The Somatic Antigens of Two Strains of *Rhizobium trifolii*

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SUMMARY

Strain-specific lipopolysaccharide extracted by hot phenol from two strains of *Rhizobium trifolii* was fully antigenic in rabbits with an injection schedule using Freund's adjuvant, and highly active in gel diffusion. A minor second diffusion line was considered to be due to a smaller fragment of the main molecule, since the antigen was converted almost entirely to this form by sodium dodecylsulphate and such disaggregated material absorbed antibodies to both forms of the antigen. Chemically the lipopolysaccharide showed some features related to those of the Enterobacteriaceae; for example, the presence of firmly bound lipid, 2-keto-3-deoxyoctonate, glucose, mannose, fucose and, in one strain, a heptose. However, the lipopolysaccharide of *R. trifolii* was unusual in its very low phosphorus content and the presence of glucuronic acid. One strain diverged even further in its high content of carbohydrate, its lack of a heptose, its anionic behaviour and the gelatinous nature of some preparations; these properties might relate to the presence of a capsule-like structure which has been observed in old cultures of this strain.

INTRODUCTION

The composition and structure of the lipopolysaccharide (LPS) somatic antigens of Gram-negative bacteria has been most studied in the Enterobacteriaceae, in which the general pattern (Lüderitz, Staub & Westphal, 1966) is that of a glucosamine-containing lipid component, linked through a heptose phosphate core to a polysaccharide containing 2-keto-3-deoxyoctonate (KDO) and bearing highly specific sugar side chains. However, recent work on *Xanthomonas* spp. (Volk, 1966, 1968*a, b*) reveals that this is not invariable. The toxic phenol-extracted LPS from 20 *Xanthomonas* species contained KDO, uronic acid, glucose, mannose and various combinations of galactose, fucose, xylose and rhamnose; in no case was a heptose detected. The present work reports properties of the LPS extracted by hot aqueous phenol from two serotypes of *Rhizobium trifolii*, with the object of comparing them with the pattern established in the Enterobacteriaceae, and determining any differences which might contribute to the antigenic distinction between the two strains.

METHODS

Organisms. The strains of *Rhizobium trifolii* were SU297/31 and TA1, the latter being maintained as line SU329 in the Sydney University collection.

Preparation of lipopolysaccharide (LPS). The bacteria were grown for 72 hr at 26°, each in two 40 l. batches of a defined medium adequate in calcium (Vincent,

1962). The harvested bacteria, totalling about 200 g. wet weight for each strain, were disintegrated by ultrasonic treatment for 5 min. in an ice bath, and the bacterial debris centrifuged, washed and used for preparation of the somatic antigen. The modification by O'Neill & Todd (1961) of the hot phenol extraction method (Westphal, Lüderitz & Bister, 1952) was used to further decrease contamination by nucleic acids. This modification involves a first extraction in the cold with 0.25 % (w/v) trichloroacetic acid (TCA) which dissolves nucleic acid besides liberating a portion of the somatic antigen in the form of a Boivin-type antigen (LPS attached to protein). The subsequent phenol extraction of the residue yields, in the aqueous phase, LPS low in protein and contaminating nucleic acid. This procedure allows two forms of the somatic antigen (Boivin-type antigen and phenol-extracted LPS) to be obtained from one batch of bacteria.

LPS extracted by the unmodified hot phenol method of Westphal *et al.* (1952) was used for some phosphorus analyses to permit comparison with figures published for other organisms.

Preparation of antisera. Rabbit antisera to whole or mechanically disintegrated organisms were prepared as described by Humphrey & Vincent (1965) by using one intramuscular injection of bacterial suspension in Freund complete adjuvant (Difco) followed 4 weeks later by an intravenous injection of bacterial suspension without adjuvant. A similar regimen was followed with phenol-extracted LPS or TCA-extracted Boivin-type antigen, using 100 µg. dissolved in saline for each injection. Blood was collected by exsanguination 1 week after the intravenous injection.

Gel diffusion and quantitative absorption. The technique for gel diffusion followed that of Dudman (1964) as detailed by Humphrey & Vincent (1965). The precipitinogen activity of preparations was assessed by determining the least concentration (µg./ml.) at which extracted material formed a precipitation line. Routinely, antisera to mechanically disintegrated organisms suspended in culture supernatant were used. In addition, gel immunoelectrophoresis was done in barbiturate buffer (pH 8.8), $I = 0.05$, for 2 hr at 350 V, which gave a current of 1 mA/cm. The method of quantitative absorption of agglutinins was as described in Vincent & Humphrey (1968).

Total phosphorus analysis. This was done by the method of Chen, Toribara & Warner (1956) with perchlorate digestion of the sample, and by the method of Ames (1966) with magnesium nitrate ashing.

Chromatography. The LPS, or the polysaccharide obtained after lipid extraction (see Results), was hydrolysed for 16 hr at 100° in N-HCl, and the HCl removed by repeated evaporation in vacuum over KOH pellets and concentrated H₂SO₄. Descending chromatography was used in solvents A (butanol + pyridine + benzene + water = 5 + 3 + 1 + 3 by vol.), B (butanol + acetic acid + water = 3 + 1 + 1 by vol.) and C (butanol + ethanol + water = 4 + 1 + 5 by vol.). Solvent A was favoured for routine analysis since it gave clear separation of glucose from galactose; the other solvents were used to confirm results from solvent A. Chromatograms were made visible with *p*-anisidine HCl for sugars, with naphthoresorcinol to confirm the presence of glucuronic acid, or with urea phosphate (Greene, 1958) to confirm the presence of heptose. For KDO, the antigens were hydrolysed for 8 min. at 100° in 0.25 N-HCl, and the chromatograms developed with solvent D (butanol + pyridine + 0.1 N-HCl = 5 + 3 + 2 by vol.), and the KDO located with sodium thiobarbiturate (Warren, 1960). For separation of glucosamine and galactosamine, solvent E

(isopropanol + water = 4 + 1, v/v) was used, with the Elson-Morgan reagent (Smith, 1960, p. 252) to locate the sugars.

Spectrophotometric identification of sugars. The cysteine + H₂SO₄ reaction (Dische, 1955) was used to confirm the identities of hexoses, heptoses and methylpentoses eluted from chromatograms. The carbazole reaction (Bowness, 1957) was used to identify and estimate the glucuronic acid in eluted spots. With whole antigen, KDO was determined by the thiobarbiturate reaction as modified by Osborn (1963) and by the semicarbazide reaction (MacGee & Doudoroff, 1954). The method of Dische & Shettles (1948) modified by Gibbons (1955) was used for deoxysugars, and that of Randle & Morgan (1956) for amino sugars. Total carbohydrate was estimated by the phenol + H₂SO₄ method (Dubois *et al.* 1956).

Materials. Authentic KDO, provided by Dr K. Knox (United Dental Hospital, Sydney, N.S.W.), had been obtained from the penta-acetyl derivative made available to him by Dr E. C. Heath (Johns Hopkins University, Baltimore, Maryland, U.S.A.). The standard heptose used was the 'α-D-glucoheptose' supplied by Light & Co. The agarose column was of Gelarose (Litex, Denmark).

Fig. 1. Schematic representation of gel-diffusion pattern shown by a broken suspension of *Rhizobium trifolii* (Ag) diffusing against homologous antiserum (AS). The 'a' antigens are on the bacterial surface (somatic) and strain-specific. The 'b' and 'c' antigens are located internally and are common to all *R. trifolii* strains which have been tested.

RESULTS

Serological reactions of the whole bacteria

The two strains were distinctive in their somatic agglutination, although sporadically cross-reacting at 1/50 dilution of sera which gave a homologous reaction at 1/3200. This slight degree of cross-agglutination was not reflected in gel diffusion of surface antigens from intact bacteria. The 'a' line corresponding to these strain-specific somatic antigens (Humphrey & Vincent, 1965) was resolved at suitable concentrations into three lines (designated a₁, a₂, a₃ according to their relative proximity to the antigen well). The a₂ line was invariably visible even with a low concentration of bacteria, and developed early. The 'b' and 'c' lines, obtained only with broken or Ca-deficient bacteria, were common to both strains but unrelated to agglutination (Fig. 1).

Immunological characteristics of the isolated antigens

Precipitinogen activity of the extracted fractions. The Boivin-type antigen, precipitated from the TCA extract by 3 vol. ethanol, the concentrated dialysed ethanol supernatant fluid of the Boivin precipitate and the phenol extract of the supernatant fluid after the initial ultrasonically treated material had been centrifuged, all showed precipitation with specific antisera. Limiting concentrations varied between 4 and 1000 $\mu\text{g./ml.}$ from batch to batch. About 0.8 % of the wet weight of the TCA-treated bacterial debris was extracted in the aqueous phase of the hot phenol, and this extract was consistently highly active, showing precipitation at 1 and 4 $\mu\text{g./ml.}$ in the two batches of SU297/31, and 8 and 16 $\mu\text{g./ml.}$ with TA1. For each strain, the two batches of these last phenol-extracted antigens were then pooled for subsequent analysis.

There was no cross-precipitation between the extracted antigens of SU297/31 and TA1. The TA1 antigen, a more slowly diffusing, presumably larger, molecule than that of SU297/31, formed its precipitation arc nearer the antigen well. The angle of precipitation line when antigen and antibody diffused at right angles to each other also indicated that the TA1 antigen was a larger molecule (Crowle, 1961, p. 80).

After 24 to 48 hr, all extracts showed only a single line in gel diffusion corresponding to the major strain specific (a_2) line of whole bacteria; but in 5 to 7 days, at higher concentrations, a faint second line began to appear, nearer the serum well and apparently identical with the a_3 line (Fig. 1). Incubation of antigen solutions for 5 days at 26° before being placed in the antigen well did not result in a quicker appearance or greater quantity of the a_3 antigen precipitate. It was concluded, therefore, that the slow appearance of this line was due to the longer time necessary for a low concentration of a smaller fragment to accumulate a precipitate to visible limits, rather than to its slow liberation from the a_2 antigen either in the well or in the first-formed a_2 line. Diffusion of antigen and antiserum at right angles to each other showed that this was not a Liesegang effect (Crowle, 1961, p. 81). The following methods were used to attempt to purify the phenol-extracted antigen:

(i) *Ethanol precipitation in the presence of Mg^{2+}* (Osborn, Rosen, Rothfield & Horecker, 1962). One hundred mg. of LPS were brought as far as possible into true solution by shaking with 25 ml. distilled water, centrifuging the undissolved material at 12,000 g, resuspending in a further 25 ml. distilled water, shaking, etc., to a total of 100 ml. The supernatant solutions from the water treatments were poured into 3 vol. ethanol containing 0.025 M- MgCl_2 , the flocculent precipitate collected, suspended in 20 ml. 0.02 M-ethylene diaminetetra-acetic acid (EDTA) and dialysed for 24 hr. The recovery of the dissolved LPS was 68 % for SU297/31 and 40 % for TA1. The activity of the recovered material in gel diffusion was unchanged with regard to the a_2 line, and the a_3 line still appeared at a concentration of 250 $\mu\text{g./ml.}$ The insoluble residue remaining after the four water treatments was lyophilized, suspended in saline (1 mg./ml.) and tested for the limiting dilution of its precipitinogen activity. SU297/31 yielded 1.5 mg. of inactive residue. The LPS of TA1 was less soluble, since there remained after the four water treatments a gelatinous pellet weighing 11.6 mg. which was as active in gel diffusion as the starting material.

(ii) *Ultracentrifugation.* Twenty mg. phenol-extracted antigen were shaken vigorously in 10 ml. distilled water to obtain an evenly opalescent suspension which was then centrifuged at 100,000 g for 1 hr. Both supernatant solution and pellet were

lyophilized, yielding, in SU297/31, 67 % in solution, 33 % in the pellet; in TA1, 27 % in solution, 73 % sedimented. The gel diffusion activity of the sedimented material was unchanged both for the a_2 and a_3 lines. The a_2 line appeared in the soluble fraction of both strains at a slightly lower concentration than with the original material (2, compared with 4 $\mu\text{g./ml.}$ for SU297/31, 8 compared with 16 $\mu\text{g./ml.}$ for TA1). The limiting concentration of the a_3 line was also unchanged in this fraction, and chromatography of the two fractions showed no qualitative difference.

Effect of sodium dodecylsulphate (DDS) on diffusion patterns. Beer, Braude & Brinton (1966) found that DDS altered the proportion of the three diffusion lines produced by a Boivin-type preparation from *Escherichia coli*. In the present work with phenol-extracted antigens, it was found that DDS, at a concentration of 0.2 % in the antigen well, similarly altered the occurrence and density of the lines. In SU297/31, the a_2 line virtually disappeared, the a_3 line was intensified, and an additional line, ' a_4 ', formed nearer to the serum well than the a_3 line. In TA1, increased density of a_3 was accompanied by a decrease in a_2 , and both lines were displaced markedly towards the serum well; a_4 did not appear. In both preparations lines lost definition and reaction became less sensitive (visible) in the presence of the detergent. Prolonged treatment (14 days at 4°) with DDS disaggregated the antigens to such an extent that they no longer formed precipitin lines.

When antisera were absorbed 'in-well' (Humphrey & Vincent, 1965) with DDS-treated antigens and diffused against untreated LPS, the formation of the a_3 line was prevented completely, and only a fine tracing of the a_2 line remained. This indicated that only a small proportion of the antibodies responsible for this line were still free (i.e. unabsorbed by the DDS-treated antigen). The antigen responsible for the a_3 line and formed from the a_2 antigen in the presence of DDS carried in the main the same antigenic groupings.

The effect of cetyltrimethylammonium chloride (cetavlon). The cetyltrimethylammonium ion will precipitate highly acidic polysaccharides at neutral pH and less acidic polysaccharides at higher pH (Scott, 1960). Phenol-extracted antigens (100 mg.) were dissolved in water with shaking, and the fraction sedimented after centrifugation at 12,000 g removed. The solutions were made 2 % (w/v) with respect to cetavlon, and the pH value was adjusted progressively with alkali. The TA1 antigen began to precipitate at pH 7 to 8; that of SU297/31 at pH 10. The opalescent solution was left overnight, the precipitate separated by centrifugation, thrice dissolved in 5 M-NaCl (30 min., 37° with shaking) and precipitated in 10 vol. ethanol to displace the cetyltrimethylammonium ion (Jann *et al.* 1965). The precipitate was freeze dried and tested in gel diffusion. The supernatant fluid from the primary cetavlon precipitation was dialysed at 4° with stirring until free from foam (7 days), concentrated in vacuum at 35° and freeze dried. In the case of SU297/31, 7 % of the starting material was recovered as precipitate (inactive), and 80 % as active material from the supernatant fluid. The latter gave a faster and more diffuse band which fused with a_3 of the parent material. It seemed that the effect of cetavlon on the SU297/31 antigen was mainly as a detergent comparable with DDS, but that, under the conditions used, the LPS was not sufficiently acidic to be precipitated by cetavlon. In the case of TA1, 60 % of the starting material was recovered in the precipitate, fully active and identical with a_2 . The 18 % recovered from the supernatant fluid formed a fast-moving diffuse precipitation line in the a_3 region.

Immunoelectrophoresis. The indication, from cetavlon precipitation, that the TAI antigen was more acidic than that from SU297/31, was confirmed by immunoelectrophoresis, both of the phenol-extracted LPS and of mechanically-disintegrated bacteria. The TAI antigen moved towards the anode, as did the a_2 component of the antigen mixture from disintegrated bacteria. The a_2 antigen of SU297/31 moved a short distance towards the cathode, probably by endosmosis. In neither case did the a_3 line become visible.

Antigenicity in rabbits. The phenol-extracted LPS and the Boivin-type antigen fractions yielded antisera showing a_2 and a_3 gel-diffusion lines, and having the following titres (determined by agglutination of homologous whole bacteria): SU297/31 phenol-extracted LPS, 3200; SU297/31 Boivin-type antigen, 3200; TAI phenol-extracted LPS, 800; TAI Boivin-type antigen, 3200. These compare well with titres of 3200 to 6400 usually obtained with antisera produced against whole bacterial cultures using the same regimen.

The antisera obtained from isolated antigen differed from those obtained from the bacteria themselves in that they lacked precipitin due to internal antigens (Humphrey & Vincent, 1965) and those agglutinins more efficiently absorbed by Ca-deficient bacteria as shown by the technique of quantitative absorption (Vincent & Humphrey, 1968). In the case of antisera produced from isolated antigen, the Ca-adequate and Ca-deficient bacteria were equally able to absorb all the antibodies, whereas with antisera to bacteria a portion of the total agglutinin resisted absorption by the Ca-adequate cells.

Chemical composition of the extracted antigens

Table 1 shows the chemical characteristics of the phenol-extracted LPS, and compares these with data published for *Xanthomonas* spp. and members of the Enterobacteriaceae. In many cases, the rhizobial antigens show similar values to other antigenic LPS which have been analysed. Notable exceptions are the low P value and the presence of glucuronic acid in both rhizobial strains; and in TAI, the high total carbohydrate content and failure to detect heptose.

Phosphorus content. It was thought that the extremely low phosphorus value (about one twentieth of the amount usually reported in the LPS of the Enterobacteriaceae), might be due to the preliminary extraction with TCA. To test this possibility, whole bacteria of SU297/31 were extracted directly by hot phenol according to the most commonly used technique (Westphal *et al.* 1952), and the resulting extract precipitated with ethanol. The precipitate was redissolved and reprecipitated twice. This material contained about 10 % nucleic acids (as estimated from the absorption peak at 260 m μ) and 1.2 % P. It was further purified successively by digestion with ribonuclease and deoxyribonuclease, dialysis and passage through a column of 8 % agarose. The column had a void volume of 50 ml. and the antigen as revealed by gel diffusion was located in fractions 12 to 23 (5 ml. fractions of 0.2 M-ammonium acetate). The nucleotides appeared in fractions 28 to 49. The antigen-containing fractions were pooled, dialysed, concentrated in vacuum and freeze dried. The recovered material (115 mg.) showed no absorption peak at 260 m μ and was highly active in gel diffusion (the a_2 line appeared at 0.5 μ g./ml., a_3 at 64 μ g./ml.). This antigen preparation contained only 0.045 % phosphorus. The fact that this highly active extract showed a lower content than the previous preparation

Table 1. *Chemical characteristics of phenol-extracted lipopolysaccharides**

<i>Rhizobium trifolii</i>				Enterobacteriaceae
	SU 297/31	TA I	Xanthomonas species ^a	
Carbon	44	46	—	45 ^b
Nitrogen	2.0	1.4	—	1.7 ^b ; 1.7 ^c
Phosphorus	0.14; 0.045†	0.07	1.8 to 6.5	2.2 ^b ; 1 to 1.5 ^c ; 3 to 4 ^d
Lipid (firmly bound)	13	10	22 to 47	46 ^b ; 10 to 16 ^c ; 32 to 50 ^e
Total Carbohydrate‡	33	71	—	35 to 48 ^c ; 28 to 35 ^f
Hexose	Glucose, mannose	Glucose, mannose	Glucose, mannose	Glucose, galactose, mannose
Heptose	10.8	n.d.§	n.d.	12 to 14 ^c ; 3 to 9 ^e ; 16 to 20 ^f ; 16 to 19 ^d
Hexosamine	2.5 (one-third in lipid)	1.1 (half in lipid)	Glucosamine (in lipid only)	10 to 13 ^c ; 4 to 7 ^e ; 5 to 7 ^f ; 3.5 to 6 ^d
KDO	1.5	1.2	1.5 to 3	9 to 10 ^e ; 2.3 ^e ; 3 to 5 ^e ; 3 to 6 ^f ; 1.7 ^d
Methylpentose	Fucose 16	Fucose 20	Rhamnose 1-31	Rhamnose 0.7 to 1.5 ^g
Di-deoxysugar	Possibly present¶	Possibly present¶	Fucose 5-15	Fucose
Uronic acid	Glucuronic acid 6	Glucuronic acid 6	n.d.	Often present
Pentose	n.d.	n.d.	Galacturonic acid 4 to 9	Rarely present ^h
			Xylose	Ribose ^e

* Numerical values are % dry wt.

† Prepared and purified by a different method (see text for details).

‡ Phenol-sulphuric acid method.

§ n.d. = not detected.

|| Compare Adams *et al.* (1967).¶ Unidentified sugar *R_G* 2.14 in Solvent A.^a Volk (1966, 1968*a, b*).^b Davies (1956).^c Kasai (1966).^d Work *et al.* (1966).^e Lüderitz, Galanos *et al.* (1966).^f Rapin and Mayer (1966).^g Knox (1966).^h Lüderitz *et al.* (1968).

made according to the method of O'Neill & Todd (1961) suggests that the small amount of P is a contaminant rather than an essential component of the antigen. The gelatinous nature of a similar direct phenol extract of TA I prevented its separation, due to its extremely slow penetration of the water phase of the agarose column. Contamination of the unfractionated TA I material with nucleic acid was estimated at 18 %.

Lipid content of the antigens. The phenol-soluble antigens (200 mg.) were extracted successively with ether, chloroform and chloroform + methanol (2 + 1). The antigens were first extracted without pre-treatment, then the residue was divided into two parts and each heated for 1 hr at 100°, either in buffered saline (pH 7.2) or in N-acetic acid. The extractions with ether, chloroform and chloroform + methanol were repeated on the heated material. The percentages of lipid material extracted at each step are shown in Table 2. The fractions heated in acid had lost both their ability to form a precipitate in gel diffusion and their ability to absorb the antibodies in 'in-well' absorption. The fractions heated at pH 7.2 and extracted with the three solvents were as active in gel diffusion as the starting material.

Table 2. *Lipid fractions extracted from rhizobial antigens*

				<i>Rhizobium trifolii</i>			
				Strain SU297/31		Strain TA I	
				% dry wt			
From unheated material	...	...	3.6		10.1		
			Neutral	Acid	Neutral	Acid	
From heated material	...	...					
Ether extract			8	7	—	4.6	
Chloroform extract			1.9	9.4	—	1.8	
Chloroform+ MeOH extract			7.7	14.5	4.8	9.4	
Total lipid extractable after heating			17.6	30.9	4.8	15.1	
Firmly bound lipid (liberated by acid hydrolysis)			13.3		10.3		
Total lipid in preparation			34.5		25.2		

Chromatography of sugars. Chromatography, in solvents A, B and C, was carried out on hydrolysates both of the original phenol-extracted LPS and the polysaccharide residue left after lipid extraction. The latter material gave cleaner separations, although both materials showed the same sugars when visualized with *p*-anisidine HCl.

The phenol-extracted antigens of both SU 297/31 and TA I appeared to contain glucose, mannose, fucose, glucuronic acid and its lactone and an unknown, fast-running spot ($R_{\text{glucose}} = 2.14$ in solvent A) which reacted with *p*-anisidine HCl but not naphthoresorcinol. The presence of glucuronic acid and its lactone was confirmed by the use of naphthoresorcinol as dipping reagent. In addition, SU 297/31, but not TA I, gave a spot in the galactose region shown to be a heptose by urea phosphate spray. A slow-moving spot in SU 297/31 was probably a hexose-heptose oligosaccharide (see below).

To confirm the identity of the sugars, and the absence of heptose in the TA I antigen, parallel strips of both polysaccharide hydrolysates and standard sugars were developed in solvent A, and then either made visible or eluted, the eluates

being tested by various identification procedures. Both the glucose spot in SU297/31 and in TA1, and the slow-moving spot in SU297/31 showed, in the cysteine + H₂SO₄ reaction, a peak at 400 m μ identical with that from the glucose standard. The unknown slow-moving spot also showed a small peak at 510 m μ , indicating the presence of a heptose. The SU297/31 spot in the galactose region showed only a clear heptose peak at 510 m μ , with no hexose peak. The TA1 eluate from this region showed no sugars. The mannose and fucose spots from both antigens showed absorption typical of the standard sugars in the cysteine + H₂SO₄ reactions, i.e. mannose showed a flat peak at 400 m μ which did not change on standing to a peak at 600 m μ (as does glucose); fucose showed a very sharp peak at 400 m μ . The fast-running sugar also gave a sharp peak at 400 m μ , suggesting a deoxy- or dideoxy-sugar, but it was not rhamnose. Eluates of the glucuronic acid and glucuronolactone spots gave typical coloration with the carbazole reaction, and were estimated at about 4 % of the total polysaccharide residue, i.e. about 6 % of the original LPS.

Glucosamine was identified, and distinguished from galactosamine, in hydrolysates of LPS in 6 N-HCl (100°, 16 hr). Chromatograms were run in Solvent E and the amino sugars detected with the Elson-Morgan reagent.

KDO was detected on chromatograms after hydrolysis of the LPS for 8 min. at 100° in 0.25 N-HCl. The thiobarbiturate-positive spots in the hydrolysates developed in solvent D (48 hr) co-chromatographed with authentic KDO.

Estimation of carbohydrate constituents. These were made on phenol-extracted antigen prepared by the method of O'Neill & Todd (1961). The results are shown in Table 1. In heptose analyses, using Osborn's 10 min. modification (1963) of the cysteine + H₂SO₄ reaction, the SU297/31 antigen developed a pink coloration with a maximum at 508 m μ , typical of heptose and equivalent to about 10 % heptose in the whole antigen. TA1, on the other hand, gave a brownish solution with an atypical broad curve having a slight maximum at 530 m μ confirming the absence of heptose. Full colour developed in these reactions only after 16 hr.

In the thioglycollic acid reaction for estimating methylpentose (Gibbons, 1955), the SU297/31 antigen developed a predominating pink colour, and showed not only a peak at 400 m μ for fucose but also a peak at 510 m μ for heptose (as did a heptose standard). The heptose content was probably high enough to interfere slightly with the fucose value (see Gibbons). TA1 developed a yellow-brown colour with thioglycollic acid, and showed a large amount of fucose (as estimated by $A_{400} - A_{430}$), and in addition a low broad absorption curve with a small maximum at 530 m μ .

Glucosamine was estimated in the whole LPS and in the lipid and polysaccharide fractions after hydrolysis in N-acetic acid and extraction with chloroform and chloroform + methanol. For these analyses, all the solvent extractions were pooled to give a total lipid fraction. The hexosamine content of the rhizobial antigens, lower than that usually reported in LPS, was distributed between polysaccharide and lipid fractions.

KDO, estimated with thiobarbituric acid according to Osborn (1963) was detected in both antigens, even without prior hydrolysis (SU297/31, 1.1 %; TA1, 0.6 %). After hydrolysis with 0.1 N-acetic acid for 30 min. at 100° the amount of KDO detected in the SU297/31 antigen increased to 1.5 %; more vigorous hydrolysis with 0.25 N-HCl for 8 min. at 100° did not augment this value. Prior hydrolysis of the TA1 antigen with 0.1 N-acetic acid did not release more KDO but hydrolysis with 0.25 N-HCl

increased the amount detected to 1.2 %. The semicarbazide reaction of MacGee & Doudoroff (1954), although less specific, did not give higher values for the estimated KDO content of either antigen.

DISCUSSION

The lipopolysaccharides of two strains of *Rhizobium trifolii* studied in the present work were fully antigenic. Boivin-type antigens have always been considered to be capable of stimulating antibody production (Boivin, Mesrobian & Mesrobian, 1933). This property was ascribed to the presence of the protein moiety; but Webster, Sagin, Landy & Johnson (1955) have shown that purification of a Boivin-type antigen with successive decrease of nitrogen content, to 0.6 %, did not diminish the antigenicity. With phenol-extracted antigens, low in nitrogen content, some workers (e.g. Davies, 1956) were unable to produce antiserum unless the antigen was coupled with a foreign protein. Others who have used phenol-extracted LPS without adjuvant as antigen, have had to adopt a programme of as many as twenty injections to obtain satisfactory response (e.g. Taylor, Knox & Work, 1966). The antisera developed with the rhizobial antigens, by using one intramuscular injection with Freund complete adjuvant, had satisfactory titres when this was followed by a single intravenous injection. The use of adjuvant is known to reduce the lower limiting molecular size associated with antigenicity (White, 1967). In a previous study of the antisera developed by whole and broken rhizobia (Vincent & Humphrey, 1968) the disorganized surface of bacteria grown in a deficiency of calcium absorbed a portion of the antibodies from all antisera more readily than did the agglutinogens of calcium-adequate bacteria. In the present study, however, the antisera produced against the isolated LPS were absorbed as effectively by normal as by calcium-deficient bacteria. This indicates that any extra antigen revealed on the calcium-deficient surface is not part of the phenol-extractable somatic antigen, but part of some underlying layer of the wall which has been exposed as a result of calcium deficiency. The concentration of phenol-extracted rhizobial antigens produced precipitation lines in gel diffusion (between 0.5 and 16 $\mu\text{g./ml.}$), comparing well with values reported by other workers (e.g. 1 $\mu\text{g./ml.}$ for *Pasteurella* antigen; Davies, 1956; 20 to 40 $\mu\text{g./ml.}$ with an *Escherichia coli* LPS preparation; Kasai, 1966).

The two lines formed in gel diffusion by the rhizobial antigens probably represent particles of different molecular weight and configuration bearing the same set of antigenic sites, rather than two antigenically distinct particles. This concept is based on the observation that the antigen responsible for the major precipitation line (a_2) was readily converted to that producing the minor line (a_3) by DDS disaggregation, and the fact that this disaggregated material was able to combine with antibodies responsible for the a_2 precipitation line. The observation that a smaller fragment was also liberated, as revealed in the a_4 line, suggests that each particle size of antigen had an optimum precipitation zone at a different distance between the two wells. The methods of purification used, including column chromatography on agarose, were not able to separate the different particle sizes encountered in the present investigation. However, other workers (Beer *et al.* 1966), with a Boivin-type antigen from *Escherichia coli*, were able to separate on sucrose density-gradients a small particle responsible for two of the three diffusion lines shown by the parent preparation, and a large particle which formed the third diffusion line. The large particle could be

disaggregated with DDS to form small particles which then showed the two precipitation lines typical of the small particles in the parent mixture, while the line due to the large particle had disappeared. Beer *et al.* considered that the two precipitation lines obtained with the apparently homogeneous small particles (with some but not all antisera) were due to two different antibodies. In the present study of rhizobial antigens, with clear evidence of conversion of one form to another, and of the ability of one form to absorb the antibodies of the other, it seems reasonable to suppose that the extracted material, in all states of aggregation, carried the same antigenic determinants, but that different particle size or configuration may either have stimulated the production of several distinct antibodies or have been responsible for different zones of optimum precipitation with the same antibody.

Chemically the rhizobial antigens, particularly from strain TA 1, differed markedly from the established structure of the phenol-extracted O-somatic antigens of the Gram-negative bacteria (Lüderitz, Jann & Wheat, 1968). The most basic and significant departure from the structure proposed for any other somatic antigens which have been studied was the extremely low phosphorus content of both rhizobial preparations. In the Enterobacteriaceae (see, for example, Holme, Lindberg, Garegg & Onn, 1968), phosphate is taken as being part of the lipid fraction, and also as a link between di-heptose units in the polysaccharide core. The value usually found for LPS phosphorus is 1 to 4 % (see Table 1). In the rhizobial LPS the highest value found (0.14 % for SU 297/31) might be decreased to 0.05 % by a different method of preparation and purification, with no loss of activity in gel diffusion. It seems possible therefore that the phosphorus is a contaminant, and in any case it is unlikely that at these low concentrations it could play the major structural role assigned to it in the Enterobacteriaceae.

The other major departure from the usual pattern, and a distinction between the two rhizobial strains studied here, was the absence of heptose sugar from the antigen of TA 1. Various heptose-less mutants have been reported in *Salmonella* (e.g. Kasai & Nowotny, 1967), but the LPS from these strains, soluble in the phenol rather than the aqueous phase of extraction, is the lipid-KDO-phosphate-glucosamine core of a normal somatic antigen which has lost its O-specific polysaccharide by mutation. Volk (1966, 1968*a, b*) isolated from twenty *Xanthomonas* species an LPS which contained all the normal constituents of an O-somatic antigen, including lipid, KDO and phosphate, but in which no heptose was detected by the cysteine + H₂SO₄ reaction, or by chromatography. However, Adams, Quadling & Perry (1967), who used urea phosphate as visualizing reagent on paper chromatograms (and also by using gas-chromatography), found heptose in chromatograms of the phenol-extracted LPS of all bacteria tested, including two of the *Xanthomonas* species (but not necessarily the same strains) studied by Volk. In the rhizobial preparations studied here, heptose was clearly present in the SU 297/31 antigen but was not detected in the TA 1 antigen by any of the tests applied, including the urea phosphate procedure used by Adams *et al.* (1967).

In other respects, the antigens of the rhizobial strains resembled those extracted from Enterobacteriaceae, although firmly bound lipid, glucosamine and KDO were present in amounts less than those usually reported. The lability of the KDO bonding in the SU 297/31 antigen makes it possible that some of this compound may be involved in the type of polysaccharide-(KDO)_n-lipid linkage proposed by Osborn

(1963). The other sugar constituents common to both TA I and SU297/31, glucose, mannose and fucose, have been reported often as the constituents of cell wall LPS, but values for methylpentoses appear higher in these rhizobia and in the *Xanthomonas* species than in the strains of Enterobacteriaceae. In the rhizobial antigens it seems probable that the unknown fast-moving sugar component (possibly a di-deoxy-sugar) is contributing to the methylpentose value in analyses of the whole antigen. Uronic acids reported rarely in LPS of Enterobacteriaceae (see Lüderitz *et al.* 1968) are suspected of being of capsular polysaccharide origin with the suggestion that sometimes O- and K-antigens are bound together with unusual firmness. Volk (1968*a*) identified the uronic acid which he had reported earlier in *Xanthomonas* spp. as galacturonic acid. In our two rhizobial antigens it seems that the glucuronic acid of SU297/31, unlike that of TA I, is linked in such a way as to decrease any negative charge on the molecule.

The low phosphorus content of the rhizobial antigens places them in a different category from other O-somatic LPS antigens. Both strains studied here showed some features (low phosphorus content, presence of acidic sugars, and, in TA I, a high percentage of neutral sugar) which related them chemically to the K-antigens of capsulated *Escherichia coli* (Ørskov, Ørskov, Jann & Jann, 1963; Jann *et al.* 1965). They also differed chemically from the K-antigen capsular polysaccharides in that they contained KDO and a higher percentage of firmly bound lipid. When Jann *et al.* (1965) extracted a mixture of O-antigens and capsular polysaccharides from capsulated *E. coli* strains by the hot phenol method, they were able to centrifuge down the O-somatic antigen LPS from the aqueous phase of phenol extraction at 105,000 g, and then precipitate acidic K-polysaccharide from the supernatant fluid with cetavlon. In this study of the rhizobial antigens it was not possible to fractionate the material at 100,000 g or by cetavlon. The phenol extract of the *E. coli* capsulated strains showed a gel-diffusion line attributable to the O-somatic LPS and another (double) line from the acidic K-polysaccharide. The rhizobial preparation consisted of one antigen, albeit in two convertible molecular forms. The *E. coli* K-polysaccharides, unlike the rhizobial antigens, were not able to stimulate antibody production, even with Freund adjuvant. The high serological activity and homogeneity of the rhizobial antigens would seem to preclude the possibility that the preparations studied in this present work were mixtures of O-antigen and K-polysaccharide. Gel-immunoelectrophoresis in particular would be expected to separate a typically acidic K-polysaccharide from a typically neutral LPS.

However, the antigen of rhizobial strain TA I requires some further consideration. This LPS had a persistently gelatinous nature when prepared from whole bacteria without prior treatment with TCA. Although it was quite different in chemical constitution, solubility and antigenic activity, its physical appearance resembled extracellular gum (Humphrey & Vincent, 1959, 1965); TA I is one of the few rhizobial strains in which Dudman (1968) observed capsules. In confirming Dudman's observations on this strain, we have been able to distinguish on TA I, but not on SU297/31, capsules morphologically distinct from the extracellular gum. They were not seen in cultures less than 15 days old. We could not show any difference in agglutination or gel diffusion with such old capsulated cultures. The preparations of TA I antigens described in this paper were from 72 hr, twice washed, and in one case ultrasonically-treated, bacteria, at which age no capsules were detected. Any extracellular gum

would have been removed from the bacteria by the washings. The gelatinous nature of the antigen extracted by hot phenol from such bacteria suggests that even before the capsules are demonstrable the surface is covered with a gelatinous material which is the somatic antigen, and which accumulates as the culture ages until forming a visible layer. The surface as well as the isolated antigens of the strain TA I are probably atypical of the species. Marshall (1967), on the evidence of whole-cell electrophoretic mobility, found that its surface appeared to be charged exclusively with carboxyl ions (unlike most other *Rhizobium trifolii* strains which showed a carboxyl- and amino-charged surface) and its surface charge density was also unusually high. Our results with gel-immunoelectrophoresis of the isolated antigens of TA I and SU297/31 paralleled the relative mobility of the whole bacteria as determined by Marshall.

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