Modification of the Antigenic Surface of *Rhizobium trifolii* by a Deficiency of Calcium

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SUMMARY

Quantitative absorption of antisera was used to study the effect of calcium on the antigenic surface of *Rhizobium trifolii*. Antisera to Ca-deficient and Ca-adequate rhizobia (whole or broken) revealed two parts in the absorption curve: *range 1* in which there was ready removal of most of the agglutinating antibody with a very small absorbing dose; *range 2* in which the remaining agglutinating antibody resisted absorption. When Ca-adequate bacteria were used for absorption, range 1 consisted of about 87% of the total titre. The corresponding figure with Ca-deficient bacteria was 95%. These values have been attributed to three types of antibody; avid 1 (readily absorbed by either kind of bacterium), avid 2 (readily absorbed by Ca-deficient bacteria), non-avid (difficult to absorb with either bacterium). The fact that avid antibody 2 was absorbed readily by Ca-deficient bacteria but with difficulty by Ca-adequate bacteria may be due to a quantitative deficiency of a particular antigen on the surface of the Ca-adequate bacterium, or to a structural condition which gives the antigen lower affinity for its homologous antibody. Absorption characteristic of Ca-deficient rhizobia was obtained with the Ca-adequate bacteria treated with EDTA under conditions known to remove 90% of Ca from the cell. Broken bacteria to some extent simulated the absorption curve found with Ca-deficient bacteria. It is suggested that Ca located in the surface lipopolysaccharide layer of the wall of rhizobium grown with a sufficiency of this element obscures or modifies an antigenic group. Glucuronic acid found in the somatic antigen fraction of this bacterium is suggested as a possible site of Ca action.

INTRODUCTION

Earlier work showed that when *Rhizobium trifolii* was grown in a defined medium deficient in calcium, its shape was altered to an enlarged sphere (Vincent & Colburn, 1961) which, although not osmotically fragile, showed a greatly increased susceptibility to lysozyme without the potentiating action of EDTA (Vincent & Humphrey, 1963), and increased permeability or fragility (Humphrey & Vincent, 1965). The small amount of Ca contaminating the medium was concentrated in the walls of the Ca-deficient bacteria and amounted to only half that of normal walls (Humphrey & Vincent, 1962). It was concluded that Ca played a role in securing wall components in a firm steric arrangement, although the more loosely knit structure consequent on a shortage of this element retained a degree of stability and function. It seemed likely that differences in the surface of Ca-adequate and Ca-deficient rhizobia would be reflected in their detailed antigenic composition. However, simple agglutination and gross cross-absorption tests failed to reveal any differences of this kind; and dif-

ferences in respect of diffusing antigens by the gel immune diffusion technique were attributable to internal antigens leaking from the Ca-deficient cells (Humphrey & Vincent, 1965). The present work is an attempt at a more critical analysis of the surface antigens of both kinds of rhizobia by relating the amount of antibody removed to the quantity of cells used for absorption. Quantitative absorption could be expected to reveal disorganization of surface antigens that would be hard to detect by other methods. This approach has the additional advantage of being applicable to the untreated bacterial surface.

METHODS

Organism and cultural conditions. *Rhizobium trifolii* (strain SU297/31) was grown in a defined liquid medium (Vincent, 1962) having mM total divalent cation made up either of 0.5 mM-Ca²⁺ and 0.5 mM-Mg²⁺ (Ca-adequate) or mM-Mg²⁺ (Ca-deficient).

Breakage of bacteria. When broken organisms were used as antigens in the preparation of antisera, whole Ca-adequate culture was shaken for 20 min. at 4° with ballotini beads in a Mickle disintegrator. Washed organisms for absorption were similarly broken in some experiments.

Preparation of antisera. Antisera to Ca-adequate, Ca-deficient and Mickle-disintegrated Ca-adequate whole organisms were produced in rabbits by the method previously described (Humphrey & Vincent, 1965). For brevity, these antisera will be referred to as 'Ca-adequate', 'Ca-deficient' and 'broken'. Agglutination titres of the antisera so obtained were greater than 3200.

Absorption of antisera. Antisera were diluted to a suitable agglutination titre (400–600 in earlier, 1000 or greater in later experiments) and absorbed, at a further dilution of 1/25 in saline (0.85%, w/v, NaCl), by a range of increasing amounts (50 to 2500 µg./ml.) of Ca-adequate and Ca-deficient bacteria. In the case of whole bacteria these had been centrifuged out of a standardized suspension of known dry weight. Disintegrated cells were not centrifuged but were added in appropriate volume to a more concentrated serum. Absorption took place under controlled conditions: shaken at 37° on a wrist shaker for 1 hr followed by standing at 4° for 18 hr. The sedimented material was then removed by centrifugation.

Determination of titre. The residual titre of the absorbed serum was determined at closely spaced dilution intervals. With sera absorbed at 1/25, the least dilution possible, using concentrated testing antigen, was 1/27. For dilutions 1/50 upwards, the testing antigen was a suspension in saline containing 280 µg. unheated, washed, Ca-adequate bacteria per ml. The suspension so prepared showed no flagellar agglutination. Agglutination was recorded after 24 hr (5 hr at 55°, 19 hr as the water bath cooled to room temperature). The end-point was taken as the highest dilution at which there was some deposit and definite reduction in the opacity of the suspension. An initial, pre-absorption, titre of 1000 was used in later experiments, rather than 400 to 600 as was first used. The higher starting level allowed definite end-points to be determined in range 2 of the residual titre:absorptive dose curve.

Ca-deficient bacteria used as testing antigen at the same concentration as Ca-adequate bacteria showed a condition of antigen excess in that the suspension showed no definite reduction in turbidity though showing a deposit of agglutinated bacteria. When the concentration of Ca-deficient test suspension was halved, the resulting absorption curve resembled that obtained with Ca-adequate testing bacteria.

Expression of results. Residual titres have been expressed as a proportion of the titre of the unabsorbed antiserum. This has proved a satisfactory way of making use of results with antisera of starting titre between 400 and 1200.

Treatment of antisera with 2-mercaptoethanol (ME) and heat. Treatment was by 0.1 M-ME in saline buffered with 0.05 M-phosphate buffer pH 7.2. Antisera were diluted 1/5 with this solution, incubated at 26° for 24 hr in sealed tubes, and diluted with saline to 1/25 prior to absorption. Heat treatment consisted of heating antisera for 30 min. at 75°, either at a dilution of 1/25 in unbuffered saline, or at a dilution of 1/5 in buffered saline, pH 7.2.

RESULTS

Form of absorption curve

Residual titres obtained in a large number of experiments, after varying degrees of absorption, are shown for Ca-adequate antiserum (Fig. 1). The same form of curves was obtained with both Ca-deficient antisera and the broken antiserum. Most of the antibody was removed by the smallest practical dose of absorbing bacteria

Fig. 1. Absorption of rhizobial antiserum by Ca-adequate (○—○) and Ca-deficient (●—●) bacteria. Points represent average values of all experiments with the antiserum developed against Ca-adequate rhizobia (see Table 1). The same form of curves was obtained with Ca-deficient and broken antisera.

(50 $\mu\text{g./ml.}$) but the remainder was absorbed much less readily so that a significant amount remained after treatment with more than 500 $\mu\text{g./ml.}$

Experiments in which the starting titre was varied between 400 and 1600 showed that 50 $\mu\text{g./ml.}$ absorbing bacteria provided considerable excess of the antigen(s) required for the removal of most of the antibody (range 1 absorption, as defined below). In these experiments more than 80% of the antibody was removed whether the initial titre was 400, 800 or 1200. The proportion removed when the starting titre was 1600 was less but represented an additional absolute amount removed by the same weight of cells.

These results have been interpreted in terms of at least two kinds of antibody: (i) the larger part with high affinity for the absorbing bacteria (range 1 absorption); (ii) a smaller part having very low affinity (range 2 absorption). From the curve shown in Fig. 1 it is apparent that the residual titre after absorption with 500 $\mu\text{g./ml.}$ bacterial suspension can be used to estimate the proportion of low affinity antibody.

Table 1. *Absorption of Ca-adequate, Ca-deficient and broken antisera by Ca-adequate bacteria (whole, broken and EDTA-treated) and Ca-deficient bacteria*

	Proportion (%) of antibody unabsorbed by 500 $\mu\text{g./ml.}$ bacterial suspension*			
	Ca-adequate antiserum	Ca-deficient antiserum†		Broken antiserum
		(1)	(2)	
Absorbing suspension				
Ca-adequate	13.1 $\pm$ 0.7 (8)	19.8 $\pm$ 1.5 (10)	8.9 $\pm$ 0.5 (20)	14.6 $\pm$ 1.1 (5)
Ca-deficient	4.7 $\pm$ 0.6 (7)	< 5 (4)	3.3 $\pm$ 0.3 (12)	6 (2)
Ca-adequate, broken	—	9.3 $\pm$ 0.7 (3)	7 $\pm$ 1 (2)	8.5 $\pm$ 0.7 (2)
Ca-adequate, EDTA-treated	—	8.0 $\pm$ 1.1 (3)	3.9 $\pm$ 0.3 (10)	7.5 $\pm$ 2.2 (2)
Ca-adequate, EDTA-treated + divalent cation	—	9.1 $\pm$ 0.2 (11)	4.8 $\pm$ 0.6 (10)	—
Partition of antibody (%)				
Type of antibody	Ca-adequate antiserum	Ca-deficient antiserum†		Broken antiserum
		(1)	(2)	
Avid 1	87	80	91	85
Avid 2 (avid with Ca-deficient bacteria)	8	> 15	6	9
Non-avid	5	< 5	3	6

* Standard errors based on deviations from the mean based on the number of determinations shown within parentheses. The two values without a standard error do not justify this treatment.

† Separate values for two antisera.

Absorption of Ca-adequate, Ca-deficient and broken rhizobia

Table 1 summarizes the results for a large number of experiments with Ca-adequate, Ca-deficient and broken antisera. Absorbing with Ca-deficient bacteria (consistently with all antisera) reduced the titre to a half or a third of the range 2 level remaining when Ca-adequate cells were used. This means that some of the antibody which was difficult to absorb with Ca-adequate bacteria was absorbed avidly by the Ca-deficient bacteria.

Because the supply of Ca-adequate bacteria was already in excess of that required for the removal of readily absorbed range 1 antibody, the capacity of Ca-deficient cells to absorb readily a further significant fraction must be attributed to a different antigen having greater affinity for this part of the antibody complex.

A sufficiently large dose (2500 $\mu\text{g./ml.}$) of Ca-adequate bacteria removed difficultly absorbable antibody to below the limit of detection (residual titre, < 27). The slopes of the range 2 curves were similar with Ca-adequate and Ca-deficient bacteria but, because they started at a lower level, the latter reached the limit of detection at 500 to 1000 $\mu\text{g./ml.}$

Mechanical breakage of Ca-adequate bacteria increased their range 1 absorption to a degree intermediate between intact Ca-adequate and Ca-deficient bacteria.

Effect of EDTA on the absorbing capacity of Ca-adequate rhizobia

EDTA under conditions which remove 90 % Ca from the wall (Humphrey & Vincent, 1962) modified the Ca-adequate bacteria in the direction of the absorbing properties of Ca-deficient bacteria (Table 1). It was not possible to obtain a significant restoration of Ca-adequate absorption characteristics by subsequent washing of the EDTA-treated bacteria (either in water, or HCl at pH 3.5) followed by exposure to Ca^{2+} , Sr^{2+} or Mg^{2+} . Rhizobia grown with a deficiency of Ca were not further affected by EDTA, nor converted to the Ca-adequate condition by exposure to Ca^{2+} after growth had occurred.

Possible role of internal antigens

The possibility that the internal antigens revealed by gel diffusion using Ca-deficient and broken rhizobia (Humphrey & Vincent, 1965) might be responsible for the agglutinin absorption pattern of Ca-deficient and broken bacteria observed in the present work was investigated. A strain of *Rhizobium trifolii* SU 157 which does not cross-agglutinate, reveals, after breakage, internal precipitinogens, identical with the homologous, when diffused in agar against antisera to *R. trifolii*, SU 297/31. Treatment with such broken SU 157 failed to reduce the agglutination titre of antiserum developed against SU 297/31. Further it did not, when used with Ca-adequate SU 297/31, increase percentage absorption in range 1. That is, the extra range 1 absorption of antibody with Ca-deficient bacteria cannot be attributed to internal antigens.

The effect of heat and mercaptoethanol (ME) on antisera

It seemed possible that the antibodies responsible for various ranges of absorbability might prove to be macroglobulin or γ -globulin, in which case macroglobulin would be distinguishable by its sensitivity to heat and ME (Pike, 1967). In the present system, however, it was not possible to show complete destruction of antibody concerned with either range by heat or ME. Antisera heated in unbuffered saline at a dilution of 1/25 showed a proportionate decrease of a half to a third of their titre, reflected evenly throughout the curve. On the other hand, the application of heat or ME to antisera diluted 1/5 in saline buffered at pH 7.2 showed partial reduction of titre specifically in range 1 (Table 2). However, range 1 was not destroyed completely and the portion destroyed (ME, 40 %; heat, 20 %) could not be related to the difficultly absorbable fractions.

Table 2. *Partial destruction of antibody by heat and ME**

	Titre			
	Unheated	Heated	Untreated	ME
Unabsorbed	1000	800	1000	600
Absorbed (500 μ g./ml.) Ca-adequate bacteria	80	90	80	113
Absorbed (500 μ g./ml.) Ca-deficient bacteria	40	34	40	40

* Both treatments were carried out a serum dilution of 1/5 in saline buffered at pH 7.2.

DISCUSSION

Quantitative absorption was used many years ago in an attempt to study the nature of the agglutination reaction (e.g. Eisenberg & Volk, 1902; Craw, 1905). Later, Wilson & Miles (1932) used it to provide a quantitative basis to the analysis of cross-reacting species of the genus *Brucella* which had two common antigens in varying proportion on their surface. Their data, like ours with *Rhizobium*, showed a marked degree of absorption with a small dose of absorbing antigen but a residual titre that required much heavier doses for absorption beyond the limit of detection. These characteristics they related to the relative amount of A and M antigens on the surface of the absorbing suspensions and the amounts of the respective agglutinins in the antiserum. However, not enough absorbing doses were used to enable the quantitative relationships to be fully expressed. The sharp inflection in the curve of the dose/response relationship between residual antibody and absorbing antigen found by Wilson & Miles (1932) and our own more detailed data contrast with the relatively smooth curves obtained by Heidelberger & Kabat (1937). In the latter case only one antigen/antibody system was thought to be operating.

We interpret the marked difference we have found in all the antisera between ready absorption of the larger part of the agglutinins (range 1) and the more difficult absorption of the residual agglutinin (range 2) as evidence that each contain at least two kinds of agglutinating antibody, distinguished by different avidity for the absorbing bacteria. Further the Ca-deficient and, to a less degree, Mickle-disintegrated bacteria have antigens in a form that permits them to combine readily with an antibody fraction non-avid for the Ca-adequate surface. On this basis antibody in the four antisera given in Table 1 can be partitioned on an average percentage basis, thus: avid antibody 1 (removed by Ca-adequate or Ca-deficient bacteria), 87%; avid antibody 2, non-avid with Ca-adequate, avid with Ca-deficient bacteria, 8%; non-avid, 5%. The consistent marked capacity of the Ca-deficient bacteria to absorb avid antibody 2 would seem to indicate a marked difference in the antigens concerned with this combination, consequent on the shortage of Ca in the cell wall. The difference in the Ca-adequate and Ca-deficient surfaces points either to a direct role of Ca in the antigenic surface layer of the *rhizobium* or to considerable but less direct modification of the detailed architecture of the surface due to absence of a specific bonding cation. That is, the difficult absorption of this fraction of antibody by Ca-adequate bacteria may be due to (i) a modification of a surface antigen in the presence of Ca-bonding, whereby only one part of an effective grouping is fully exposed, resulting

in lowered affinity for a corresponding antibody; or (ii) fewer but whole particular antigenic sites on the surface of the bacterium when the surface is both intact and firmly bonded. EDTA conversion of the surface to simulate the absorbing characteristics of the Ca-deficient bacteria suggests a fairly direct role for Ca but failure to restore *in vitro* the Ca-adequate condition to the Ca-deprived or EDTA-treated bacteria argues for a more complex structural relationship.

The fact that Mickle-breakage, as well as Ca removal or absence (due to nutritive deficiency), makes the absorbing surface more avid for a significant proportion of difficultly absorbable antibody suggests that Ca obscures rather than combines directly with an antigenically active group. It is also apparent that the surface of the Ca-adequate rhizobia is modified to provide this antigenic form when inoculated into the rabbit.

The lipopolysaccharide somatic antigen extracted by the phenol method (O'Neill & Todd, 1961) from the strain of *Rhizobium trifolii* used in the present study (Humphrey & Vincent, unpublished data) while showing sugars similar to the 'core' and 'side-chain' sugars of lipopolysaccharides of the Enterobacteriaceae (Lüderitz, Staub & Westphal, 1966) was unusual in that it included a significant amount of glucuronic acid and was sufficiently acid to be precipitated by the cetyltrimethyl ammonium ion. This would provide a ready site for Ca-combination, which might either directly prevent glucuronic acid acting as an antigenic determinant group or, by forming a cross link, block a potential antigenic site and render it subsurface.

These results add to a growing body of evidence which points to the outer lipopolysaccharide/lipoprotein layers as a probable site of fixed divalent cations. It is an advantage of the present investigation over those that depend solely on EDTA treatment for the removal of divalent cation, that by studying deprivation by both methods one can observe parallelism in behaviour, and distinguish, in the case of *Rhizobium*, an effect for Ca not substituted by Mg. The fact that it has not been possible to show the same specific requirement for Ca in the growth of *Escherichia* and *Aerobacter* (A. Chan, P. Y. Yao & J. M. Vincent, unpublished data) parallels the experience of others with *Escherichia* and *Pseudomonas* for which the requirement is a more general one for divalent cation, including Ca^{2+} , Mg^{2+} , Zn^{2+} (Asbell & Eagon, 1966). Compared to the wall of *Pseudomonas* (Eagon, Simmons & Carson, 1965), that of *Rhizobium trifolii* contains twice as much Ca, but only one-fifth the Mg (Humphrey & Vincent, 1962).

The several effects of EDTA on walls of Gram-negative bacteria: potentiation of lysozyme action (Repaske, 1956; Salton, 1958; Noller & Hartsell, 1961); increased permeability to toxic reagents (Gray & Wilkinson, 1965; Leive, 1965); and leakage of intracellular components (Gray & Wilkinson, 1965) could be due to disturbance of the lipopolysaccharide/lipoprotein outer layer. In fact, Leive found that up to 50 % of the wall lipopolysaccharide of *Escherichia coli* could be liberated by EDTA, an effect not attributable to any alkaline detergent action. Relating these effects more directly to shortage of divalent cation we were able to show parallel effects such as immediate lysozyme sensitivity (Vincent & Humphrey, 1963) and leakage of intracellular antigens (Humphrey & Vincent, 1965) in rhizobia that had been deprived of Ca during growth.

Recent work on the respective effects of lysozyme and EDTA on the rigidity of the wall of *Pseudomonas* (Carson & Eagon, 1966; Eagon & Carson, 1965) and on the conversion of rods of *Escherichia* to spheres by phospholipase C (Weinbaum *et al.*

1967) points to the disruption of divalent cation cross-links involving phospholipid. It would seem from these studies with other bacteria that one need not invoke deficiency of Ca in the mucopeptide layer itself to account for the changed shape of Ca-deficient rhizobia. The present experiments make it seem probable that some at least of the Ca is situated either at the antigenic surface or, if below the surface, so as to affect the configuration of the lipopolysaccharide. Failure to detect any difference in electrophoretic mobility between Ca-adequate and Ca-deficient rhizobia (Humphrey, Marshall & Vincent, 1968) suggests either that the general configuration of the lipopolysaccharide is affected without exposing additional groups or that any such additional charged groups, freed because of Ca-deficiency, are too deeply situated to contribute to the charge on the surface.

The method of quantitative absorption described in this paper is applicable to other types of investigation of the bacterial surface. For example, a cross-reacting strain could be expected to absorb more slowly in range 1, displacing the curve to the right, if it had an antigen (or antigens) related to the principal antigen(s) of the homologous bacterium but not identical and with lower affinity for the corresponding antibody. On the other hand, a sharp but incomplete range 1 absorption would indicate that the bacteria shared some, but not all, high affinity antigens. Modification of antigenically active groups, either chemically or as a result of lysogenization can also be studied by this method. Investigations along these lines are in progress in this laboratory.

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