Carbohydrate Content and Enzymic Activities in the Membrane of *Spiroplasma citri*

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INTRODUCTION

The spiroplasmas constitute a newly discovered group of wall-less prokaryotes, resembling mycoplasmas in morphology of colonies, ultrastructure, filtrability, requirement for cholesterol, absolute resistance to penicillin and low guanine and cytosine content of cellular deoxyribonucleic acid. Actively growing spiroplasma cultures differ from those of classical mycoplasmas in being composed mostly of long helical filaments which have a screw-like rotational and flexional movement (Cole *et al.*, 1973). Our previous investigation (Razin *et al.*, 1973) showed that the cell membrane of *S. citri* can be readily isolated by osmotic lysis of the organisms. In gross chemical composition and ultrastructure it resembled the cell membrane of the classical mycoplasmas. The purpose of the present investigation was to provide more information on the enzymic activities and carbohydrate moieties of the *S. citri* membrane.

METHODS

Organisms and membranes. *Spiroplasma citri* (Morocco strain, ATCC27556) was grown in the medium of Saglio *et al.* (1973) and *Acholeplasma laidlawii* (oral strain) in modified Edward medium (Razin & Rottem, 1976). Both media were supplemented with 5 % (v/v) horse serum. The organisms were harvested, washed and osmotically lysed as described previously (Razin *et al.*, 1973; Razin & Rottem, 1976). The membranes were separated from the cytoplasmic fluid at 4 °C by centrifugation at 32 000 g for 30 min, and were washed once in water and suspended in a small volume of β -buffer (Razin & Rottem, 1976) diluted 1:20 in deionized water.

Binding of lectins. Wheat germ agglutinin (WGA), *Ricinus communis* agglutinin (RCA) and concanavalin A (ConA) were isolated, purified and iodinated, and their binding to membranes was estimated as described by Kahane & Tully (1976). In general, ¹²⁵I-labelled lectins (10 μ g) were incubated for 15 min at 37 °C with membrane suspension (400 μ g protein in 0.4 ml 0.05 M-Tris/HCl, pH 7.2). The membranes were washed three times in the same buffer and their radioactivity was estimated. Specific displacement of membrane-bound lectins by simple sugars was tested by incubating the labelled membranes for 1 h at 37 °C in 0.1 M solutions of *N*-acetyl-D-glucosamine, D-galactose or α -methyl-D-mannoside for WGA, RCA and ConA, respectively. Non-specific displacement of these lectins by sugars was tested by the same procedure, but using D-galactose, α -methyl-D-mannoside and *N*-acetyl-D-glucosamine for WGA, RCA and ConA, respectively (Kahane & Tully, 1976).

Analytical procedures. Protein was determined according to Lowry *et al.* (1951). Carbohydrates were analysed by gas-liquid chromatography (Packard, model 7300) using a 3 % SE-30 column (2.5 m $\times$ 3.2 mm diam.) as described by Clamp (1974). Hexosamines were also determined in the amino-acid analyser (Spira, 1972). Enzymic activities were measured as described by Ne'eman & Razin (1975). NADH oxidase (EC 1.6.99.3) activity was measured spectrophotometrically by following the decrease in absorbance at 340 nm on addition of NADH to the reaction mixture. Results were expressed as decrease in absorbance at 340 nm (mg protein)⁻¹ min⁻¹. Adenosinetriphosphatase (EC 3.6.1.3) activity was measured by following the release of inorganic phosphate from ATP. Results were expressed as μ mol inorganic phosphate released (mg protein)⁻¹ (30 min)⁻¹. *p*-Nitrophenylphosphatase activity was measured spectrophotometrically by determining the intensity of the yellow colour of the liberated *p*-nitrophenol. The reaction mixture (1.0 ml) consisted of 100 mM-Tris/HCl, pH 7.6, 5 mM-MgCl₂, 10 mM-*p*-nitrophenylphosphate and 400 μ g membrane

protein. The reaction was stopped after 30 or 60 min incubation at 37 °C by adding 2 ml 0.5 M-Tris (not neutralized) in 5 % (w/v) trichloroacetic acid. The precipitate was removed by centrifuging and the intensity of the yellow colour in the supernatant fluid was determined at 420 nm. Results were expressed as increase in absorbance at 420 nm (mg protein)⁻¹ h⁻¹. Ribonuclease and deoxyribonuclease activities were determined by measuring the absorbance at 260 nm of soluble nucleotides released from the nucleic acids. Results were expressed as increase in absorbance at 260 nm (mg protein)⁻¹ (6 min)⁻¹.

RESULTS AND DISCUSSION

Carbohydrates in membranes

The presence of hexosamines in *S. citri* membranes has been reported by us (Razin *et al.*, 1973) and confirmed by Bebear *et al.* (1974). In the present study we tried to determine whether the hexosamines form a polymer of the kind found in *A. laidlawii* (Gilliam & Morowitz, 1972) or whether they are attached to other membrane components. The absence of a polyhexosamine polymer in *S. citri* is indicated by our finding that the hexosamines could not be concentrated by extraction of the membranes with 75 % (v/v) aqueous ethanol and precipitation with acetone, according to the method of Gilliam & Morowitz (1972). Moreover, the quantities of glucosamine and galactosamine detected in *S. citri* by the amino-acid analyser varied quite markedly in different batches of membranes and were only about one-eighth to one-third of those found associated with *A. laidlawii* membranes. The glucosamine content of *S. citri* membranes ranged from 3.6 to 7.8 µg (mg protein)⁻¹, and that of galactosamine ranged from 1.9 to 5.3 µg (mg protein)⁻¹. The addition to 250 ml of *S. citri* growth medium of 5 µCi of [1-¹⁴C]glucosamine (58 mCi mmol⁻¹) or [1-¹⁴C]galactosamine (51.4 mCi mmol⁻¹) resulted in slight radioactive labelling of the membranes. The values ranged from 500 to 700 c.p.m. (mg protein)⁻¹, compared with 4000 to 9600 c.p.m. (mg protein)⁻¹ in membranes of *A. laidlawii* grown in the same medium. The hexosamine content of *S. citri* membranes was not affected by the removal of membrane lipids by extraction with chloroform/methanol (2:1, v/v). However, proteolytic digestion of membrane proteins decreased the hexosamine content, particularly glucosamine, in proportion to the release of membrane proteins. Thus, the release of about 55% of membrane proteins by pronase caused a decrease of 45 % of the glucosamine and about 20 % of the galactosamine content of the membranes.

Lectins were used to detect exposed carbohydrate residues on the *S. citri* membrane. Substantial amounts of ConA and RCA were bound to the membranes (Table 1). The specificities of the binding, measured by their displacement with specific sugars, were 94.5 % and 99 %, respectively. Only about 10 % of the bound lectins were displaced by the non-specific sugars. Carbohydrate analysis indicated the presence in the membranes of the sugars specific for the lectins, suggesting that at least some of these sugars are exposed on the membrane surface. Although the membranes contained *N*-acetylglucosamine, there was no binding of WGA, the lectin specific for this sugar. Treatment of *S. citri* membranes with proteolytic enzymes markedly decreased the lectin binding (Table 1). The decrease in binding of both RCA and ConA was proportional to the loss of protein and corresponded to the decrease in carbohydrate residues from the membranes (Table 1). On the other hand, small amounts of WGA did bind to the membranes after proteolysis. The amount of WGA bound after removal of 45 % of the membrane proteins was greater than that bound to membranes from which 70 % of the proteins had been removed. In the first case the *N*-acetylglucosamine content of the membrane was not affected, but the more extensive digestion removed almost 75 % of this sugar (Table 1).

The binding of lectins to *S. citri* membranes indicates that some of their mannose, glucose and galactose residues are exposed on the membrane surface, while the *N*-acetylglucosamine residues are masked in native membranes. The partial removal of membrane proteins by proteolysis exposes some of the *N*-acetylglucosamine residues to WGA. More extensive proteolysis decreases the hexosamine residues and WGA binding. Whereas proteolysis of

Table 1. *Effect of proteolytic treatment on the binding of lectins and the carbohydrate content of S. citri membranes*

Membranes were digested with 2 μg pronase ml^{-1} for 30 min or with 50 μg trypsin ml^{-1} for 2 h at 37 °C. For measurement of lectin binding, ^{125}I -labelled lectins (10 μg) were incubated with membranes (400 μg protein) and the amount of bound lectin was determined as described in Methods. Carbohydrates were estimated by the method of Clamp (1974): Fuc, fucose; Man, mannose; Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine.

Proteolytic treatment	None	Pronase	Trypsin
Protein released (%)	0	45	70
Lectin bound (%)			
WGA	0	1.9	0.8
RCA	30.9	21.2	14.6
ConA	43.3	28.8	9.4
Carbohydrate [nmol (mg protein) $^{-1}$]			
Fuc	?	0	0
Man	27	12	6
Gal	27	21	14
Glc	24	27	21
GlcNAc	7	7	2
GalNAc	trace	trace	0

* Minor peak, close but apparently not identical to that of fucose.

Mycoplasma and *Acholeplasma* membranes usually increased their lectin-binding capacity, suggesting that binding sites are glycolipids or other protease-resistant compounds (Kahane & Tully, 1976), the progressive digestion of *S. citri* membrane proteins resulted in a proportional decrease in the lectin-binding sites (Table 1), suggesting that most of the carbohydrate residues in the *S. citri* membrane are attached to protein. Glycoproteins are not common constituents of the plasma membrane of prokaryotes (Razin, 1975). Among mycoplasmas, however, they have been detected in *M. pneumoniae* (Kahane & Marchesi, 1973) and *M. gallisepticum* (Goel & Lemcke, 1975). Our results suggest that glycoproteins are also present in *S. citri* membranes. However, we failed to detect glycoproteins in *S. citri* by electrophoresis in sodium dodecyl sulphate–polyacrylamide gels stained with the periodic acid–Schiff reagent. This failure may be due to the fact that the staining procedure we followed (Segrest & Jackson, 1972) is most effective with glycoproteins containing sialic acid (I. Kahane, unpublished results) which is apparently absent from *S. citri* membranes (Table 1). Nevertheless, the possibility that the carbohydrate moieties in *S. citri* membranes are part of other macromolecules, loosely attached to the membrane proteins and released following the removal of these proteins, cannot be ruled out.

Enzymic activities

The localization of several enzymic activities in cell fractions of *S. citri* and *A. laidlawii* are compared in Table 2. In general, the specific activities were lower in *S. citri*, particularly those of the *p*-nitrophenylphosphatase. As in *A. laidlawii* and in all other mycoplasmas tested so far (Razin, 1975), the *S. citri* adenosinetriphosphatase activity was localized in the membrane. It could not be released by washing the membranes in buffers of decreasing ionic strength, with or without ethylenediaminetetraacetate, according to the method used by Ne'eman & Razin (1975). Hence, the adenosinetriphosphatase of *S. citri* appears to be an integral membrane enzyme. It thus resembles the adenosinetriphosphatase of *M. mycoides* subsp. *capri* and of *A. laidlawii*, and differs from the adenosinetriphosphatases of the wall-covered bacteria which behave like peripheral membrane proteins in being released with low-ionic strength buffers and ethylenediaminetetraacetate (Razin, 1975). As in the sterol-requiring *Mycoplasma* species, but unlike *Acholeplasma* species (Razin, 1975), the NADH oxidase activity was localized in the cytoplasmic fraction of *S. citri*, suggesting the presence

Table 2. *Localization of enzymic activities in S. citri and A. laidlawii cell fractions*

Data are expressed as specific activities, as defined in Methods.

Preparation	Adenosine-triphosphatase	NADH oxidase	p-Nitrophenyl-phosphatase	Ribonuclease	Deoxyribo-nuclease
<i>S. citri</i>					
Membranes	2.30	0.03	0.17	1.30	0.62
Cytoplasmic fluid	0.15	0.62	0.00	0.66	0.00
<i>A. laidlawii</i>					
Membranes	7.80	1.50	6.00	7.70	1.34
Cytoplasmic fluid	0.02	0.02	0.15	3.90	4.02

of a truncated electron transport system in spiroplasmas. Another interesting difference between *S. citri* and *A. laidlawii* was the absence of detectable deoxyribonuclease activity in the cytoplasmic fraction of *S. citri* (Table 2). The presence of NADH oxidase activity in the cytoplasm, the requirement for cholesterol (Freeman *et al.*, 1976; Saglio *et al.*, 1973) and the inability to synthesize fatty acids from acetate (Freeman *et al.*, 1976; S. Razin & S. Greenstein, unpublished data) place *S. citri* closer to the *Mycoplasma* than to the *Acholeplasma* species.

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