Immunochemical Studies on Cell-wall Polysaccharide of Rough Mutants of *Salmonella typhimurium*

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SUMMARY

Immunological studies were made in order to characterize the lipopolysaccharides of rough mutants of *Salmonella typhimurium* and *S. minnesota*. Groups of mutants, specified by their phage pattern, also showed group homogeneity with respect to their immunological specificity, especially when using the passive haemagglutination inhibition technique. Cross-reactions between mutants belonging to different phage groups could generally be explained on the basis of the carbohydrate composition of the lipopolysaccharide.

INTRODUCTION

Lipopolysaccharides isolated from rough strains of different *Salmonella* species have been serologically differentiated by means of haemagglutination and haemagglutination inhibition techniques (Beckmann, Lüderitz & Westphal, 1964; Lüderitz, Beckmann & Westphal, 1964; Lüderitz *et al.* 1966*a, b*). In contrast to the numerous O-specificities encountered in the wild-type O-antigens of different *Salmonella* strains only a few specificities are found in R-antigens regardless of the serotype from which the mutant has been derived. Rough mutants of *Salmonella* strains have also been defined by their sensitivity to rough-specific phages (Subbaiah & Stocker, 1964; Wilkinson & Stocker, 1964; Lüderitz *et al.* 1966*a*; Brandis, 1966). Wilkinson & Stocker used a collection of eight R-specific phages, of which four were obtained from Brandis, in an attempt to separate rough mutants of *Salmonella typhimurium* strain LT2. More than eight different phage patterns could be established. Results obtained by Lüderitz *et al.* (1966*a*) indicated a certain relationship between the phage pattern, the sugar composition of the lipopolysaccharide and the reactivity with R-antisera in groups of rough mutants from *S. minnesota* and *S. riuu*. The present paper reports on the further characterization of the *S. typhimurium* rough mutants, investigated chemically in a preceding paper (Holme, Lindberg, Garegg & Onn, 1968), with the aid of serological techniques and the sensitivity to different phages.

METHODS

Strains. The *Salmonella typhimurium* and *S. minnesota* strains were the same as used in the preceding paper by Holme *et al.* (1968).

Lipopolysaccharides. The strains were grown and the lipopolysaccharides extracted

from the bacteria by the phenol+water procedure (Westphal, Lüderitz & Bister, 1952). The lipopolysaccharides were purified by three cycles of washing and ultracentrifugation at 105,000g for 4 hr in a Christ Omega preparative ultracentrifuge. The pellet was lyophilized and the lipopolysaccharide resuspended in saline before use.

Antisera. Antisera were obtained by injecting rabbits intravenously three times a week for 9 weeks with heat-killed suspensions of the bacteria. Serum specific for the O-antigenic factor 4 of group B was obtained from the Salmonella Centre, National Bacteriological Laboratory, Stockholm. Serum against *Salmonella typhimurium* TV 119, obtained by immunization of a horse, was obtained from Dr M. Raynaud (Institut Pasteur, Paris). All sera were inactivated at 56° for 30 min., absorbed with packed washed sheep red cells at 37° for 2 hr and stored at -20° until use.

Passive haemagglutination. Sheep red cells were collected aseptically in sterile Alsever solution and washed three times in phosphate buffered saline before use. The lipopolysaccharides were treated with sodium hydroxide to enhance the affinity to red cells. The procedure was done according to Beckmann *et al.* (1964).

For sensitization 10 ml. saline containing 250 µg. alkali-treated lipopolysaccharide were added to 1.25 ml. of a 4% (v/v) suspension of washed sheep red cells. After incubation at 37° for 30 min. the sensitized red cells were washed 3 times with saline and finally diluted to 10 ml. with phosphate-buffered saline. Serial twofold dilutions of serum were prepared with phosphate-buffered saline (pH 7). To each tube containing 0.2 ml. serum dilution an equal volume of sensitized red cells was added. The tubes were kept at room temperature and read after 3 and 18 hr. The haemagglutinating titre was determined as the last tube showing macroscopically visible haemagglutination. Serum with unsensitized sheep red cells, and sensitized red cells in saline only, were used as controls in each experiment. The reactions were identical or deviated in only one dilution step on repeated tests.

Haemagglutination inhibition. A twofold dilution series of the lipopolysaccharide used as inhibitor was added to appropriately diluted sera. The serum dilution used was 4-8 haemagglutinating units (serum diluted 4-8 times less than the highest dilution causing haemagglutination). Serial twofold dilutions of the lipopolysaccharide with concentrations ranging from 256 to 1 µg./ml. were prepared in saline; 0.2 ml. of each lipopolysaccharide dilution was added to an equal volume of the appropriate serum dilution. The mixtures were incubated at 37° in a water bath for 30 min. Thereafter 0.2 ml. of sensitized sheep red cells was added to each mixture. Haemagglutination was read after incubation for 30 min. at 37° and 3 hr at room temperature. The lowest concentration of lipopolysaccharide giving a total inhibition of the haemagglutination was determined. The haemagglutination titre without inhibition was determined in each experiment. Homologous inhibition with the lipopolysaccharides used for sensitization was also done, as well as the controls described in the preceding section.

Phage classification. The strains were characterized by their pattern of sensitivity to a collection of phages. The phages used were classified as smooth-specific (P 22), rough specific (P 221, 6 SR, Br 2, Ffm, Br 60 and C 21) and phages active on smooth and rough strains (P 22h and FO-1). The phages were obtained from Professor H. Brandis (Hygiene-Institut der Universität Göttingen) and Professor B. A. D. Stocker (Dept. of Medical Microbiology, Stanford, California, U.S.A.). The phages were propagated by the soft-agar layer method of Adams (1959) or in broth culture. The

lysates of the phages contained 10^8 – 10^{10} plaque-forming units (p.f.u.)/ml. Freshly prepared lawns of the strains on nutrient agar (Difco) were tested for sensitivity to tenfold dilution steps of the phages by the spot technique. Readings were carried out after 5 and 18 hr incubation at 37° .

RESULTS

Phage classification of the strains

Drs Wilkinson & Stocker (personal communication) used a collection of phages for grouping rough mutants of *Salmonella typhimurium*. Differences in the sensitivity

Table 1. Phage patterns of (1) wild-type *Salmonella typhimurium* 395MS and derived mutants, (2) known mutants of *S. typhimurium* LT2 and (3) known mutants of *S. minnesota* S99. Phages: smooth specific (P 22), smooth and rough specific (P 22h, FO-1) and rough specific (P 22I, 6 SR, Br 2, Ffm, Br 60, C 21).

Phage patterns obtained by using the collection of nine phages in spot test on the <i>S. typhimurium</i> and <i>S. minnesota</i> strains. +, Clear or turbid confluent lysis with the phage undiluted, scattered plaques in higher dilutions. —, No lytic effect of the phages.										
Strain	Phage									Classification
	P22	P22h	FO-I	P22I	6 SR	Br 2	Ffm	Br 60	C 21	
<i>S. typhimurium</i>										
395MS	+	+	+	—	—	—	—	—	—	Smooth
395MR0	—	—	+	+	+	+	+	+	—	Rough sensitive
395MR1	—	—	—	+	—	+	+	+	—	Rough resistant-1
395MR2	—	—	+	+	+	+	+	+	—	Rough sensitive
395MR3	—	—	+	+	+	+	+	+	—	Rough sensitive
395MR4	—	—	+	+	+	+	+	+	—	Rough sensitive
395MR5	—	—	—	+	—	—	+	+	—	Rough resistant-2
395MR6	—	—	—	+	—	—	+	+	—	Rough resistant-2
395MR7	—	—	—	—	—	—	+	+	—	Rough resistant-2
395MR8	—	—	—	+	—	—	+	+	—	Rough resistant-2
395MR9	—	—	±	+	—	—	+	+	+	Galactose-epimerase-less
395MR10	—	—	—	+	—	—	+	+	+	Glucosyl-I-transferase-less
TV119	—	—	+	+	+	+	+	+	—	Rough sensitive
TV148	—	—	—	+	—	—	+	+	—	Rough resistant-2
TV160	—	—	—	+	—	+	+	+	—	Rough resistant-1
TV161	—	—	—	+	—	+	+	+	—	Rough resistant-1
TV163	—	—	+	+	+	+	+	+	—	Rough sensitive
TV166	—	—	—	+	—	+	+	+	—	Rough resistant-1
TV208	+	+	+	+	+	—	—	+	—	Not recognized
TV225	—	—	+	+	+	+	+	+	—	Rough sensitive
TV226	—	—	+	+	+	+	+	+	—	Rough sensitive
TV227	—	—	+	+	+	+	+	+	—	Rough sensitive
<i>S. minnesota</i>										
S99	—	—	+	—	—	—	—	—	—	Not recognized
R60	—	—	+	—	—	—	+	+	—	Rough sensitive
R345	—	—	—	—	—	—	+	+	—	Rough resistant-1

to the various phages may depend on the composition of the cell-wall lipopolysaccharide of the mutants. Thus, the sensitivity to phage P 22 was limited to the smooth strains of *Salmonella* having the O-antigen 12 (Zinder & Lederberg, 1952). Phage FO-1 lysed smooth strains and mutants belonging to chemotype Ra and was therefore designated as a SR-specific phage. Six phages lysed only rough mutants and were

named R-specific. The strains used in the present work were grouped on the basis of their sensitivity pattern to nine phages. The phage patterns are represented in Table 1. The designation of the patterns is in accordance with Wilkinson & Stocker (1964).

Smooth specific. The parent strain, *Salmonella typhimurium* 395MS was lysed by the smooth specific phage P 22 and the smooth and rough specific phages P 22h and FO-1. The phage pattern of strain TV208 was not recognized since the strain was lysed by phages from all three groups.

Rough sensitive. Nine of the ten strains included in this group displayed a pattern characterized by lysis with the phages FO-1, P 221, 6 SR, Br 2, Ffm and Br 60. *Salmonella minnesota* R60 was lysed only by the phages FO-1, Br 2, Ffm and Br 60, but adsorbed the phages P 221 and 6 SR. Therefore this strain was classified as rough sensitive.

Rough resistant-1. Mutants in this group were lysed by the rough specific phages P 221, Br 2, Ffm and Br 60. This group contained four strains: R1, TV160, TV161 and TV166. *Salmonella minnesota* R345 was included in this group since it adsorbed phage P 221.

Rough resistant-2. The four strains in this group, R5, R6, R8 and TV148, were separated from the rough resistant-1 group by their resistance to phage Br 2. The mutant 395MR7 was included in this phage pattern although it was resistant to phage P 221 since the receptor structure for this phage appears to be independent of the polysaccharide structure (unpublished).

UDP-Galactose-4-epimerase-less mutant pattern. Only one strain, R9, was represented in this class. This galactose non-fermenter was sensitive to phages P 221 Ffm, Br 60 and C 21.

Glucosyl-I-transferase-less mutant pattern. The mutant R10 showed the same phage pattern as strain R9. The designation glucosyl-I-transferase-less is based on the phage pattern, its ability to ferment galactose and the presence of S-specific polysaccharides in the cytoplasmic fraction.

Haemagglutination and haemagglutination inhibition reactions

The lipopolysaccharides of the *Salmonella typhimurium* and *S. minnesota* strains were tested against 11 antisera with passive haemagglutination technique (Table 2). The homologous haemagglutination titres ranged between 1/640 and 1/1280. Duplicate or triplicate determinations were made in all experiments with positive reactions. The variability did not in any case exceed one dilution step. Sera drawn from rabbits before immunization showed no reactions in dilution exceeding 1/40. Titres 1/160 or above were considered as significantly positive reactions.

The lipopolysaccharides were further investigated in haemagglutination inhibition tests against eight homologous systems, representative for the smooth form and the different groups of rough mutants. Homologous inhibition was observed with the lipopolysaccharides in concentrations ranging from less than 1 to 4 $\mu\text{g./ml.}$ (Table 3).

The results of the immunological studies will be presented in a following section of this paper.

Table 2. *Passive haemagglutination of Salmonella antisera with Salmonella lipopolysaccharides*

Haemagglutination systems. Data recorded are the highest dilution of serum, before addition of the lipopolysaccharide sensitized erythrocytes, giving visible haemagglutination. Entries left blank indicate negative reaction or reaction only in the dilution 1/40.

Lipopolysaccharide	Salmonella factor	Reciprocal of titre of antiserum to <i>S. typhimurium</i>						<i>S. minnesota</i>		
		O-4	TV208	TV119	TV163	TV160	R6	R9	R60	R345
<i>S. typhimurium</i>										
395MS	1280	160	320	80	80	.	160	.	.	
395MR0	.	.	1280	640	.	.	.	80	.	
395MR1	.	80	.	.	160	160	80	.	80	
395MR2	.	.	1280	640	.	.	.	160	.	
395MR3	.	80	1280	640	.	80	.	320	.	
395MR4	160	.	1280	640	.	.	.	160	.	
395MR5	160	80	80	.	160	160	160	.	.	
395MR6	.	.	.	.	.	640	.	.	.	
395MR7	.	.	.	.	.	320	.	.	.	
395MR8	.	.	.	.	.	160	.	.	.	
395MR9	320	.	.	.	80	320	640	.	.	
395MR10	.	.	.	.	.	.	.	.	.	
TV160	.	.	.	.	640	80	.	.	80	
TV163	320	80	1280	1280	160	80	80	.	.	
TV208	640	640	160	.	160	80	160	80	.	
<i>S. minnesota</i>										
R60	.	.	1280	1280	80	80	.	640	.	
R345	.	.	.	.	160	80	.	.	640	

Table 3. *Haemagglutination inhibition of Salmonella antisera with Salmonella lipopolysaccharides*

Haemagglutination inhibition systems: homologous lipopolysaccharide and serum. Data recorded are the minimal concentration ($\mu\text{g./ml.}$) of lipopolysaccharide from the *S. typhimurium* and *S. minnesota* strains needed for inhibition of the homologous system. Entries left blank indicate that lipopolysaccharide concentrations higher than $256 \mu\text{g./ml.}$ were needed for the inhibition.

Lipopolysaccharide	<i>S. typhimurium</i>					<i>S. minnesota</i>	
	TV208	TV163	TV160	R6	R9	R60	R345
<i>S. typhimurium</i>							
395MS	16	.	.	.	.	.	.
395MR0	.	8	.	.	.	.	.
395MR1	.	.	8	.	.	.	.
395MR2	.	8	.	.	.	.	.
395MR3	.	4	.	.	.	.	.
395MR4	.	8	.	.	.	.	.
395MR5	.	.	.	4	.	.	.
395MR6	.	.	.	< 1	64	.	.
395MR7	.	.	.	32	.	.	.
395MR8	.	.	.	4	32	.	.
395MR9	.	.	.	.	< 1	.	.
395MR10	.	.	.	.	128	.	.
TV160	.	.	4	.	.	.	.
TV163	.	4	.	.	.	.	.
TV208	4	.	.	.	.	.	.
<i>S. minnesota</i>							
R60	.	8	.	.	.	< 1	.
R345	.	.	.	.	128	.	< 1

Table 4. *Salmonella typhimurium* and *S. minnesota* smooth and rough strains: phage pattern, composition of cell-wall polysaccharide and serological cross-reactions

values are given in % of total polysaccharide. The antisera causing haemagglutination in a dilution 1/160 are recorded as well as the homologous lipopolysaccharide-antiserum systems being inhibited by the lipopolysaccharides tested.														
Strain	Phage pattern	Approximate molar ratios of sugars							GN (%)	Haemagglutination of antiserum	LPS-antiserum system	Haemagglutination inhibition of LPS-antiserum system		
		Hep	Glu	Gal	Man	Rha	Abe							
<i>S. typhimurium</i> 395 MS	Smooth	< 1	1	0.7	0.7	0.6	0.3	3.6	0-4, R9, TV119, TV208	TV208	nt			
<i>S. minnesota</i> s99	Not recognized	1	1	2.4	nd	nd	nd	nt	nt	nt	TV208			
<i>S. typhimurium</i> TV208	Not recognized	1	1.3	4.9	3.4	2.4	1.3	5.2	0-4, R9, TV119, TV160, TV208	TV208	nt			
<i>S. typhimurium</i> 395 MR 0 395 MR 2 395 MR 3 395 MR 4 TV163 TV225 TV R60	Rough sensitive	1	1	1	0.1	< 0.1	< 0.1	16.7	TV119, TV163	TV163	TV208			
		1	0.6	1	< 0.1	< 0.1	< 0.1	2.6	TV119, TV163, R60	TV163	TV163			
		1	0.5	0.5	< 0.1	< 0.1	< 0.1	5.0	TV119, TV163, R60	TV163	TV163			
		1	1	1	0.2	0.1	< 0.1	5.0	0-4, TV119, TV163, R60	TV163	TV163			
		1	1	1	0.3	0.3	0.1	3.3	0-4, TV119, TV160, TV163	TV163	TV163			
<i>S. minnesota</i> R60		1	0.9	1	0.2	0.1	< 0.1	2.7	nt	nt	nt			
		1	1.4	1.5	nd	nd	nd	nt	TV119, TV163, R60	TV163, R60	TV163, R60			
<i>S. typhimurium</i> 395 MR 1 TV160 TV161	Rough resistant-1	1	0.5	0.8	0.1	< 0.1	< 0.1	1.4	R6, TV160	TV160	TV160			
		1	0.7	1	0.2	0.2	< 0.1	1.1	TV160	TV160	TV160			
		1	0.6	0.9	0.3	0.1	< 0.1	0.6	nt	nt	nt			
<i>S. minnesota</i> R345		1	0.7	1.7	nd	nd	nd	nt	TV160, R345	R9, R345	R9, R345			
<i>S. typhimurium</i> 395 MR 5 395 MR 6 395 MR 7 395 MR 8	Rough resistant-2	1	0.8	1.1	0.8	0.5	0.2	1.8	0-4, R6, R9, TV160	R6	R6			
		1	0.7	1	0.4	0.3	0.2	1.0	R6	R6	R6			
		1	0.6	0.5	0.2	< 0.1	< 0.1	2.1	R6	R6	R6			
		1	0.4	0.4	0.1	< 0.1	< 0.1	0.8	R6	R6	R6			
<i>S. typhimurium</i> 395 MR 9	Galactose-epimerase-less	1	1.1	0.1	0.1	< 0.1	< 0.1	0.7	0-4, R6, R9	R9	R9			
<i>S. typhimurium</i> 395 MR 10	Glucosyl-I-transferase-less	1	< 0.1	0.1	0.2	< 0.1	< 0.1	1.3	—	R9	R9			

nd = not detected; nt = not tested; — = negative results

*Correlation between chemical composition, sensitivity to phages
and immunochemical investigations*

In Table 4 the results of the different methods used for characterization of the rough mutants are summarized. The phage patterns which were obtained have been used as a basis for classification because they offered the most simple overall view.

Smooth specific pattern. The cell-wall polysaccharide from the parent strain *Salmonella typhimurium* 395MS contained small amounts of ribose and the S-specific sugars. The phage pattern was smooth specific. In haemagglutination tests positive reactions were observed with sera against the O-antigen 4, the Ra mutants TV119 and TV208 as well as the Rc mutant R9. Haemagglutination inhibition showed a cross-reaction with the TV208 system. *Salmonella minnesota* S99, only lysed by the FO-1 phage, had heptose:glucose:galactose ratios of 1:1:2.4.

Salmonella typhimurium TV208. This strain was originally described as a Ra mutant (Nikaido, Nikaido, Subbaiah & Stocker, 1964). The lipopolysaccharide contained great amounts of glucose, galactose, mannose, rhamnose and abequose compared to the other Ra-mutants. In serological tests TV208 showed cross-reactions with S-specific as well as several R-specific sera. Also in phage typing the strain behaved as both a smooth and rough strain. Its sensitivity to the phage P 22 indicated the presence of antigen 12 of the S-specific repeating unit. In contrast to the smooth form the strain was sensitive to three of the rough-specific phages.

Rough sensitive pattern. Four of the mutants in this group, R0, R4, TV163 and TV225, had heptose:glucose:galactose molar ratios of 1:1:1 whereas for three of the mutants the figures varied from 1:0.5:0.5 for R3, 1:0.6:1 for R2 and 1:1.4:1.5 for *Salmonella minnesota* R60. The content of *N*-acetyl-glucosamine in the R0 mutant was much higher than in any of the other *S. typhimurium* strains. Small amounts of mannose, rhamnose and abequose were found in all the mutants with the exception of *S. minnesota* R60. In serological tests the strains were fairly regular with one notable exception. The *S. minnesota* R60 homologous system was not inhibited by any of the lipopolysaccharides from the six *S. typhimurium* mutants of this group.

Rough resistant-1 pattern. This group contained four strains, *Salmonella typhimurium* 395MR1, TV160, TV161 and *S. minnesota* R345. The polysaccharide composition differed from that of the rough sensitive group as the strains contained a significantly smaller amount of glucosamine. Again the S-specific sugars were detected in small amounts. In serological tests cross-reactions were detected with the TV160 antiserum. Antiserum against *S. minnesota* R345, the test strain for RI specificity according to Lüderitz *et al.* (1966a), showed weak reactions in the haemagglutination test with the R1 and TV160 lipopolysaccharides.

Rough resistant-2 pattern. The phage pattern of strains in this group differed from the rough resistant-1 group as the strains were not lysed by phage Br 2. *Salmonella typhimurium* 395MR5 and R6, selected for their resistance to phage FO-1, had heptose:glucose:galactose molar ratios of approximately 1:0.75:1 and appreciable amounts of mannose, rhamnose and abequose. In haemagglutination tests lipopolysaccharide from R5 cross-reacted with O-factor 4, R6, R9 and TV160 sera whereas R6 only showed the homologous reaction. In haemagglutination inhibition tests lipopolysaccharide from R6 inhibited the R9 system. The mutants R7 and R8, selected for their resistance to the Lilleengen phage 4, had heptose:glucose:galactose molar ratios of approxi-

mately 1:0.5:0.5 with smaller amounts of the S-specific sugars than the strains R5 and R6. Lipopolysaccharide from R7 and R8 cross-reacted with R6 antisera in haemagglutination tests. The homologous systems R6 and R9 were both inhibited by lipopolysaccharide from R8.

UDP-Galactose-4-epimerase-less pattern. The only strain in this class, R9, was characterized by its sensitivity to phage C21 in addition to the phages P 221, Ffm and Br 60. The polysaccharide of this strain contained heptose and glucose in a 1:1 ratio and small amounts of galactose and S-specific sugars. Upon addition of galactose the strain R9 developed smooth specific characteristics as judged by colony morphology, agglutination reactions and phage pattern. Sometimes this strain showed an unexpected sensitivity to phage FO-1 in galactose-free media but not to the phages 6 SR and Br 2 at the same time. In haemagglutination tests the lipopolysaccharide cross-reacted with O-factor 4 and R6 antisera.

Glucosyl-I-transferase-less mutant pattern. *Salmonella typhimurium* 395MR10 was a galactose-fermenter with the same phage pattern as the UDP-galactose-epimerase-less mutant R9. Its polysaccharide contained small amounts of galactose, mannose and traces of glucose, rhamnose and abequose. The R10 lipopolysaccharide did not show cross-reactions in haemagglutination tests but inhibited the R9 system in high concentrations.

DISCUSSION

Attempts to classify rough mutants of *Salmonella* according to both the chemical composition, serological specificity and sensitivity to phages were made on *Salmonella minnesota* and *S. riu* mutants by Lüderitz *et al.* (1966a). The sugar composition of the lipopolysaccharides of the rough mutants separated them into five groups. Haemagglutination inhibition tests revealed that many antisera cross-reacted with lipopolysaccharides extracted from rough mutants belonging to different chemotypes.

The results presented in a preceding paper (Holme *et al.* 1968) showed that the rough mutants of *Salmonella typhimurium* in addition to the sugars of the basal core also contained the S-specific sugars in varying amounts. It was not possible, on the basis of the molar ratios of the sugar components calculated for the different *S. typhimurium* and *S. minnesota* rough mutants, to separate the strains into distinct groups. In haemagglutination and haemagglutination inhibition tests, presented in this paper, the lipopolysaccharides of the different rough strains showed a variety of cross-reactions which also made a separation between the mutants difficult. The overall picture was therefore based on the phage patterns obtained when applying the collection of phages used in an investigation of the phage susceptibility of mutants of *S. typhimurium* LT2 by Drs Wilkinson & Stocker (personal communication).

With the use of this collection of nine phages the rough mutants of *Salmonella typhimurium* were separated into five groups. Two of the *S. typhimurium* mutants had phage patterns which had not been recognized earlier. The three *S. minnesota* strains also showed phage patterns not recognized earlier but were included into the groups as they adsorbed the corresponding phages. Each phage pattern was stable when the strains were retyped several times as well as when the strains were grown both in the absence and in the presence of glucose. This indicated that the mutants were stable rough forms as revertants to the smooth form would have given growth in the area lysed by the R-specific phages in spot tests.

The mutant *Salmonella typhimurium* TV208 was lysed by both the S-specific and some of the R-specific phages. This strain was originally described as a Ra mutant defective in the synthesis of TDP-rhamnose (Nikaido *et al.* 1964). These workers also suggested that strain TV208 showed a high frequency of reversion to the smooth form. The molar ratios of the sugar components of the TV208 polysaccharide revealed high amounts of the S-specific sugars as compared to the other Ra mutants. In serological investigations the strain showed both R and S specificity. The results would be compatible with the assumption that the observed S-specificity could be due to smooth revertants. In virulence tests, however, no mice were killed by strain TV208; if smooth revertants were frequent in cultures of τ , ν , 208 no mice would have survived. It appears that the strain TV208 is a non-virulent rough mutant with unusually high content of S-specific sugars, which confer susceptibility to the S-specific phage P 22 and serological cross-reactions with the smooth form. In the rough sensitive group the sugar composition was similar for four of the strains—R0, R4, TV163, TV225—with heptose:glucose:galactose ratios of 1:1:1, more than 2.5% of glucosamine in the polysaccharide and small amounts of mannose, rhamnose and abequose. Two of the mutants—R2 and R3—did not show the 1:1:1 ratios. The observed differences in carbohydrate composition were, however, not reflected in the serological reactions where the strains were quite homogeneous with the exception of *Salmonella minnesota* R60.

Fig. 1. Possible structure of core of *Salmonella* lipopolysaccharide. Hep = heptose, glu = glucose, gal = galactose, GNac = *N*-acetyl glucosamine, X = unidentified unit, possibly involved in RII specificity.

The lipopolysaccharides of all the Ra mutants inhibited the homologous *S. typhimurium* TV163 system in haemagglutination inhibition tests whereas the homologous *S. minnesota* R60 system was not inhibited by any of the *S. typhimurium* lipopolysaccharides. This non-reciprocal inhibition could be attributed to the possible attachment of an acid-labile unit 'X' to the terminal *N*-acetylglucosamine of the core polysaccharide (Fig. 1). This unit is supposed to be involved in the production of the complete core by *rfb* mutants of the antigenic R II character, i.e. *S. minnesota* R60 (Lüderitz *et al.* 1966a). The *rfa* mutant *S. typhimurium* TV163 contains both the glucosamine of the core and the cytoplasmic S-specific polysaccharide. The latter has been demonstrated by gel precipitation (unpublished). This strain therefore should not possess the 'X'-unit, which is indicated by the haemagglutination inhibition tests. However, as evidenced by the uniformity in phage pattern within this group the presence of the 'X'-unit does not appear to have any influence on the susceptibility of the strains to the different phages.

The eight mutants classified as chemotype Rb were complex in phage pattern, carbohydrate composition and serological specificity. The *Salmonella typhimurium* mutants with the rough resistant-1 pattern had heptose:glucose:galactose ratios of approximately 1:0.5:1, which indicates that the core ended with the gal I-residue (Fig. 1). The *S. minnesota* R345 mutant did not show the same sugar ratios and differed also in serological specificity. The mutants 395MR5, R6, R7 and R8 showed the rough resistant-2 pattern and were rather homogeneous in haemagglutination inhibition

tests. The heptose:glucose:galactose ratios for the R7 and R8 mutants were approximately 1:0.5:0.5, which could be due to that either the gal I or gal II residue of the core is lacking. This would explain the difference between the two classes within the chemotype Rb with respect to phage pattern and serological specificity. The heptose:glucose:galactose ratios for the R5 and R6 mutants were not the same as for the R7 and R8 mutants, which could be due to a high degree of leakiness resulting in the synthesis of a few S-specific side chains. The side chains do not, however, prevent the reaction with R-specific antisera.

The UDP-galactose-4-epimerase-less mutant R9 contained small amounts of galactose and S-specific sugars. Possible explanations of this finding were discussed in the preceding paper by Holme *et al.* (1968). The assumption that rhamnose, mannose and abequose were linked in a configuration determining O-specificity was supported by the finding of cross-reactions between the anti R9 serum and lipopolysaccharides from the smooth specific strain *Salmonella typhimurium* 395MS and *S. typhimurium* TV208. The lipopolysaccharide from R9 also reacted with the O-factor 4 serum in haemagglutination tests. Furthermore this strain showed an unexpected sensitivity to the FO-1 phage on some occasions. This FO-1 sensitivity was regularly accompanied by a lysis with phage C 21 which otherwise has been reported to lyse only mutants of the Rc and Rd chemotypes (Shedlovsky & Brenner, 1963; Stocker, Wilkinson & Mäkelä, 1966). No lysis with phage P 22 or P 22h was ever observed. The fact that this strain could be sensitive to both FO-1 and C 21 indicates that the FO-1 receptor structure can be present without conferring a masking effect on the C 21 receptor structure. The same inconstancy in phage pattern has also been observed for the UDP-galactose-4-epimerase-less mutant *S. typhimurium* SL1070. Thus for the *S. typhimurium* mutants the phage pattern was constantly rough, the only exception being the strain TV208. The presence of small amounts of the S-specific sugars was not accompanied by any adsorption of phages P 22 or P 22h.

Naide *et al.* (1965) described a class of mutants in *Salmonella* intermediate between the smooth and the rough form both in biological properties and lipopolysaccharide composition. They were named semi-rough (SR) mutants and displayed a smooth colony type on solid media, the O-antigen 4 was present in all mutants and their recombinants, and the O-antigen 5 was detected in some. The S-specific sugars were present in small amounts in all the strains. Some of the strains used in the present investigation have characteristics which could indicate that they were SR-mutants, namely R4, R5 and TV163. SR mutants display typical phage patterns in addition to the characteristics described above (Drs Wilkinson & Stocker, personal communication). Both the mutants R4 and TV163 showed a phage pattern which was recognized as specific for the rough sensitive group. The mutants R5 and TV163 synthesize the soluble hapten which was not found in any of the SR-mutants (Naide *et al.* 1965). The results indicate that none of the mutants could be classified as semi-rough using the definition of Naide *et al.* (1965).

In haemagglutination tests cross-reactions were observed between mutants belonging to different phage groups. The presence of the cross-reacting antigens was reflected in the monosaccharide composition of the lipopolysaccharide. With the haemagglutination inhibition technique the reactions were more specific for each phage group.

Additional information on the structure of the polysaccharide core may be obtained

by the use of the different R-specific phages in inhibition experiments with lipopolysaccharides extracted from the rough mutants. The changing susceptibility to the rough specific phages observed for the different mutants indicates that the monosaccharide components determine the specificity of the phage receptors.

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