also provided evidence for the presence of a lectin in clover seed extracts capable of binding to isolated capsular antigen and to infective—but not the noninfective—strains of rhizobia. These results led Dazzo and Hubbell (6) to propose that the clover lectin provides a bridge between common antigen structures for the preferential adsorption of infective strains of *R. trifolii* to the root surface of the host.

Wolpert and Albersheim (20) isolated lectins from four different legumes and obtained lipopolysaccharide preparations from four strains of *Rhizobium*, each capable of nodulating one of the legume species. The authors reported that the rhizobial lipopolysaccharides interacted with the host lectins, but not with the lectins from the nonhost legumes.

Dazzo and Hubbell (5) also examined the binding of FITC-labeled concanavalin A, the lectin of jack bean seeds, to several strains of rhizobia. They were unable to find evidence for preferential binding of this lectin to *Rhizobium* strains capable of nodulating jack bean. Chen and Phillips (3) have recently reported a similar lack of specific lectin binding in a brief survey of several lectins and rhizobial strains.

Several of these investigations have provided good correlative evidence in support of the hypothesis that lectins can function as important determinants of recognition or host range specificity in *Rhizobium*-legume symbiosis. At the same time, the existing literature is confusing, with a number of apparently

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³ Abbreviations: SBL: soybean lectin; FITC: fluorescein isothiocyanate; PBS: phosphate-buffered saline.

conflicting or anomalous results and interpretations. The diversity of methods and organisms used in these studies has made the direct comparison of results virtually impossible.

We have reexamined the binding of SBL to various strains of *Rhizobium*, and have attempted to develop a quantitative methodology appropriate for such studies. Our results generally agree with those of Bohlool and Schmidt (2). In addition, we have found that the biochemically specific binding sites for SBL appear to be transient rather than constitutive components of the *R. japonicum* cell surface.

MATERIALS AND METHODS

Commercial soybean flour (Soya Fluff 200W) was obtained from Central Soya Chemurgy, Chicago, Ill. Sugars and fluorescein isothiocyanate were purchased from Sigma Chemical Co. Cyanogen bromide and 6-amino caproic acid were purchased from Aldrich Chemical Co. Sepharose 4B was purchased from Pharmacia Fine Chemicals, and Aquasol and potassium ³H-borohydride were purchased from New England Nuclear.

Radioactivity was measured with a Packard Tri-Carb model 2002 liquid scintillation counter. A Leitz Ortholux II instrument with a Ploemopak 2.2 vertical fluorescence illuminator and FITC filter module H (2 × KP490/TK510/K515) was used for microscopic examinations.

Preparation of Soybean Lectin. Seed flour was defatted and extracted with 20 ml of phosphate-buffered saline (0.43 g KH₂PO₄, 1.48 g Na₂HPO₄, 7.20 g NaCl/l [pH 7.2]) per g of flour. After centrifugation and filtration, 1 ml of affinity-adsorbant beads (Sepharose-N-caproylgalactosamine [1]) per g of flour was added to the extract, stirred gently for 1 to 2 hr and washed extensively with PBS on a sintered glass filter. The washed beads were transferred to a column and the column was eluted with PBS until the eluate contained negligible A at 280 nm. The column was then eluted with 100 mM galactose until the lectin was fully desorbed. The eluted lectin was dialyzed extensively against PBS to remove galactose and stored frozen at -10 C.

Radiolabeled soybean lectin (³H-SBL) was prepared by periodate oxidation and borotritide reduction of the carbohydrate portion of the lectin (9). The radiolabeled lectin was repurified by affinity chromatography prior to use.

Fluorescent-labeled soybean lectin (FITC-SBL) was prepared according to Udenfriend (16). The FITC-labeled lectin was then repurified by affinity chromatography, dialyzed against PBS to remove the eluting sugar, and stored frozen at -10 C.

The purity of all of the labeled and unlabeled soybean lectin preparations was routinely checked by disc gel electrophoresis of 50-μg samples at pH 4.3 according to the procedures of Gabriel (7). The gels always showed one major band corresponding in position to unaggregated SBL, and usually showed a minor band corresponding in position to aggregated SBL (see 9), as well as a very faint band which migrated just ahead of the major band in the gels.

Rhizobium Cultures. *R. japonicum* strains 3Ilb 24, 29, 31, 38, 46, 62, 71a, 110, 117, 123, 125, 126, 135, 138, 140, 142, 143, and Wisc 505 were obtained through the courtesy of D. Weber, USDA, Beltsville, Md. *R. japonicum* strains 61A72, 61A76, 61A93, and 61A96, and strains of *Rhizobium* isolated from jack bean (22A1, 22A2, 22A4) and lima bean (127E12, 127E15) were the generous gifts of J. Burton, The Nitragin Company, Milwaukee. *R. phaseoli* 127K17, *R. trifolii* 162P17, *R. meliloti* 102F71, and *R. lupini* 96B9 were obtained from D. Keister, C. F. Kettering Research Laboratory.

Stock cultures were stored at 4 C on agar slants containing the medium of Valera and Alexander (17). Cultures in liquid medium were initiated by adding 1 ml of a sterile distilled H₂O suspension of bacteria from the stock slant (0.8 A at 660 nm) to 100 ml of growth medium in a 250-ml culture flask. Cultures

were grown at 24 C on a rotary shaker at 120 rpm and harvested by centrifugation at 4 C. In some cases, where noted, liquid cultures were initiated by transferring bacteria from stationary phase cultures to fresh media. Cultures which had just entered stationary phase were harvested, washed by centrifugation with sterile medium, and diluted with fresh sterile medium to give an A of 1.0 at 660 nm. Aliquots (1 ml) of this suspension were added to 250-ml flasks containing 100 ml of fresh culture medium.

Liquid cultures of *R. trifolii*, *R. lupini*, *R. meliloti*, and *R. phaseoli* were grown in the defined medium described by Vincent (18). Liquid cultures of *R. japonicum* were grown in a defined medium containing 0.5% sodium gluconate and 0.5% mannitol. In addition, this medium contained, per liter, 0.15 g NaH₂PO₄, 2.5 g KNO₃, 0.135 g (NH₄)₂SO₄, 0.25 g MgSO₄·7 H₂O; 1 ml of stock containing g MnSO₄·H₂O, 0.3 g H₃BO₃, 0.2 g ZnSO₄·7 H₂O, 0.025 g Na₂MoO₄·2 H₂O, 0.0025 g CuSO₄·5H₂O, and 0.0025 g CoCl₂·6 H₂O/100 ml; 1 ml of stock containing 100 mg nicotinic acid, g thiamine·HCl, 100 mg pyridoxine·HCl, and 10 g myo-inositol/100 ml; 1 ml of stock containing 75 mg KI/100 ml; and 1 ml of a sterile solution containing 16 g CaCl₂·2 H₂O/100 ml added to the foregoing medium after the medium had been autoclaved.

Bacterial cell populations were determined by averaging the cell counts from three replicate samples in a Petroff-Hausser bacterial counting chamber with phase contrast optics. Samples were diluted so as to contain between 150 and 300 cells/counting field.

FITC-SBL-binding Assay. For most of the FITC-SBL-binding studies reported in this paper, 100-μl samples of bacterial suspensions containing 2 × 10⁹ washed, living cells/ml PBS were mixed with an equal volume of FITC-SBL stock solution containing 3.0 mg FITC-SBL/ml PBS. The ratio of the absorbances of this stock at 493 nm and 280 nm was A₄₉₃/A₂₈₀ = 1.4. A few minutes after mixing, the sample was diluted with 5 ml PBS, centrifuged, resuspended in 200 μl PBS, and examined under the microscope. The proportion of cells in a bacterial population exhibiting fluorescence after exposure to FITC-SBL was determined by averaging replicate counts of both fluorescent and nonfluorescent bacteria. All fluorescing spots were routinely checked with phase contrast optics to confirm that they corresponded to bacterial cells. Cells in small aggregates of bacteria were counted individually, whereas large cell aggregates were removed prior to mixing with lectin by careful resuspension or, occasionally, by filtration through a plug of glass wool. The agglutination of rhizobia into large aggregates by SBL was minimized by using saturating concentrations of FITC-SBL in the binding experiments. The biochemical specificity of FITC-SBL binding to rhizobia was routinely checked by repeating the assays in the presence of 5 mM N-acetyl-D-galactosamine. (Solutions of N-acetyl-D-galactosamine in PBS deteriorate fairly rapidly, even when stored frozen or at 4 C, so that hapten activity is lost. The daily preparation of fresh solutions is essential.)

³H-SBL-binding Assay. Cultures of bacteria grown in liquid media were harvested and washed by centrifugation (10 min at 20,000g, 4 C). The cells were routinely washed twice with 30 ml PBS, three times with 30 ml PBS containing 50 mM galactose, then twice again with 30 ml PBS. After careful resuspension in a small volume of PBS (3-5 ml) the bacteria were counted and diluted with cold PBS to give a suspension containing 2 × 10⁹ cells/ml. Solid N-acetyl-D-galactosamine was added to half of the bacterial suspension to give a concentration of 10 mM.

Stock solutions of radiolabeled soybean lectin (³H-SBL) were prepared by diluting unaggregated (9) ³H-SBL with unlabeled SBL to give solutions (in PBS) which contained between 250 and 1,000 μg SBL/ml and 50,000 to 65,000 net cpm/ml. These stock solutions were stored frozen at -10 C.

Aliquots of the ³H-SBL stock solutions, 150 to 500 μl, were

added to duplicate sets of conical polycarbonate centrifuge tubes with a Rainin Pipetman adjustable micropipet. Reproducibility of delivery was improved by wetting the tips two or three times with the stock ^3H -SBL solution before dispensing aliquots into the assay tubes. PBS was then added to each tube as needed to bring the total volume to 500 μl .

One set of tubes then received 500- μl aliquots of the bacterial suspension containing 10 mM N-acetyl-D-galactosamine, and the duplicate set of tubes received 500- μl aliquots of the suspension to which no N-acetyl-D-galactosamine had been added. Frequently, a third set of duplicate tubes was assayed concurrently. This set received 500 μl of PBS rather than bacterial suspension (no bacteria control). Mixing of the bacterial suspensions (or PBS) with the ^3H -SBL solutions in the tubes was accomplished by rapid delivery of the former. After mixing, the samples were incubated in a 25 C water bath for 30 min and centrifuged for 10 min at 20,000g. Duplicate 400- μl aliquots of the supernatant solution were then taken for counting in 5 ml of Aquasol.

Swirling or shaking the tubes to effect mixing resulted in an increased and variable adsorption of the lectin to the inner surfaces of the assay tubes. The addition of 1 to 2 mg/ml of BSA reduces this nonspecific adsorption, but makes reproducible pipetting of solutions more difficult. Washing the cells repeatedly prior to the binding assays has been found to improve the reproducibility of the binding curves. Five washes with PBS alone are quite sufficient in this regard. It may also be noted that cells that have been frozen bind significantly less lectin at a given concentration of ^3H -SBL than cells from the same culture which have not been frozen.

Binding curves were plotted as the reciprocal of the molar concentration of bound lectin versus the reciprocal of the molar concentration of the free lectin, as suggested by Steck and Wallach (15). The concentrations of lectin protein in the ^3H -SBL stock solutions were determined by the method of Lowry *et al.* (10) using BSA as the standard.

RESULTS AND DISCUSSION

Binding of FITC-SBL to Rhizobia. Cultures of various *Rhizobium* strains were grown in defined liquid medium for 84 hr, then harvested and washed as described for the ^3H -SBL-binding assay. Shortly after washing, suspensions of living cells were mixed with highly purified FITC-SBL and examined under the microscope for binding of FITC-SBL to the bacteria (Table I).

Fifteen of the 22 *R. japonicum* strains which were tested showed binding of FITC-SBL. The remaining strains of *R. japonicum*, and the several strains representative of species which do not nodulate soybean, did not show detectable levels of FITC-SBL binding. Strain 3I1b 123, designated at (\pm) in Table I, was tested on several occasions with variable results, sometimes negative (no fluorescent cells observed), sometimes intermediate (1–30% fluorescent cells), and sometimes positive (>30% fluorescent cells).

The effect of the monosaccharide hapten, N-acetyl-D-galactosamine, on FITC-SBL binding was examined. N-acetyl-D-galactosamine is an effective monosaccharide hapten (*i.e.* competitive analog inhibitor) of SBL binding in hemagglutination assays (8) and fully inhibits the binding of SBL to *R. japonicum* cells at concentrations greater than 1 mM. No fluorescent cells could be detected after mixing FITC-SBL with *R. japonicum* cells suspended in 5 mM N-acetyl-D-galactosamine. Similarly, the addition of 5 mM N-acetyl-D-galactosamine to cells previously exposed to FITC-SBL caused a rapid and apparently complete reversal of lectin binding. This hapten also prevents or reverses the agglutination of *R. japonicum* cells which results from lectin binding.

The specificity of SBL binding to *R. japonicum* was also examined by testing a variety of other sugars (D-glucose, D-galactose, D-mannose, D-xylose, L-arabinose, D-galacturonic

Table I. Binding of FITC-SBL to Strains of *R. japonicum*

Liquid cultures of *R. japonicum* strains were initiated from agar slant cultures, harvested after 84 hr, and assayed for FITC-SBL binding as described under "Materials and Methods." A strain was designated as SBL-positive (+) if at least 30% of the cells in the culture exhibited 4+ fluorescence due to FITC-SBL binding. Strains were designated as SBL-negative if none of the cells in the culture exhibited fluorescence due to FITC-SBL binding. Strain 3I1b 123, designated as (\pm), was variable with respect to the proportion of FITC-SBL-positive cells. The results of FITC-SBL-binding experiments reported by Bohlool and Schmidt (2) are also indicated in Table I as B & S. They have used 3+ and 4+ to indicate intensity of fluorescence for strains with high proportions of FITC-SBL-positive cells, and either + or ++ to designate strains with small proportions of FITC-SBL-positive cells. Strains that appear visually to produce slime on agar slants or in liquid cultures are indicated with a + in the table. ND means not determined.

Strain	B & S	Slime	Strain	B & S	Slime
3I1b 24	+ 4+	+	3I1b 126	+ ++	+
3I1b 29	+ +	-	3I1b 135	+ +	\pm
3I1b 31	+ +	\pm	3I1b 138	+ 4+	+
3I1b 38	+ 4+	-	3I1b 140	+ 4+	+
3I1b 46	- ND	ND	3I1b 142	+ ND	+
3I1b 62	+ +	+	3I1b 143	+ ND	+
3I1b 71a	+ ND	+	Wisc 505	- -	-
3I1b 110	+ 4+	+	61 A 72	- -	-
3I1b 117	+ +	+	61 A 76	- ND	-
3I1b 123	(\pm) 3+	+	61 A 93	- -	-
3I1b 125	- ND	ND	61 A 96	- ND	-

acid, D-glucuronic acid, D-galactosamine·HCl, D-glucosamine·HCl, and N-acetyl-D-glucosamine) as potential haptens. Of these sugars, only D-galactose was an effective hapten of FITC-SBL binding to *R. japonicum* strain 3I1b 138 cells at a concentration of 50 mM, although D-fucose and D-galactosamine both gave partial inhibition at this concentration.

Binding of ^3H -SBL to Rhizobia. The difficulties of quantitating accurately the binding of FITC-SBL to rhizobia led us to develop a radiolabel-binding assay. Soybean lectin can be readily radiolabeled with either tritium (9) or ^{125}I (J. Paxton and K. Keegstra, personal communications) and repurified by affinity chromatography.

A time course of the binding of ^3H -SBL to cells from 84-hr-old, slant-initiated cultures of *R. japonicum* strain 3I1b 138 is shown in Figure 1. Comparison of the samples with the hapten controls and the no bacteria controls indicated that 2 to 4% of the added ^3H -SBL bound nonspecifically to the polycarbonate assay tubes, and that 96 to 98% of the total ^3H -SBL which bound to the bacteria could be prevented from binding by the sugar hapten.

Changes in Lectin Binding with Culture Age. Attempts to obtain quantitative measures of the number of lectin molecules bound/bacterial cell and to determine the affinity constant for lectin binding to the bacteria were initially unsatisfactory because the results varied considerably from one set of cultures to the next. Since the variability did not seem to be due to contamination of the cultures, we investigated the possibility that the lectin-binding properties of the bacteria were changing rapidly with culture age.

Stationary phase cultures (9-day) of *R. japonicum* strain 3I1b 138 were transferred to fresh medium and grown as described under "Materials and Methods." After harvesting and washing the cells as described for the ^3H -SBL-binding assay, the cell suspensions were diluted appropriately and counted. The percentage of cells in the population which bound FITC-SBL and the average number of ^3H -SBL molecules bound/cell were measured. Saturating or near saturating concentrations of the lectin (300–500 $\mu\text{g/ml}$) and appropriate hapten controls were used for these determinations.

The pattern of variation in lectin-binding properties with

culture age, seen in Figure 2, is quite reproducible for strain 3Ilb 138 provided that the culture conditions and inocula are made as nearly the same as possible. The stationary phase cell suspensions used to initiate the cultures contained no cells exhibiting fluorescence after exposure to FITC-SBL, and no binding of ^3H -SBL to these cell suspensions could be detected. When the number of cells had increased two to eight times in the fresh medium, however, a small proportion of the cells (5–20%) exhibited maximum (*i.e.* 4+) fluorescence after exposure to FITC-SBL, and modest binding of ^3H -SBL to the suspensions could be measured. After 24 hr the percentage of cells binding FITC-SBL increased rapidly, reaching a maximum of about 70% for cultures in the midlog phase of growth (4 days). Thereafter, the percentage of cells binding FITC-SBL decreased fairly rapidly, returning to zero for 9-day-old, stationary phase cultures. It may be noted that the cells from older cultures (6–8 days) generally appeared less intensely fluorescent (3+ to 1+) after exposure to FITC-SBL than cells from younger cultures. Also, older cultures frequently yielded fluorescent globs or particles of FITC-labeled material which did not appear to be attached to bacterial cells. These fluorescent globs or particles may be aggregates of receptor material which have become detached from the cells and precipitated by FITC-SBL. In contrast, all of the FITC-SBL fluorescence in younger cultures is clearly associated with particular cells.

Figure 2 also shows that the average number of ^3H -SBL molecules bound/cell increases abruptly between 24 and 36 hr, then decreases abruptly by 48 hr. This spike in the number of lectin molecules bound/cell has been observed repeatedly, and always occurs very soon after the lag phase of growth ends. The mean generation time of *R. japonicum* 3Ilb 138 at this stage of growth is approximately 12 hr. After this spike, the number of SBL-binding sites/cell increases gradually to a maximum at 4

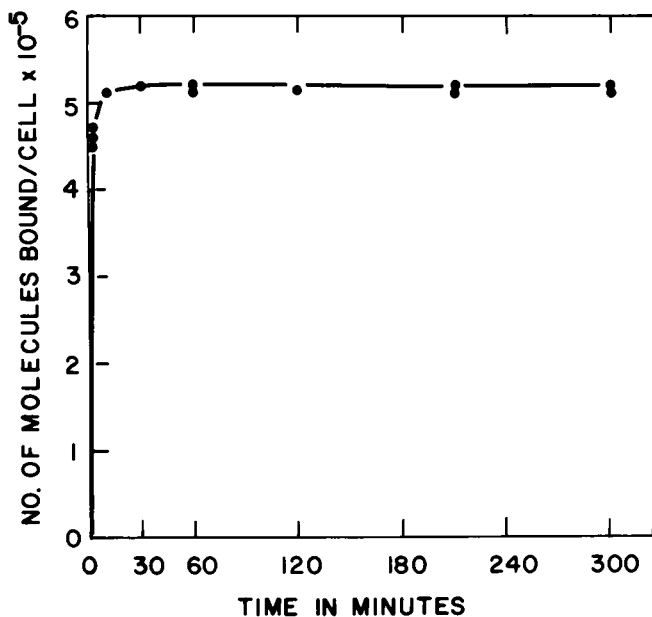


FIG. 1. Time course of ^3H -SBL binding to living *R. japonicum* cells. Liquid, slant-initiated, cultures of *R. japonicum* strain 3Ilb 138 were harvested after 84 hr and washed as described under "Materials and Methods" for ^3H -SBL-binding experiments. A suspension of the cells (2×10^9 cells/ml) in PBS was mixed with an equal volume of ^3H -SBL stock containing 228 μg SBL/ml and incubated at 25 C. Aliquots (1,000 μl) were withdrawn at various times and centrifuged at 20,000g for 10 min at 4 C. Duplicate 400- μl aliquots of the supernatant solutions were taken for counting in Aquasol. Hapten controls (containing 2 mM N-acetyl-D-galactosamine) and "no bacteria" controls were assayed concurrently. Figure 1 shows the average number of SBL molecules bound/cell. The concentration of SBL used in the experiment was not saturating.

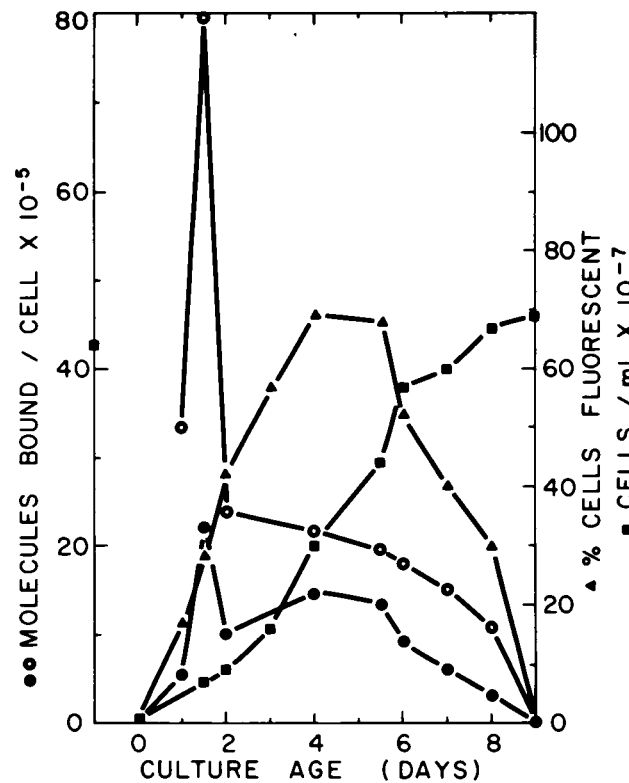


FIG. 2. Changes in lectin-binding properties of *R. japonicum* strain 3Ilb 138 with culture age. Stationary phase 3Ilb 138, diluted 100-fold, were used to initiate new cultures in duplicate flasks. These cultures were grown, harvested at intervals, and the cells washed for ^3H -SBL-binding experiments as described under "Materials and Methods." The number of cells/ml in the cultures (■), the percentage of cells exhibiting fluorescence due to FITC-SBL binding (▲), and the average number of SBL molecules bound/cell at saturating concentrations of ^3H -SBL (●) were determined as described under "Materials and Methods." The average number of SBL molecules bound/lectin-positive cell (○) was calculated by dividing the average number of SBL molecules bound/cell by the proportion of FITC-SBL-positive cells in the population. The binding of SBL shown in Figure 2 is that which could be inhibited by the presence of 5 mM N-acetyl-D-galactosamine (94–99% of the total SBL bound).

days (midlog phase) then decreases gradually to zero again once the cultures have reached stationary phase (9 days). The average number of ^3H -SBL molecules bound/fluorescent (or lectin-positive) cell varies in a similar manner with culture age. There is however no increase in the number of SBL-binding sites/lectin-positive cell as the cultures enter midlog phase.

Several other strains of *R. japonicum* (3Ilb 29, 38, 62, 110, and 143) give the same pattern of variation in the percentage of fluorescent cells with culture age (or growth phase). At least one strain of *R. japonicum*, 3Ilb 123, however, has a radically different pattern of variation. Cultures of this strain have FITC- and ^3H -SBL-binding cells in the stationary, lag, and early log phases of growth, but none in the midlog and late log growth phases. This is the strain designated as "±" with respect to lectin binding in Table I. The rather abrupt loss of lectin receptors from cells of this strain during the transition from early to midlog growth phase probably explains why cultures harvested at 84 hr sometimes contained FITC-SBL-binding cells and sometimes did not.

Two other strains of *R. japonicum*, 61 A 93 and Wisc 505, designated as negative in Table I, were tested with FITC-SBL at various culture ages. No FITC-SBL-binding cells could be detected in cultures of these strains, regardless of culture age or growth phase. Cultures of these strains grown on a modified yeast-mannitol (19) or a glutamate (14) medium were likewise FITC-SBL-negative at all culture ages tested.

³H-SBL-binding Curves. In order to obtain quantitative estimates of the affinity of soybean lectin for the receptors on cells of *R. japonicum* strains, suspensions of washed, living bacteria were incubated with varying concentrations of ³H-SBL as described under "Materials and Methods." Representative binding curves are shown in Figure 3.

The only binding curve that can be interpreted in a straightforward manner is curve A, which shows binding of SBL to cultures initiated with a cell suspension from a 4-week-old agar stock slant and harvested after 84 hr. Curve A shows a simple linear relationship between $1/[SBL]_{bound}$ and $1/[SBL]_{free}$ over the range of concentrations employed. The affinity constant for the binding of SBL to the bacteria, obtained by extrapolation of curve A, is $4 \times 10^7 M^{-1}$. The average number of lectin molecules bound/cell at saturating concentrations is 5×10^5 .

The other binding curves cannot be readily interpreted because they appear to be biphasic. Curves B and C show the binding of SBL to midlog phase cultures of strain 3IIb 138. Curve B was obtained with cells from the 4-day-old cultures used for the experiment shown in Figure 2. A virtually identical curve, displaced somewhat toward the abscissa, was obtained with the cells from this same experiment harvested after 36 hr. The cells used in the experiment giving curve C were from cultures initiated from 1-week-old agar stock slants and harvested after 4 days. Very similar binding curves have been obtained from midlog phase, slant-initiated cultures of strains 3IIb 110 and 3IIb 143. Curve D is representative of binding curves obtained from late log phase (6-day), slant-initiated cultures of both strain 3IIb 138 and strain 3IIb 110 (shown).

No experimentally verified explanation for the biphasic character of the majority of these binding curves can be offered. It seems unlikely that negative cooperativity of SBL binding can be the correct explanation since monophasic curves (such as curve A) have been obtained. *R. japonicum* strain 3IIb 138, which was used for most of the binding experiments, was reisolated from single cell colonies of the original stock. Similar biphasic binding curves have been obtained repeatedly with

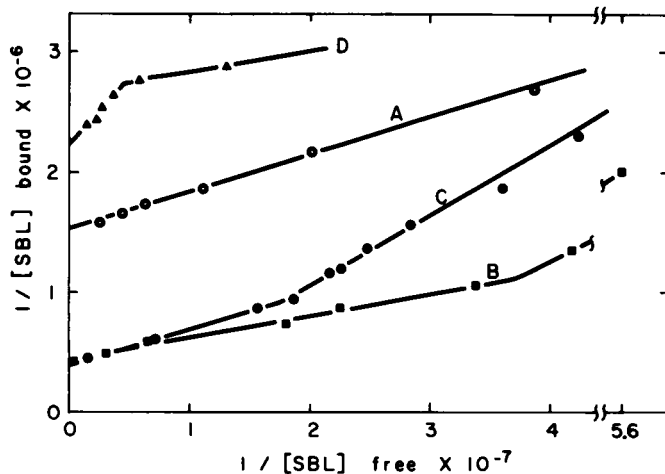


FIG. 3. Concentration dependence of ³H-SBL binding to living *R. japonicum* cells. The binding of ³H-SBL to *R. japonicum* cells at various lectin concentrations was determined as described under "Materials and Methods." The concentration dependence of SBL binding to the cells is shown as a double reciprocal plot of the molar concentrations of free and bound lectin, as described by Steck and Wallach (15). The concentration dependence of SBL binding to cells from 84-hr, slant-initiated cultures of strain 3IIb 138 is shown by curve A (○). Curve B (■) shows the binding to cells from the midlog phase (4-day) culture of 3IIb 138 obtained from the experiment shown in Figure 2. Curve C (●) shows the binding to cells of a midlog phase (4-day) slant-initiated culture of this strain. Curve D (▲) shows the binding to cells from a late log phase (6-day) culture of strain 3IIb 110. All curves show the binding inhibitable with 5 mM N-acetyl-D-galactosamine.

serial subcultures of strain 3IIb 138 and with cultures of two other *R. japonicum* strains (3IIb 110 and 143). These considerations make it unlikely that contaminating microorganisms are the cause of the biphasic binding curves. The existence of two classes or types of SBL-binding sites on the bacterial cells could also account for the biphasic binding, and is perhaps the most likely explanation.

Inhibition of ³H-SBL Binding by Culture Filtrates. The complete and rather rapid loss of lectin-binding sites from *R. japonicum* cells as the cultures enter stationary phase suggested that the receptors might be liberated into the medium in a more or less intact form. In order to investigate this possibility, culture filtrates from stationary phase cultures of strain 3IIb 138 were prepared. When a small volume of culture filtrate was preincubated briefly with FITC-SBL, and the mixture added to washed cells of a midlog phase culture of this strain, the binding of FITC-SBL to the bacteria was clearly diminished relative to the control without culture filtrate.

The ability of stationary phase culture filtrates of strain 3IIb 138 to inhibit the binding of radiolabeled SBL to the Sepharose-N-caproylgalactosamine affinity beads used for lectin purification was also examined. The results (Table II) show that stationary phase culture filtrates of this *R. japonicum* strain do contain an effective inhibitor of lectin binding, and that the inhibition of binding is roughly proportional to the concentration of culture filtrate in the assay mixture. Similar results have been obtained in inhibition assays using frozen, midlog phase *R. japonicum* 3IIb 138 cells rather than the affinity beads. Relatively little is known as yet about the nature and stability of this inhibitor. The culture filtrates do retain inhibitory activity at least 8 weeks after dialysis and freezing.

FURTHER DISCUSSIONS AND CONCLUSIONS

The data in Table I indicate good agreement between our results and those of Bohlool and Schmidt (2). In addition to the *R. japonicum* strains, nine strains of rhizobia that do not nodulate soybean were examined, and all failed to bind FITC-SBL. This finding is in agreement with the results reported by Bohlool and Schmidt. Chen and Phillips (3) however, recently reported that FITC-SBL binds to five of seven strains of rhizobia that are incapable of nodulating soybean. We agree with their characterization of *R. japonicum* 61A96 and *R. lupini* 96B9 as SBL-negative. However, we find that both *R. phaseoli* 127K17 and *Rhizobium* sp. 22A1 are SBL-negative at all phases of growth in our cultures. Bohlool and Schmidt have also characterized these two strains, as well as *R. leguminosarum* 128C53, as SBL-negative. Unfortunately, Chen and Phillips did not discuss the instances where their data conflicted with those of Bohlool and Schmidt. It may be significant that the binding of FITC lectins reported by Chen and Phillips was of uniformly low intensity (1+ or 2+) and frequency (always less than 8% of the cells, and often less than 1%).

Several features of the appearance and disappearance of SBL-binding sites on *R. japonicum* may be noted because of their potential importance in understanding the nature of the binding sites and in appreciating some potential complexities of the *in vivo* interactions between the symbionts. (a) The appearance of SBL-binding sites on *R. japonicum* depends on different conditions or control mechanisms for different strains of the organism. Strains 3IIb 29, 38, 62, 110, 138, and 143 all appear to follow a very similar pattern of variation in SBL-binding properties with culture age. Strain 3IIb 123 has a distinctly different pattern of variation, as indicated previously. Strains Wisc 505 and 61A93 differ by having no SBL-positive cells at any phase of growth under the conditions described. If one assumes that SBL-negative strains of *R. japonicum* have the genetic potential to synthesize SBL-binding sites, then these strains would comprise a third class of isolates with respect to

Table II. Inhibition of ^3H -SBL Binding to Sepharose-N-Caproylgalactosamine Affinity Beads by *R. japonicum* Culture Filtrate

Aliquots of ^3H -SBL stock solution (400 μl) were mixed with dialyzed culture filtrate (c.f.) from a stationary phase (9-day) culture of *R. japonicum* strain 311b 138 and incubated at 25 C for 15 min. One hundred μl of an affinity bead (1) suspension was then added and the total volume adjusted to 850 μl with PBS. The beads were kept in suspension by frequent withdrawal and return of liquid with individual pipet tips. Samples were incubated for 30 min at 25 C, centrifuged, and 300- μl aliquots of the supernatant solution taken for counting in Aquasol.

Additions	Supernatant	^3H -SBL b d
	net cpm	%
N-Acetylgalactosamine (to 5 mM)	5,194	0
None	835	84
50 μl dialyzed C.F.	1,659	68
100 μl dialyzed C.F.	2,143	59
200 μl dialyzed C.F.	3,021	42
350 μl dialyzed C.F.	3,565	31

the culture conditions or control mechanisms that determine the appearance of lectin-binding sites. (b) The lectin-binding sites on *R. japonicum* seem to appear in an all-or-none fashion on individual bacterial cells. (c) The average number of SBL-binding sites/lectin-positive cell is not constant.

It is evident that the regulation of the appearance and disappearance of lectin-binding sites on these bacteria is a many faceted and little understood phenomenon. While earlier investigators have largely ignored such regulation as a possible source of variation in the lectin-binding properties of rhizobial strains, such regulation must now be regarded as an important experimental consideration. In this regard, we are currently attempting to develop a suitable methodology for studying the lectin-binding properties of *R. japonicum* growing in the rhizosphere-rhizoplane environment of soybean plants. This approach could be useful in studying the role of the host plant in regulating the appearance of specific lectin-binding sites on the rhizobia, and offers the further advantage that both the lectin-binding properties and infectivity of the rhizobia can be examined concurrently and *in vivo*.

There appears to be no consensus regarding the biochemical nature and location of the lectin-binding sites on rhizobia. Wolpert and Albersheim (20) have suggested that legume lectins bind to the lipopolysaccharides of symbiotic rhizobia, and have presented some evidence for specific lipopolysaccharide-lectin interactions. Dazzo and Hubbell (6) on the other hand reported that the lectin from white clover appears to bind to capsular polysaccharide of the clover symbiont *R. trifolii*. Planqué and Kinje (13) have found that the lectin from pea binds to a polysaccharide species from the pea symbiont, *R. leguminosarum*, which is neither the lipopolysaccharide nor the exopolysaccharide. These results are not necessarily in conflict. Different rhizobia may have different—or multiple—receptor structures. The biphasic SBL-binding curves seen in Figure 3 may be due to the presence of two types of receptor structures on *R. japonicum*. However, it is difficult to be certain that lipopolysaccharide and exopolysaccharide preparations are free of cross-contamination or the presence of glycans such as the one described by Planqué and Kinje. A comparison of the surface polysaccharides obtained from lectin-positive and lectin-negative cells may be quite helpful in distinguishing which type of polysaccharide is involved in lectin binding. In this regard we have found that simple centrifugation separates *R. japonicum* cell suspensions into two visually distinct layers in the pellet. The top layer consists almost exclusively of cells that will bind SBL, whereas cells in the bottom layer are SBL-negative.

The results of our binding experiments may provide some useful insights regarding the nature and location of the lectin

receptors of *R. japonicum*. At saturating concentrations the number of SBL molecules bound/lectin-positive cell in actively growing cultures of *R. japonicum* strain 311b 138 varies between 2×10^6 and 8×10^6 . Rough calculations (assuming cell dimensions of $1 \times 3 \mu\text{m}$ and a molecular cross-section of $10 \times 10 \text{ nm}$ for SBL) indicate that only about 1×10^5 molecules of SBL could be fitted onto the outer membrane of a cell. This is 20- to 80-fold lower than the number of SBL molecules actually bound/lectin-positive cell. A discrepancy of this magnitude suggests that the SBL-binding sites are not located primarily on the outer membranes of the cells, but rather on some structure extending beyond the outer membrane. In addition, there is an excellent correlation between SBL binding to *R. japonicum* strains and the appearance of slime in slant or liquid cultures of the strains (Table I).

The results presented here and in the previous literature are clearly inadequate either to prove or disprove the validity of the hypothesis that lectins are important determinants of host range specificity in legume-rhizobia interactions. However, the demonstration that SBL binds specifically, with high affinity and in substantial amounts to many strains of the soybean symbiont, *R. japonicum*, helps to make the hypothesis more attractive. The existence of lectin-negative strains of the rhizobial symbiont seems less of an obstacle to acceptance of the hypothesis not that it has been shown that the lectin-binding sites on *R. japonicum* can appear or disappear depending on the growth condition of the organisms. We are thus encouraged to believe that further, intensive investigations of the lectin recognition hypothesis are justified. At the same time, we feel certain that the interactions which determine host range specificity are far more complex than anyone has imagined.

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