

Analyses of Lipopolysaccharides Extracted from Penicillin-Resistant, Serum-Sensitive *Salmonella* Mutants

By B. W. NELSON AND R. J. ROANTREE

*Department of Medical Microbiology, Stanford University,
 Stanford, California, U.S.A.*

(Accepted for publication 1 March 1967)

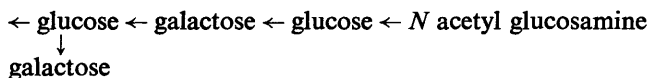
SUMMARY

Serum-sensitive mutants have been derived from serum-resistant smooth virulent *Salmonella typhimurium* and *S. enteritidis* strains by selection for resistance to cephalosporin or penicillin. Chemical analyses of the lipopolysaccharides of these mutants reveal that they belong to at least three different rough or semi-rough classes. Partial or total loss from the lipopolysaccharide of the sugars responsible for O antigenicity resulted in loss of virulence, as well as increased sensitivity to the bactericidal effect of antibody plus complement. However, such loss is not necessary for serum sensitivity because two serum-sensitive mutants possessed lipopolysaccharides indistinguishable from the smooth serum-resistant parents and were nearly as virulent.

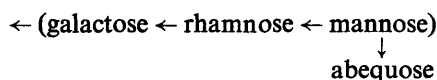
INTRODUCTION

Rough mutants derived from smooth virulent strains of *Salmonella* species are less virulent than their parents (Lingelsheim, 1913; Topley & Ayrton, 1924) and are more susceptible to killing by antibody and complement (Thjøtta & Waaler, 1932; Rowley, 1956). Recently much has been learned about the composition of cell walls of Enterobacteriaceae, particularly those of *Salmonella* species (Lüderitz, Staub & Westphal, 1966), and it seems appropriate to reinvestigate the effect of various mutations to roughness on the virulence and serum-sensitivity of some *Salmonellas*.

New knowledge of the polysaccharide substructure of the lipopolysaccharides (LPS) of *Salmonellas* is largely attributable to a combination of chemical and immunochemical analyses (Lüderitz, Kauffmann, Stierlin & Westphal, 1960; Staub, 1960) and the investigation of mutants unable to synthesize or attach one or another of the monosaccharides which constitute the polysaccharide (Osborn *et al.* 1964; Lüderitz & Westphal, 1966). The results of these studies make it reasonably certain that the lipopolysaccharides of some *Salmonella* species of different O antigenic groups contain a common rough polysaccharide core. This consists of a polyheptose phosphate backbone (probably attached to lipid by a keto-deoxyoctonate) and an attached pentasaccharide side-chain of the composition:



In fully smooth strains of *Salmonella typhimurium* a repeating unit is attached to the distal end:



It has been shown (Staub, Tinelli, Lüderitz & Westphal, 1959) that the monosaccharides abequose, O-acetyl galactose and rhamnose are those principally responsible, respectively, for the O antigenic factors 4, 5, and 12 in the Kauffmann-White classification. The antigenic factor 1 is present in those *Salmonella typhimurium* strains lysogenized with P22 or other A phages and is dependent upon a glucose attached to the galactose in the repeating unit (Stocker, Staub, Tinelli & Kopacka, 1960). The repeating unit of *S. enteritidis* differs from that of *S. typhimurium* in containing tyvelose in place of abequose.

If one disregards the mutants unable to synthesize the activated donor forms of the proximal sugars of the rough core side-chains, two main classes of rough mutants have been described.

Strains of one class are unable to synthesize the oligosaccharide repeating unit of the distal part of the side-chains responsible for O specificity. The LPS of such mutants has the antigenic character RII (Beckmann, Subbaiah & Stocker, 1964) presumably determined by the exposed terminus of the proximal part ('R-stub') of the side-chain. In *Salmonella typhimurium*, inability to manufacture the O repeat unit may result either from mutation in the *rouB* gene cluster near *his* (Subbaiah & Stocker, 1964) or, less commonly, from a mutation mapping between *gal* and *try* resulting in loss of ability to synthesize phosphomannose isomerase and consequent loss of the mannose component of the repeating unit (Rosen *et al.* 1965).

Mutants of the other main rough class are unable to attach to their LPS the O-specific polysaccharide which is found in the supernatant (L-1) fraction after the LPS has been sedimented in the Westphal extraction procedure (Beckmann *et al.* 1964). It is presumed (or in some instances proven) that this class is unable to complete the synthesis of the 'R-stub' to which the O-specific polysaccharide is usually attached. Many mutants of *Salmonella typhimurium* belonging to this class map in the *rouA* cluster near *xyl* (Subbaiah & Stocker, 1964); some, but not all, make LPS of antigenic character RI (Beckmann *et al.* 1964).

In addition, two forms of 'semi-rough' mutants have been described (Naide *et al.* 1965). Their LPS contains much less of the oligo-saccharide responsible for O antigenicity than does that of smooth strains. The LPS of one type of semi-R mutant probably has only one O-specific unit attached to each of the rough stubs whereas that of the other has some long O-specific side-chains, but has also many R stubs which lack repeating units.

An opportunity to study the virulence of some classes of rough strains occurred when it was found that certain penicillin-resistant mutants derived from virulent, serum-resistant *Salmonella enteritidis* and *S. typhimurium* strains were sensitive to serum. Most of them were less virulent for mice than the parent strains (Roantree & Steward, 1965). Among these mutants, some showed typical character of rough strains, i.e. growth as a deposit in broth, auto-agglutinability in physiologic saline, loss of specific agglutinability in anti-O sera and rough colonial appearance on eosin-methylene-blue agar. Other mutants, although serum-sensitive, could not be distinguished from the smooth parents in these respects; one single-step mutant of *S. enteritidis* grew in broth like a rough strain but retained its O-agglutinability. The penicillin-resistant, serum-sensitive strains were isolated as series of mutants, each one more antibiotic-resistant than its predecessor, by serial passage on gradient plates containing either benzylpenicillin or α -aminobenzylpenicillin. Subsequent work with

the virulent parent strains of *S. typhimurium* and *S. enteritidis* has shown that if picks from single colonies of the parent strain were grown overnight in broth cultures and 0.1 ml. portions of these spread on gradient plates containing just one concentration of penicillin or cephalosporin, a variety of single-step mutants were isolated, some of which were serum-sensitive and less virulent than the parent. This method avoided the confusion of multiple mutations to antibiotic resistance.

This paper describes the chemical composition of the polysaccharide of the cell walls of these serum-sensitive mutants. Some previously reported observations on the virulence of the mutants and their sensitivity to serum are recapitulated here (Roantree & Steward, 1965).

MATERIALS

Bacterial strains

Salmonella enteritidis 173 (O = 1, 4, 5, 12) was originally obtained from Professor D. Rowley, University of Adelaide, Adelaide, Australia.

Salmonella enteritidis 203 (O = 9, 12) was obtained from the Division of Laboratories, California Department of Public Health.

Mutants derived on penicillin gradient plates are given the prefix P-; those on α -aminobenzylpenicillin (ampicillin) the prefix BR, and those on cephalothin the prefix K. The numeral immediately following the prefix denotes the serial mutational step to antibiotic resistance represented by the strain. A small letter following the strain number indicates that the strain is one of a series of mutants. For example, the designation BR3-173a means that this is a third-step mutant derived by passage on α -aminobenzylpenicillin and is a member of the 'a' series. A capital letter following the strain number indicates a particular one-step mutant to antibiotic resistance.

Testing of resistance to penicillin

Nutrient agar plates containing various concentrations of benzylpenicillin or α -aminobenzylpenicillin were inoculated with 100–200 organisms of the strain to be tested. The greatest concentration of antibiotic allowing the consistent appearance of colonies was taken as the value for degree of resistance. Mutant strains within any one series were plated on the same batches of agar on the same day. Concentrations of antibiotic were varied by increments of 5 u./ml. for benzylpenicillin and 2 μ g./ml. for α -aminobenzylpenicillin in testing strains of *Salmonella typhimurium* (Table 1). Increments of α -aminobenzylpenicillin used to test *S. enteritidis* strains were 0.2 μ g./ml. (Table 2).

Determination of virulence of bacteria for the mouse

Ten mature CF-1 mice housed together were each inoculated intraperitoneally with the same volume of one serial 10-fold dilution in saline of an overnight broth culture. Sufficient 10-fold dilutions were used to permit the calculation of the LD₅₀ from survivals to 30 days (Reed & Muench, 1938). The LD₅₀, when greater than 100, is reported to the closest whole power of 10.

Determination of sensitivity to serum

This has been described in detail (Roantree & Steward, 1965). Briefly, 0.1 ml. amounts of 10^{-2} and 10^{-5} dilutions of overnight broth cultures were added to 0.4 ml. amounts of active human serum and incubated at 37°. The number of bacteria

surviving in 0.1 ml. volumes of the suspensions was determined by colony counts from pour plates made at 1 and 2 hr. The number of bacteria at zero time was determined by colony counts made from pour plates prepared from 0.1 ml. volumes of 10^{-6} dilutions of overnight culture. For the purpose of the present paper, strains showing a survival of greater than 10 % from an inoculum of 10^8 organisms per 0.1 ml. after 2 hr are considered resistant; those showing survival of less than 0.1 % from an inoculum of 10^6 are considered sensitive.

Table 1. *Characteristics of parent and mutant strains of Salmonella typhimurium and the sugars related to the O antigens found in their lipopolysaccharides*

Strain	Penicillin resistance*	Sensitivity to serum†	O Anti-gens (1, 4, 5, 12)	LD 50 for mice	Deposit in broth	Rhamnose	Abequose	Mannose	Galactose
173	1 µg. 5 u.	Res.	++	10^1	0	+++	+++	+++	+++
P 1-173	10 u.	Res.	++	10^2	0	+++	+++	+++	+++
P 2-173	15 u.	Sen.	+	10^4	0	+	+	+	++
P 3-173	40 u.	Sen.	++	10^6	0	+	+	+	++
P 5-173	50 u.	Sen.	++	10^6	0	+	+	+	++
BR 1-173 a	2 µg.	Res.	++	10^1	0	+++	+++	+++	+++
BR 2-173 a	4 µg.	Sen.	0	n.d.	±	0	0	0	++
BR 3-173 a	8 µg.	Sen.	0	n.d.	++	0	0	0	++
BR 6-173 a	10 µg.	Sen.	0	10^7	++	0	0	0	++
BR 1-173 c	2 µg.	Res.	++	n.d.	0	+++	+++	+++	+++
BR 2-173 c	8 µg.	Res.	++	n.d.	0	+++	+++	+++	+++
BR 4-173 c	16 µg.	Res.	++	n.d.	0	+++	+++	+++	+++
BR 6-173 c	20 µg.	Res.	++	10^6	0	+++	+++	+++	+++
P 173 C	10 u.	Sen.	++	10^1	0	+++	+++	+++	+++
K 173 A		Sen.	0	10^4 ‡	++	0§	0§	0§	++§

*u. = units benzylpenicillin/ml. nutrient agar. µg. = micrograms α -aminobenzylpenicillin/ml. nutrient agar.

†Serum sensitivity is defined as less than 0.1 % survival of an inoculum of 10^6 organisms in 0.1 ml. of active human serum in 2 hr; serum resistance as greater than 10 % survival of an inoculum of 10^2 .

‡ 10^4 was highest challenge dose used.

§These sugars were present in the supernatant (L-1) fraction but not in the LPS.

Table 2. *Characteristics of parent and mutant strains of Salmonella enteritidis and the sugars related to the O antigens found in their lipopolysaccharides*

Strain	Penicillin resistance†	Sensitivity to serum†	O Anti-gens (9, 12)	LD 50 for mice	Deposit in broth	Rhamnose	Abequose	Mannose	Galactose
203	1.0 µg.	Res.	++	10^2	0	+++	+++	+++	+++
BR 1-203 b	2.0 µg.	Sen.	+	10^6	+++	+	+	+	++
BR 203 C	1.8 µg.	Sen.	++	10^2	0	+++	+++	+++	+++

*µg. = micrograms α -aminobenzylpenicillin/ml. nutrient agar.

†Serum sensitivity is defined as less than 0.1 % survival of an inoculum of 10^6 organisms in 0.1 ml. of active human serum in 2 hr; serum resistance as greater than 10 % survival of an inoculum of 10^2 .

Analysis of lipopolysaccharides

Mass cultures of the organisms were grown on brain-heart infusion agar (Difco) in Roux bottles at 37° for 18 hr. Bacteria were washed from the surface with saline, centrifuged in the cold, resuspended in fresh saline and again centrifuged. They were then killed by immersion in approximately 10 vol. acetone for 24 hr.

Lipopolysaccharide was extracted from acetone-dried organisms by the method of Westphal, Lüderitz & Bister (1952). Approximately 10 g. dried organisms were suspended in 600 ml. of 45 % phenol at 65–68° and agitated for 20 min. The water phase was separated from the phenol phase by centrifugation in the cold; the phenol phase was re-extracted with an additional 300 ml. water. The water-phase material from both extractions was washed with ether and dialysed overnight against running tap water. After concentration of the water phase by pervaporation and removal of debris by low-speed centrifugation, the LPS was separated from contaminating nucleic acid, etc., by spinning the concentrate for 2 hr at 100,000 g in a Beckman preparative ultracentrifuge. The pellet containing the LPS was resuspended in distilled water and the ultracentrifugation repeated twice. Sediment from the last ultracentrifugation was lyophilized. Greater than fivefold differences in yields (mg. LPS/g. dry wt bacteria) from different smooth strains occurred. The supernatant from the first ultracentrifugation (L_1 fraction of Beckman *et al.* 1964) was saved and lyophilized.

Degraded polysaccharide was prepared directly from dried cells by the method of Freeman (1942). The crude polysaccharide was extracted with dilute acetic acid at 100°. Purification consisted of 6 alcoholic and 3 acetic acid fractionations except that the LPS from p2–173 failed to precipitate in the designated concentrations of acetic acid so ten alcoholic fractionations were substituted in this instance.

To determine the sugars present in either the LPS, polysaccharide or L_1 fraction, 10–20 mg. dried material was hydrolysed for 4 hr in 1 ml. $N-H_2SO_4$ at 100° in a sealed ampoule. Samples were neutralized with saturated $Ba(OH)_2$, dried and then redissolved in a small volume (0.2–0.4 ml.) of water: 5 and 10 μ l. samples were then spotted on plates for thin-layer chromatography (TLC). The silica gel on the plates had been emulsified with 0.1 M-boric acid. The solvent systems were butanol+acetic acid 4+1 (v/v), and butanol+pyridine+water 6+4+3 (v/v); the plates were developed with a diphenylamine, aniline mixture. The butanol+pyridine+water system was also used for paper chromatography. Sugars were identified by comparison with commercially available sugars with the exception of abequose and tyvelose. These sugars were identified by (1) comparison of experimental R_F values with published values (Kabat & Mayer, 1961); (2) a failure of the material eluted from TLC plates to produce an absorption band in the Dische reaction, and (3) the close similarity of the R_F value of the spot identified as tyvelose to that of a spot from hydrolysates containing colitose. Both spots were of characteristic purple colour and migrated considerably farther than rhamnose. Good separation of rhamnose and dideoxyhexose from the other sugars was obtained by thin-layer chromatography. Paper chromatography for 18 hr at room temperature was used to separate mannose, glucose, and galactose. Mannose was also determined by paper electrophoresis of the LPS in a Spinco cell at 320 v for 4 hr. The buffer used was 0.025 N-sodium tetraborate (pH 9.3) and the sugars were detected by spraying the paper with aniline hydrogen phthalate.

Glucose was determined quantitatively by the glucose oxidase test (Glucostat, Worthington Biochemical Co.) and galactose by the galactose oxidase test (Galactostat, Worthington Biochemical Co.). Rhamnose was determined quantitatively and heptose both qualitatively and quantitatively by the 10 min. modification of the Dische Cysteine-sulphuric acid reaction (Dische, 1953). The sugar standard used for the determination of heptose was α -D-manno-heptose.

RESULTS

Certain characteristics of the virulent *Salmonella typhimurium* parent strain 173, three series of mutants and two single-step mutants derived from it are included in Table 1. Sugar contents of their lipopolysaccharides were estimated from the results of thin-layer chromatography and paper electrophoresis by comparing the densities of spots obtained from equal amounts by weight of the LPS from each member of the group of mutants.

The first-step mutant in the P173 series differed from the parent strain mainly in its greater resistance to penicillin, but the second-step strain, P2-173, was sensitive to serum and relatively avirulent. It was considered a semi-rough mutant because its LPS contained much less rhamnose, mannose and abequose and somewhat less galactose than the parent. Further multi-step mutants in this line led to no change in the tested characteristics, other than greater resistance to penicillin.

Similarly, in the BR173a series, obtained by passage of 173 on α -aminobenzylpenicillin, the first-step mutant differed little from the parent strain but the second-step mutant showed the qualities of a completely rough strain and its LPS lacked detectable rhamnose, mannose or abequose, and galactose appeared reduced. Further mutants in this series shared these rough characteristics and the sixth-step mutant was tested and found avirulent for mice.

The BR173c series originated from a different colony on the same initial gradient plate from which the colony giving rise to the BR173a series was picked. Although the sixth mutant in the 'c' line was more resistant to α -aminobenzylpenicillin than its counterpart in the 'a' line (20 to 10 μ g./ml.), the 'c' line showed no loss of smooth characteristics or 0 specific sugars and remained resistant to serum. The sixth-step mutant in the 'c' line was much less virulent than the parent, but its LD₅₀ (10^6) was considerably less than that of the rough BR6-173a (10^7).

The P173C one-step mutant seemed identical to the parent in the characteristics tested except for being more resistant to penicillin and sensitive to serum.

The one-step mutant to cephalosporin resistance, K173A, was sensitive to serum, culturally rough, and relatively avirulent. Although its LPS lacked the smooth-specific sugars, the strain differed from the rough mutants described above because O-specific sugars were found in the L1 supernatant fraction.

Characteristics of the virulent serum-resistant *Salmonella enteritidis* 203 and two one-step mutants resistant to α -aminobenzylpenicillin are summarized in Table 2. The BR1-203b strain looked like a typical rough strain when grown in broth, but weak agglutination was obtained with anti-09 and -012 sera. The LPS contained substantially reduced amounts of smooth-specific sugars; it was therefore considered to be a semi-rough mutant.

Salmonella enteritidis BR203C is like the P173C strain in the *S. typhimurium* group because its LPS seems to contain the same amounts of sugars as that of the parent strain even though it is sensitive to serum.

The lipopolysaccharides of the more interesting strains were analysed further by obtaining absorption spectra of the products of the Dische reaction. In Fig. 1, curves from analyses of the LPS derived from the smooth *Salmonella typhimurium* 173, the semi-rough mutant P2-173, and the rough mutant BR6-173a are compared. The sharp peak at 400 m μ represents rhamnose and the broader peak at 505 m μ is mainly

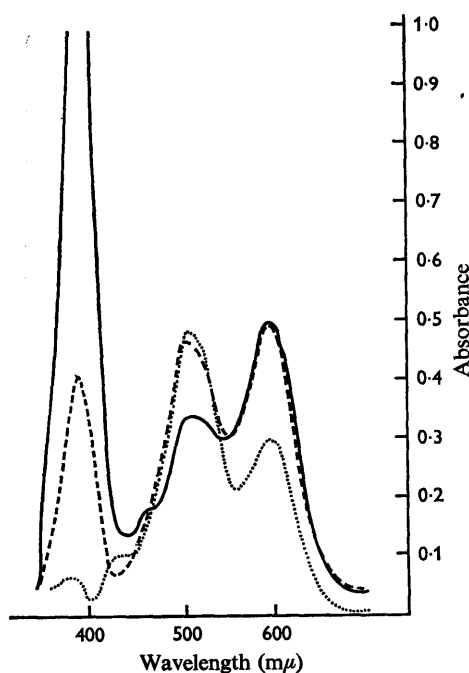


Fig. 1

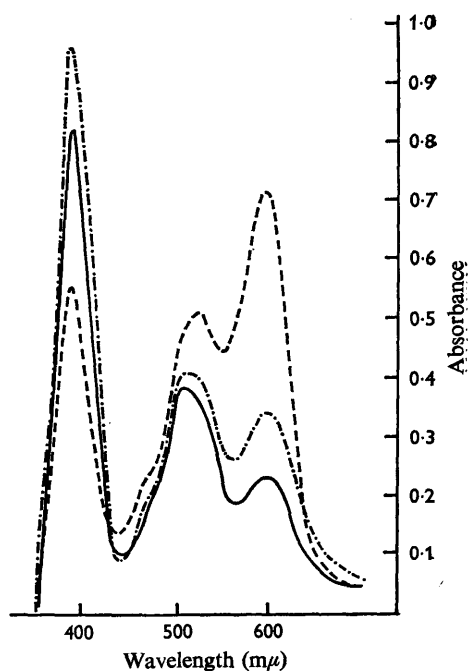


Fig. 2

Fig. 1. Absorption spectra of products derived from 750 μ g. LPS 24 hr after the Dische reaction. Peak at 400 $m\mu$ indicates the amount of rhamnose; that at 505 $m\mu$ is principally caused by heptose. LPS from *Salmonella typhimurium*: —, smooth parent 173; — — —, semi-rough mutant P2-173;, and rough mutant BR6-173a.

Fig. 2. Absorption spectra of products derived from 750 μ g. LPS 24 hr after the Dische reaction. Peak at 400 $m\mu$ indicates amount of rhamnose; that at 505 $m\mu$ is principally caused by heptose. LPS from *Salmonella enteritidis*: —, smooth parent 203; — . —, smooth serum-sensitive mutant BR203C; — — —, semi-rough mutant BR203b.

Table 3. Rhamnose, glucose and heptose composition of strain P2-173

Lipopolysaccharide	Molar ratios			
	Rhamnose	Glucose	Galactose	Rhamnose
	Heptose	Heptose	Heptose	Glucose
Parent strain 173	2.0	2.0	3.5	1.0
Mutant strain P 2-173	0.23	0.72	0.82	0.3
Polysaccharide				
Parent strain 173	3.1	3.6	5	0.85
Mutant strain P 2-173	0.50	0.84	0.78	0.55

caused by heptose. It is evident that the rhamnose:heptose ratio is greatly reduced in the P2-173 LPS as compared with the parent and that no rhamnose peak is detectable in the LPS from the rough mutant BR6-173a. The curve obtained from the LPS of the smooth serum-sensitive mutant P173C is not included since it is virtually identical with that of the parent.

LPS and degraded polysaccharides isolated from the parent 173 and the semi-rough

mutant P2-173 were analysed for their glucose and galactose contents, and the results are shown in Table 3, together with the rhamnose:heptose ratios derived from the curves in Fig. 1. These values confirm the previous results in indicating that the LPS of P2-173 contains reduced amounts of the sugars determining O specificity.

Absorption spectra of the products from the Dische reactions upon lipopolysaccharides from *Salmonella enteritidis* 203, its serum-sensitive smooth mutant, BR203C, and the suspected semi-rough BR1-203b, are shown in Fig. 2. The greatly diminished rhamnose:heptose ratio of BR1-203b as compared with the parent is evident, confirming its classification as a semi-rough strain. Curves of the two smooth strains are very similar.

DISCUSSION

At least five classes of mutants have been derived from virulent serum-resistant *Salmonella typhimurium* and *S. enteritidis* strains by selection of colonies resistant to antibiotics which interfere with the synthesis of the bacterial cell wall.

(1) The late mutants in the BR173a series are rough and neither their LPS nor their L₁ fractions contain detectable amounts of O-specific material as evidenced by the absence of mannose, rhamnose and abequose. By chemical analysis, this class could fit into the hapten-negative (*rouB*) class (Beckmann *et al.* 1964), but antigenic analyses of LPS from BR6-173a, kindly done by Dr O. Lüderitz, did not identify it as of RII antigenic character or other rough antigenic subclasses (Lüderitz & Westphal, 1966). Tests of the sensitivity of this mutant to various R-specific phages by Dr Peter Gemski confirmed the classification of roughness, but did not aid in sub-classification. It is not a mutant lacking ability to synthesize phosphomannose isomerase because it can ferment mannose (see Rosen *et al.* 1965).

(2) The K173A one-step mutant to cephalosporin resistance represents a rough class different from the above because it apparently makes polysaccharide containing the ← galactose ← rhamnose ← mannose ← abequose repeating unit but does not attach it. In this respect it is similar to the hapten-positive (*rouA*) mutants of *Salmonella typhimurium* (Beckmann *et al.* 1964). However, antigenic analyses by Dr Lüderitz and phage analyses by Dr Gemski did not conclusively subclassify this type of mutant.

(3) The two mutants, *Salmonella typhimurium* P2-173 and *S. enteritidis* BR1-203b, clearly belong in the semi-rough category (Naide *et al.* 1965). The two are very different from each other; BR1-203b would probably be classified as rough by traditional criteria whereas P2-173 is indistinguishable from the smooth parent by such criteria.

(4) The existence of semi-rough, serum-sensitive strains, such as P2-173 which are very like smooth strains, made us suspect that every seemingly smooth strain sensitive to serum was actually some form of semi-rough. However, the mutants *Salmonella typhimurium* P173C and *S. enteritidis* BR203C represent a chemically smooth class markedly more sensitive to antibody and complement than the parent.

(5) The *Salmonella typhimurium* BR173c line represents a group of penicillin-resistant mutants which show no significant changes in sensitivity to serum, antigenic character or sugar composition of the lipopolysaccharide, although they are less virulent for mice than the parent strain.

Selection of mutants of enteric bacilli resistant to penicillin reveals a minority which are more sensitive to serum than the parent strain (Michael & Braun, 1958; Roantree

& Steward, 1965). The present work shows that among the serum-sensitive mutants are rough and semi-rough strains as well as 'chemically smooth' mutants. It is possible that the latter strains have a chemical change in their lipopolysaccharide too minor to detect by our means of analysis.

Our work with these mutants and a number of rough strains not obtained by selection for antibiotic resistance indicates that any loss of sugars from the lipopolysaccharide of enteric bacilli is accompanied by increased sensitivity to the bactericidal effect of antibody and complement. The rough strains are uniformly avirulent (see also Herzberg & Green, 1964) whereas the semi-rough strains, *Salmonella enteritidis* and *S. typhimurium* P2-173, are of intermediate virulence.

The smooth serum-sensitive mutants are nearly as virulent for mice and guinea pigs as the parent serum-resistant strains (Roantree & Steward, 1965; Steward, Collis & Roantree, 1966). The investigation of their virulence uncovered the fact that most guinea pigs and mice used in this laboratory did not possess measurable natural bactericidal antibody against *Salmonella typhimurium* or *S. enteritidis*. Immunization with the homologous species led to high levels of bactericidal antibody and excellent protection against either the serum-resistant parents or the serum-sensitive mutants (Steward *et al.* 1966). Present information, then, indicates that virulence of the serum sensitive smooth mutants is very like that of the parent strains. Perhaps a difference in virulence might be observed if animals with intermediate levels of antibody were tested.

It is well established that penicillin interferes with the synthesis of the mucopeptide portion of the cell wall (Park & Strominger, 1957; Rogers & Mandelstam, 1962; Izaki, Matsushashi & Strominger, 1966). We have derived serum-sensitive mutants by selection with cephalosporin and bacitracin, as well as with penicillin. Less extensive selections on neomycin, streptomycin and chloramphenicol have yielded no such mutants.

In our present state of ignorance of the relationship of the rigid mucopeptide layer to the lipopolysaccharide, it is difficult to see why some mutations to penicillin resistance should involve a change in the latter layer.

This investigation was supported by United States Public Health Service Research Grant no. 02755 and Training Grant no. 82, from the National Institute of Allergy and Infectious Diseases.

REFERENCES

- BECKMANN, I., SUBBAIAH, T. V. & STOCKER, B. A. D. (1964). Rough mutants of *Salmonella typhimurium*. 2. Serological and chemical investigations. *Nature, London*. **301**, 1299.
- DISCHE, Z. (1953). Qualitative and quantitative colorimetric determination of heptoses. *J. biol. Chem.* **204**, 983.
- FREEMAN, G. G. (1942). The preparation and properties of a specific polysaccharide from *Bact. typhosa* Ty 2. *Biochem. J.* **36**, 340.
- HERZBERG, M. & GREEN, J. H. (1964). Characteristics of cell walls of smooth strains of *Salmonella typhimurium* and derived rough variants. *J. gen. Microbiology*. **35**, 421.
- IZAKI, K., MATSUHASHI, M. & STROMINGER, J. L. (1966). Glycopeptide transpeptidase and D-alanine carboxy-peptidase: a penicillin-sensitive enzymatic reactions. *Proc. natn. Acad. Sci., U.S.A.* **55**, 566.
- KABAT, E. A. & MAYER, M. M. (1961). *Experimental Immunochemistry*, 2nd ed. Springfield, Illinois: C. C. Thomas.
- LINGELSHIM, W. v. (1913). Zur Frage der Variation der Typhusbacillen und verwandter Gruppen. *Zentbl. Bakt. ParasitKde, Abt. I Orig.* **68**, 577.

- LÜDERITZ, O., KAUFFMANN, F., STIERLIN, H. & WESTPHAL, O. (1960). Zur Immunochemie der O-Antigene von Enterobacteriaceae. II. Vergleich der Zuckerbausteine von Salmonella S-, R-, und T-Formen. *Zentbl. Bakt. ParasitKde, Abt. T. Orig.* **179**, 180.
- LÜDERITZ, O., STAUB, A. M. & WESTPHAL, O. (1966). Immunochemistry of O and R antigens of Salmonella and related Enterobacteriaceae. *Bact. Rev.* **30**, 192.
- LÜDERITZ, O. & WESTPHAL, O. (1966). The significance of enterobacterial mutants for the chemical investigation of their cell-wall polysaccharides. *Angew. Chem. int. Edit.* **5**, 198.
- MICHAEL, J. G. & BRAUN, W. (1958). Relationships between bacterial resistance to serum and penicillin. *Proc. Soc. exp. Biol. Med.* **97**, 104.
- NAIDE, Y., NIKAIKO, H., MÄKELÄ, P. H., WILKINSON, R. G. & STOCKER, B. A. D. (1965). Semi-rough strains of Salmonella. *Proc. natn. Acad. Sci., U.S.A.* **53**, 147.
- OSBORN, M. J., ROSEN, S. M., ROTHFIELD, L., ZEZEZNICK, L. D. & HORECKER, B. L. (1964). Lipopolysaccharide of the Gram-negative cell wall. *Science, N.Y.* **145**, 783.
- PARK, J. T. & STROMINGER, J. L. (1957). Mode of action of penicillin. Biochemical basis for the mechanism of action of penicillin and for its selective toxicity. *Science, N.Y.* **125**, 99.
- REED, L. J. & MUENCH, H. (1938). A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.* **27**, 493.
- ROANTREE, R. J. & STEWARD, J. P. (1965). Mutations to penicillin resistance in Enterobacteriaceae that affect sensitivity to serum and virulence for the mouse. *J. Bact.* **89**, 630.
- ROGERS, H. J. & MANDELSTAM, J. (1962). Inhibition of cell-wall-mucopolysaccharide formation in *Escherichia coli* by benzylpenicillin and 6 [D(-)- α -aminophenylacetamido] penicillanic acid (ampicillin). *Biochem. J.* **84**, 299.
- ROSEN, S. M., ZEZEZNICK, L. D., FRAENKEL, D., WIENER, I. M., OSBORN, M. J. & HORECKER, B. L. (1965). Characterization of the cell wall lipopolysaccharide of a mutant of *Salmonella typhimurium* lacking phosphomannose isomerase. *Biochem. Z.* **342**, 375.
- ROWLEY, D. (1956). Rapidly induced changes in the level of nonspecific immunity in laboratory animals. *Br. J. exp. Path.* **37**, 223.
- STAUB, A. M. (1960). Bases chimiques de la spécificité immunologique des antigènes des Salmonella. *Annls Inst. Pasteur, Paris* **98**, 814.
- STAUB, A. M., TINELLI, R., LÜDERITZ, O. & WESTPHAL, O. (1959). Role de quelques sucres et en particulier des 3-6 dideoxyhexoses dans la spécificité des antigènes O du tableau de Kauffmann-White. *Annls Inst. Pasteur, Paris* **96**, 303.
- STEWART, J. P., COLLIS, L. R. & ROANTREE, R. J. (1966). Effects of immunization of guinea pigs lacking bactericidal antibody against *Salmonella enteritidis*. *J. Immunol.* **97**, 224.
- STOCKER, B. A. D., STAUB, A. M., TINELLI, R. & KOPACKA, B. (1960). VI. Étude de l'antigène I présent sur deux Salmonella des groupes B et E. *Annls Inst. Pasteur, Paris* **98**, 505.
- SUBBAIAH, T. V. & STOCKER, B. A. D. (1964). Rough mutants of *Salmonella typhimurium*. 1. Genetics. *Nature, Lond.* **201**, 1298.
- THJØTTA, R. & WAALER, E. (1932). Dissociation and sensitiveness to normal serum in dysentery bacilli of type III. *J. Bact.* **24**, 301.
- TOPLEY, W. C. C. & AYRTON, J. (1924). The segregation of biological factors in *B. enteritidis* (Aertrycke). *J. Hyg., Camb.* **22**, 305.
- WESTPHAL, O., LÜDERITZ, O. & BISTER, F. (1942). Über die Extraktion von Bakterien mit Phenol/Wasser. *Z. Naturforsch.* **7b**, 148.