IMMUNOCHEMICAL INVESTIGATIONS ON LIPOPOLYSACCHARIDES AND ACIDIC POLYSACCHARIDES FROM SERUM-SENSITIVE AND SERUM-RESISTANT STRAINS OF *ESCHERICHIA COLI* ISOLATED FROM URINARY-TRACT INFECTIONS

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It is well established that normal human serum possesses bactericidal activity against a wide range of gram-negative bacteria. Although some aspects of killing by serum are not well understood, bactericidal antibody and at least some of the components of complement are known to interact with structures in the outer membrane of the bacterial cell (Inoue, 1972). The killing of both smooth and rough strains usually requires antibody directed against the polysaccharide side chain (O) or core (R) antigenic determinants of lipopolysaccharide (LPS; Muschel, 1960; Chedid *et al.*, 1968); activated complement components appear to be fixed by the lipid A moiety of LPS (Mergenhagen *et al.*, 1968; Galanos *et al.*, 1971; for a review of LPS structure see Lüderitz *et al.*, 1971).

The LPS in serum-sensitive strains is assumed to be freely available to interact with antibody and complement. Studies of resistance to serum have therefore been concerned with cell-surface structures that might impede the attachment or subsequent activity of the serum factors. Mutations leading to reduction or loss of O side-chains of LPS result in a marked increase in serum sensitivity (Nelson and Roantree, 1967; Dlabač, 1968; Muschel and Larsen, 1970); it has therefore been suggested that long or numerous O side-chains interfere with the functioning of complement and are thus responsible for resistance to serum. Furthermore, phenotypic modification from serum resistance to serum sensitivity is accompanied by a striking reduction in the ratio of O side-chain to core sugars (Feingold, 1969). In these studies the structural changes were extensive since the serum-sensitive cells showed rough characteristics. Many naturally occurring serum-sensitive strains are completely smooth by cultural, morphological and serological criteria and are indistinguishable in these respects from serum-resistant organisms.

Because larger amounts of LPS can be extracted from smooth serum-resistant strains than from rough serum-sensitive strains, some workers have suggested that serum resistance is due to large amounts of LPS in the envelope (Wardlaw, 1963; Reynolds and Pruul, 1971).

It was established by Muschel (1960) that the serum resistance of *Escherichia coli* is partially correlated with inagglutinability in O antisera. Glynn and Howard (1970) investigated the possibility that polymers known to play a role in O inagglutinability might also be responsible for the resistance of *E. coli* to antibody and complement. They found that crude homogenates of serum-resistant strains were in general more able to inhibit non-specifically

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TABLE I
Smooth strains of *Escherichia coli* used in this study

Serotype	Serial numbers of strains giving the indicated response to serum		
	Sensitive (Grades 1, 2)	Intermediate (Grades 3, 4)	Resistant (Grades 5, 6)
O2	LP1173 (a)* LP2362 (b)	LP1624 (a) ...	LP2017 (b) ...
O6	LP944 (c) LP1894 (d) LP2707 (e) LP856a (f) ...	LP898 LP1174 LP1339 LP2155 LP2671	LP1107 (c) LP1123 (f) LP2214 (d) LP2706 (e) ...
O7	LP903 LP1674 (g)		LP1170 (g) ...
O9	LP609 LP729 LP1977 (h)		LP1255 (h)
O18	LP2212 (i) LP2506 (j)		LP1301 (i) LP1395 (j)

* Pairing of strains for statistical analysis is indicated by the letters in parentheses.

the agglutination of red cells by anti-red-cell serum than homogenates of serum-sensitive strains. This inhibition was interpreted as being due to K antigens in the preparations. Moreover, purified K antigen from a serum-resistant strain had greater inhibitory activity than equal amounts of K antigen from a serum-sensitive strain.

A series of immunochemical and genetic investigations were recently undertaken in this laboratory in an attempt to define the basis of serum resistance of *E. coli*. In this study urinary strains of similar O serotype but differing in serum sensitivity were examined to determine whether current theories could explain the behaviour of these strains in normal human serum.

MATERIALS AND METHODS

Strains. Twenty-eight culturally smooth strains of *E. coli*, isolated from confirmed cases of urinary-tract infection and belonging to serogroups O2, O6, O7, O9, and O18, were selected for investigation. The sensitivity of the strains to normal human serum as determined by the technique of Taylor, Roberts and Gower (1972) is shown in table I. The results are categorised into six grades, grade 1 being the most serum sensitive and grade 6 the most serum resistant. For convenience and comparison, strains in grades 1 and 2 were considered to be serum sensitive and strains in grades 5 and 6 to be serum resistant; grades 3 and 4 were taken as intermediate (Taylor, 1974).

E. coli strains F470, F576 and F653, obtained from Dr G. Schmidt, Max-Planck-Institut für Immunbiologie, Freiburg, Germany, are rough strains with complete *E. coli* R1, R2 and R3 LPS cores respectively (Schmidt, Jann and Jann 1969; Schmidt, Fromme and Mayer, 1970).

Sensitivity to bacteriophages. Acidic polysaccharides and LPS O side-chains are known to interfere with the attachment of rough-specific phages. The phage sensitivity of the bacterial strains was therefore determined with six phages (nos. F0, Br2, Br10, Fp1, 6SR and C21) which are actively lytic against certain R forms of *E. coli* (Schmidt *et al.*, 1969). Phages were propagated on suitable host strains and bacterial sensitivity was determined by applying drops of phage containing approximately 10^8 p.f.u. per ml on to surface-inoculated nutrient-agar plates. The phages and their host strains were supplied by Dr G. Schmidt.

Preparation of LPS and acidic polysaccharides. The bacteria were grown in 4×700 -ml amounts of Nutrient Broth (Oxoid no. 2) in 2.5-litre conical flasks. The cultures were incubated in an orbital incubator (120 orbits per min.) at 37°C for 16 h, harvested by centrifugation (0°C), washed twice with 0.4% NaCl and dried to constant weight over P_2O_5 .

Two batches of each strain were prepared. One was extracted with 45% phenol at 68°C (Westphal and Jann, 1965) and the other with 0.9% NaCl at 60°C as described by Jann *et al.* (1970). Extracts obtained by both methods were subjected to fractional cetrimide precipitation according to procedure III of Westphal and Jann (1965). Fractions representing acidic polysaccharides and LPS were freeze dried and weighed. The yield was expressed as the weight of antigen (mg) per g dry weight of bacterial cells. Phenol-soluble LPS has been found by Hickman and Ashwell (1966) and by Raff and Wheat (1968). Therefore, all phenol layers from phenol-water extractions were examined for LPS.

Hydrolysis of LPS. Optimal conditions for release of neutral sugars were determined by subjecting LPS from an *E. coli* O7 strain (no. LP903) to hydrolysis in 0.1N HCl, 0.5N H_2SO_4 , and 1N H_2SO_4 for varying periods at 100°C . The highest values were obtained after hydrolysis for 48 h in 0.1N HCl; lower values for L-glycero-D-mannoheptose and rhamnose were found when other hydrolysis conditions were applied. The same optimum was established by Schmidt *et al.* (1970) for the release of neutral sugars from *Salmonella greenseide* R LPS.

Determination of neutral sugars of LPS. LPS (2 mg) was hydrolysed (100°C , 48 h) in sealed tubes containing 0.1N HCl (2 ml); 0.1 ml of 0.005M xylose was then added as an internal standard (Schmidt *et al.*, 1970) and the hydrolysate neutralised with Amberlite IRA 410 (HCO_3^-). The neutral solution was treated with *E. coli* alkaline phosphatase (Sigma Chemical Co. Ltd, St Louis, Mo, USA) to remove heptose-linked phosphate residues (Hämmerling *et al.*, 1971) and then reduced with sodium borohydride (5 mg) overnight at room temperature. Excess borohydride was destroyed with Dowex 50W-X8 (H^+), the hydrolysate dried and boric acid removed by three reduced-pressure distillations with methanol (Holme *et al.*, 1968). The syrups were dried and then heated at 100°C for 10 min. in 1 ml of acetic anhydride + pyridine (1 : 1); excess acetic anhydride was destroyed by adding water and the solutions were dried over P_2O_5 . The resulting alditol acetates were dissolved in 0.2 ml of chloroform and analysed by gas-liquid chromatography (Sawardeker, Sloneker and Jeanes, 1965). Samples (1 μ litre) were injected into a Pye Unicam Series 104 chromatograph fitted with 2.7×0.6 cm glass columns containing 3% ECNSS-M on 100/120 mesh Gas-Chrom Q (Applied Science Laboratories Inc., State College, Penn., USA). Separations were obtained isothermally at 180°C by the use of a nitrogen-carrier gas-flow rate of 45 ml per min.; the injection port temperature was 250°C . Peaks were identified by comparison with authentic alditol acetates prepared from the corresponding acid-treated (0.1N HCl, 48 h, 100°C) neutral sugars.

Pentoses, methylpentoses and hexoses were obtained from BDH Chemicals Ltd, Poole, Dorset; persitol, the reduction product of L-glycero-D-mannoheptose, was provided by Dr E. Zissis, National Institutes of Health, Bethesda, Md., USA. D-glycero-D-mannoheptose was a gift from Dr M. Perry, National Research Council of Canada, Ottawa. Under the chromatographic conditions used, alditol acetates of all neutral sugars known to be present in the *E. coli* LPS considered in this study were readily separated.

Other estimations. Hexosamine was determined according to Strominger, Park and Thompson (1959) with glucosamine as standard; lipid A was removed after hydrolysis in 1% acetic acid at 100°C for 90 min.

3-deoxy-D-manno-octulosonic acid (KDO) was estimated by a modification of the thio-

barbiturate method described by Ellwood (1970); synthetic KDO ammonium salt, prepared by the condensation of D-arabinose and oxalacetic acid (Ghalambor, Levine and Heath, 1966), was used as standard.

Hexuronic acids were determined according to Dische (1947); neuraminic acid was estimated with the modified Ehrlich procedure (Barry, Abbott, and Tsai, 1962). The method of Lowry *et al.*, (1951) was used for protein estimation.

Chromatography of degraded polysaccharides. The method of Müller-Seitz, Jann and Jann (1968) was followed. LPS (50 mg) from one strain (no. LP729) was degraded in 1% acetic acid at 100°C for 90 min. After removal of lipid A, degraded polysaccharides were fractionated on Sephadex G50 (Pharmacia AB, Uppsala, Sweden) with pyridine acetate as eluant (Schmidt *et al.*, 1969). The sugar content of 6-ml fractions was determined by the phenol-H₂SO₄ technique (Dubois *et al.*, 1956).

Passive haemagglutination (HA). O side-chains are known to interfere with the attachment of anti-R antibodies to the LPS core in HA and haemagglutination-inhibition tests (Schmidt *et al.*, 1969, 1970). LPS from serum-sensitive and serum-resistant strains were therefore examined for differences in O side-chain content which might be correlated with serum-sensitivity.

E. coli R antisera were prepared in rabbits according to Schlecht and Westphal (1967). LPS (1 mg) was treated with 0.02N NaOH for 18 h at 37°C and, after neutralisation, was added to 6 ml of 0.5% sheep RBC. After incubation for 1 h at 37°C the cells were washed and resuspended in 0.9% NaCl. Serial two-fold dilutions of antiserum (0.1 ml) were made in WHO trays with 0.9% NaCl as diluent and 0.1 ml of sensitised sheep RBC were added. The trays were incubated at 37°C for 1 h and haemagglutination titres (highest dilution showing complete agglutination) recorded after 1 h at room temperature.

Haemagglutination inhibition (HAI). Serial two-fold dilutions (0.1 ml) of LPS (250 µg per ml) were made in WHO trays. R antiserum (4 haemagglutination units; 0.1 ml) was added to each well and the trays were incubated for 1 h at 37°C; 0.2-ml amounts of sensitised sheep RBC (0.5%) were then added. After incubation at 37°C for 1 h the lowest inhibitor concentration causing total inhibition of haemagglutination was recorded.

Inhibition of red-cell agglutination (Glynn and Howard, 1970). Serial two-fold dilutions (0.5 ml) of acidic polysaccharide (1 mg per ml) were made in phosphate-buffered saline (PBS). Equal amounts of 1% sheep RBC in PBS were then added. After incubation at 37°C for 30 min., the cells were washed three times in PBS. Serial two-fold dilutions (0.1 ml) of rabbit anti-sheep red-cell serum (Wellcome Reagents Ltd, Beckenham, Kent) were prepared in the horizontal rows of a WHO tray with PBS as diluent. The sheep RBC sensitised with various amounts of inhibitor substance were then added in vertical rows, thus forming a chequerboard titration. The plates were incubated at 37°C for 1 h and then kept at 4°C overnight. The HAI activity was expressed as the amount of inhibitor necessary to double the minimal agglutinating dose of the anti-red-cell serum.

Statistical analyses. When results from all serum-resistant and serum-sensitive strains were compared, regardless of O specificity, the significance of the difference of the means was assessed by means of Student's *t* test.

As serum sensitivity may be related to the O serotype (Taylor, 1974), in some cases comparisons were made between randomly-paired strains of the same O serotype. The pairing is indicated in table 1 by the letters in parentheses following the strain number. In these cases the significance of the mean difference was assessed using Student's *t* test except for the comparison of titres, when the Wilcoxon signed rank (T) test was used (Wilcoxon, 1945).

RESULTS

Bacteriophage sensitivity of the E. coli strains

The phages designated F0, Br2, Br10, Fp1, 6SR and C21 interact with receptors in the core of the bacterial LPS; absorption of these phages may

TABLE II
Phage-sensitivity patterns of smooth and rough strains of E. coli

Strain no.	Serotype	Reaction obtained* with R-specific phage no.					
		FO	Br2	Br10	Fp1	6SR	C21
LP2362	O2 : K? : H6	—	—	—	+	—	—
LP1894	O6 : K? : H1	—	—	—	(+)	—	—
LP609	O9	—	—	—	+	—	+
LP729	O9	(+)	—	—	—	—	—
F470	R1	—	—	+	+	+	+
F576	R2	+	+	+	+	+	—
F653	R3	—	+	+	+	+	—

* = Complete lysis; (+) = partial lysis; — = no lysis.

be blocked by the presence of O side-chains or acidic polysaccharide (Rapin and Kalckar, 1971).

The 15 serum-resistant or serum-intermediate strains were resistant to all six phages, and although four of the 13 serum-sensitive strains were sensitive to at least one phage (table II) there was no clear relationship between serum sensitivity and sensitivity to these R-specific phages.

Strains LP2362 and LP1894 were sensitive to phage Fp1, which has its receptor deep in the LPS core (Rapin and Kalckar, 1971). Strain LP609 was lysed by phages Fp1 and C21, indicating that this strain probably possesses a R1 core; strain LP729 was sensitive to phage FO and is thus likely to have a R2 core. No smooth strains were sensitive to phages 6SR and Br2; it has been found that even very small amounts of O side chain material render strains resistant to these phages (Rapin and Kalckar, 1971).

Effect of growth conditions on the serum sensitivity of the E. coli strains

The response to serum of the strains selected for this study was determined on small batch cultures in the early log phase of growth. For the preparation of antigens, bacteria were cultivated in relatively large volumes and harvested just before the stationary phase. Cells harvested at different times during the growth cycle may vary in their sensitivity to serum (Rowley and Wardlaw, 1958), so the response to serum of the strains at the time of harvesting was estimated. After the cells were washed in 0.4% NaCl, a standardised inoculum in Tris-HCl, pH 8.4, was added to serum and sensitivity determined in the usual way (Taylor *et al.*, 1972). Most of the results obtained with this modified bactericidal test agreed with those obtained with the standard bactericidal test. All three *E. coli* O7 strains, however, showed a marked change in response to serum. The two strains previously classed as sensitive were fully resistant, whereas the one resistant strain was clearly susceptible. Slightly modified

responses to serum were also observed with three of the intermediate O6 strains.

Studies on the lipopolysaccharide (LPS)

Yield. There was no significant difference in the mean yield of LPS from serum-sensitive and serum-resistant strains (table III) as determined by either the standard test or the modified test, whether extracted by phenol-water (means 54.30–59.05 mg per g; $t < 0.800$; $P > 0.4$) or by saline (means 46.55–50.59 mg per g; $t < 0.740$; $P > 0.4$). Small amounts of LPS were extracted from strain LP1624. No phenol-soluble LPS was isolated from any of the strains.

Passive haemagglutination. LPS preparations from the *E. coli* strains were examined by HA with R antisera prepared against the *E. coli* R1, R2 and R3 reference strains. These tests, in addition to assessing the extent to which O side-chains interfere with the attachment of antibodies to the LPS core, identify the LPS core type.

Titres of 5120 were obtained against the homologous R LPS with all three antisera. As previously noted by Schmidt *et al.* (1969, 1970), R2 and R3 LPS cross-react. LPS from 26 smooth strains reacted strongly with anti-R1 serum and are likely to have R1 cores; LPS from the remaining two strains, LP729 and LP1977, reacted with anti-R2 serum and therefore probably possess the R2 core-structure. No preparations reacted to any extent in anti-R3 serum. Some slight reactivity of S LPS in heterologous R antisera was found. Only three preparations reacted to the full titre of the appropriate anti-R serum; these were all from serum-sensitive strains. The lowest titre, 320, was found with LPS from three serum-resistant strains. However, 41 of the 56 preparations gave titres of 1280 or 2560. There appears, therefore, to be little difference in the extent to which LPS from serum-sensitive, -intermediate and -resistant strains react with anti-R sera in passive haemagglutination tests.

Haemagglutination inhibition. The ability of LPS from the *E. coli* strains to inhibit the R1, R2 and R3 haemagglutination systems was determined. The R1, R2 and R3 systems were completely inhibited by the lowest doses of homologous R LPS used (0.5 μ g per ml). LPS from strain F576 inhibited the R1 and R2 systems, although it did not react with anti-R1 serum in HA tests; this is because for HA, LPS is pre-treated with alkali whereas it is not when used as an inhibitor of HA. Thus, the groups that render F576 LPS reactive in anti-R1 sera are removed by alkali treatment (Schmidt *et al.*, 1969).

The identification of the LPS core types by HA was confirmed by HAI. LPS with the R2 core did not inhibit the R1 haemagglutination system. LPS preparations varied markedly in their ability to inhibit the various R systems. Preparations from two strains, LP1173 and LP2362, were as effective as the homologous R LPS and there was a tendency for LPS from serum-sensitive strains to be more effective inhibitors than those from serum-resistant strains; the difference, however, was not statistically significant (phenol-water extracts: $T = 16$; $P > 0.05$; saline extracts: $T = 14$; $P > 0.05$).

TABLE III

Chemical analysis of lipopolysaccharides (LPS) extracted with phenol-water (PW) and warm saline (Sal) from E. coli strains

Strain no.	Extract	Yield: mg per g of cell mass	Molar ratio of the product						KDO*	Percentage of hexosamine
			Rhamnose	Mannose	Galactose	Glucose	D-glycero- D-manno- heptose	L-glycero- D-manno- heptose		
LP1173	PW	66.3	2.27	0	3.12	6.12	0	1.00	2.40	9.2
	Sal	35.5	1.53	0	3.36	5.22	0	1.00	1.46	5.2
LP2362	PW	35.6	0.28	0	1.56	1.66	0	1.00	1.18	3.0
	Sal	35.5	0.29	0	2.37	3.00	0	1.00	1.47	0.7
LP1624	PW	14.0	1.49	0	2.97	5.27	0	1.00	2.46	10.3
	Sal	17.0	4.73	0	2.31	1.14	0	1.00	1.32	9.2
LP2017	PW	57.9	2.11	0	1.24	1.08	0	1.00	1.85	12.0
	Sal	44.2	5.66	0	2.75	1.62	0	1.00	1.41	4.0
LP944	PW	58.3	0	3.70	1.68	3.91	0.58	1.00	1.49	14.1
	Sal	42.8	0	3.03	1.37	3.02	0.36	1.00	1.05	5.7
LP1894	PW	66.6	0	1.87	1.64	1.93	0.57	1.00	1.24	8.8
	Sal	79.0	0	2.66	1.74	3.72	0.22	1.00	1.24	2.0
LP2707	PW	71.7	0	4.10	2.08	4.39	0.97	1.00	1.08	13.5
	Sal	27.6	0	6.09	1.96	5.39	0.48	1.00	1.08	8.1
LP856a	PW	65.5	0	3.95	2.40	3.97	0.88	1.00	1.53	15.5
	Sal	62.0	0	4.72	3.22	4.65	0.75	1.00	0.88	5.0
LP1107	PW	62.6	0	3.29	1.95	3.19	0.49	1.00	0.59	9.6
	Sal	64.7	0	4.48	3.47	5.15	0.41	1.00	1.76	4.9
LP1123	PW	71.8	0	3.81	1.27	3.14	0.53	1.00	0.96	10.5
	Sal	58.1	0	3.83	2.93	3.80	0.57	1.00	0.82	4.5
LP2214	PW	69.3	0	2.98	1.90	2.05	0.75	1.00	1.00	13.5
	Sal	54.5	0	4.83	2.16	4.99	0.76	1.00	1.71	6.2
LP2706	PW	59.6	0	5.33	2.30	4.56	1.36	1.00	1.54	14.5
	Sal	43.7	0	3.87	2.17	3.64	0.60	1.00	1.07	7.4
LP898	PW	73.5	0	2.49	1.87	2.36	0.64	1.00	1.15	11.7
	Sal	66.6	0	2.90	2.32	3.77	0.53	1.00	1.18	4.0
LP1174	PW	57.4	0	4.19	2.30	4.65	1.29	1.00	1.57	14.5
	Sal	39.3	0	4.11	1.18	3.65	1.15	1.00	0.84	7.2
LP1339	PW	41.1	0	3.96	1.11	3.47	0.38	1.00	0.81	15.6
	Sal	43.8	0	2.97	1.38	2.96	0.85	1.00	0.73	8.1
LP2155	PW	45.2	0	6.35	2.45	4.16	1.18	1.00	1.28	10.0
	Sal	45.4	0	4.25	1.91	4.30	0.64	1.00	0.99	6.4
LP2671	PW	60.9	0	3.41	1.78	3.52	0.90	1.00	1.31	9.2
	Sal	35.0	0	4.43	2.44	4.54	0.29	1.00	1.50	6.2
LP903	PW	44.8	1.80	1.78	2.58	1.10	0	1.00	0.75	9.4
	Sal	44.4	1.83	2.24	4.21	1.62	0	1.00	0.88	3.7
LP1674	PW	42.4	0.78	1.53	2.18	0.71	0	1.00	0.77	12.7
	Sal	36.2	2.33	2.71	5.65	2.15	0	1.00	1.06	6.2
LP1170	PW	64.5	2.57	1.81	3.14	0.89	0	1.00	1.13	11.2
	Sal	39.0	2.70	2.47	6.04	0.92	0	1.00	1.16	6.0
LP609	PW	74.5	0	9.49	1.86	1.14	2.80	1.00	1.26	2.1
	Sal	35.7	0	10.21	1.76	1.46	1.75	1.00	0.59	1.8
LP729	PW	64.6	0	13.13	1.13	0.98	2.41	1.00	1.27	4.5
	Sal	54.6	0	13.82	2.60	2.21	3.93	1.00	0.94	1.4
LP1977	PW	57.4	0	9.61	2.01	1.33	3.35	1.00	0.46	4.1
	Sal	51.7	0	8.18	2.08	1.44	1.69	1.00	2.15	1.8
LP1255	PW	60.6	0	10.85	1.80	1.10	3.64	1.00	0.92	3.0
	Sal	64.1	0	15.05	2.16	1.47	2.35	1.00	0.71	1.7
LP2212	PW	59.7	1.04	0	2.79	2.83	0	1.00	0.88	14.0
	Sal	53.5	1.50	0	4.46	2.80	0	1.00	0.88	5.0
LP2506	PW	39.4	0.41	0	2.31	2.07	0	1.00	1.36	9.1
	Sal	46.6	0.75	0	4.10	3.37	0	1.00	2.02	2.5
LP1301	PW	63.4	1.31	0	3.43	3.45	0	1.00	1.25	18.5
	Sal	58.5	0.79	0	3.88	3.40	0	1.00	0.83	9.0
LP1395	PW	50.9	0.93	0	3.02	2.66	0	1.00	0.76	16.0
	Sal	62.1	1.41	0	3.95	3.40	0	1.00	1.16	7.9
F470	PW	16.9	0	0.11	1.35	1.44	0	1.00	1.31	...
F576	PW	10.1	0	0.25	1.00	1.18	0	1.00	1.05	...
F653	PW	10.5	0	0.21	0.70	1.67	0	1.00	1.40	...

*KDO = 3-deoxy-D-manno-octulosonic acid.

Chemical analysis. The sugar composition of LPS from the three rough and 28 smooth strains are shown in table III. Glucose, galactose, L-glycero-D-mannoheptose and KDO were found as major components of the polysaccharide portion of the LPS from strains F470, F576 and F653; small amounts of mannose were also detected. The major core components were found in all S-LPS preparations. In addition, LPS from *E. coli* O2 and O18 strains contained rhamnose; O6 and O9 LPS contained mannose; and both rhamnose and mannose were found in LPS from O7 strains. However, in all preparations from *E. coli* O6 and O9 strains, but in none from O2, O7 and O18 strains, an additional peak with a retention time relative to glucose (R glu) of 2.09–2.12 was found (fig. 1). Previous studies (Adams, Quadling and Perry, 1967; Dmitriev *et al.*, 1971) indicate that this peak might be the alditol acetate of D-glycero-D-mannoheptose. The alditol acetate derived from an authentic sample of D-glycero-D-mannoheptose gave a R glu value of 2.10. The authentic sugar was added to hydrolysates of phenol-extracted LPS from strain LP729 and alditol acetates were prepared; the heptose and the unidentified component appeared in chromatographs as a single peak. The sugar was therefore tentatively identified as D-glycero-D-mannoheptose; further characterisation of this component is in progress.

The presence of D-glycero-D-mannoheptose raises the question of whether it is associated with the core or the O side-chain. Phenol-extracted LPS was therefore subjected to mild hydrolysis with acetic acid and the resulting degraded polysaccharides were fractionated by gel filtration on Sephadex G50. Previous studies have shown that gel filtration of degraded polysaccharides from S LPS gives three peaks (Schmidt *et al.*, 1969), peak I containing predominantly O-side-chain material, peak II representing the core moiety and peak III consisting of mono-, di- and trisaccharides. The elution profile obtained with degraded polysaccharides from strain LP729 is shown in fig. 2. Peak I was excluded from the column with the void volume; subsequent peaks were small, indicating that in LPS from this strain the majority of core "stubs" are substituted with O side-chains. The neutral sugar composition of the various peaks is shown in table IV; D-glycero-D-mannoheptose was associated exclusively with the O-side-chain fractions (peak I).

Several minor peaks were evident in some chromatograms of S-LPS hydrolysates. A peak with a retention time slightly greater than that of hexa-O-acetyl glucitol was frequently found (peak X; fig. 1); this peak represents a conversion or degradation product of L-glycero-D-mannoheptose during acid hydrolysis (Schmidt *et al.*, 1970). It is assumed that the small ribitol-penta-acetate peak that was found in a few of the chromatograms was due to slight contamination with ribonucleic acid.

The relative molar amounts of rhamnose (serogroups O2, O7 and O18) or mannose (serogroups O6 and O9) and L-glycero-D-mannoheptose were taken as a measure of the ratio of O-side-chain sugar to core sugar. There was a tendency for this ratio to be greater in serum-resistant strains than in serum-sensitive strains (table III). The difference in ratios given by LPS extracted with phenol-water from paired serum-sensitive and serum-resistant

FIG. 1.—Gas-liquid chromatography on ECNSS-M columns of alditol acetates derived from hydrolysates of LPS from *E. coli* strain no. LP729. For chromatographic details, see *Methods*. Peaks represent derivatives of: 1, xylose (internal standard); 2, mannose; 3, galactose; 4, glucose; 5, D-glycero-D-mannoheptose; 6, L-glycero-D-mannoheptose; X, degradation product of L-glycero-D-mannoheptose.

FIG. 2.—Fractionation of degraded polysaccharides from LPS of *E. coli* strain no. LP729 on Sephadex G50. Fractions were examined by the phenol- H_2SO_4 technique and the absorbance at 445 nm was measured.

TABLE IV

Analysis of degraded polysaccharides from the phenol-extracted LPS (50 mg) of E. coli strain no. LP729

Peak*	Fractions	Weight (mg)	Percentage amount of material in each peak				
			Mannose	Galactose	Glucose	D-glycero-D-mannoheptose	L-glycero-D-mannoheptose
I	15-33	21.8	61.9	4.0	5.3	16.8	3.3
II	34-47	2.6	10.6	20.6	24.8	0	19.0
III	48-60	0.7	9.6	20.4	6.4	0	0

* Material was fractionated on Sephadex G50 and fractions representing peaks I, II and III were pooled.

strains—as determined by the standard bactericidal test—was probably significant (mean difference = 0.67; $t = 2.301$; $P < 0.05$), but it was not when the modified serum responses were taken into account (mean difference = 0.31; $t = 0.880$; $0.5 > P > 0.4$). No significant mean differences were found with LPS extracted by the saline technique (standard test : mean difference = 1.63; $t = 1.801$; $0.2 > P > 0.1$; modified test : mean difference = 1.55; $t = 1.693$; $0.2 > P > 0.1$). The ratios obtained with the intermediate O6 strains were similar to those found with other strains of this O serogroup.

There were no significant mean differences in total sugar content of LPS from serum-sensitive and serum-resistant strains whether they were extracted with phenol-water (standard test : mean difference = 2.61%; $t = 0.650$; $0.6 > P > 0.5$; modified test : mean difference = 6.19%; $t = 1.763$; $0.2 > P > 0.1$) or with saline (standard test : mean difference = 2.83%; $t = 1.263$; $0.3 > P > 0.2$; modified test : mean difference = 3.67%; $t = 1.763$; $0.2 > P > 0.1$). Total sugar did, however, account for significantly more of the phenol-water than of the saline preparations (mean difference = 29.7%; $t = 14.145$; $P < 0.001$).

Studies on acidic polysaccharides

Yield. There was considerable variation in the amount of acidic polysaccharide extracted from the smooth strains, ranging from 0.3% of the cell mass for the resistant strain LP2214 to 9% for strain LP903 (table V). There were no significant differences in the mean yields of phenol-extracted acidic polysaccharide from serum-sensitive and serum-resistant strains as determined by either the standard test or the modified test (means 22.49 and 31.80 mg per g; $t < 1.315$; $P > 0.05$). Similarly, the difference in the mean yields of saline-extracted acidic polysaccharide was not significant when strains were classified as sensitive or resistant on the basis of the standard test (means 24.28 and 34.04 mg per g; $t = 1.036$; $0.4 > P > 0.3$). However, when the modified serum responses were taken into account, a significant difference in the mean yields was found (means 16.14 and 42.04 mg per g; $t = 3.348$; $P < 0.005$).

TABLE V

Analysis of acidic polysaccharides obtained by cetrimide precipitation from phenol-water (PW) and warm saline (Sal) extracts of smooth E. coli strains

Strain no.	Serotype	Grade of serum sensitivity in		Extracting agent	Yield: mg per g of cell mass	Presence of		Amount (μ g per ml) required to double the minimal haem-agglutinating dose*
		standard test	modified test			hexuronic acid	neuraminic acid	
LP1173	O2	1	1	PW	18.8	—	—	250
LP2362	O2 : K ? : H6	1	1	Sal	6.0	—	—	> 1000
				PW	12.8	—	+	500
LP1624	O2	4	4	Sal	17.6	—	+	> 1000
				PW	19.0	+	—	0.1
LP2017	O2 : K ? : H—	6	6	Sal	20.8	+	—	0.2
				PW	30.6	—	+	4
LP944	O6	1	1	Sal	36.6	—	+	> 1000
				PW	25.1	+	—	0.5
LP1894	O6 : K ? : H1	1	1	Sal	22.6	+	—	32
				PW	46.0	—	—	> 1000
LP2707	O6 : K ? : H—	1	1	Sal	15.8	—	—	> 1000
				PW	23.2	+	—	16
LP856a	O6	1	1	Sal	18.8	+	—	4
				PW	27.1	—	—	62
LP1107	O6	6	6	Sal	32.0	—	—	250
				PW	43.6	—	—	16
LP1123	O6	6	6	Sal	26.2	—	—	32
				PW	28.7	—	—	16
LP2214	O6 : K ? : H31	6	6	Sal	38.0	—	—	> 1000
				PW	9.5	—	—	1000
LP2706	O6 : K ? : H31	6	6	Sal	3.2	—	—	> 1000
				PW	31.8	—	—	1
LP898	O6	4	2	Sal	25.5	—	—	125
				PW	34.7	—	—	32
LP1174	O6	4	4	Sal	29.8	—	—	250
				PW	43.4	+	—	2
LP1339	O6 : K ? : H1	4	4	Sal	59.3	+	—	4
				PW	36.1	+	—	1
LP2155	O6 : K ? : H1	4	5	Sal	42.8	+	—	4
				PW	48.9	+	—	0.5
LP2671	O6 : K2ac : H1	4	1	Sal	60.3	+	—	32
				PW	45.4	—	—	0.5
LP903	O7	1	6	Sal	26.4	—	—	4
				PW	49.7	—	+	8
LP1674	O7	1	6	Sal	89.8	—	+	1000
				PW	20.9	—	+	16
LP1170	O7	5	1	Sal	56.7	—	+	4
				PW	27.6	+	—	250
LP609	O9	1	1	Sal	24.5	+	—	250
				PW	11.6	—	—	> 1000
LP729	O9	1	1	Sal	3.9	—	—	> 1000
				PW	18.0	—	—	> 1000
LP1977	O9 : K26a : H10	1	1	Sal	3.5	—	—	> 1000
				PW	11.8	—	—	> 1000
LP1255	O9	6	6	Sal	3.3	—	—	> 1000
				PW	43.0	+	—	> 1000
LP2212	O18ac : K ? : H5	1	1	Sal	54.1	+	—	> 1000
				PW	10.6	—	—	> 1000
LP2506	O18ac : K ? : H—	1	1	Sal	15.5	—	—	> 1000
				PW	37.3	—	+	32
LP1301	O18	6	5	Sal	30.2	—	+	32
				PW	37.4	+	—	0.5
LP1395	O18	6	6	Sal	39.7	+	—	4
				PW	35.6	+	—	1
				Sal	71.8	+	—	4

* See *Methods*.

Similar values were obtained when mean differences were assessed. More material was extracted from serum-intermediate *E. coli* O6 strains than from serum-sensitive or serum-resistant O6 strains (table V).

Chemical analysis. Polysaccharides from 10 strains were found to contain hexuronic acid (table V). Large amounts of neuraminic acid were found in acidic polysaccharides from five strains. Neither hexuronic acid nor neuraminic acid could be detected in polymers from the remaining 13 strains. There was

no obvious relationship between the serum sensitivity of a strain and the type of polymer it produced. Chromatography and spectrophotometry indicated that a few of the acidic polysaccharides were contaminated with 1–5% of ribonucleic acid. Small amounts of protein were found in all saline-extracted polymers.

Inhibition of red cell agglutination. Some serum-sensitive and serum-resistant strains produced acidic polysaccharides with high HAI activity; both groups also contained strains that produced polymers with no detectable activity (table V). There was no significant mean difference between the activity of preparations from serum-sensitive strains and serum-resistant strains, whether they were extracted with phenol-water (standard test: $t = 1.427$; $0.2 > P > 0.1$; modified test: $t = 2.008$; $0.1 > P > 0.05$) or with saline (standard test: $t = 0.539$; $0.7 > P > 0.6$; modified test: $t = 0.850$; $0.5 > P > 0.4$). The majority of hexuronic-acid-containing polymers were very effective inhibitors. The preparations from the serum-intermediate *E. coli* O6 strains were more inhibitory than those from either the serum-sensitive or serum-resistant O6 strains (table V). The phenol-water preparations appeared to be more effective inhibitors than the saline preparations ($t = 2.721$; $P < 0.025$).

DISCUSSION

It is evident that no one theory of serum resistance explains the responses to serum of all the *E. coli* strains examined in this study.

There was little difference in the amount of LPS which could be extracted from the bacteria by either the phenol-water or the saline techniques. No attempt was made to determine the proportion of LPS remaining in the bacterial mass after extraction.

In general, more O-side-chain material was associated with LPS from serum-resistant than from serum-sensitive strains. In only one of four statistical analyses, however, were the differences significant. LPS from some resistant strains, such as no. LP2214, contained smaller amounts of O-side-chain sugars than sensitive strains of the same O serotype (table III). The quantitative analysis presented in table III gives no indication of the degree of substitution of LPS core " stubs " by O side-chains, and it is possible that a higher proportion of stubs are substituted in LPS from serum-resistant than from serum-sensitive strains. This is supported by the observation that some serum-sensitive strains are resistant to R-specific phages. Fractionation of degraded polysaccharides suggested, however, that in at least one serum-sensitive strain, no. LP729, the LPS contained few unsubstituted core stubs. This strain was also sensitive to phage F0; N-acetylglucosamine is an essential part of the receptor for this phage (Lindberg and Holme, 1969) and is present in the *E. coli* R2 core as a short side chain attached to the distal glucose unit (Hämmerling *et al.*, 1971). It appears, therefore, that even substantial amounts of O-side-chain material may not always prevent some phages from interacting with core components. In addition, with all LPS preparations examined, anti-R antibodies were able to react with antigenic determinants in the core despite the presence of varying

amounts of O side-chains. These results suggest that O-specific side-chains may not be able to prevent macromolecules, such as activated complement components, from reaching sites deeper in the outer membrane of the cell wall. The increase in serum sensitivity that accompanies the loss by mutation of O side-chains (Dlabač, 1968) may, therefore, be related to the simultaneous decrease in protein content of the outer membrane (Ames, Spudich and Nikaido, 1974). A small number of serum-sensitive mutants derived from serum-resistant salmonellae by Nelson and Roantree (1967) retained the cultural characteristics of the smooth parent strains and showed unaltered LPS O-side-chain-sugar to core-sugar ratios.

There was wide variation in the amount of extractable acidic polysaccharide in serum-sensitive and serum-resistant strains, and the lowest yields were obtained from a serum-resistant strain, no. LP2214 (table V). Although only very small amounts of such substances were obtained from a number of strains, such as no. LP729, they functioned as K antigens in tests with anti-O and anti-OK sera (P. W. Taylor, unpublished data). No statistically significant differences were found between the HAI activity of polymers from serum-sensitive and serum-resistant strains. There was also no obvious relationship between the amount of polymer that could be extracted and its inhibiting activity, the combined effects of which might be expected to determine the total activity of crude homogenates. Many of the more effective inhibitors contained hexuronic acid, suggesting that the inhibitory activity might be related to the charge carried by this polymer. Whether this is related to the ability of the polymer to repel cationic macromolecules or to a higher affinity for the red-cell surface is not known. The lower haemagglutination-inhibiting activity of the saline-extracted polymers may be due to their containing small amounts of protein which might reduce the total negative charge or the red-cell-binding capacity of the polymers.

Some serum-resistant strains contained neither large amounts of acidic polysaccharides nor LPS O-side-chain material and it is likely that resistance in these strains is due to other factors. Jann *et al.* (1970) found an O-inagglutinable *E. coli* strain with no polysaccharide representing a distinct K antigen and suggested that the cell surface might carry a protein "inagglutinability factor."

The results of the present study indicate, therefore, that serum resistance in *E. coli* may have a multifactorial basis. The observation that changes in cultural conditions may modify the response to serum raises the possibility that these factors could be identified and characterised in individual strains by the effect of various growth environments on the cell surface and serum sensitivity.

In contrast to the salmonellae, a number of distinct LPS core types have been recognised among the *E. coli* serotypes. Passive-haemagglutination and haemagglutination-inhibition tests indicated that most of the strains in the present study probably possess the R1 core structure; two strains have the R2 core and none have the R3 core.

Recently, two additional *E. coli* core types have been recognised (Schmidt,

1973; Schmidt, Jann and Jann, 1974). The K12 core is serologically distinct from the R1, R2 and R3 cores (Schmidt, 1973). Although LPS from rough strains possessing the complete R4 core cross react to some extent in anti-R1 sera, extensive cross reactivity in R antisera is not apparent in tests with LPS from smooth strains (Schmidt *et al.*, 1969). The finding of a sugar resembling D-glycero-D-mannoheptose in hydrolysates of many of the LPS from smooth strains was unexpected, because this sugar is not generally recognised as being a constituent of *E. coli* antigens (Lüderitz *et al.*, 1971). The sugar was found in all preparations from serogroups O6 and O9 but was absent from O2, O7, O18 and R LPS (table III); it was also associated exclusively with high-molecular-weight O-side-chain fractions after Sephadex G50 chromatography of degraded polysaccharides from strain LP729 (table IV). Therefore, although direct evidence is lacking, it seems likely that D-glycero-D-mannoheptose is linked to the O-side-chain moiety of the LPS in some strains of *E. coli*. D-glycero-D-mannoheptose has been found in LPS extracted from *Proteus mirabilis* (Kotelko, Lüderitz and Westphal, 1965) and was considered by Bagdian *et al.* (1966) to be a component of the O side-chain in strains of this species. Adams *et al.* (1967) found D-glycero-D-mannoheptose in LPS from seven species of gram-negative bacteria, including *E. coli*, and considered this heptose to be a common component of LPS. However, Lehmann *et al.* (1973) examined LPS from some of the strains tested by Adams *et al.* (1967) and did not find D-glycero-D-mannoheptose in any of them.

Thirteen of 28 strains produced polymers that formed complexes with cetrimide, and thus carried a net negative charge, but contained no detectable hexuronic acid or neuraminic acid. Relatively large amounts of these polymers, frequently with high haemagglutination-inhibiting activity, were obtained from a number of strains. Glycerol phosphate is known to confer acidic properties on exopolysaccharides from some strains of *E. coli* (Jann *et al.*, 1970); further studies will be necessary to determine whether any of the strains in the present study produce polymers of this nature.

SUMMARY

Factors that may determine the variable resistance of urinary strains of *Escherichia coli* to the bactericidal activity of normal human serum have been analysed. No statistically significant difference was found in the amount of lipopolysaccharide (LPS) that could be extracted from serum-sensitive and serum-resistant strains by either the phenol-water or warm-saline techniques. The ratio of LPS O-side-chain sugars to core sugars was not found to be significantly greater in serum-resistant than in serum-sensitive strains. A sugar resembling D-glycero-D-mannoheptose was found in LPS from some of the strains; in one case the sugar was shown to be associated with the O-side-chain moiety. Lipopolysaccharides from all but two of the strains contained the *E. coli* R1 core structure.

No consistent difference was observed between serum-sensitive and serum-resistant strains in either the amount of acidic polysaccharide extracted or its

red-cell agglutination-inhibiting activity; nor was a clear relationship found between sensitivity to serum and sensitivity to R-specific bacteriophages.

It is concluded that no one mechanism of serum resistance explains the response to serum of the *E. coli* strains examined in this study.

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