Calcium in Cell Walls of *Rhizobium trifolii*

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SUMMARY

The cell walls from *Rhizobium trifolii*, grown under both 'normal' and 'calcium-deprived' conditions, were analysed in an attempt to detect a chemical cause for the apparent weakness or looseness of the walls in calcium-deprived organisms. The organic components typical of Gram-negative cell walls were present in normal and calcium-deprived cells. The latter concentrated most of the small amount of available calcium in the walls, which, however, contained only 60 % of that present in the walls of normal organisms. Magnesium was not able to substitute for calcium as a wall component.

INTRODUCTION

Calcium-deprived cells of *Rhizobium trifolii* developed an abnormal morphology (Vincent & Colburn, 1961). They became swollen and much more spherical in form, apparently distorted by a huge vacuole. Although a double-layered wall structure was seen in the calcium-deprived as well as in the calcium-sufficient cells, it appeared to be appreciably weakened in its sectioned appearance. The effect could be one of a loosening of wall structure, and in this paper we have looked for some chemical evidence of this. We have particularly considered the possibility that calcium may be structurally involved.

METHODS

Organism. *Rhizobium trifolii* SU 297/31.

Cultural conditions. The organism was grown in a defined medium (Vincent, 1962) having a total concentration of divalent cations of 1 mM. This was made up of either 0.5 mM-Ca²⁺ + 0.5 mM-Mg²⁺ ('normal') or 1 mM-Mg²⁺ ('calcium-deprived'). All salts were A.R. grade. Cultures were harvested after 72 hr. incubation, with shaking, at 25°.

Preparation of cell walls. Early attempts at a clean cell-wall preparation were frustrated by the presence of up to 50 % poly- β -hydroxy-butyric acid in the cells (Vincent, Humphrey & North, 1962). It was found best to remove this polymer first, by lyophilizing the organism and extracting with chloroform for 24 hr. at room temperature. Such extracted organisms gave a satisfactory cell-wall preparation by the usual method of Mickle disintegration, trypsin digestion and differential centrifugation.

Identification of wall components. For the identification of sugars, walls were hydrolysed at 100° for 20 hr. in 2N-HCl. Chromatography was in butanol:acetic acid:water, 6 + 1 + 2 descending, and the papers were developed by *p*-anisidine-HCl.

For the identification of amino acids and amino sugars, walls were hydrolysed

for 20 hr. at 100° in 6N-HCl. Chromatograms were developed with ninhydrin after two-dimensional chromatography in pyridine + water (4 + 1) and butanol + acetic acid + water (3 + 1 + 1).

Calcium and magnesium analyses. These were carried out by the method of atomic absorption spectrophotometry. Five mg. whole cells or cell walls were ashed overnight at 450° in an electric muffle furnace in covered platinum crucibles. Two ml. of 3N-HNO₃ were added to the residue and evaporated to dryness on a water bath. The crucibles were again placed in the muffle furnace overnight, after which no visible ash remained. The residue was taken up in 0.1 ml. 6N-HCl and diluted to 5 ml. with water containing 1500 p.p.m. strontium to suppress phosphate interference. The atomic absorption of the solution was measured and read against a standard curve for Ca²⁺ prepared at the same time. Values lay within the range of 0.2–1.2 p.p.m. calcium. Duplicates were within 10 %, as were different batches of the organism. The same solution, sometimes with further dilution, could be used for Mg²⁺ analyses within the range of 0.1–1.5 p.p.m.

RESULTS

Composition of cell walls

Removal of the poly- β -hydroxy-butyric acid by extraction with chloroform enabled us to obtain satisfactorily clean preparations of cell walls, free from high refractive index material as judged by phase and electron microscopy. Walls prepared in this way contained the 'R' layer components (Weidel, Frank & Martin, 1960)—glucosamine, muramic acid, glutamic acid, alanine and diaminopimelic acid, as well as the usual wide range of amino acids found in Gram-negative walls; in this case, lysine, aspartic acid, glycine, serine, valine, methionine, leucine and tryptophane were present, together with glucose and rhamnose.

Cell-wall composition in relation to calcium nutrition

The peculiarly swollen appearance of cells of *Rhizobium trifolii* (Vincent & Colburn, 1961) led us to believe that the shape-determining rigidity of the cell wall had been lost. The 'R' layer components were, however, found to be qualitatively alike, whether the organism had been grown in the presence or absence of Ca²⁺, and all the other amino acids and sugars were also present in the calcium-deprived walls. It seemed therefore that the deficiency might lie in the Ca²⁺ itself, or in the organization of the components.

Calcium and magnesium analyses of whole cells and cell walls

Table 1 summarizes the results of Ca²⁺ and Mg²⁺ analyses of cells and cell walls. Those grown in the presence of calcium were thereafter deprived of Ca²⁺ by ethylene diamine tetra-acetic acid (freeze-dried normal material was exposed to M/10 EDTA in M/200 tris buffer, value pH 7.0, at 0° overnight); those grown in the absence of calcium were then exposed to Ca²⁺ (freshly harvested deprived cells were exposed either to normal medium containing 0.5 mM-Ca²⁺ for 1 hr. at 25° or to 10 mM-CaCl₂ in M/100 tris buffer, value pH 8, at 0° for 72 hr.). In each case, where organisms were exposed to Ca²⁺, they were washed five times with water, and a cell-wall preparation was then made.

The following conclusions could be drawn: (1) In 'normal' organisms the Ca^{2+} concn. in the cell wall was 25 % greater than that in the cell as a whole. (2) The total Ca^{2+} in deprived cells was very low (about 10 % of normal) and this was probably almost all accounted for by the amount retained in the walls (approximately 60 % of the normal wall Ca^{2+} content). (3) The deficiency of Ca^{2+} in the whole cell was compensated by additional Mg^{2+} . This did not apply to the walls. Those of deprived cells still had less than 75 % of the total divalent cations of normal walls. (4) EDTA removed most of the cations, Mg^{2+} as well as Ca^{2+} , from normal whole cells and walls. The small part resistant to removal would seem to be more strongly bound in the structure of the cell and wall. (5) Exposure of the deprived cells and walls to Ca^{2+} (in normal medium and as dilute CaCl_2) led to its uptake, more strikingly in the whole cells than in the walls. The concentration of Ca^{2+} in the walls remained appreciably below the value of walls of cells grown in the presence of Ca^{2+} .

Table 1. *Calcium and magnesium content of cells grown in the presence and absence of calcium*

+ = normal cells grown in 0.5 mM- Ca^{2+} + 0.5 mM- Mg^{2+} . — = calcium deprived cells grown in 1 mM- Mg^{2+} .

Fraction	Calcium status and treatment	mm/g. dry wt. of cells		
		Calcium	Magnesium	Total
Whole cells	+	0.062	0.064	0.126
	—	0.007	0.154	0.161
Cell walls	+	0.081	0.016	0.097
	—	0.047	0.024	0.071
Whole cells	+	0.008	0.005	0.013
	EDTA treated*			
Cell walls	+	0.010	0.005	0.015
	EDTA treated			
Whole cells	—	0.019	.	.
	Exposed 1 hr.* to normal medium			
Cell walls	—	0.054	.	.
	Exposed 1 hr. to normal medium			
Whole cells	—	0.058	.	.
	Exposed 72 hr.* to M/100 CaCl_2			
Cell walls	—	0.063	.	.
	Exposed 72 hr. to M/100 CaCl_2			

* See text for details.

Observations by phase and electron microscopy of the EDTA-treated organisms did not reveal the striking morphological abnormalities seen in cells deprived of Ca^{2+} during their growth. The electron micrographs of cells shadowed with platinum did, however, indicate some loss of structure in that those treated with EDTA had lost their smooth contours and had the walls wrinkled and collapsed in an angular manner over the cytoplasm. Phase observations of deprived cells exposed to Ca^{2+} failed to reveal any convincing evidence of morphological recovery.

DISCUSSION

Calcium deprivation could affect the cell wall in various ways:

(1) Calcium may have a catalytic role in the manufacture of the rigid-layer components. However, in the case of rhizobium, the walls of cells grown in the absence of added Ca^{2+} had the same 'R' layer components, amino acids and sugars, as those grown in its presence. It seems therefore that dependence of 'R' layer precursors on Ca^{2+} is not the explanation of the observed condition.

(2) Calcium may be needed for organizing the 'R' layer or other components into a rigid unit. Primosigh, Pelzer, Maass & Weidel (1961) pointed out that the peptide side chains of the 'R' layer which contain diaminopimelic acid would carry an excess negative charge which would need to be neutralized before they could be packed into a tight, rigid structure. It is feasible to visualize the specificity of Ca^{2+} being associated with its having, unlike Mg^{2+} , the dimensions that permit it to fit structurally into such a network. Recent growth observations by one of us (J. M. V.) have shown that Sr^{2+} , but not Ba^{2+} , could replace Ca^{2+} , though less efficiently, to support the growth of cells of normal morphology. If the dimension of the ion is involved, this observation fits with the respective place of Mg, Ca, Sr and Ba in the group of alkaline earths. There is some evidence that Ca^{2+} may be involved structurally in bacterial cell walls, apart from that presented in this paper. Keerer & Gray (1960) found up to 70 % of radioactive Ca^{2+} incorporated into the cell-wall fraction of *Listeria monocytogenes*. Murti (1960) prepared spheroplasts of *Vibrio cholerae* and *Escherichia coli* by suspending the cells in EDTA with lysozyme. Lysozyme attacked the polysaccharide backbone of the 'R' layer (Salton & Ghuysen, 1960) and the function of the EDTA could be to withdraw Ca^{2+} from the structure.

(3) Calcium may affect the stability and permeability of proteins present in the wall. Takahashi & Gibbons (1959) found that Ca^{2+} and Mg^{2+} protected the cells of *Micrococcus halodenitrificans* from swelling under unfavourable salt concentrations. Nermut (1960) found that *Proteus* large bodies became permeable to Alcian blue after treatment with lauryl sulphate and EDTA, suggesting that removal of Ca^{2+} has altered the permeability of the cell membrane. Brown (1961) found that the ionic strength of the medium, and particularly the concentration of divalent cations, determined the amount of cell envelope protein formed in a marine pseudomonad. Organisms growing on media of low ionic strength contained low concentrations of cell-wall protein and became spherical. Levy & Slobodiansky (1950) suggested that under conditions of unfavourable ionic strength the amount of protein synthesized in a system was reduced since proper hydrogen bonding did not occur, and the entropy of the system was too high for synthesis to continue. In rhizobium the effect of Ca^{2+} and Sr^{2+} was more specific since the swollen cells were seen in the presence of excess Mg^{2+} , sufficient to keep the total molarity of divalent cation constant. It is possible, however, that here the Ca^{2+} was specifically involved in stabilizing cell wall protein and that in the absence of Ca^{2+} , this wall protein was present in reduced quantity.

We were not able to simulate the morphological appearance of calcium-deprived cells by treatment of normal cells with EDTA, nor to restore normal morphology by exposure of deprived cells to Ca^{2+} . These observations, combined with the difficulty in restoring normal Ca^{2+} content to walls of deprived cells, compared with the

relative ease with which the whole cell took up the ion, suggested that there was an active incorporation of Ca^{2+} into the wall during growth, and that this was what was required for normal morphology. Alternatively, the Ca^{2+} could have been required to assist the normal formation of wall protein during cell growth. Cells grown in insufficient Ca^{2+} could not then be restored to normal by later addition of Ca^{2+} , nor would the removal of Ca^{2+} at that stage have any effect on a wall already synthesized. Noller & Hartsell (1961) studied the effect of pre- and co-lytic treatment of *Escherichia coli* and *Aerobacter* with lysozyme. They suggested that the action of EDTA and other agents (butanol, heat) in potentiating the action of lysozyme was by dissociation of the lipoprotein rather than the 'R' layer component of the wall. It is possible that in rhizobium also, Ca^{2+} may be concerned as much with the stability of the lipoprotein as with that of the 'R' layer portion of the wall.

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