

## Specific Lipopolysaccharides of *Bordetella*

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In many species of Gram-negative bacteria one of the surface components of the bacterial cell is a material composed of polysaccharide, protein and phospholipid residues (Boivin, Mesrobianu & Mesrobianu, 1933; Morgan, 1937, 1949; Miles & Pirie, 1939*a*; Morgan & Partridge, 1940, 1941, 1942; Freeman, 1943; Davies, Morgan & Mosimann, 1954). These substances are antigenic, toxic and pyrogenic and are thermostable with respect to these properties; the dominant immunological specificity is determined by the polysaccharide moiety. They have been referred to as endotoxins or Boivin antigens or, in special cases, as O somatic antigens, but these terms, especially endotoxin, are also used with other meanings.

Although little is known either of the chemical composition or the biological properties of the endotoxins of *Bordetella pertussis* (*Haemophilus pertussis*), *B. parapertussis* and *B. bronchisepticus*, there is already ample evidence for the occurrence of biologically active polysaccharides in organisms of these species. Cruickshank & Freeman (1937) digested phase I *B. pertussis* cells with trypsin and precipitated with ethanol from the soluble digest a material containing polysaccharide. This preparation also gave positive reactions in the Millon's, ninhydrin and biuret tests. It had an immunizing potency for mice equal to that of whole cells when immunity was measured by intraperitoneal challenge. Eldering (1941, 1942) isolated lipopolysaccharides from strains of the three *Bordetella* spp. and obtained grossly similar products whether she used a modification of the trichloroacetic acid method (Boivin *et al.* 1933) or the hydrochloric acid or tryptic-digestion methods of Felton & Kauffmann (1938). The preparations were soluble in water, giving opalescent solutions; the Mølich test was positive and reducing sugars were detected with Benedict's solution after acid hydrolysis. Tests for protein were negative. The *B. bronchisepticus* lipopolysaccharide was toxic for mice and an insoluble toxic component could be obtained from it by 0.2*N*-acetic acid hydrolysis. The three substances appeared to be serologically distinct but immunization of mice with any one of them conferred substantial protection against intraperitoneal challenge with *B. bronchisepticus*. It has been suggested more recently, from the results of agglutination and agglutinin-absorption tests, that

the three *Bordetella* spp. have a common thermostable O antigen (Andersen, 1952; Eldering, Hornbeck & Baker, 1957).

The detailed chemical composition of these polysaccharide materials is not known although the substances described by Eldering (1941, 1942) are clearly of an 'endotoxin nature.' However, the presence of mannose, galactose and a 'fructose-like' substance in a polysaccharide extracted with '50% pyridine' from defatted cells of *B. pertussis* has been reported (Akiya, Takahashi, Kuriyama & Ogawa, 1951). Although this method of extraction is known to yield endotoxin when applied to certain other bacteria (Goebel, Binkley & Perlman, 1945), the absence of any reference to biological or serological properties in this instance leaves the precise nature of the material in doubt.

In the present study the chemical composition and biological properties of the lipopolysaccharide components of the *Bordetella* endotoxins are described. A preliminary report has already appeared (MacLennan, 1957). These toxic substances are distinct from the well-known thermostable toxin produced by *Bordetella* spp. (A. P. MacLennan, unpublished work).

### MATERIALS AND METHODS

*Bacteria.* The *B. bronchisepticus* strains N.C.T.C. 452, 454, 8759 and 8760, and the *B. parapertussis* strain N.C.T.C. 8250, were obtained from the National Collection of Type Cultures, Colindale, London. Of the four *B. bronchisepticus* strains, 452 and 454 were avirulent (A. P. MacLennan, unpublished work), but a third avirulent strain, 8759Av, was derived from N.C.T.C. 8759 by repeated transplanting of broth cultures. A phase IV *B. pertussis* strain, G 154E, and an acetone-dried preparation of phase I organisms were kindly provided by Dr Jean Dolby, The Lister Institute, Elstree, Herts.

*Cultivation and recovery of bacteria.* Strains of *B. bronchisepticus*, *B. parapertussis* and phase IV *B. pertussis* were cultivated in tryptic-meat broth for 40–64 hr. on a reciprocating shaker at 37°. The culture medium was dispensed in Thompson bottles (A. Gallenkamp and Co. Ltd., London, E.C. 2) in 250 ml. amounts and each bottle was inoculated with a suspension in broth of the cells from an 18 hr. confluent growth on about 15 cm.<sup>2</sup> of tryptic-meat broth-agar. Organisms were recovered by centrifuging at 20 000 g, resuspended evenly in an equal volume of water and poured into 10 vol. of acetone at –20°. Several days later the bacteria were sedimented by centrifuging, washed

three times with cold dry acetone and dried *in vacuo* over  $H_2SO_4$ . They were then either extracted at once or stored at 0–2° until required. Acetone-dried cells of the phase I *B. pertussis* strain were prepared by Dr Jean Dolby from a 2-day shake culture in Cohen & Wheeler (1945) medium.

*Preparation of materials for analysis.* Samples were thoroughly dialysed against distilled water at 0–2° and dried from the frozen state. They were further dried to constant weight *in vacuo* at 78°.

*Total nitrogen.* The Kjeldahl method was used, with the distillation apparatus of Markham (1942) and the mixed bromocresol green–methyl red indicator of Ma & Zuazaga (1942).

*Amino nitrogen and  $\alpha$ -amino acid nitrogen.* Amino N was determined by measuring the  $N_2$  liberated on treatment with  $HNO_3$  in the Van Slyke–Neill manometric apparatus (Peters & Van Slyke, 1932). The  $\alpha$ -amino acid N estimations were made by the ninhydrin– $CO_2$  method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) at pH 2.5 and with a reaction time of 10 min.

*Phosphorus.* Determinations were made on samples containing 5–30  $\mu g.$  of P by the method described by Martland & Robison (1926).

*Sugars.* The following methods were employed: hexosamine (Rondle & Morgan, 1955); aldoheptose (Dische, 1953); hexose (Dische, Shettles & Osnos, 1949). The sugars employed as standards in the estimations were D-glucosamine, D-glycero-D-galactoheptose and D-glucose.

*Nucleic acid.* The nucleic acid content of preparations was estimated in relation to a reference standard of purified ribonucleic acid by measuring the absorption at 260  $m\mu$  in a Unicam ultraviolet spectrophotometer.

*Other analyses.* Determinations of C, H, acetyl and of acid function by direct titration were made by Weiler and Strauss, Oxford.

*Chromatography.* Polysaccharide samples were hydrolysed in sealed ampoules at 100° with  $N-H_2SO_4$  or  $N-HCl$  for 16 hr. For application to paper chromatograms salt-free preparations were made from  $H_2SO_4$  hydrolysates by  $Ba(OH)_2$  neutralization followed by filtration and evaporation *in vacuo* to dryness. Acid was removed from HCl hydrolysates by repeated evaporation *in vacuo* in the presence of NaOH pellets. Whatman no. 1 paper was used with butan-1-ol–pyridine–water (6:4:3, by vol.). Papers were sprayed with ninhydrin or with Ehrlich's reagent (Partridge, 1948) to reveal hexosamine, or with *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950), which distinguishes by colour the different classes of reducing sugars.

*Ultracentrifuging.* Preparative ultracentrifuge runs were made in a Spinco model L ultracentrifuge at 2–4°. Figures quoted for the force in *g* refer to the force exerted at the centres of the tubes and are average values. Analytical ultracentrifuge runs were carried out in a Spinco model E machine.

*Serological activity.* Precipitin reactions in fluid media were performed by addition of the solution under test (0.25 ml.) to an equal volume of antiserum and mixing. The tests were read after incubation at 37° for 4 hr. and leaving at 0–2° overnight. The agar-diffusion precipitin method of Ouchterlony (1948, 1953), as described by Crumpton & Davies (1956), was also used.

*Antisera.* Groups of rabbits were injected weekly by the intravenous route with 1 ml. amounts of suspensions in

0.9% NaCl of 18 hr. cultures grown on tryptic-meat broth-agar and containing about  $10^{10}$  organisms/ml. With *B. bronchisepticus* two injections of heat-killed cells (55°/30 min.) were given first. Satisfactory antibody titres were frequently achieved after 10–12 injections of live cells and the rabbits were then bled out. An antiserum to phase I *B. pertussis* cells was provided by Dr Jean Dolby; this was prepared by the intravenous injection of rabbits with an aqueous extract of disintegrated phase I cells, detoxified with formalin. Several courses, each of ten injections, were given before the animals were bled and the sera pooled.

*Pyrogenicity.* The method used was that described by Davies (1956).

## EXPERIMENTAL AND RESULTS

### *Extraction of lipopolysaccharides*

The phenol method of Westphal, Lüderitz & Bister (1952) was chosen because it had already been applied successfully to the preparation of lipopolysaccharides from a number of bacterial spp. (Westphal *et al.* 1952; Davies, 1956, 1958; MacLennan & Rondle, 1957*a*) including *Pasteurella pestis* (Davies, 1956), in which diethylene glycol and trichloroacetic acid had proved of no value.

Preliminary experiments showed that it was unnecessary to remove protein from cells by extraction with 0.9% NaCl in order to obtain substantial yields of lipopolysaccharide by the phenol method. Acetone-dried cells were suspended in water (10 g./100 ml.), warmed to 65° and treated with an equal volume of aq. 90% (w/v) phenol soln. at 65°; the mixture was stirred for 20–45 min. at this temperature and then cooled to 0–2°. Upon centrifuging, a clear separation of water and phenol phases occurred. The phenol phase was discarded. The clear, rather viscous aqueous phase was poured into 10 vol. of acetone at –10°, left overnight at this temperature and centrifuged. The deposit was washed three times with cold acetone, dialysed exhaustively and freeze-dried. The preparations contained large amounts of nucleic acid (Table 1), which in most cases was readily removed by centrifuging an aq. 1% (w/v) soln. at 100 000 *g* for 4 hr.; the colourless transparent deposit retained very little nucleic acid (Table 2, Fig. 1). The results in Table 1 show that the average yield of lipopolysaccharide from *B. bronchisepticus* cells of strain N.C.T.C. 8760 was 6.3%, a value similar to those found for 'Smooth' O somatic antigens of *Shigella* spp. (Morgan, 1949; Goebel *et al.* 1945; Davies *et al.* 1954). Smaller yields of lipopolysaccharide were obtained from *B. pertussis* (3.4%) and *B. paraptussis* (2.7%). The substances extracted from the phase IV *B. pertussis* strain and from avirulent strains of *B. bronchisepticus* differed from those of virulent organisms and are considered separately below.

The products obtained from the virulent *B. bronchisepticus* strain N.C.T.C. 8760 (BR; a pool of preparations 2 and 3, see Table 1), from the phase I strain of *B. pertussis* (PE) and from *B. paraptussis* strain N.C.T.C. 8250 (PA), were white powders giving opalescent solutions in water. At 5 mg./ml. only BR was completely soluble in water and in 0.9% NaCl. The substances were biuret-negative and Mölisch's test-positive. Measurement of ultraviolet absorption at 260  $m\mu$  showed that less than 2% of nucleic acid was present in PE and PA (Table 2); BR appeared to be free from nucleic acid.

Table 1. Cell yields from broth growths of *Bordetella* spp. and yields of products on aqueous phenol extraction

Strain	Cell yield (g. of acetone- dried cells/l. of medium)	Wt. of acetone-dried cells extracted (g.)	Wt. of phenol extract (g.)	Nucleic acid (%)	Lipopoly- saccharide in cells* (%)
<i>Bordetella bronchisepticus</i>					
N.C.T.C. 8760	3	11	0.68	12	5.4
N.C.T.C. 8760	2	25	1.73	20	5.5
N.C.T.C. 8760	2	25	2.73	26	8.1
N.C.T.C. 454	3.6	36	0.60	62	0.6
8759 Av	3.6	36	0.99	85	0.4
<i>Bordetella pertussis</i>					
'Phase I vaccine'	—	13	0.65	32	3.4
Phase IV G 154 E	1.2	5	0.01	30	—
Phase IV G 154 E	0.7	12	0.51	45	—
<i>Bordetella parapertussis</i>					
N.C.T.C. 8250	0.6	10	0.34	20	2.7

\* Maximum values, calculated on the assumption that extracts are composed solely of lipopolysaccharide and nucleic acid.

Table 2. Composition of lipopolysaccharides obtained from *Bordetella* spp.

All values are expressed as percentages.

Lipopolysaccharide preparation	N	P	Nucleic acid	Aldoheptose	Hexosamine	Hexose
<i>B. bronchisepticus</i> (BR/A)	7.7	2.1	0	18	16.6	5
<i>B. pertussis</i> (PE)	3.9	2.5	1.4	34	20.3	6
<i>B. parapertussis</i> (PA)	8.2	2.6	0.6	18	16.5	5

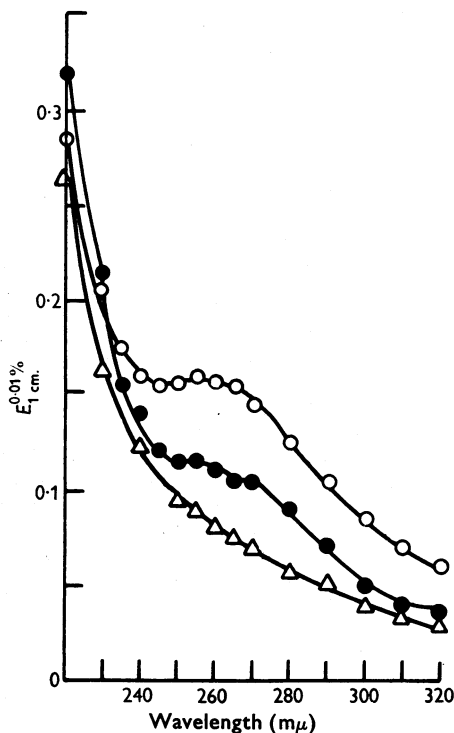


Fig. 1. Ultraviolet-absorption spectra of lipopolysaccharides.  $\Delta$ , *Bordetella bronchisepticus* (BR/A);  $\circ$ , *B. pertussis* (PE);  $\bullet$ , *B. parapertussis* (PA).

*Purification of BR.* An aq. 1% (w/v) soln. of BR (1.24 g.) at 0–2° was fractionated by the addition of acetone at –10° in 5% increments of concentration (v/v) in the presence of about 0.3% of potassium acetate. Virtually the whole of the material precipitated at 65% (v/v) acetone concentration. It was then reprecipitated twice at this level, redissolved and centrifuged from aq. 1% (w/v) soln. at 100 000 g for 4 hr. to remove traces of nucleic acid, dialysed exhaustively and freeze-dried (yield, 1.0 g.). The analytical figures of the purified material (BR/A) differed only slightly from BR; for this reason and because only small amounts of the *B. pertussis* and *B. parapertussis* preparations were available they were not fractionated in this way.

#### Examination for physical homogeneity

In the analytical ultracentrifuge a 1% (w/v) soln. of BR/A in phosphate–NaCl buffer at pH 8.0 (phosphate, 1.0; NaCl, 1.0) gave a very rapidly sedimenting sharp peak ( $S$  92 s), which was preceded by some material which accumulated on the base of the cell (Fig. 2). This behaviour is similar to that of the lipopolysaccharide obtained from *P. pestis* (Davies, 1956), where evidence was adduced from ultracentrifuge studies on aqueous solutions that the more rapidly sedimenting substance was itself composed of lipopolysaccharide which had not truly dissolved in salt solutions. With BR/A prolonged centrifuging of an aq. 1% (w/v) soln. at about 2500 g in an 'angle' centrifuge also caused a separation of a cloudy viscous lower layer from a less viscous opalescent supernatant fluid. The materials obtained by freeze-drying the two layers did not differ significantly in composition as measured by N, P and aldoheptose (see below) analyses nor

in their serological activity. It is therefore probable that the two components detected by the ultracentrifuging of buffered solutions were not chemically different but that the more rapidly sedimenting material was a more highly aggregated fraction that was not truly in solution. The particle size of both components is evidently very large and of a similar order to that of the O somatic antigen of *Shigella dysenteriae* (Davies *et al.* 1954).

The *B. pertussis* and *B. parapertussis* preparations were not examined because of their low solubility in water and in salt solutions.

#### Examination for immunological homogeneity

*Bordetella bronchisepticus*. The cells from an 18 hr. culture of strain N.C.T.C. 8760 grown on tryptic-meat broth-agar were washed once, resuspended in 0.9% NaCl (about 100 mg. dry wt./ml.) and examined by diffusion-precipitin analysis in agar. The photograph (Fig. 3) shows that a number of lines of precipitation were formed by these cells when they reacted with a homologous bacterial-cell antiserum. The strongest of these lines, that nearest the antigen reservoir, was shown on suitably designed plates to be identical with the strong line given by BR/A (1 mg.) against the same antiserum (Fig. 3). Characteristically, the line due to the lipopolysaccharide formed close to the antigen reservoir and reached maximum intensity after development for 5-7 days; unlike the lines due to other *B. bronchisepticus* antigens (MacLennan, unpublished work) it could not be detected until the third or fourth day of development. The line was formed also by cells or BR/A solutions heated at 100° for 30 min.

The formation of a single strong line of precipitation by BR/A indicates the absence of other native *B. bronchisepticus* antigens from this preparation. As further evidence for immunological homogeneity (Davies, 1956) it was necessary to demonstrate the absence of other antigenic substances from BR/A, such as might have been produced by the alteration of native antigens during the phenol

extraction of cells. An artificial antigenic complex of BR/A was therefore prepared by coupling this substance with the conjugated protein component of the O somatic antigenic complex of *S. dysenteriae* (Partridge & Morgan, 1940; Morgan, 1943; Davies, 1956). Antisera were then prepared in rabbits by alternate subcutaneous and intravenous injections of the BR/A complex (50-100 µg.) at intervals of 4 days. After a total of twelve injections the antisera obtained precipitated with a 1/128 000 dilution of BR/A and gave a line of precipitation on diffusion plates against both BR/A and strain N.C.T.C. 8760 cells (Fig. 3). On suitably designed plates these lines were found to be identical. However, it is apparent (Fig. 3) that a second weak line of precipitation was formed by the cells against an antiserum to the artificial BR/A complex and, although not clear from Fig. 3, an identical second line was formed by BR/A. Moreover, with both cells and BR/A, the continuity of each of the two lines formed against antiserum to the artificial complex with the apparently single line formed against the bacterial-cell antiserum demonstrates that this line must itself be compound. The significance of the two lines is uncertain. Both were formed by heated preparations (100°/30 min.) of strain N.C.T.C. 8760 cells and of BR/A. In addition, neither of the lines was formed against antisera prepared with BR/A alone, suggesting that the antigens responsible for both are polysaccharide in nature, requiring coupling with *S. dysenteriae* conjugated protein to become antigenic. The presence of immunologically inactive contaminants in BR/A cannot, of course, be detected by these tests.

*Bordetella pertussis* and *Bordetella parapertussis*. Each of the preparations PE and PA (1 mg.) gave a single faint diffuse line of precipitation on diffusion plates against homologous bacterial-cell antisera which linked with one of the several weak lines formed by whole cells. Like the line given by BR/A the lipopolysaccharide lines were formed also by heated cells, were slow to develop and were close to the antigen reservoir. The weakness of the reactions might be due either to the low solubility of the antigens or to a low antibody titre in the antisera.

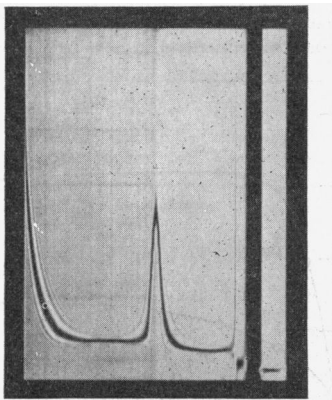


Fig. 2. Sedimentation pattern of *B. bronchisepticus* lipopolysaccharide (BR/A). Initial concentration 1.0% (w/v) at pH 8.0 (phosphate, 1.0; NaCl, 1.0). Centrifugal force 60 000 g. Migration from right to left. Exposure 8 min. after reaching full speed. Phase plate angle, 50°.

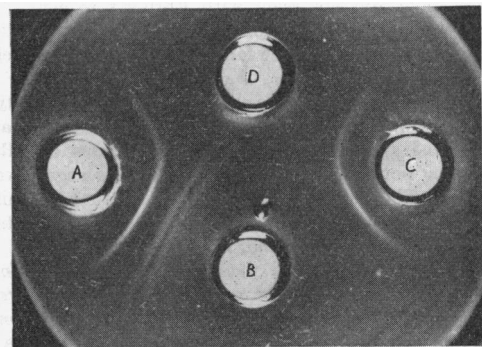


Fig. 3. Agar-diffusion precipitin pattern of *B. bronchisepticus* cells and lipopolysaccharide. Reservoir A, cells (20 mg. dry wt.) of *B. bronchisepticus* (strain N.C.T.C. 8760); reservoir B, a homologous antiserum (0.2 ml.); reservoir C, purified lipopolysaccharide (BR/A, 1 mg.); reservoir D, specific lipopolysaccharide antiserum (0.2 ml.).

### Chemical composition

Analytical figures for BR/A, PE and PA with respect to sugar and other components discussed below are recorded in Table 2. Additional analyses of BR/A gave C, 42.5; H, 6.4; O-acetyl, nil; ash, 7.2%;  $[\alpha]_D^{25} = -36^\circ$  in water (c, 1). The reducing value (Somogyi) of BR/A was 7%, calculated as glucose, after hydrolysis for 24 hr. with 0.5N-HCl. The low reducing value is in sharp contrast with the large amounts of reducing sugars detected in BR/A (Table 2) by methods involving more drastic hydrolysis, and it is probable therefore that BR/A is unusually resistant, when compared with many other bacterial lipopolysaccharides, to dilute acid hydrolysis.

**Paper chromatography.** Two major components were detected on chromatography of 24 hr. N-HCl or N-H<sub>2</sub>SO<sub>4</sub> hydrolysates of BR/A, PE and PA. One of these reacted with Ehrlich's reagent, ninhydrin and anisidine and the spot corresponded in position and colour with glucosamine. The second component occupied a position between glucose and galactose on chromatograms. When sprayed with the anisidine reagent and heated at 140° for 2-3 min. the characteristic colour reaction (purple with grey-green margin) of an aldoheptose was obtained. This heptose corresponded in both position and colour to D-glycero-L-mannoheptose, or its optical enantiomorph, but its identity could not be established by this means. An unidentified component ( $R_{\text{glucose}} 1.3$ ) giving an immediate bright yellow on spraying with anisidine and a brown with the orcinol reagent of Bevenue & Williams (1951) was sometimes noted in hydrolysates of BR/A and PA.

Hydrolysis of BR/A with 6N-HCl for 16 hr. liberated a number of substances reacting with ninhydrin and Ehrlich's reagent. With ninhydrin five spots were obtained on chromatograms. The strongest of these was due to glucosamine and a much weaker spot corresponded to galactosamine. These two spots, and a third slow-moving, ninhydrin-positive component, reacted with Ehrlich's reagent. This third substance might be an oligosaccharide-containing hexosamine, arising from incomplete hydrolysis of BR/A. Two ninhydrin-positive components, giving faint diffuse spots, did not react with Ehrlich's reagent. The virtual absence of contaminating protein from the BR/A preparation is shown by the failure to identify amino acids on these chromatograms.

A noticeable feature of the chromatographic examination was the faint colour reactions given by aldoheptose and hexosamine spots when N-H<sub>2</sub>SO<sub>4</sub> hydrolysates of BR/A containing nominally large amounts of these sugars were examined. This behaviour was quite unlike that of similar hydrolysates of other lipopolysaccharides known to contain aldoheptose and hexosamine, namely those produced by strains of *P. pestis* and *Pasteurella pseudotuberculosis* (D. A. L. Davies, personal communication), *Pasteurella septica* (MacLennan & Rondle, 1957a) and *Chromobacterium violaceum* (MacLennan & Davies, 1957), and is no doubt due to the relative resistance of the *Bordetella* preparations to dilute acid hydrolysis.

**Hexosamine.** In Fig. 4 the release of hexosamine from BR/A during N-HCl and 6N-HCl hydrolysis at 100° is shown. A much greater proportion of the total hexosamine content of BR/A remained unreleased by N-HCl hydrolysis for 24 hr. than with the lipopolysaccharide of *P. pestis*. Both preparations contain an aldoheptose, hexose and

hexosamine. It appears that there are two distinct types of hexosamine linkage in BR/A, one of which is resistant to dilute acid hydrolysis. A period of hydrolysis for 6 hr. with 6N-HCl gave maximum release of hexosamine from BR/A and the figures recorded in Table 2 for BR/A, PE and PA were obtained from 6 hr. hydrolysates.

The absorption spectrum of the pink chromophore produced by these hydrolysates in the Elson & Morgan reaction (Rondle & Morgan, 1955) was identical with that of authentic D-glucosamine in the region 500-570 m $\mu$ . A 6 hr. 6N-HCl hydrolysate was further analysed for amino sugars by chromatography on a column of Zeo-Karb 225 resin (The Permutit Co. Ltd., London), eluted with 0.33N-HCl by a modification (Rondle & Morgan, 1955) of the method of Gardell (1953). This analysis, kindly carried out by Dr M. J. Crumpton, revealed glucosamine and galactosamine in the ratio of between 4:1 and 5:1. There was no indication of any other amino sugar. The 16.6% total hexosamine of BR/A (Table 2) is therefore made up of glucosamine and galactosamine in this ratio.

**Aldoheptose.** The chromatographic evidence for the presence of aldoheptose was confirmed by the H<sub>2</sub>SO<sub>4</sub>-cysteine reaction (Dische, 1953), carried out on the three lipopolysaccharides and also on material eluted from paper chromatograms of N-HCl hydrolysates of BR/A in the region giving an aldoheptose colour with anisidine. With BR/A, PE and PA the 3 min. heating period recommended for the estimation of free heptose was insufficient to give the maximum colour reaction as measured by either absorption at 505 m $\mu$  or the difference in absorption 505-540 m $\mu$ . This heating period was sufficient, however, for almost maximum colour development with a number of other bacterial lipopolysaccharides that contain aldoheptose, and the anomalous behaviour of the *Bordetella* preparations in this regard appears to be another instance of the difficulty with which these substances are hydrolysed. Fig. 5 illustrates the increase in cysteine-reacting chromogen corresponding to aldoheptose as the heating period of BR/A with H<sub>2</sub>SO<sub>4</sub> is increased from 3 to 15 min. The aldoheptose figures of Table 2 are based on 10 min. heating periods. Since there is a decrease in the intensity of the colour produced by free authentic heptose (D-glycero-D-galactoheptose) with increase in heating period (Fig. 5), it

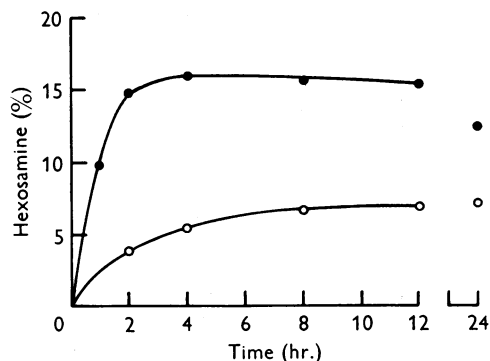


Fig. 4. Hydrolysis curves of *B. bronchiseptica* lipopolysaccharide (BR/A) showing release of amino sugar estimated as glucosamine in the Elson & Morgan reaction; ○, N-HCl; ●, 6N-HCl.

appears that the estimated heptose content of *Bordetella* preparations (10 min. heating) must represent a balance of chromogen formation and destruction. Depending on whether the rate of chromogen destruction is greater or less for the standard aldoheptose than for the lipopolysaccharides, the estimated heptose contents of the latter will

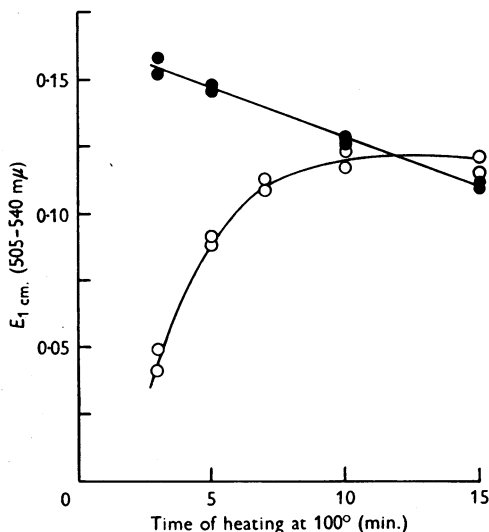


Fig. 5. Influence of time of heating on amount of aldoheptose colour (505-540 mμ) produced by *B. bronchiseptica* lipopolysaccharide (BR/A) and by free aldoheptose after treatment with H<sub>2</sub>SO<sub>4</sub>-cysteine; O, BR/A (500 μg.); ●, D-glycero-D-galactoheptose (50 μg.).

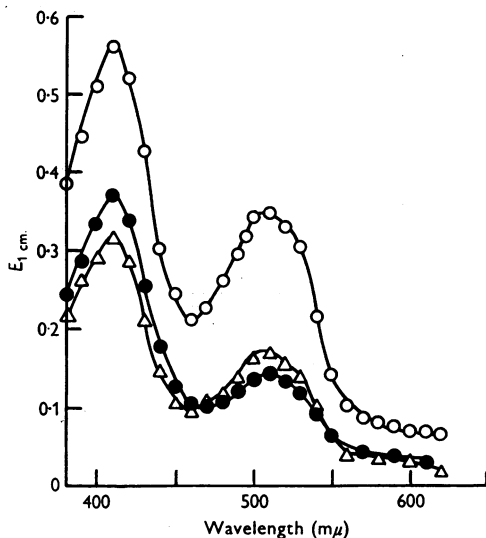


Fig. 6. Absorption spectra of lipopolysaccharides. Δ, *B. bronchiseptica* (BR/A); O, *B. pertussis* (PE); ●, *B. parapertussis* (PA) after treatment of 500 μg. amounts with H<sub>2</sub>SO<sub>4</sub>-cysteine; readings were made at 22 hr. The absorption maximum at 410 mμ is due to hexose and that at 505 mμ to aldoheptose.

be higher or lower respectively than the correct value. The accuracy of the figures recorded in Table 2, which are calculated in terms of D-glycero-L-mannoheptose, also depends on the correctness of this identification because different aldoheptoses give different colour intensities in the test (Dische, 1953; Davies, 1957a).

In addition to an aldoheptose the presence of a hexose, undetected on paper chromatograms, was revealed by the H<sub>2</sub>SO<sub>4</sub>-cysteine test. Fig. 6 shows the characteristic hexose- and heptose-absorption curves of BR/A, PE and PA with peak absorptions at 410 and 505 mμ respectively. These curves were obtained after a heating period of 3 min., because at 10 min. the hexose peak is destroyed, although, as already stated, the intensity of the heptose peak is increased. It was concluded that the hexose is not galactose from the CyR<sub>11</sub> reaction of Dische *et al.* (1949). The hexose values of Table 2 were obtained from the H<sub>2</sub>SO<sub>4</sub>-cysteine test (3 min. heating).

*Other sugars.* Titration of BR/A in boiling 50% (v/v) ethanol with phenolphthalein as indicator gave a neutral equivalent of 1150. This figure agrees with the 20% hexuronic acid detected by manometric measurement of CO<sub>2</sub> evolved by heating BR/A at the temperature of boiling toluene with 12% (w/w) HCl for 5 hr. (Tracey, 1948). On the other hand, hexuronic acid was not detected in the Tollen's naphtharesorcinol test (Hanson, Mills & Williams, 1944) nor in the carbazole test (Dische, 1947), nor on chromatograms sprayed with a bromophenol blue indicator. The nature of the substance evolving CO<sub>2</sub> on treatment with HCl therefore remains in question. The reaction was not confined to the *B. bronchiseptica* lipopolysaccharide but was given also by the degraded polysaccharide (Davies, 1956) of *P. pestis* (22% hexuronic acid), which, like BR/A, did not give a positive carbazole test. A preparation of the degraded polysaccharide of *S. dysenteriae* (Davies, Morgan & Record, 1955) reacted to a lesser extent (5% hexuronic acid). Synthetic mixtures of D-glucosamine, D-glucose and D-glycero-D-galactoheptose, in the proportions in which hexosamine, hexose and aldoheptose have been detected in BR/A, gave a negligible reaction (0.3% of hexuronic acid). Control estimations on purified mammalian hyaluronic acid and the capsular polysaccharide of *Klebsiella pneumoniae* gave values for hexuronic acid similar to those reported by other workers.

Tests for keto sugars (Dische & Borenfreund, 1951; Bevenue & Williams, 1951) and for sugar alcohols (Bradfield & Flood, 1950) were negative. Chromatograms showed no sign of 3:6-dideoxyhexoses (Fromme, Himmelpach, Lüderitz & Westphal, 1957).

*Ash.* Emission spectroscopy revealed that the high ash content of BR/A was due in large part to Ca, Mg and P (of the order of 1% of each) with some K, Zn and Pb (of the order of 0.1% of each).

#### Component with high nitrogen content

The most noteworthy feature of BR/A and PA is their high N content (Table 2), since these materials do not contain significant amounts of protein or nucleic acid (Fig. 1) and their hexosamine content is much too small to account for the total N. In contrast, PE, with a smaller N content, has a greater proportion of aldoheptose and hexosamine.

Analysis of BR/A samples hydrolysed with 6N-HCl at 100° for 16 hr. gave amino N, 3.2%, and α-amino acid N, 0.12%. In addition, free ammonia N, 3.1%, was detected

in the hydrolysates by the Conway diffusion method (Conway, 1947). The amino N figure is roughly equal to the sum of the values of hexosamine N (1.3%),  $\alpha$ -amino acid N and ammonia N (under the conditions of the test about 50% of the ammonia was estimated as amino N). Therefore, since BR/A contains 7.7% of N (Kjeldahl), an amount of N (3.2%) equal to the amount giving rise to ammonia must occur in BR/A in a form other than ammonia-forming or amino N.

The infrared-absorption spectrum of BR/A was grossly similar to those of a number of other bacterial lipopolysaccharides but differed from them in having absorption bands at 3.27, 6.05 and 6.5  $\mu$ , suggesting the presence of bonded NH groups.

#### Degradation of the lipopolysaccharide

A sample of BR/A (252 mg.) at 1% (w/v) concentration in 1% (v/v) acetic acid was heated for 4 hr. at 100° in an atmosphere of N<sub>2</sub> gas (Morgan & Partridge, 1940). A precipitate formed which was removed by centrifuging. The sediment was washed once with water and the washings were added to the acid-soluble fraction. Both the soluble and the insoluble fractions were dried from the frozen state. The insoluble material (64 mg.) constituted 25% of BR/A; successive extractions with ether and CHCl<sub>3</sub> yielded soluble fractions and an insoluble residue. Hexosamine was detected in the ether- and CHCl<sub>3</sub>-soluble lipids. The acid-soluble material (175 mg.) was a fluffy white powder which dissolved very readily in water to give a clear solution. Analysis gave C, 39.9; H, 6.1; N, 8.6; P, 2.0; aldoheptose, 24; hexosamine, 16.4%;  $[\alpha]_D^{18} = -64^\circ$  (c, 1).

To confirm the rather unexpected observation that the component with high N content was associated with the acid-soluble degraded polysaccharide, a second sample of BR/A (220 mg.) was hydrolysed with 15% (v/v) acetic acid,

all other conditions being the same as before. Similar results were obtained and the yields and compositions of the various fractions are recorded in Fig. 7.

Hexosamine was detected in both the ether-soluble lipid and the CHCl<sub>3</sub>-soluble phospholipid. Chromatographic examination of a 6N-HCl hydrolysate of the ether- and CHCl<sub>3</sub>-insoluble material (9 mg., Fig. 7) revealed the presence of glycine, alanine, leucine, valine, lysine, glutamic acid and aspartic acid, in addition to hexosamine. This insoluble substance might well account for the unidentified weakly reacting ninhydrin-positive spots produced by hydrolysed BR/A on chromatograms, and for the low but definite  $\alpha$ -amino acid N content (0.12%) of BR/A.

The acid-soluble degraded polysaccharide contained a greater proportion of both N and aldoheptose than did undegraded BR/A. Moreover, these proportions were further increased, and the absence of combined P from the polysaccharide was shown, when diffusible substances such as inorganic phosphate were removed by dialysing the preparation against distilled water for two periods of 48 hr. This procedure also showed that the component of high N content was not liberated as a freely diffusible substance by acetic acid hydrolysis of BR/A (Fig. 7). Titration of the dialysed polysaccharide with alkali after repeated drying from the frozen state over NaOH to remove acetic acid, gave a neutral equivalent of 1290. Examination in the analytical ultracentrifuge of the degraded polysaccharides produced by both 1 and 15% (v/v) acetic acid hydrolysis showed a single peak which failed to resolve completely from the meniscus after 2 hr. at 240 000 g. There was no trace of either of the components previously detected in BR/A (Fig. 2).

#### Biological properties

*Antigenicity.* As already noted, when BR/A was coupled with *S. dysenteriae* conjugated protein it was a potent antigen and precipitating antisera were readily prepared.

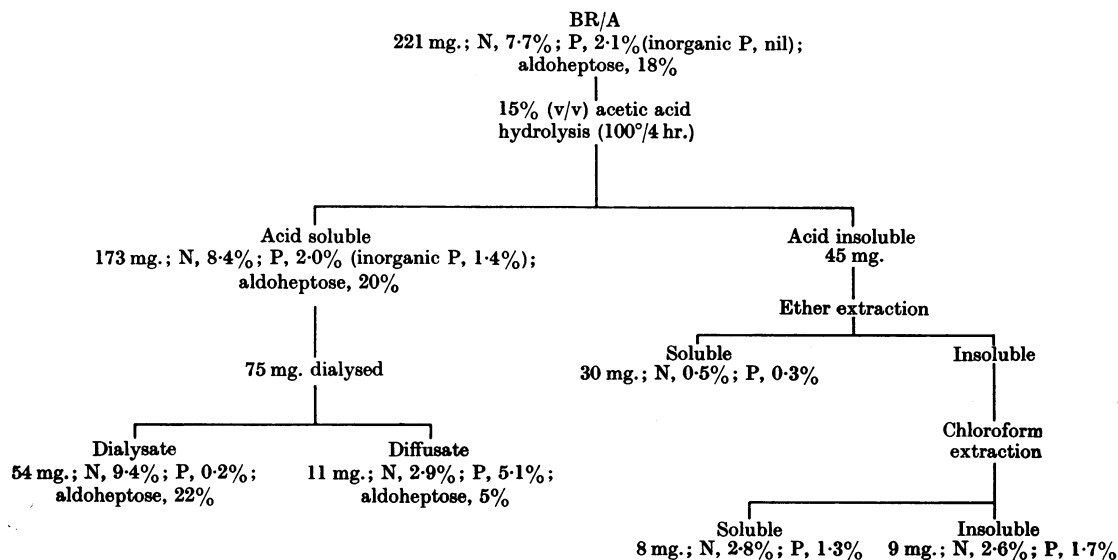


Fig. 7. Products of hydrolysis of *B. bronchisepticus* lipopolysaccharide (BR/A) by 15% (v/v) acetic acid. For further explanation see the text.

It is doubtful, however, whether uncombined BR/A is antigenic. When three rabbits were injected with BR/A, following precisely the method of immunization used with the BR/A artificial complex, precipitins could be detected in only one of the antisera obtained. Since *B. bronchisepticus* is a natural pathogen for rabbits it is possible that a subclinical infection could be responsible for the production of low-level precipitins in this one rabbit.

**Serological reactions.** BR/A precipitated at a dilution of 1/128 000 with an antiserum prepared against the artificial antigenic complex, and at dilutions between 1/128 000 and 1/1 000 000 with a number of antisera prepared against live cells of strain N.C.T.C. 8760. At equal antigen dilutions in the two tests a much more copious precipitate was formed with the bacterial-cell antisera than with the antiserum to the artificial antigen. On the other hand, antisera prepared against live cells of the avirulent strain N.C.T.C. 452 did not precipitate with dilutions of BR/A as low as 1/500 nor did they give a line of precipitation with this substance on diffusion plates. The antibody content of these antisera with respect to other *B. bronchisepticus* antigens was, however, at least equal to that of antisera to cells of strain N.C.T.C. 8760, so far as could be judged by agglutination tests and by diffusion-precipitin analysis (MacLennan, unpublished work). As with *P. pestis* (Davies, 1956), antibody to the specific lipopolysaccharide BR/A did not agglutinate cells of *B. bronchisepticus* strains.

The degraded polysaccharide prepared by acetic acid hydrolysis of BR/A did not precipitate with the antisera that precipitated high dilutions of the latter substance. The degraded polysaccharide (2.5 mg.) from the 15% (v/v) acetic acid hydrolysis was dissolved in 2.5 ml. of an antiserum to strain N.C.T.C. 8760 cells and was left at 20° for 2 hr. and then at 0–2° for 18 hr. This mixture and a sample of the untreated antiserum were then tested against dilutions of BR/A in a tube-precipitin test. A 1/250 000 dilution of BR/A, and all lower dilutions, precipitated equally well with the treated and untreated antisera. Thus the serological specificity of BR/A had evidently been lost during the mild hydrolysis.

Precipitin tests were carried out with the preparations PE, PA and BR/A, both in tubes and on diffusion plates, each material being examined against antisera to live cells of the three *Bordetella* spp. In tube tests the homologous precipitin titres of PE and PA were low, due no doubt in part to the poor solubility of these substances. Despite these low titres it nevertheless appeared from cross-precipitation tests that the three substances are serologically distinct. An antiserum to *P. pestis* cells which contained high-titre antibody to the *P. pestis* lipopolysaccharide (Davies, 1956) did not precipitate with BR/A despite the similarity in chemical composition between the two materials.

On agar-diffusion plates, PE, PA and BR/A each gave a line of precipitation with their homologous bacterial-cell antiserum which linked with one of the several lines given by whole cells. PE and PA gave much weaker homologous reactions than did BR/A. However, BR/A did not precipitate at all with antisera to *B. pertussis* and *B. paraper-tussis* cells, even when these antisera were artificially concentrated or the distance between the antiserum and antigen reservoirs on the diffusion plates was reduced. Similarly, PE and PA did not form a line of precipitation with the potent antiserum to cells of *B. bronchisepticus*.

**Toxicity.** Amounts between 10 µg. and 1 mg. of each lipopolysaccharide were injected intravenously into pairs of rabbits and intraperitoneally into groups of mice, white rats and guinea pigs. BR/A killed some animals of each species at the 1 mg. dose, but rabbits and mice alone at 100 µg. All deaths occurred within 24 hr. of injection and animals that survived 100 µg. (10 µg. in mice) or more appeared very ill during this period. PA killed only mice and rats at the 1 mg. dose. PE was not lethal.

**Dermonecrosis.** A lethal dermonecrotic toxin, which is extremely thermolabile with respect to these properties, is produced by organisms of the genus *Bordetella* (Evans & Maitland, 1937, 1939; Brueckner & Evans, 1939). Aqueous solutions of BR/A (200 µg.–1 mg.), either untreated or heated at 80°/30 min., and injected intradermally into rabbits, gave rise to a necrotic lesion which progressed to ulceration with 1 mg. doses. This lesion was similar in appearance, in time of formation and in subsequent development to that caused by smaller amounts (30 µg.) of the thermolabile toxin prepared from cells of the *B. bronchisepticus* strain N.C.T.C. 8760 by the freezing and thawing method of Evans & Maitland (1939). PE and PA were not examined.

**Pyrogenicity.** Despite the variation in toxicity the three preparations were pyrogenic and thus resembled the lipopolysaccharides and O somatic antigens of the Enterobacteriaceae (Westphal, Lüderitz, Eichenberger & Keiderling, 1952) and Pasteurellae (Davies, 1956, 1958; MacLennan & Rondle, 1957a). A marked response was obtained by the intravenous injection of rabbits with 2.5 µg./kg. body wt. (Fig. 8).

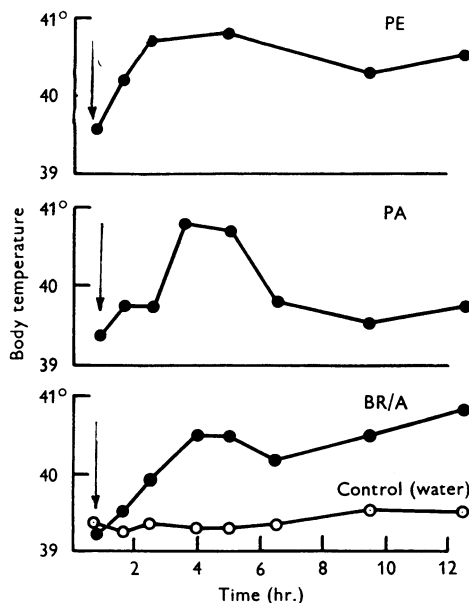


Fig. 8. Influence of intravenous injections of the lipopolysaccharides of *B. bronchisepticus* (BR/A), *B. paraper-tussis* (PA) and *B. pertussis* (PE) on the rectal temperature of rabbits. At the time indicated by the arrow the dose (2.5 µg./kg. body wt.) was injected.



*Properdin inactivation.* PE (3 mg.) was found (L. Pillemer, personal communication) to inactivate 8 units of properdin in a standard system (Pillemer, Schoenberg, Blum & Wurz, 1955). This degree of activity is similar to that of a number of other bacterial lipopolysaccharides. PA and BR/A were inactive at the 3 mg. dose.

#### *Phenol extracts of avirulent variants*

Warm 45% phenol extraction of the avirulent *B. bronchisepticus* strain N.C.T.C. 454 yielded a small amount of material (Table 1) which, like BR/A, dissolved slowly in water to give an opalescent solution. The colourless transparent pellet obtained by centrifuging an aq. 1% (w/v) soln. at 100 000 g for 4 hr. was freeze-dried to a white powder which still contained 36% of nucleic acid. This material (N 11.3%) contained an aldoheptose and a hexose and the increase in heptose chromogen with increase in the heating period of the H<sub>2</sub>SO<sub>4</sub>-cysteine test suggested that, like BR/A, it was resistant to acid hydrolysis. Biological properties were not examined. On agar-diffusion plates the material (1 mg.) did not give a line of precipitation with antiserum to strain N.C.T.C. 8760, which reacts strongly with BR/A, but a clear single line of precipitation, visible only after 4 days' plate development, was formed by untreated and by heated (80°/30 min.) preparations with an antiserum to the avirulent strain N.C.T.C. 452. This antiserum, as has been noted above, does not precipitate BR/A in tube-precipitin tests nor does it form a line of precipitation with BR/A on agar-diffusion plates (A. P. MacLennan, unpublished work).

The *B. bronchisepticus* strain N.C.T.C. 8759 is virulent and possesses a lipopolysaccharide of identical serological specificity (A. P. MacLennan, unpublished work) to BR/A. From this strain the avirulent variant 8759 Av was derived. Aqueous phenol extraction of this variant gave a preparation containing 85% of nucleic acid (Table 1), which could not be removed by ultracentrifuging. Material resembling either BR/A or that obtained from strain N.C.T.C. 454 could not be detected by chemical or serological tests.

From the phase IV strain G154E of *B. pertussis* a substance was extracted which gave a clear viscous solution in water and was composed of a polyglucose and nucleic acid (Table 1) which could not be separated by ultracentrifuging. The acetone-dried cell preparation from which this material was extracted differed from all others examined in that the cells were extensively lysed. Material resembling PE in chemical composition was not detected.

#### DISCUSSION

The observations reported here confirm and extend those of Eldering (1941, 1942) in showing that close resemblances exist between the physical, chemical and biological properties of the lipopolysaccharides of *Bordetella* spp. obtained by phenol extraction and those of the well-characterized endotoxins of several other Gram-negative bacteria (Morgan, 1949; Van Heyningen, 1950; Westphal & Lüderitz, 1954). These latter endotoxins when extracted from bacteria by suitably gentle means (Morgan, 1937; Miles & Pirie, 1939a) consist of polymole-

cular aggregates of phospholipid, protein and polysaccharide. Warm phenol extraction (Westphal *et al.* 1952) leads to loss of the protein component so that the absence of combined protein from the *Bordetella* lipopolysaccharides does not imply that protein is absent from the native antigens. The amounts of lipopolysaccharide present in the virulent strains of *B. bronchisepticus* and *B. pertussis* and in the *B. paraptussis* strain are comparable with those reported for virulent strains of many other Gram-negative bacteria.

The significance of the results obtained from an examination of the *B. bronchisepticus* lipopolysaccharide for physical and immunological homogeneity is uncertain. The material is apparently polydisperse but the two components detected in the analytical ultracentrifuge are unlikely to be distinct chemical and immunological entities since it was possible by centrifuging an aqueous solution of the lipopolysaccharide to separate two components which could not be distinguished by chemical or serological tests. Moreover, acetic acid hydrolysis led to loss of both components, as shown by ultracentrifuge studies; the degraded polysaccharide gave a single broad peak which failed to resolve completely from the meniscus. Both Goebel & Jesaitis (1952) and Davies (1956) have reported the separation of chemically and serologically homogeneous endotoxins into fractions of widely different particle size.

Serological analysis of the *B. bronchisepticus* lipopolysaccharide, more particularly diffusion-precipitin analysis in agar, gave evidence for inhomogeneity, but it is nevertheless clear that if two components were present in the preparation then they were substances of very similar properties. The lipopolysaccharides of *P. pestis* and *P. pseudotuberculosis* (Davies, 1956, 1958) and of *P. septica* (MacLennan & Rondle, 1957a) gave rise to only one line with homologous antisera under the conditions used for the examination of *B. bronchisepticus*.

As with many other endotoxins which have been investigated, acetic acid hydrolysis of *B. bronchisepticus* lipopolysaccharide liberates a soluble degraded polysaccharide and inorganic phosphate, and also an acid-insoluble material which can be separated into three fractions by successive extraction with ether and chloroform. The ether-soluble material contains little phosphorus whereas that soluble in chloroform but not in ether appears to be phospholipid. The solvent-insoluble residue contains a number of amino acids and probably accounts for the  $\alpha$ -amino acid nitrogen detected in the lipopolysaccharide. The possibility that these amino acids arise from contamination of the preparation with traces of combined conjugated protein similar to that found in other endotoxins receives some support from the apparent absence of

sulphur-containing amino acids (Morgan & Partidge, 1940; Goebel *et al.* 1945). The total amount of lipid in the lipopolysaccharide is about 25%, a figure that falls within the rather broad range of lipid contents of O somatic antigens quoted by Landy, Trapani & Clark (1955). The lipid content of the *B. parapertussis* endotoxin is probably similar to that of *B. bronchisepticus* because of the quantitative similarity of these substances with respect to other components, particularly phosphorus. The lipid content of the *B. pertussis* preparation is more in doubt because of the differences in other respects (Table 2) between this lipopolysaccharide and those of the other organisms, although again the phosphorus content suggests a similar value.

Aldoheptoses have been detected, and in some cases identified, as components of the specific polysaccharides of a large number of Gram-negative bacteria (references quoted by Davies, 1957*b*). The aldoheptose of the *Bordetella* spp. may be D-glycero-L-mannoheptose, or its optical enantiomorph, but the chromatographic evidence presented here does not permit a firm identification.

The extremely high nitrogen content of *B. bronchisepticus* lipopolysaccharide (7.7%) and its degraded polysaccharide (9.6%) is exceptional among endotoxins so far isolated, since it is not due to contaminating protein or nucleic acid as both chemical and physical examination has shown. The *B. parapertussis* lipopolysaccharide (nitrogen, 8.2%) almost certainly contains a similar high nitrogen component. Miles & Pirie (1939*a, b*) isolated a formylated polyhydroxyamino compound from the somatic antigen of *Brucella melitensis*, which contained 7–8% of nitrogen, but, unlike the *B. bronchisepticus* endotoxin, 90% of this nitrogen was present in amino groups. The amino nitrogen content of *B. bronchisepticus* lipopolysaccharide can be accounted for in terms of the measured hexosamine and  $\alpha$ -amino acid nitrogen plus the contribution made by ammonia present as ammonium salts in acid hydrolysates. The ammonia nitrogen itself accounts for 40% of the total Kjeldahl nitrogen and a roughly equal quantity appears to be present in a form other than ammonia or amino nitrogen. The formation of ammonia on acid hydrolysis of the lipopolysaccharide suggests an amide type of compound which would agree with the bonded NH absorption bands noted in the infrared spectrum.

In biological properties the *Bordetella* lipopolysaccharides are akin to other bacterial endotoxins. All three substances are pyrogenic, but whereas that of *B. bronchisepticus* is strongly toxic, *B. parapertussis* endotoxin is less so and the *B. pertussis* material was not toxic at the highest level tested. Pyrogenic but relatively non-toxic lipopolysaccharides have been isolated from organisms

of the genus *Pasteurella* by Davies (1956) and by MacLennan & Rondle (1957*a*). The *B. bronchisepticus* endotoxin causes dermonecrosis when injected intradermally into rabbits, as noted previously by Eldering (1941). Dermonecrotic activity is possessed also by the thermolabile toxin of *Bordetella* spp. but there is no good reason at present for supposing that the two toxins are materially related; apart from the great differences in their properties the thermolabile toxin occurs in certain variants which do not produce the lipopolysaccharide (A. P. MacLennan, unpublished work).

The *B. bronchisepticus* lipopolysaccharide is a potent antigen when coupled with the conjugated protein of *S. dysenteriae*, although it is doubtful whether the uncombined material is antigenic. The poor solubility of the *B. pertussis* and *B. parapertussis* preparations makes the interpretation of the serological work uncertain, but it appears from the results of diffusion-precipitin analysis that the three lipopolysaccharides are serologically distinct since homologous but not heterologous reactions were observed. Most significantly, the soluble *B. bronchisepticus* lipopolysaccharide did not give a line of precipitation with an antiserum to *B. pertussis* cells as did the less soluble *B. pertussis* preparation. Tube-precipitin tests did not give clear-cut results but nevertheless supported the conclusions drawn from agar-diffusion precipitin analysis. These conclusions appear to be opposed to those of Andersen (1952) and of Eldering *et al.* (1957), who have inferred from agglutination and agglutinin-absorption tests the presence of a common O somatic antigen in the three *Bordetella* spp.

The specific polysaccharide isolated from strain N.C.T.C. 454, an avirulent strain of *B. bronchisepticus*, is thought to be analogous to the 'Rough' lipopolysaccharides of *P. pseudotuberculosis* (Davies, 1958) and *P. septica* (MacLennan & Rondle, 1957*a, b*). It differs sharply in serological specificity from the lipopolysaccharide of the virulent organism and is present in the cell in very much smaller amount. The avirulent *B. bronchisepticus* strain 8759 Av did not yield either 'Smooth' (strain N.C.T.C. 8760) or 'Rough' (strain N.C.T.C. 454) lipopolysaccharides by aqueous phenol extraction and, in the light of Kauffmann's (1956) observations with *Salmonella* spp., may represent a transient stage in the passage from 'Smooth' to 'Rough'.

#### SUMMARY

1. Specific lipopolysaccharides were extracted with warm aqueous phenol (45%, w/v) from acetone-dried cells of strains of *Bordetella pertussis* (*Haemophilus pertussis*), *B. parapertussis* and *B. bronchisepticus* and purified by differential ultracentrifuging.

2. The preparations contained an aldoheptose, a hexose and hexosamine, and were unusually resistant to acid hydrolysis. The *B. bronchisepticus* preparation contained a small amount of galactosamine.

3. The high nitrogen content (7.7%) of the lipopolysaccharide of *B. bronchisepticus*, and probably also that of *B. parapertussis*, could not be accounted for in terms of amino nitrogen or of nucleic acid nitrogen. Acid hydrolysis liberated ammonia, which accounted for about half of that nitrogen present in a form other than amino nitrogen.

4. Evidence bearing on the presence of sugar acid in *B. bronchisepticus* lipopolysaccharide was contradictory but the amount of carbon dioxide liberated by hot mineral acid would correspond to about 20% of hexuronic acid.

5. The *B. bronchisepticus* lipopolysaccharide sedimented as two components in the ultracentrifuge, each of very large particle size. These components could not be distinguished chemically or serologically.

6. The lipopolysaccharides of the *Bordetella* spp. were active in serological tests with homologous bacterial-cell antisera and appeared to differ among themselves in serological specificity.

7. *B. bronchisepticus* lipopolysaccharide was antigenic in rabbits when combined with the conjugated-protein component of the O somatic antigen of *Shigella dysenteriae*. Antisera prepared against the complex formed two lines of precipitation with the uncombined lipopolysaccharide when examined by diffusion-precipitin analysis in agar.

8. The lipopolysaccharides were pyrogenic. The *B. pertussis* preparation differed from the other two in being non-lethal for laboratory animals and in having a higher properdin activity.

9. Acetic acid hydrolysis of *B. bronchisepticus* lipopolysaccharide liberated a water-soluble degraded polysaccharide, inorganic phosphate and an insoluble material which could be separated into three fractions by successive extractions with ether and with chloroform.

10. The component with high nitrogen content was concentrated in the water-soluble degraded polysaccharide, which did not precipitate with homologous bacterial-cell antisera nor did it inhibit the precipitation of the intact lipopolysaccharide with these antisera.

11. The chemical and biological properties of the *Bordetella* lipopolysaccharides are similar to those of the well-characterized endotoxins of certain other Gram-negative bacteria.

12. Avirulent strains of *B. bronchisepticus* and *B. pertussis* did not yield the lipopolysaccharides characteristic of virulent strains but a small amount of material was extracted with phenol from

one avirulent *B. bronchisepticus* strain; this was similar in chemical composition to the lipopolysaccharide of the virulent strain but had a different serological specificity.

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## Pyruvate Utilization in Castor-Bean Endosperm and Other Tissues

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Although pyruvate occupies a pivotal position in metabolism, its utilization by intact higher plant tissues has not been intensively studied. There is, however, ample evidence for its production by soluble glycolytic systems, for its oxidation by mitochondria and for its participation in other reactions such as transamination, reduction and decarboxylation by isolated enzyme systems. The fact that it is usually found in only small amounts in plant tissues attests to the activity of the various consuming reactions *in vivo*; if for any reason the internal pyruvate concentration increases to a level above that which can be accommodated by the oxidation systems, ethanol and carbon dioxide or, occasionally, lactate accumulate. It is for this reason that when substrate levels of pyruvate are

supplied to plant tissues the usual response is a large increase in the respiratory quotient, although stimulations in uptake of oxygen have been observed (e.g. Laties, 1949*a, b*).

The availability of labelled pyruvate has made possible experiments in which its metabolism can be followed under conditions where gross gas exchanges are unaffected, and specifically where internal levels are not increased to the point where ethanol is formed.

Pyruvate samples specifically labelled with  $^{14}\text{C}$  in individual carbon atoms were used in the present experiments. The fates of the carbon atoms were determined by measuring the respiratory release of  $^{14}\text{CO}_2$  and analysing samples of tissue at suitable intervals. In most of the experiments virtually all of the added pyruvate was accounted for as carbon dioxide, organic acids, amino acids or carbohydrates.

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