Antigenic Analysis of *Rhizobium japonicum* by Immunodiffusion

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Immunodiffusion reactions were studied with seven strains of *Rhizobium japonicum* and three strains of the cowpea miscellany by using antisera against eight of the strains. Most strains yielded only weak precipitin bands when untreated cell suspensions were used as antigens in the diffusions. Ultrasonic disruption or heat treatment of the cells led to stronger bands, and immersion in boiling water for 20 min was used as the standard procedure for preparing these bacteria for immunodiffusion analysis. Heat-labile antigens were detected in only a few strains; the major antigens of all of the strains appeared to be heat-stable. Many of the strains cross-reacted, sometimes in a nonreciprocal manner; unheated cell suspensions cross-reacted more widely but more weakly than the heated suspensions. Heat-treated crushed nodule preparations reacted well in immunodiffusions. The antigens of cultured cell and nodule extract (bacteroid) forms of three strains were compared. In one of these strains, an antigen present in the cultured cells was absent from the bacteroids. Unknown strains present in soybean root nodules were readily identified by immunodiffusion.

The agglutination technique has been widely used to identify nodule bacteria of the *Rhizobium japonicum* and cowpea groups grown in culture (3, 6, 11, 14, 16, 22, 28, 29) or present in the bacteroid form in the juice of crushed root nodules (5, 18, 23). The results of inoculation experiments and field ecological investigations with soybeans and *R. japonicum* strains identified in this way are frequently expressed in terms of serogroups (4, 5, 14, 15). The agglutination technique, however, lacks the analytical resolving power of immunodiffusion, especially in distinguishing between antigenically identical and closely related nonidentical strains. Because of the greater confidence that can be placed in identification of strains made by immunodiffusion than by agglutination (12, 19, 21, 26), it would be helpful in ecological studies if immunodiffusion could be used conveniently for the identification of *R. japonicum* isolates, especially directly from the crushed nodules.

Recently, Škrdleta has shown that immunodiffusion can be applied to *R. japonicum* strains, has detected the presence of heat-stable and heat-labile antigens (26), and has used the technique to identify strains present in soybean root nodules (24, 25, 27). However, when immunodiffusion experiments with *R. japonicum* were performed in this laboratory, with untreated concentrated cell suspensions as antigens, difficulties were encountered in obtaining reproducible, satisfactory results with different strains. Škrdleta had used freeze-dried cell suspensions (26) and crushed nodule preparations with (25) and without (24) heat treatment as the antigens in immunodiffusions but without any comment on differences in their usefulness. It was therefore decided that the immunodiffusion behavior of this organism should be examined more closely. The present paper describes experiments which show that *R. japonicum* is readily amenable to analysis by immunodiffusion after suitable pretreatment.

MATERIALS AND METHODS

Organisms. Seven strains of *R. japonicum* and three strains belonging to the cowpea miscellany were studied (Table 1). They were maintained on yeast extract-mannitol-agar slopes. For immunization of rabbits, they were grown on the defined agar medium of Bergersen (2).

Antisera. Well-grown cultures, generally 7 to 9 days old at 25 C, were dashed off the agar medium with physiological saline, and samples were emulsified with equal volumes of Freund's complete adjuvant (Difco). Rabbits were given intramuscular injections of 0.5 ml of the mixture, rested for 4 weeks, and then given subcutaneous injections of 0.2 ml of the culture without adjuvant. They were bled from the marginal vein of the ear on the 5th, 7th, 9th, and 12th day after the second injection, taking approximately 10 and 20 ml

TABLE 1. *Rhizobium strains used in the present study*

Organism[a]	Host	Antiserum prepared
R. japonicum		
CC110[b]	Glycine max	+
123[b]	G. max	+
127[c]	G. max	+
129[c]	G. max	+
CC705	G. max	−
CC711	G. max	−
CC1809	G. max	+
Cowpea miscel- lany		
CB376	Lotononis bainesii	+
CB627	Desmodium uncinatum	+
CB756	Dolichos africanus	+

[a] Prefixes CC and CB denote the formal strain numbers in the culture collections of Division of Plant Industry, CSIRO, Canberra, and the Division of Tropical Pastures, CSIRO, Brisbane, respectively.

[b] Strain used in previous agglutination study (references 4–6, 14, 16, 17).

[c] Strain used in previous agglutination study (reference 6).

of blood at alternate bleedings. The antisera from the separate bleedings were tested by immunodiffusion and pooled when found to give similar precipitin bands.

Antigens. Dense suspensions of cells, containing 50 to 100 mg of cell dry matter per ml, were prepared by centrifuging cultures diluted with saline to reduce their viscosity. To prepare crushed nodule suspensions, single washed nodules were used if they were large or several together were used if they were small and known to have originated from one strain; the nodules were crushed in narrow tubes with a glass rod in the presence of minimal amounts (ca. 0.1 ml per nodule) of saline.

Heat treatment of antigens consisted of immersion of the bacterial suspensions or nodule preparations in long, narrow glass tubes (0.6 by 10 cm; made from Pasteur pipettes) in a boiling-water bath for the desired time. The tubes were not stoppered and there was little evaporative loss. Ultrasonic disruption was performed with a Bronwill Biosonik I probe-type disintegrator, operated at 50% power output, on small (1 to 2 ml) samples in vials immersed in ice.

Immunodiffusion. Immunodiffusions were performed in gels made with 0.75% Ionagar no. 2 (Oxoid) in 0.85% sodium chloride and 0.025% sodium azide, in layers 4 mm in thickness with wells 4 mm in diameter, spaced at 4-mm distances from edge to edge. The gel plates were kept at 4 C. All of the plates shown in the figures were photographed after diffusion for 3 or 4 days.

Antisera were absorbed directly in the gels by first placing the absorbing antigen in the well designated to receive the absorbed antiserum, keeping the gel plate at 4.C overnight and removing any liquid remaining in the well on the following day before adding the antiserum. The other antigens were added to their wells at the same time as the antiserum. A serum was considered to be properly absorbed when it no longer formed bands with the absorbing antigen in the subsequent immunodiffusion. The necessary controls were always included in the immunodiffusions. Attempts to prepare absorbed antisera in tubes by adding concentrated intact washed cells to the sera were not successful; the use of heat-treated or sonically disrupted cells in this type of absorption was precluded by the complication of having mixtures of soluble antigens.

RESULTS

Establishment of optimum conditions. Preliminary immunodiffusion experiments with the *Rhizobium* cultures or concentrated cell suspensions showed that most strains reacted weakly with their homologous antisera. *R. japonicum* strain 123 was an exception and reacted well, but the other strains gave weak, indistinct reactions with the precipitin bands close to the wells, making comparisons with bands from adjacent wells difficult (Fig. 1, top well in each pattern). This suggested that the antigens were not readily diffusible. Ultrasonic disruption of the cells for periods of as long as 10 min led to the appearance of more bands, and even stronger bands were obtained when the disrupted suspensions were immersed in boiling water for periods of as long as 2 hr. Most strains did not seem to have heat-labile antigens; only strains 756 and 1809 possessed significant heat-labile antigens, destroyed by the heat treatment within 5 min.

Heat treatment by itself was found to be as effective as heat treatment preceded by ultrasonic disruption, yielding identical precipitin patterns (Fig. 1), and heat treatment alone was therefore chosen as the simplest effective procedure. Subsequent to this, all cultures, and later all crushed nodule preparations, were immersed for 20 min in a boiling-water bath before analysis by immunodiffusion

The merit of the preliminary heat treatment was clearly shown by making doubling dilutions of suspensions of cells with and without heat treatment and observing the highest dilution at which precipitin bands were visible. Figure 2 shows a typical result. Not only were the bands detectable at higher dilutions after heat treatment but also they were visible much earlier.

The minimum concentration of cell dry matter giving rise to visible bands with most of these strains was in the region of 0.1 to 0.4 mg/ml, equivalent to 0.5×10^9 to 2×10^9 cells/ml. Of course, to observe the interaction of bands from
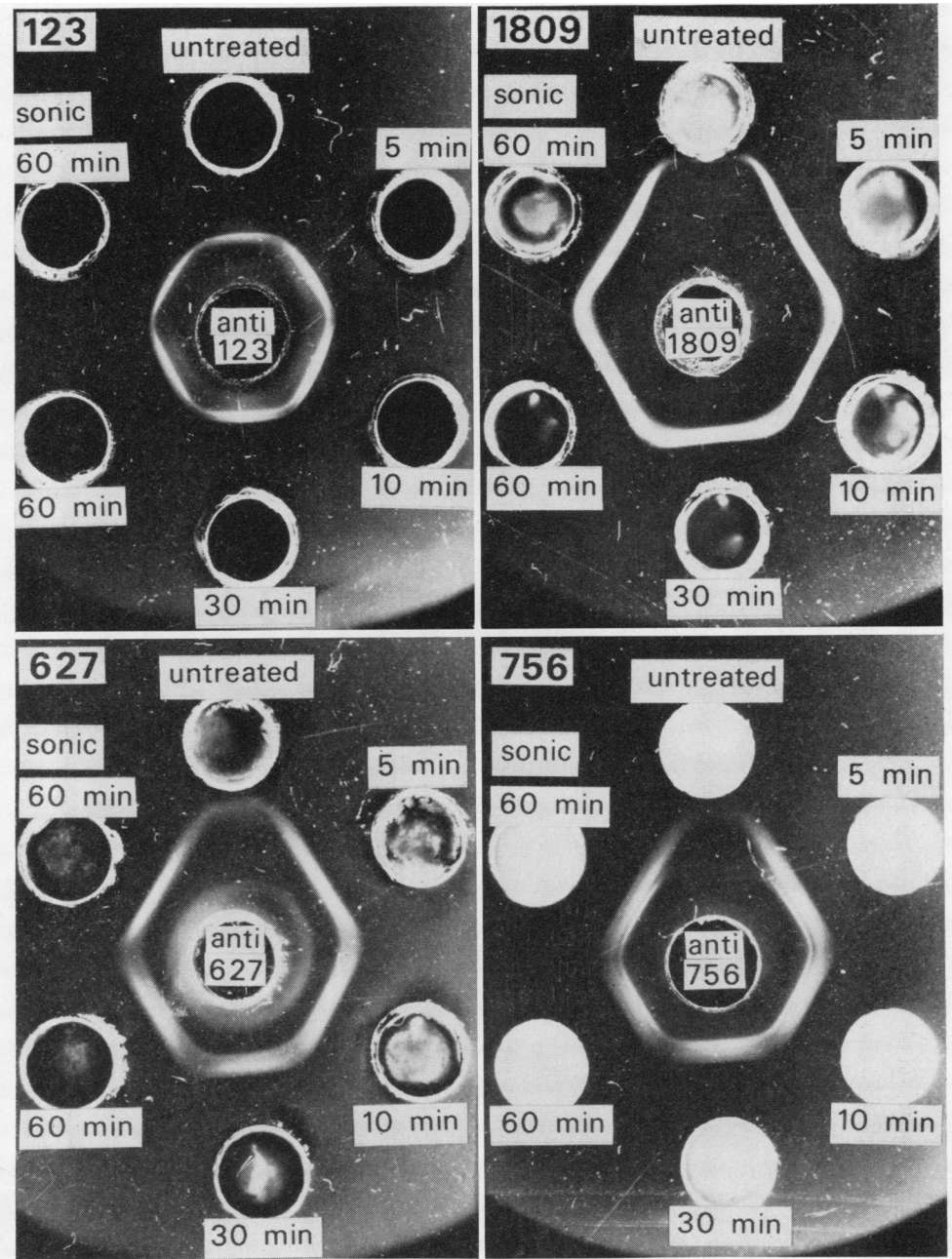
FIG. 1. *Effect of heat treatment (100 C for the times shown) on the homologous immunodiffusion patterns of intact cell suspensions of Rhizobium japonicum strains 123 and 1809 and cowpea miscellany strains 627 and 756. Untreated suspensions (top wells) and ultrasonically disrupted (8 min) cells heated for 60 min (top left wells) were included for comparison.*

adjacent wells, higher antigen concentrations are necessary.

R. japonicum strain 1809. The antigens of this strain are of particular interest because some are heat-labile in contrast to most of the other strains examined here which seem to possess only heat-stable antigens. After ultrasonic disruption of strain 1809 cells, both types of antigens were released, heat-labile antigens giving rise to diffuse bands near the antiserum well (Fig. 3, wells 3, 4,
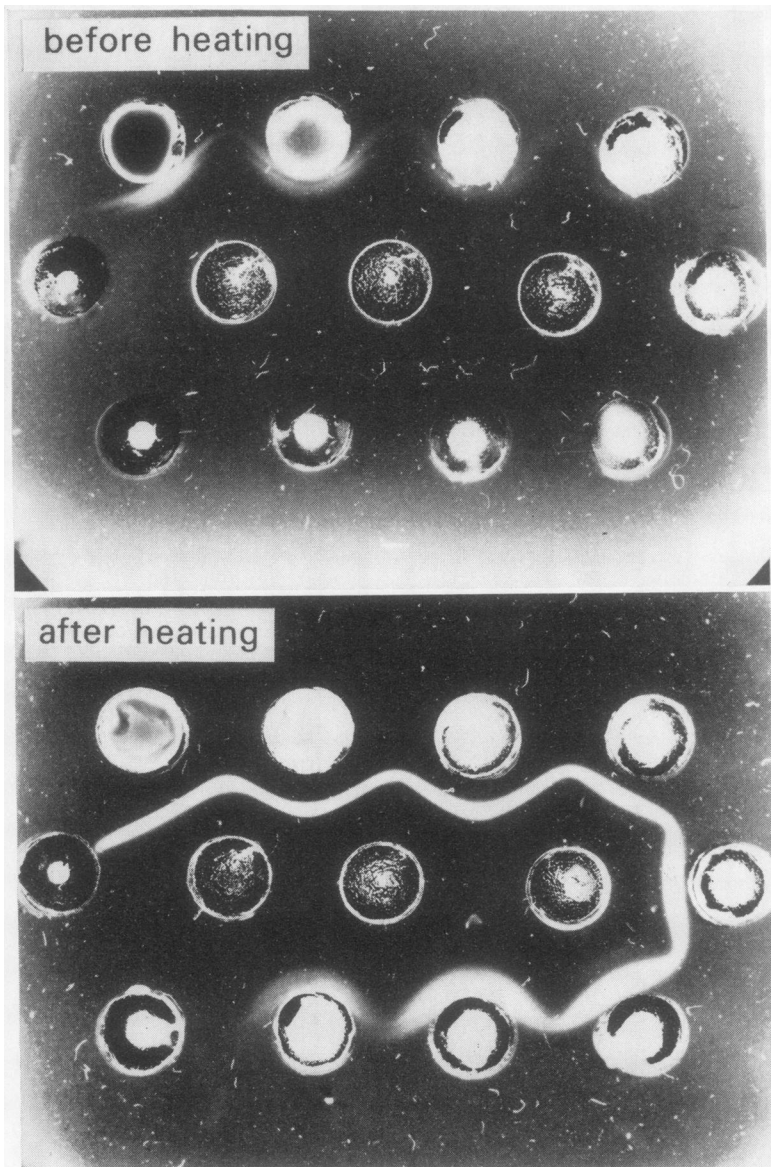
FIG. 2. *Demonstration of increased detectability of antigens after heating cell suspensions (100 C for 20 min). Identical dilution series of strain 1809 before and after heating. The most concentrated suspension (48 mg of cell dry matter/ml, equivalent to 220 × 10⁹ cells/ml was in the top left-hand well; the outer wells contained doubling dilutions in a clockwise manner. Anti-1809 antiserum was in the middle wells.*

5) and the heat-stable antigens being undetectable except for the distinctive curvature of the precipitin bands of this antigen from the adjacent wells (Fig. 1). Heat treatment destroyed the labile antigens and led to increased diffusion of the stable antigen (Fig. 3, wells 1, 2). Absorption experiments confirmed this by demonstrating that absorption with untreated native cells left both the antilabile and antistable antibodies, whereas after absorption with sonically disrupted cells only the antistable antibodies remained in the antiserum and after absorption with heated cells only the antilabile antibodies remained (Fig. 3).

The increased diffusion of the heat-stable antigen obtained after heat treatment of the cells of strain 1809 could be ascribed to changes in its diffusion coefficient or in its concentration, resulting from breakdown of the antigen to smaller size or from its increased release from the cells, respectively, because either of such changes would
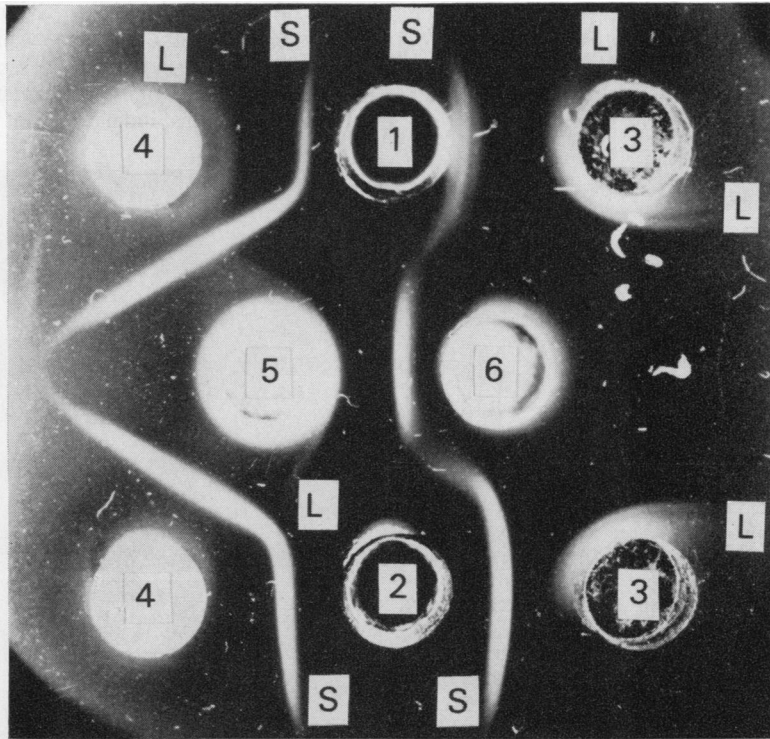
FIG. 3. *Absorption of anti-1809 antiserum in the gel with cells of strain 1809 which were untreated, ultrasonically disrupted, and heat-treated, respectively. 1, Ultrasonically disrupted 1809 cell antigen; 2, heat-treated 1809 cell antigen; 3, anti-1809 antiserum; 4, anti-1809 antiserum absorbed with untreated cells; 5, anti-1809 antiserum absorbed with heat-treated cells; 6, anti-1809 antiserum absorbed with ultrasonically disrupted cells. L = heat-labile antigens, S = heat-stable antigens. Anomalous bands were formed between wells 3 and 6, wells 4 and 5, and wells 5 and 6 because of excess antigens not bound by antibodies during absorption.*

affect the rate of diffusion of the antigen. The diffusion coefficients of the antigens were estimated by the immunodiffusion method of Allison and Humphrey (1) which uses linear wells of antigen and antiserum placed at right angles to each other. In the sonically treated cell preparation, the diffusion coefficient of the heat-labile antigens was of the order of 10×10^{-7} cm²/sec, and that of the small amount of detectable heat-stable antigen was found to be 1.2×10^{-7} cm²/sec. The diffusion coefficient of the major stable antigen released by the heat-treated cells was only 2.1×10^{-7} cm²/sec, indicating that the large increase in the amount of detectable antigen was not due to a large change in the diffusion coefficient and, hence, must be the result of an increase in the effective concentration arising from increased release of the antigen by the cells.

Cross-reactions between strains. Heat-treated cell suspensions of all of the strains in Table 1 were examined by immunodiffusion with each of the antisera and many cross-reactions were found (Table 2); most of them were weaker than the corresponding homologous reactions. There

was no difficulty in distinguishing between homologous and cross-reactions except in one instance. Strains 129 and 1809 gave identical precipitin bands in diffusion with anti-1809 antiserum, but the two strains were not identical with respect to anti-129 antiserum, as was shown by the formation of a spur at the junction of their precipitin bands (Fig. 4). The relative affinity of these two strains was confirmed by cross-absorbing the two antisera; absorption of anti-1809 with strain 129 left almost no detectable amount of homologous antibody, whereas anti-129 cross-absorbed with 1809 cells was still distinctly reactive towards the homologous strain (Fig. 4).

An unusual nonreciprocal cross-reaction was observed between strains 123 and 127 and strain 627. Anti-627 antiserum reacted strongly with the three strains, whereas the antisera of the other two strains reacted sometimes very weakly with the 627 cells but more often not at all (Fig. 5). The most interesting feature of this cross-reaction was the double spur that formed at the junction of the bands from 127 and 627 cells in diffusions against anti-627, indicating the un-

TABLE 2. *Summary of maximum number of precipitin bands observed between heat-treated cell suspensions and antisera[a]*

Antigen	Antiserum							
	110	123	127	129	1809	376	627	756
110	3[b]	—[c]	1w	—	—	—	—	—
123	—	3	1w	1s	—	—	2s	—
127	—	1s	3	—	—	—	2s	—
129	—	1s	3w	3	2(1s,1w)	—	—	—
705	—	—	1w	—	—	—	—	—
711	—	—	1w	1w	—	—	—	—
1809	—	—	—	2w	1	—	—	—
376	—	1w	—	—	—	3	—	—
627	—	1w	1w	—	—	—	3	—
756	—	2w	1w	1w	—	1w	—	2

[a] On different occasions, diffusions of the more weakly cross-reacting antigens and antisera gave fewer precipitin bands than those shown above and sometimes even none at all, as a result, most probably, of using antigen suspensions that were too dilute (see Discussion and references 12 and 26).

[b] Homologous reactions are shown in boldface type; other reactions are described as strong (s) or weak (w).

[c] Dashes indicate no reaction.

related nature of the two antigens responsible. Absorption of anti-627 antiserum with cells of strain 123, 127, and 627 separately caused the progressive selective removal of antibodies (Fig. 6), but absorption with even the homologous 627 cells did not prevent the formation of a precipitin band with cells of strain 127, arising from the unrelated antigen in 127 responsible for the double spur (Fig. 6d). Identical results were obtained with anti-627 antiserum from another rabbit.

Wider cross-reactions were observed among intact untreated cells than were obtained with the heat-treated suspensions. Thus, for example, untreated cells of strains 123 and 127, in addition to those of 129, cross-reacted with anti-1809 antiserum, but after they had been heated only the cells of strain 129 remained reactive.

Antigenic relationship between cultured cells and nodule preparations. Nodules produced by three strains (123, 127, and 129) were examined. They were from soybean plants (cv. Shelby) grown under bacteriologically controlled conditions in the Canberra phytotron, inoculated with these *R. japonicum* strains 7 days after sowing and harvested 26 days later. The nodules were picked and stored frozen at −15 C until examined 6 weeks later. Crushed nodules yielded dense suspensions of bacteroids that reacted with

their homologous antisera to give precipitin bands close to the antigen wells. Ultrasonic disruption for periods from 30 sec to 4 min led to the appearance of more and stronger bands in all of the strains, indicating that slow-diffusing
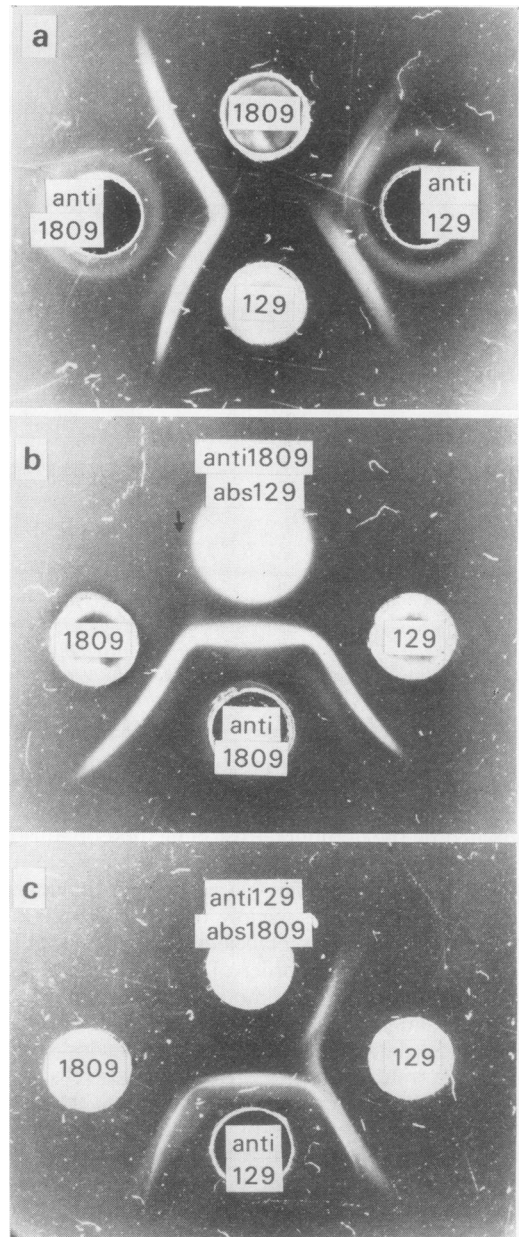

FIG. 4. *Cross-reactions between strains 129 and 1809, demonstrated with heat-treated cells. (a) Unabsorbed antisera; (b) anti-1809 absorbed with 129 cells; and (c) anti-129 absorbed with 1809 cells. Note the spur in (a) and faint specific band in (b).*
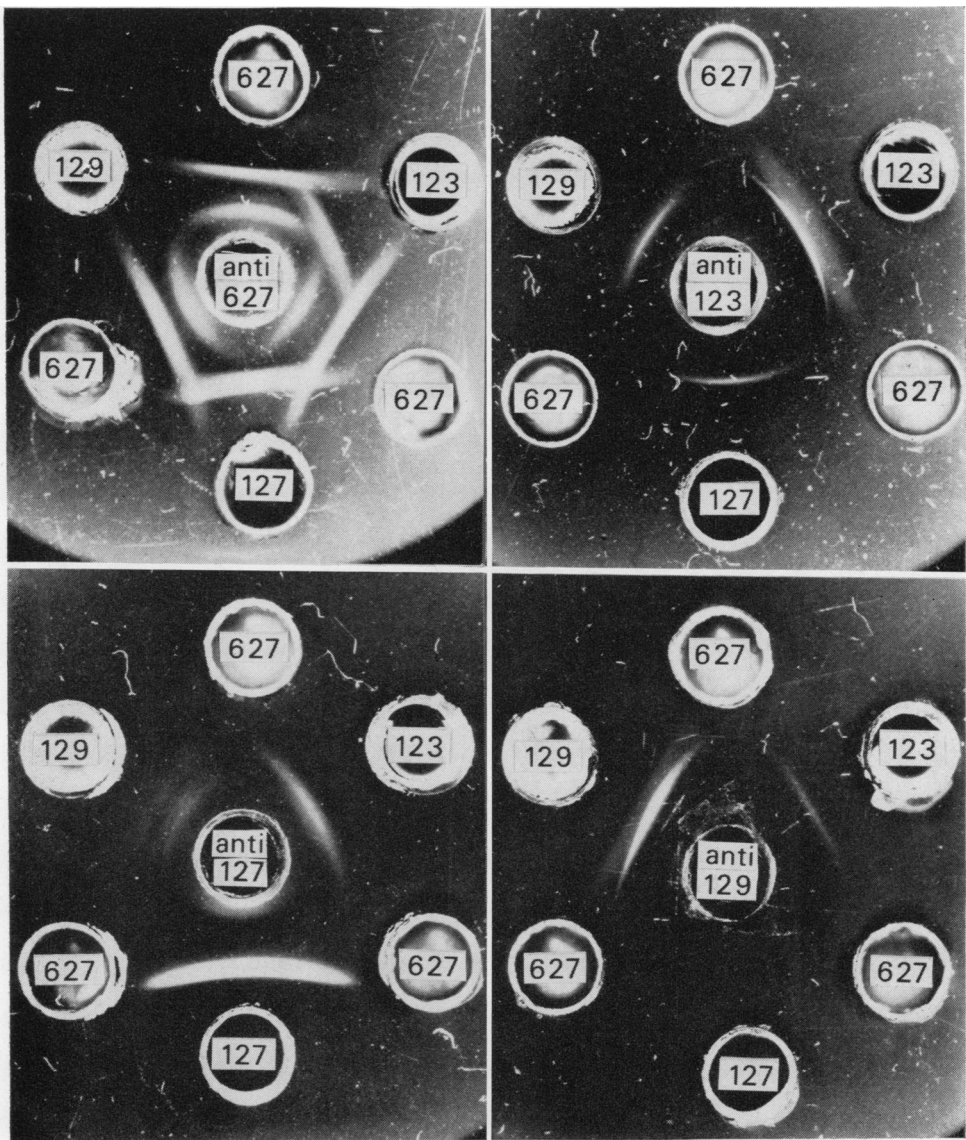
FIG. 5. *Cross-reactions between strains 123, 127, 129, and 627. Same arrangements of heat-treated antigens in outer wells of all plates diffused against different antisera in the middle wells. The cells of strain 627 did not react with anti-123 or anti-127 antisera in these plates but sometimes very weak bands were obtained in other plates (see Table 2).*

antigens were being released (Fig. 7a). Precipitin bands of greater density were formed from the crushed nodules after immersion of the suspension in boiling water for 15 min; longer heat treatment for as long as 2 hr caused no further changes in the immunodiffusion patterns (Fig. 7b, c). In strains 123 and 127, heated cultured cells possessed at least one more antigen than their respective crushed nodule preparations; cells of strain 123 sometimes yielded an additional band

(Fig. 7d), whereas the cells of strain 127 always formed distinct spurs at the junction of their precipitin bands with those of the crushed nodules (Fig. 7b, d). No detectable difference between cells and bacteroids was found in strain 129. Absorption experiments showed that heat-treated crushed nodules of strain 123 were capable of absorbing all of the antibodies in anti-123 antiserum, but in strain 127 the heat-treated nodule preparations were not and it was necessary to use
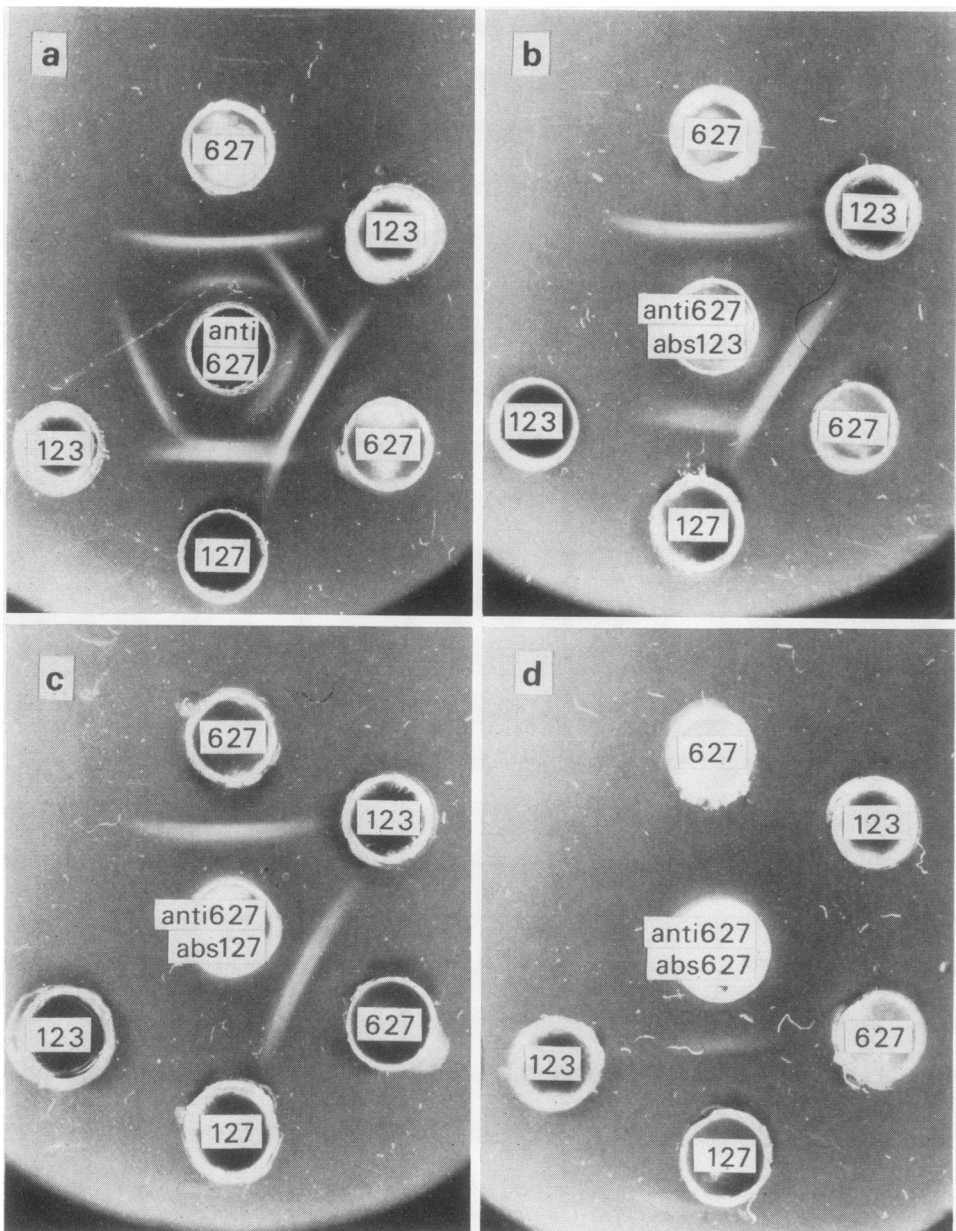
FIG. 6. *Absorption of anti-627 antiserum with heat-treated cell suspensions of strains 123, 127, and 627, respectively. Identical arrangements of antigens in outer wells of all plates, with anti-627 antiserum in middle wells: (a) not absorbed, (b) absorbed with strain 123, (c) absorbed with strain 127, and (d) absorbed with strain 627.*

heat-treated cultured cells before all of the antibodies were completely absorbed. The result for absorption by crushed nodules of strain 123 was anomalous but could have arisen because the anti-123 antiserum used was relatively weak and contained only a low concentration of antibodies specific for the cultured cells; after absorption,

the specific band could have been too weak to detect.

In diffusions against the cross-reacting anti-627 antiserum, crushed nodule preparations of strains 123 and 127 gave precipitin patterns identical with those formed by their respective cultured cells, including the double-spurred
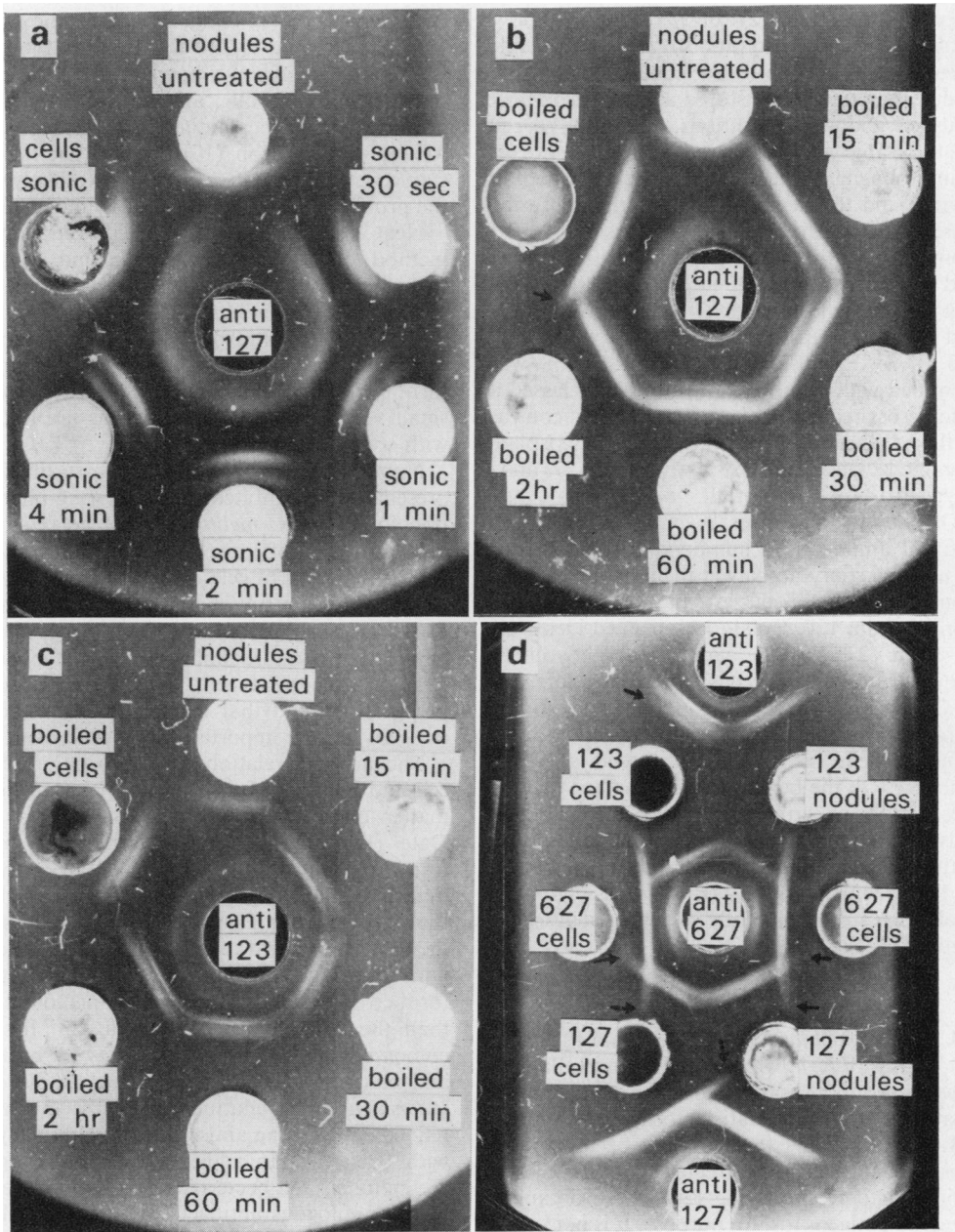
FIG. 7. *Immunodiffusion patterns with crushed nodule preparations variously treated. (a) Effect of ultrasonic disruption and (b) effect of heating to 100 C on nodule preparations of strain 127 compared with cultured cells (top left wells). (c) Effect of heating on nodule preparation of strain 123. (d) Comparison of cultured cell and crushed nodule forms of strains 123 and 127 in diffusions with anti-627 antiserum. Note the additional antigens detected by anti-123 (in d) and anti-127 (in b and d) in homologous reactions with the cultured cells and also the double spur given by anti-627 with both forms of the 127 antigen.*

junction between the 127 crushed nodule preparation and 627 cells (Fig. 7d). Similarly, both the cultured cells and crushed nodules of strain 129 gave identical reactions with the cross-reacting anti-1809 antiserum.

Identification of strains in nodules. The use of heated crushed nodule suspensions for the serological identification of *R. japonicum* strains in soybean was examined with 20 strains isolated from field-grown plants and believed to be re-

lated to strains 123, 127, or 129. These unknown strains were inoculated onto soybean plants under bacteriologically controlled conditions, and the nodules obtained were stored as described above. Crushed nodule preparations, and cultured cells of the same strains grown on yeast extract-mannitol-agar, were heated in boiling water for 20 min and then examined by immunodiffusion. Three separate nodule preparations were made from each strain. All three nodule preparations and the cultured cells of each strain gave consistent results; within 48 hr, 3 isolates were identified as being serologically identical with strain 123, 1 was identified with strain 127, 3 were identified with strain 129, and 13 were classed as being cross-reacting unknown strains. In contrast to this, agglutination reactions with these isolates identified 11 as strain 123, 6 as strain 127, and 3 as strain 129 (Gibson et al., *in press*).

Of the seven isolates reacting strongly with anti-127 antiserum in immunodiffusion, six gave an unusual spur anomaly at the junction of bands from their cultured cells and the standard suspension of strain 127. Isolate 17A reacted identically as strain 127 (Fig. 8), with a spur between the nodules and cultured cells, whereas isolate 17B, as an example of one of the other strains, formed a spur at the junction of its cultured cells with the standard cells of strain 127 instead of at the junction with the bands from its crushed nodules. Absorption experiments with heated cultured cells of these isolates showed that all anti-127 antibodies were absorbed by isolate 17A but not by 17B, confirming that 17B was a closely related strain cross-reacting with but not identical to strain 127 (Fig. 8)

DISCUSSION

Unlike *R. meliloti* (7) and *R. trifolii* (9, 12, 13, 21), strains of *R. japonicum* and the miscellaneous cowpea group require some form of preliminary treatment to react satisfactorily in immunodiffusions, but after the cells have been disrupted ultrasonically or heat-treated, or have been subjected to freeze-drying (26), these organisms are good antigens for this purpose. It is not known why the antigens of these groups of *Rhizobium* strains should be relatively insoluble or non-diffusible until released by some form of disruptive treatment, but it may be associated with the insoluble nature of their extracellular polysaccharides, which remain firmly bound to the cells even in shaken cultures grown in liquid media. Microscopic observation by the India ink negative staining method showed the cells to be embedded in amorphous masses of insoluble polysaccharide (8). Whether the polysaccharides

of *R. japonicum* are themselves antigenic remains to be proven, but even if they are not they could be extremely effective barriers to diffusion by other macromolecular antigens. It is pertinent, however, that the antigens were heat-stable; Means and Johnson (17) believe that the heat-stable antigens of *R. japonicum* are likely to be the protein-polysaccharide-lipid O antigens.

Heat treatment is the simplest and preferred method of disrupting the cells and rendering them antigenic. It has the advantage of being convenient for handling large numbers of strains, in contrast to ultrasonic disruption which requires individual treatment of each sample in turn. Furthermore, there is no difficulty regarding small volumes of samples, such as are obtained with crushed nodule suspensions.

None of the soybean or cowpea strains in the present study exhibited the interesting phenomenon found in *R. meliloti* and *R. trifolii* of the disappearance of precipitin bands after ultrasonic disruption of cells (10, 20). The present results confirm those of Škrdleta (24–27) in showing that immunodiffusion is applicable to the identification of *R. japonicum* strains as cultured cells or as crushed nodule preparations and that heat-stable slow-diffusing antigens are the important diagnostic ones.

The antigenic relationship between the cultured cell and bacteroid forms of any given strain is a matter of great interest. Means, Johnson, and Date (18) examined the cultured and nodule forms of 17 strains of *R. japonicum* by agglutination and found no detectable difference between the two forms in 15 strains. But in one strain, the bacteroids failed to react with the homologous antiserum, and, in another strain, the bacteroid form cross-reacted with a wider range of antisera than the cultured cells. Škrdleta (23) has also compared the antigenic properties of bacteroid and cultured cell forms of three strains of *R. japonicum* by agglutination, with the added refinement of using antisera from rabbits injected with bacteroids in addition to antisera against the cultures. The bacteroids of all strains reacted with homologous antibacteroid and anticultured cell antisera, but the cultured cells of two of the strains reacted with only the anticultured cell and not the antibacteroid antisera, indicating a non-reciprocal relationship.

In the present work, the nodule antigens of strain 127 consistently formed spur reactions at the junction of the precipitin bands from the homologous cultured cells, indicating that the bacteroids of this strain lack the full array of antigens of the cultured cells. This was confirmed by absorption experiments. No differences were
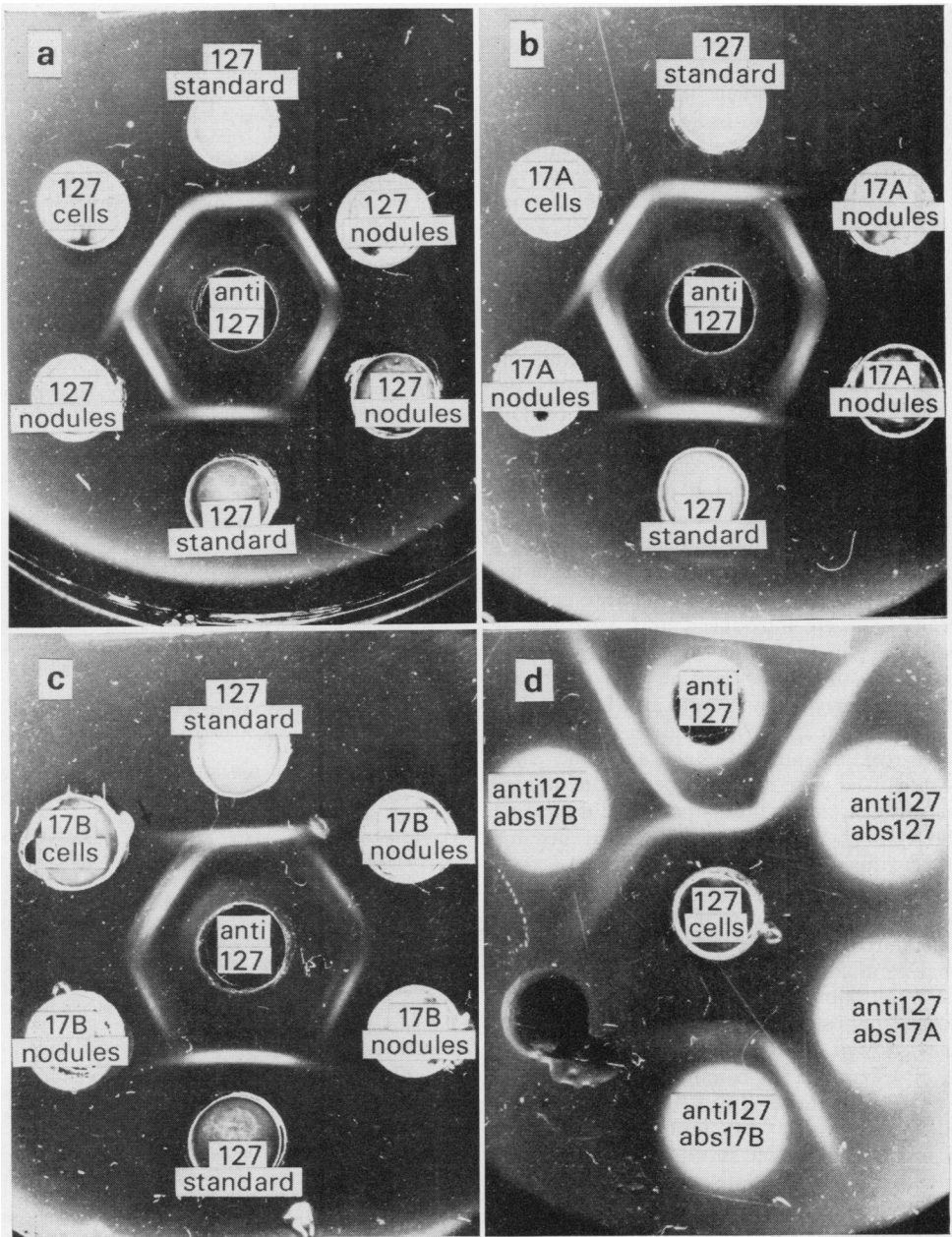
FIG. 8. *Identification of unknown isolates, by immunodiffusion, of cultured cells and crushed nodule preparations, all heat-treated. (a) Strain 127 was used as the control. (b) Isolate 17A, identical with strain 127. (c) Isolate 17B, cross-reacting with anti-127 antiserum. Note the anomalous spur at the junction with the band from the standard 127 suspension. (d) Absorption of anti-127 antiserum with cells of strain 127 and isolates 17A and 17B.*

ever found between the nodule and cultured cell antigens of strain 129. In strain 123, the results were equivocal; in most immunodiffusions, no antigenic differences were detected, but, on some occasions, the cultured cells appeared to yield

an additional precipitin band (e.g., Fig. 7d). This may have been because of the relative weakness of the antiserum in the antibodies responsible for the additional band, but also it is a common experience in immunodiffusion experiments to

find some variation in the number of bands obtained with a given antigen and antiserum on different occasions (cf. 12, 26).

Cross-reactions between the eight available antisera and the 10 strains examined confirmed the close relationship between strains 123, 127, and 129 reported by Date and Decker (6) and revealed some interesting new cross-reactions between anti-627 antiserum and strains 123 and 127 and between strains 1809 and 129, respectively. The reaction between strains 127 and 627 is especially remarkable: not only is the reaction strong and one-sided, with the 127 cells reacting very strongly with anti-627 antiserum and the converse reaction being negative or barely detectable, but the anti-627 appears to contain antibodies specific for strain 127 with which the 627 cells themselves cannot react. It is difficult to advance an explanation for this phenomenon at the present time. There was no possibility of contamination of the 627 antigen with cells of strain 127 before immunization of the rabbits; the anti-627 serum was prepared before cultures of strain 127 were introduced into Australia. A similar nonreciprocal reaction was observed between antilysozyme antiserum and carboxymethylated lysozyme by Young and Leung (30), who showed that the cross-reaction was not due to cross-reacting antibodies because the antibodies involved were not absorbed by the homologous antigen; these authors proposed that some of the lysozyme molecules may have become distorted, during immunization, into the configuration of carboxymethylated lysozyme molecules, thus giving rise to some antibodies specific for the latter protein and unreactive with the original immunizing antigen.

The results obtained in the examination of 20 *R. japonicum* strains isolated from soybean nodules showed that, although all were isolates positively identified as belonging to strain 123, 127, or 129 by the agglutination technique, 13 were distinguishable by immunodiffusion as cross-reacting unknown strains. It is concluded that, with the appropriate standards and controls, the immunodiffusion technique can be used with confidence for the rapid and convenient identification of strains of *R. japonicum* and the cowpea group, both from cultures and from root nodules. The results are available within 48 hr, and sometimes 24 hr, of setting up the immunodiffusion plates.

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