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MOLECULAR IMMUNOLOGICAL HETEROGENEITY OF THE *Salmonella zuerich* [1, 9, 12, (46), 27] CELL-WALL POLYSACCHARIDES*

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ABSTRACT

Extraction of O specific polysaccharide from *S. zuerich* leads to three fractions (ZA, ZB, ZC). Polysaccharide ZB carries specificities 1, 27, and 46, present on the *Salmonella* cells. It exhibits a factor 27 that is very similar to that present on the *S. typhi* T_2 1^- 27^+ polysaccharide, a factor 1 that is close to that present on *S. senftenberg* polysaccharide, and a factor 46 that gives a very weak cross-reaction with anti-46 antibodies. Polysaccharides ZA and ZB are immunologically different and ZB contains two distinct fractions: ZB 1^- devoid of O factor 1 and carrying the specificities 46 and 27 mostly, if not completely, on the same molecule (46, 27); and ZB 1^+ carrying O factors 1, (46), 27. ZB 1^+ is composed of at least two different molecules: [1, (46)], precipitable with anti-1 antibodies but only coprecipitable with anti-46 antibodies; and (1, 27) precipitable with both anti-1 and anti-27 antibodies. Molecules [1, (46)] precipitate only part of the anti-1 antibodies precipitable by (1, 27). The smaller precipitation of anti-27 antibodies (when factor 27 is present together with factor 1 on the same molecule) and the coprecipitation, instead of precipitation, of anti-46 antibodies (when factors 46 and 1 are present on the same molecule) may be explained by a sterical hindrance between O-factors 1 and 27, and 1 and 46. The molecular, immunological heterogeneity of the polysaccharides extracted from *S. zuerich* would result from the presence on the cells of two kinds of O polysaccharides: one with, the other without O factor 1, which is related to the presence of a side-chain of an α-D-glucosyl residue. A structure for *S. zuerich* polysaccharide is proposed.

INTRODUCTION

In *Salmonella*, Gram-negative *Enterobacteriaceae*, the lipopolysaccharide located in the cell-wall is composed of a "smooth" or "O specific" polysaccharide, linked to a core polysaccharide that is bound to "lipid A". While the core region is common to all *Salmonella*, the O specific polysaccharide, which consists of repeating

*Dedicated to Professor Michael Heidelberger in honor of his 87th birthday.

units of oligosaccharides, is characteristic of the *Salmonella* strain and supports the serological specificities of the O antigen[1]. *S. typhi* T_2 (9, 12_2^-, 27^-) (D_1 group)[2] and *S. strasbourg* (9, 46) (D_2 group)[3,4] possess the same repeating-unit, which consists of a tetrasaccharide –(D-Gal→[Tyv]→D-Man→L-Rha)→D-Gal–, but differ in the linkage of the D-Gal→D-Man residue and in the anomeric configuration of the D-mannose residue. After conversion by phage 27, *S. typhi* T_2 27^+ acquires[5] the D-Gal-(1→6)-D-Man residue that is present on *S. strasbourg*. Thus, both polysaccharides share the same main-chain, except that the anomeric configuration of the D-mannose residue is α for *S. typhi* T_2 27^+ (O factor 27) and β for *S. strasbourg* (O factor 46) (Fig. 1).

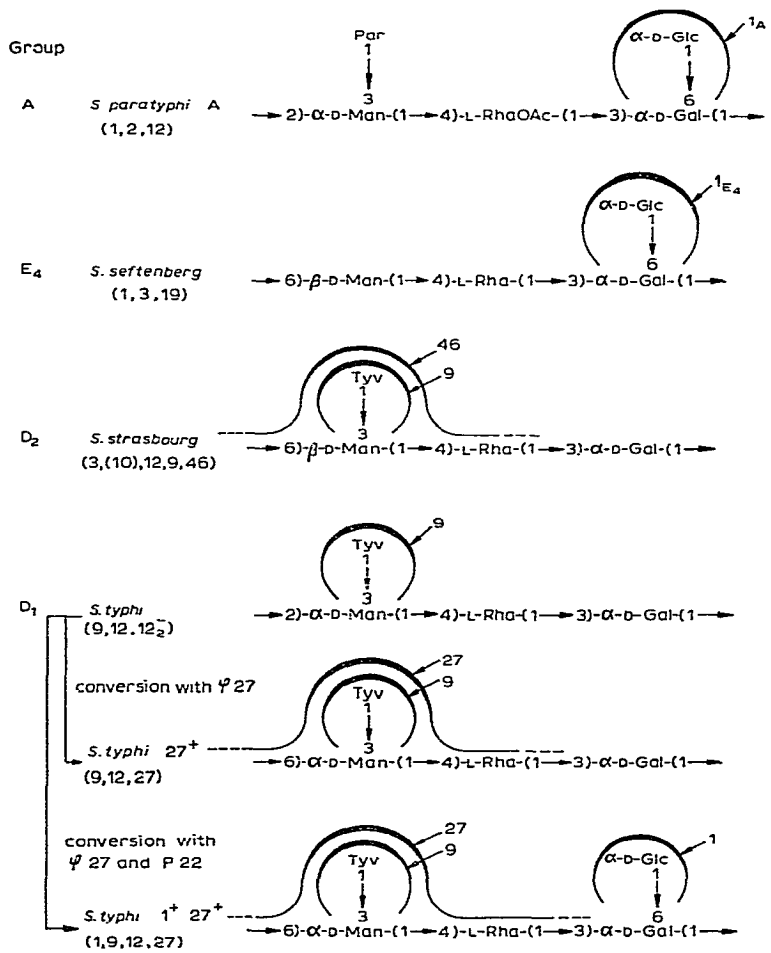

Fig. 1. Structure of some *Salmonella* O specific chains. Not common abbreviations: Par (3,6-dideoxy-D-*ribo*-hexose; paratose); Tyv (3,6-dideoxy-D-*arabino*-hexose; tyvelose).

S. zuerich [1, 9, 12, (46), 27] (D_3 group) is particularly interesting in the *Salmonella* evolution. Indeed, it possesses, according to the results of agglutination, at

the same time three factors: A factor (46) that is related to factor O 46 present on *S. strasbourg* (D_2 group, wild type); a factor 27 that is unrelated to any phage[6], although it is present on *S. typhi* only after phage φ (27) conversion; and a factor 1 that can also exist in a wild type, as in *S. senftenberg* (E_4 group)[7,8], although it is often related to the presence of a prophage, as in *S. typhi* T_2 1^+ 27^+ (D_1 group) or in *S. paratyphi* A (1, 2, 12) (A group)[1].

In this study, we have attempted to determine: (a) The degree of similarity between O factors 27, 46 of *S. zuerich* and those factors present on the polysaccharides of *S. typhi* T_2 27^+ and *S. strasbourg*, and (as regards to O factor 1) to which *Salmonella* groups is *S. zuerich* close: to *S. senftenberg* wild type, to P 22 & φ 27 converted *S. typhi*, or to P 22 converted *S. paratyphi* A. (b) Whether the *S. zuerich* O antigen consists of molecules containing chains having randomly distributed α and β-D-mannose residues, or chains containing only one kind of linkage (α or β), or of molecules containing both kinds of D-mannose residues present on distinct chains, since polymerization occurs by the transfer of a D-galactosyl residue from a phosphate ester to a D-mannose residue[9]. (c) Finally, the distribution of the O factor 1 on these different molecules.

MATERIALS AND METHODS

Organisms. — The strains *S. zuerich*, *S. strasbourg*, *S. typhi* T_2 1^- 27^+, *S. typhi* T_2 1^+ 27^+, *S. senftenberg*, and *S. paratyphi* A (1, 2, 12) were obtained from the Centre International des Salmonelles de l'Institut Pasteur (Dr. Le Minor).

Polysaccharides. — Mass cultures were grown on agar. The polysaccharides were extracted by the Freeman acetic acid technique[10], and the solutions were dialyzed and lyophilized, except for the polysaccharides of *S. zuerich*, which were obtained by precipitation from solutions with 1–10 vol. of ethanol and then reprecipitated with 98% acetic acid.

Extraction of specific polysaccharides. — Polysaccharides were specifically precipitated with monospecific antisera in the presence of a slight antigen excess; the precipitate was washed with saline solution at 4°, and then suspended in saline solution containing 1.5M sodium thiocyanate, and the suspension heated for 7 min at 100°. After being cooled, the suspension was centrifuged, and the supernatant was dialyzed against water, and then concentrated to give the "specifically" extracted polysaccharide[11].

Immunochemical techniques. — Rabbits without natural agglutinins were intravenously injected with heat-killed (2 h at 100°) bacteria, three times each week during two weeks, with increasing doses from 10^9 to $4 \cdot 10^9$ cells. Blood was taken from the ear vein three to seven days later. Further series of injections, which took place one month after the last injection, were always preceded by 2 subcutaneous injections to prevent an anaphylactic shock. Pooled and individual rabbit immune sera were used. In the study of bacterial agglutination, only qualitative agglutination tests were performed with *S. zuerich* cells and rabbit monospecific immune sera to detect the

eventual O factors on the cells. The quantitative precipitation tests were performed according to Heidelberger and Kendall[12], and the protein content was estimated with the method of Lowry *et al.*[13]. Rabbit γ-globulins were used as standards. In the study of absorption of sera, precipitation curves were established with heterologous polysaccharides and absorption of sera was effected at the concentration of polysaccharide that gave maximum precipitation. The absorption was repeated until all the undesirable antibodies were eliminated, thus leading to a monospecific antiserum. Immune electrophoresis was performed according to Grabar and Williams[14]. Paper electrophoresis was performed in a 5:2:193 (v/v) pyridine–acetic acid–water pH 5.5 buffer. A current of 50 mA under 2200 V was applied for 135 min.

Analytical methods. — Polysaccharides were hydrolyzed with 0.5M sulfuric acid for 4 h. Heptose was determined with sulfuric acid–cysteine[15], D-glucose with glucose oxidase[16], D-galactose with galactose oxidase[17], L-rhamnose with sulfuric acid–cysteine[18], tyvelose with periodate–thiobarbituric acid[19], and D-mannose with mannose isomerase[20,21] (the interference due to heptose was not taken into account). The total amount of sugars was estimated with anthrone[22,23].

RESULTS AND DISCUSSION

Polysaccharide extraction and analysis. — The O specific polysaccharide extracted from *S. zuerich* with acetic acid[10] is composed of three fractions precipitated by different proportions of alcohol and water (v/v): 1:1 (0.18 g, ZA); 5:1 (4 g, ZB); and 10:1 (0.013 g, ZC). ZC was discarded, since it behaved like ZB in precipitation tests and was obtained in a very small yield.

TABLE I

SUGAR COMPONENTS OF *S. zuerich* POLYSACCHARIDES

Components[a]	*Polysaccharides*	
	ZA	*ZB*
D-Galactose	2.2	1.3
D-Mannose	1.1	1.1
L-Rhamnose	1.0	1.0
Tyvelose[b]	0.7	1.0
D-Glucose	3.7	0.6
Heptose	[c]	+

[a]Proportions relative to L-rhamnose. [b]Tyvelose: 3,6-dideoxy-D-*arabino*-hexose. [c]Not determined.

The proportions of the components of ZA and ZB are reported in Table I. The high proportions of D-galactose (2.2) and especially of D-glucose (3.7) in ZA, as compared to that of L-rhamnose, may indicate the presence of such impurities as D-glucan or D-galactan in this fraction. ZA contains also a smaller proportion of tyvelose, probably due to its loss during acid hydrolysis. The proportion of D-glucose

in ZB, as compared to that of L-rhamnose, is only 0.6, which may indicate that not all D-galactosyl residues of the ZB main-chain are substituted by a D-glucosyl group. Both ZA and ZB do not migrate during electrophoresis in a pyridine–acetic acid buffer.

Similarities between the O factors of polysaccharides ZB and ZA and those of other Salmonella *groups.* — The results of the quantitative precipitation tests of pooled rabbit anti-*S. zuerich* antisera with some O specific polysaccharides are listed in Table II. Most of the antibodies that are precipitated by polysaccharide ZB from this antiserum are directed against the sites 9, 27 (65% are precipitated with *S. typhi* T_2 1^- 27^+). The antiserum contains anti-1 antibodies [40% precipitated with

TABLE II

PRECIPITATION OF RABBIT ANTI-*S. zuerich* ANTIBODIES BY SOME *Salmonella* O SPECIFIC POLYSACCHARIDES

Polysaccharide	*Group*	*O Factors involved*	*Antibodies (µg/0.1 ml of serum)*	*Antibodies[a] precipitated*
				(%)
S. typhi T_2 1^- 27^+	D_1	9, 27	52	65
S. typhi T_2 1^+ 27^+	D_1	1, 9, 27	48	61
S. strasbourg	D_2	9, 46	17	21
S. senftenberg (1, 3, 19)	E_4	1_{E_4}	32	40
S. paratyphi A	A	1_A	13	16
S. zuerich	D_3	1, 9, 12, (46), 27		
ZA			40	50
ZB			80	100

[a]The amount of anti-*S. zuerich* antibodies precipitated with ZB was considered as 100%.

S. senftenberg (1, 3, 19)], which leaves a very small quantity, if any, of anti-46 antibodies; indeed, the serum reacted very poorly (21%) with a *S. strasbourg* polysaccharide that carries factors 9 and 46. These results, obtained with a pool of 6 different antisera, represent an average situation. With a few individual anti-*S. zuerich* antisera tested, the titer of anti-1 antibodies was higher than that of anti-27, and once reached 75% of the titer of the antibodies precipitated by polysaccharide ZB; but the anti-46 antibodies were generally present in low proportions (15 to 20%). It is very surprising to detect no significant difference between the *S. typhi* T_2 1^- 27^+ and T_2 1^+ 27^+ polysaccharides, since the latter one carries an additional factor (factor 1). Because the anti-*S. zuerich* antiserum contains ~40% of anti-1 antibodies, this lack of difference cannot be attributed to a lack of anti-1 antibodies in the anti-*S. zuerich* antiserum. It may be due, however, either to a bad conversion on the *S. typhi* cells, which would lead to a number of 1 sites on the polysaccharide so small that anti-1 precipitation could not occur with the anti-*S. zuerich* antiserum, or it may be due to a steric hindrance towards sites 27 after the addition of sites 1 on the *S. typhi* T_2 1^+ 27^+ O polysaccharide molecules; or both effects can play a role. The second hypothesis is also supported by the flatter slope given by the *S. typhi* T_2 1^+ 27^+ polysaccharide,

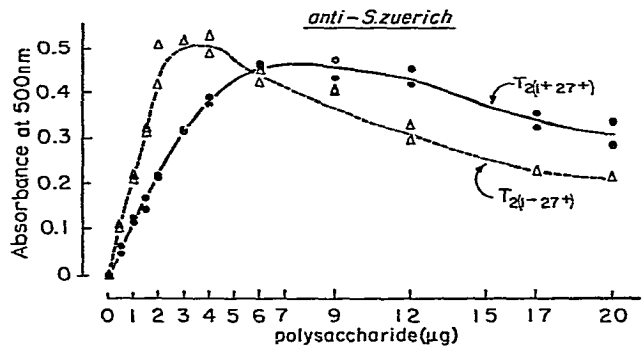

Fig. 2. Precipitation of pooled rabbit anti-*S. zuerich* antisera (100 µl) with O polysaccharides extracted from *S. typhi* T_2 1^- 27^+ (△) and T_2 1^+ 27^+ (●).

as compared to that obtained with *S. typhi* T_2 1^- 27^+ (Fig. 2). The highest proportion of precipitation of anti-1 antibodies was observed with *S. senftenberg* O polysaccharide, which indicates that the O-factor 1 present on *S. zuerich* is closest to the one of the E_4 group. This is to be expected since the same *O*-D-galactosyl-(1→6)-D-mannosyl residue, close to site 1, is present on the main chain of the polysaccharides extracted from *S. strasbourg*, *S. typhi* T_2 27^+, and *S. senftenberg*, and is different from the *O*-D-galactosyl-(1→2)-D-mannosyl residue present on *S. paratyphi* A polysaccharide (*cf.* Fig. 1). From the two *S. zuerich* polysaccharides, ZA precipitates much less (50%) anti-*S. zuerich* antibodies than did ZB, and the curve of precipitation of ZA shows a much flatter slope than that of ZB (Fig. 3). These differences may be due to the lack of specific sites or to the presence of inhibitors and possibly of impurities in polysaccharide ZA, which shows high ratios of D-galactose to L-rhamnose (2.2:1) and of D-glucose to L-rhamnose (3.7:1) (Table I).

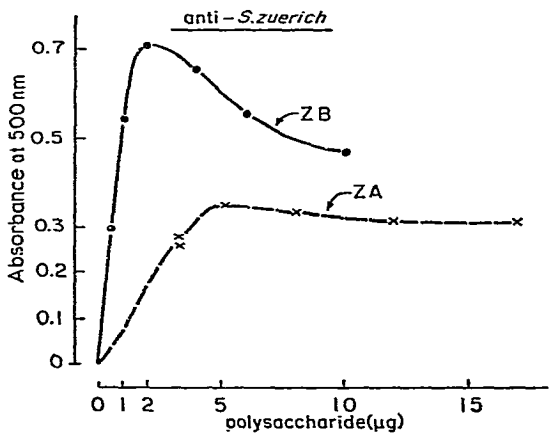

Fig. 3. Precipitation of pooled rabbit anti-*S. zuerich* antisera (100 µl) with *S. zuerich* O polysaccharides ZA (×) and ZB (●).

From the pooled anti-*S. typhi* $T_2 1^- 27^+$ antisera, 45% of the antibodies precipitated by the homologous polysaccharide $T_2 1^- 27^+$ were precipitated by polysaccharides ZB, as opposed to 15% precipitated by polysaccharide ZA (see Table III). When anti-9 antibodies were removed by saturation with a polysaccharide extracted from *S. typhi* $T_2 1^- 27^-$, the difference was more marked, and

TABLE III

CROSS-REACTION BETWEEN *S. zuerich* POLYSACCHARIDES (ZA, ZB) AND VARIOUS ANTIBODIES

Group	*Rabbit antisera (pools)*	*Factors involved*	*Cross-reaction (%) with*	
			ZA	*ZB*
D_1	Anti-T 35-52 (9, 12)	9	6	15
	Anti-*S. typhi* $T_2 1^- 27^+$	9, 27	15	45
	Anti-*S. typhi* $T_2 1^- 27^+$ saturated by $T_2 1^- 27^-$	27	6	67
D_2	Anti-*S. strasbourg*	9, 46	6	12
	Anti-*S. strasbourg* saturated by $T_2 1^- 27^-$	46	3–5	11
E_4	Anti-*S. senftenberg*	1, 3, 19	25	29

polysaccharide ZB precipitated 67% of the anti-27 antibodies (precipitated by the polysaccharide $T_2 1^- 27^+$), whereas polysaccharide ZA precipitated only 6%. Other anti-$T_2 1^- 27^+$ antibodies (obtained from individual rabbits) were all shown to have a high titer of antibodies precipitable by polysaccharide ZB, though not always as high as 67%. In contrast, the precipitations with anti-46 antibodies were very weak (11% cross-reaction with polysaccharide ZB and 3–5% with ZA). This weak cross-reaction was also observed for six out of nine other anti-*S. strasbourg* (9, 46) antisera, even before any anti-9 antibody had been removed. The same observation was made for anti-9 antibodies with pooled anti-T 35.52 antisera (6% precipitated with ZA and 15% with ZB). For the O factor 1, the gap between polysaccharides ZA and ZB was much smaller and both precipitated nearly the same amount of anti-*S. senftenberg* (1, 3, 19) antibodies (25% from pooled antisera with ZA and 29% with ZB). Two other anti-*S. senftenberg* antisera gave 34 and 26% cross-reaction with polysaccharide ZB.

The cross-reactions observed between ZB and different antisera (Table III), and the heterologous precipitations observed between anti-*S. zuerich* antiserum and polysaccharides extracted from D_1, D_2, and E_4 *Salmonella* groups (Table II) indicate a great similarity between the O factor 27 carried by polysaccharide ZB and that carried by the specific polysaccharide of *S. typhi* $T_2 1^- 27^+$. In contrast, the O factor 46 present on polysaccharide ZB seems different from that present on the specific polysaccharide of *S. strasbourg*.

In agreement with the results obtained with anti-*S. zuerich* antisera, polysaccharide ZA precipitated very small amounts of the heterologous anti-27 and anti-46 antibodies. However, it precipitated nearly the same quantities of heterologous anti-1 antibodies as did polysaccharide ZB, but the slope of the curve of precipitation was much flatter (Fig. 4). These results indicate the presence of impurities in polysaccharide ZA and suggest that the lack of determinants or the presence of inhibitors in polysaccharide ZA (as shown by the results obtained with anti-*S. zuerich* antibodies) are mostly related to O factors 27 and 46.

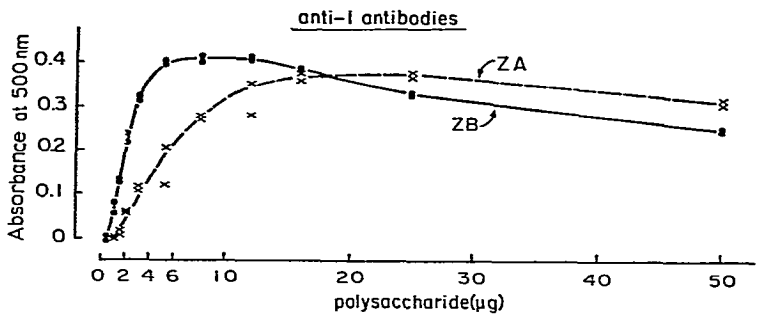

Fig. 4. Precipitation of pooled rabbit anti-*S. senftenberg* (1 ,3, 19) antisera (100 µl) with *S. zuerich* O polysaccharides ZA (×) and ZB (●).

Immunological heterogeneity of S. zuerich *cell-wall.* — Polysaccharide ZA precipitated nearly the same amount of anti-*S. senftenberg* antibodies as did ZB, but much less anti-27, anti-46, and anti-*S. zuerich* antibodies. In order to explain this difference, precipitation of an anti-*S. zuerich* immune serum was performed with an excess of polysaccharide ZA. The supernatant, which no longer reacted with polysaccharide ZA, still precipitated with polysaccharide ZB. The reciprocal situation was not observed, *i.e.* an anti-*S. zuerich* antiserum precipitated with polysaccharide ZB no longer contained antibodies precipitable by polysaccharide ZA. This result supports the hypothesis that the inhibitors in polysaccharide ZA cannot completely inhibit the precipitation of polysaccharide ZB with anti-*S. zuerich* antisera and that some antigenic sites in polysaccharide ZA are lacking; thus suggesting that the fractions extracted from the *S. zuerich* cell-wall contain heterogeneous, immunologically O specific polysaccharides. This was confirmed by (a) the immunoelectrophoretic pattern of anti-*S. zuerich* antisera and polysaccharide ZB (Fig. 5), which showed two precipitation lines indicating the presence of at least two different O antigenic fractions in polysaccharide ZB, and (b) after precipitation of an anti-*S. senftenberg* antiserum (anti-1) by polysaccharide ZA, the supernatant, in the antibody-excess zone, did not react with any of the antisera tested, thus indicating that all the antigenic determinants were present on the same molecule. In contrast, after precipitation of the same anti-1 antiserum with polysaccharide ZB, the supernatant, in the antibody-

excess zone, gave no positive ring-test with anti-1 antibodies, but it still reacted with the other immune sera (anti-*S. typhi* T_2 1^- 27^+, anti-*S. strasbourg*, and anti-*S. zuerich*). These observations were confirmed by quantitative precipitation tests.

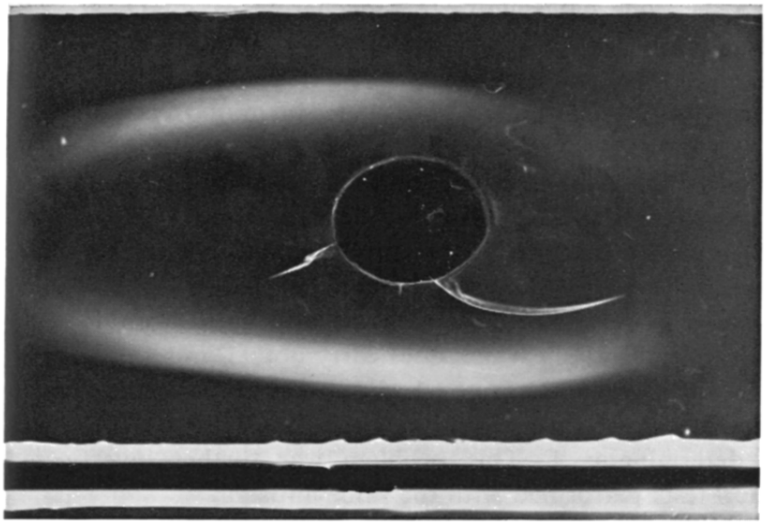

Fig. 5. Immunoelectrophoretic analysis of *S. zuerich* ZB O polysaccharide. The well contained pooled rabbit-*S. zuerich* antisera. Troughs contained *S. zuerich* ZB O polysaccharide.

These results suggest that polysaccharide ZB is composed of two fractions[24]: one (ZB 1^+) carries the O factor 1 and, possibly, the other O factors 9, 46, 27; this fraction may be similar to that present in ZA. The other fraction (ZB 1^-) is devoid of O factor 1, but carries the O antigenic determinants 9, 46, and 27. It seems highly improbable that this surprising heterogeneity of the *S. zuerich* cell-wall O polysaccharides results from a mutation, in view of the relative proportion of the two fractions. ZA contains, in addition to impurities and inhibitors, at least 1/3 of the molecules that carries factor 1 (Fig. 4). Since polysaccharides ZA and ZB (which contains also the factor 1) were isolated in the proportion 4.5:100, the ratio of molecules carrying factor 1 to molecules not carrying this factor is certainly higher than 1.5:100. If such a ratio were due to a mutation, this would happen very early in the bacteria culture. Agglutination tests of 200 *S. zuerich* colonies with anti-1, anti-9, anti-46, and anti-27 antisera indicated that all of them carried factor 1, as well as the other factors, which eliminates a form variation, as observed in the case of the 12_2 O factor[25]. Nevertheless, the possibility of form variation cannot be completely discarded, since a similar result would be obtained if the variation rate were high enough to occur during the growing time of a colony.

On the basis that the serological specificity depends on an immunodominant sugar residue located in an oligosaccharide unit, the immunological heterogeneity of the *S. zuerich* cell-wall O polysaccharides can be related to their chemical hetero-

geneity. As the O factor 1 depends on a D-glucopyranosyl side group (Fig. 1), the *S. zuerich* cell-wall carries at least two O specific polysaccharides (ZB 1^+ and ZB 1^-), which differ from each other by the presence or absence of such side-groups.

Molecular distribution of factors 27 and 46. — The 46 and 27 antigenic determinants may be present in the same molecule of polysaccharide ZB 1^+ (1, 46, 27), or in different molecules, (1, 46) and (1, 27). The same two alternatives exist for polysaccharide ZB 1^-.

To clear up this point, polysaccharide ZB was added to monospecific rabbit anti-27, anti-46, and anti-1 antisera in small antigen excess. The "specifically" precipitated polysaccharide fraction was extracted, and then tested for the O antigenic determinants 27, 46, and 1, with the ZB polysaccharide as a control (Table IV). Polysaccharide D [27] was found to carry factors 1 and 46, together with factor 27. Vice-versa, both polysaccharides A [46] and E [1] exhibited factor 27 antigenic determinants. Paradoxal results were obtained with polysaccharides A [46] and E [1]. In contrast to polysaccharide A [46], which does carry both 1 and 46 antigenic sites, polysaccharide E [1], which carries O factor 1, did not precipitate the anti-46 antibodies, which indicates a lack of precipitable antigenic sites 46 on molecules containing the antigenic determinants 1. This problem was further investigated by quantitative precipitation tests performed with the same antisera.

Precipitation with anti-27 and anti-46 antibodies. Polysaccharide E [1] precipitated anti-27 antibodies (although much less than did polysaccharide ZB), but not the anti-46 antibodies (which corroborates the ring-test) (Table IV). The precipitation curve with anti-27 antibodies was shifted towards higher antigen concentrations, indicating a decrease of O factors 27 (Fig. 6). The maximum amount of precipitation, much smaller than those from polysaccharides ZB, and A [46] or D [27], shows that polysaccharide E [1] did not precipitate all the antibodies precipitable by polysaccharide ZB, and *a fortiori* by polysaccharides A [46] or D [27]. These data suggest that polysaccharide E [1], which does not carry any precipitable O antigenic site 46, may carry a modified O antigenic site 27. Both polysaccharides D [27] and A [46] gave two precipitation curves that are nearly identical either with anti-27 or with anti-46 antibodies (Figs. 6 and 7). The same shift towards smaller antigen concentrations, observed in the precipitation curves for both of them, indicates an identical enrichment of both O factors 46 and 27 in the two polysaccharides. The amounts of maximum precipitate, identical for polysaccharides D [27] and A [46], are higher than that obtained with polysaccharide ZB, which suggests the presence of specific anti-27 and anti-46 inhibitors in the latter polysaccharide. The identity of the two curves, obtained with polysaccharides D [27] and A [46] with both anti-46 and anti-27 sera, was rather surprising since polysaccharide D [27] was expected to be more enriched in determinants 27 than was polysaccharide A [46] and vice-versa for the other determinant. This result suggests that, in our preparation, most of the determinants 27 and 46 are carried by the same molecule. The presence of molecules carrying only one of the two determinants cannot be ruled out. However, the number of such molecules may be so low or the factor so modified that their influence on the displacement of the curve to the left of the

TABLE IV

RING TEST OBSERVED BETWEEN VARIOUS ANTIBODIES AND *S. zuerich* O POLYSACCHARIDES EXTRACTED FROM SPECIFIC PRECIPITATES FROM ZB

Antibodies used for polysaccharide ZB precipitation	*Polysaccharides extracted from the precipitate*	*Immune sera*				*Possible polysaccharide structures*
		Anti-27[a]	*Anti-46*[b]	*Anti-1*[c]	*Anti-S. zuerich*	
Anti-27	D [27]	+	+	+	+	27, 46 27, 1 27, 1, 46
Anti-46	A [46]	+	+	+	+	46, 27 46, 1 46, 1, 27
Anti-1	E [1]	+	−	+	+	1, 27
Controls	ZB	+	+	+	+	
	Sodium chloride (0,9%)	−	−	−	−	

[a]Anti-*S. typhi* T_2 1^- 27^+ saturated by O polysaccharides extracted from *S. strasbourg* and *S. senftenberg*. [b]Anti-*S. strasbourg* saturated by O polysaccharides extracted from *S. senftenberg* and *S. typhi* T_2 1^- 27^+. [c]Anti-*S. senftenberg* saturated by O polysaccharides extracted from *S. strasbourg* (this antiserum did not react with *S. typhi* T_2 1^- 27^+ O polysaccharides).

graph (as expected) could not be detected by our precipitation technique. The molecule having both factors 46 and 27 does not carry O factor 1, otherwise polysaccharide E [1], which contains the polysaccharides characterized by such factor, would have precipitated anti-27 and anti-46 antibodies, as did polysaccharides A [46] and D [27]. In the absence of specificity 1, most if not all the antigenic determinants 46 and 27 are present in the same polysaccharide molecule (46, 27).

Precipitation with anti-1 antibodies. Polysaccharide E [1] precipitated more antibodies than did polysaccharide ZB, which suggests the presence of anti-1-inhibitors in the latter (Fig. 8). The shift of the precipitation curve towards lower antigen-concentrations indicates an enrichment in O factors 1. Polysaccharide D [27] precipitated about the same amount of antibodies as did polysaccharide ZB, but the curve is shifted towards higher antigen-concentrations, thus indicating a decrease of these O factors 1. These data and those obtained with polysaccharide E [1] and anti-27 antibodies (Fig. 6) suggest the presence of factors 1 and 27 on the same molecule (1, 27). As for polysaccharide A [46], the same discrepancy as just mentioned (Table IV) was observed: It precipitated anti-1 antibodies and, therefore, contains in the same molecule both O factors 46 and 1, whereas the data obtained with polysaccharide E [1] indicate that this preparation, which contains all the molecules precipitable by their O factor 1, does not carry any precipitable 46 O antigenic site, thus suggesting the absence of molecules (1, 46) in polysaccharide ZB. This quite surprising observation is best explained by the hypothesis that polysaccharide ZB contains a fraction [1, (46)], which possesses very few available 46 O antigenic determinants; thus, it can be coprecipitated with anti-46 antibodies only in the presence of another fraction rich in factor 46, present in A [46] and absent in E [1].

In the study of the presence of factors 27 on molecules [1, (46)] and, vice-versa, of factors 46 on molecules (1, 27), polysaccharides A [46] and D [27] gave two identical responses with anti-27 and with anti-46 antibodies, respectively (Fig. 6, 7), owing to the presence of the same molecule (46, 27) in both of them. When tested with an anti-1 antiserum, both polysaccharides gave two precipitation curves completely distinct, both in their slopes and in the amounts of maximum precipitate (Fig. 8). These results suggest that polysaccharide A [46] contains a structure [1, (46)] distinct from the structure (1, 27) present in polysaccharide D [27]. In contrast to their presence in the same molecule (46, 27) devoid of factor 1, the factors 46 and 27 are not detected (at least as precipitable sites) in the same molecule when it carries O factor 1. The presence, in polysaccharide ZB, of at least three different polysaccharides {(46, 27); [1, (46)]; and (1, 27)} is in complete agreement with the data obtained by ring tests. After precipitation, in the antibody-excess zone, of anti-1 antibodies by polysaccharide ZB, the polysaccharide remaining in the supernatant, which still reacts with anti-27 and anti-46 antibodies, can be identified as (46, 27); the polysaccharides precipitated with anti-1 antibodies can be identified as [1, (46)] and (1, 27).

On the basis of the presently known biosynthetic mechanism of cell-wall polysaccharides of *Salmonella*[26,27], the simultaneous presence of factors 46 and 27 only in molecules of polysaccharide ZB 1^- that are factor 1 negative is difficult to under-

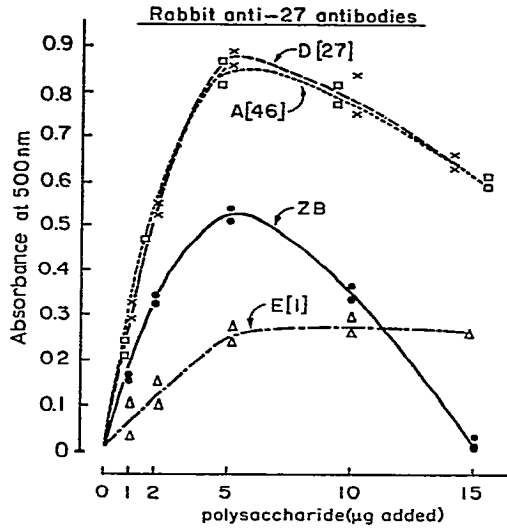

Fig. 6. Precipitation of rabbit (254) anti-27 antibodies (70 µl) by polysaccharides "specifically" (see text) extracted from ZB: ZB (●); D [27] (×); A [46] (□); and E [1] (△). Anti-27 antibodies were obtained after saturation of an anti-*S. typhi* T_2 1^- 27^+ (9, 27) immune serum by O polysaccharides extracted from *S. strasbourg* (9, 46) and *S. senftenberg* (1, 3, 19), which slightly cross-react with this serum.

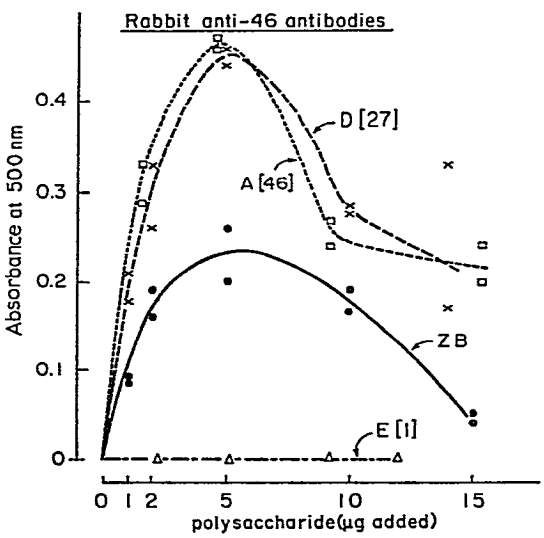

Fig. 7. Precipitation of rabbit (206) anti-46 antibodies (100 µl) by polysaccharides "specifically" extracted from ZB: ZB (●); D [27] (×); A [46] (□); and E [1] (△). Anti-46 antibodies were obtained after saturation of an anti-*S. strasbourg* (9, 46) immune serum by O polysaccharides extracted from *S. typhi* T_2 1^- 27^+ (9, 27) and *S. senftenberg* (1, 3, 19), which slightly cross-react with this serum.

stand. It is possible that molecules (1, 46, 27) are either so modified or present in so small a quantity, that they play a negligeable role in the precipitation of the rabbit anti-27 and anti-46 antibodies.

Reciprocal steric hindrance between factors 1 and 46, and between 1 and 27. — Two monospecific anti-1 antisera were precipitated, at the maximum of precipitation, by polysaccharides A [46] and D [27], more antibodies being precipitated by polysaccharide D [27] than by polysaccharide A [46] (Table V), which confirms the

TABLE V

PRECIPITATION OF ANTI-1 ANTIBODIES BY POLYSACCHARIDES A [46] AND D [27]

Polysaccharides extracted	*Anti-1 antibodies*	
	Rabbit 398[a] *(μg Ab/20 μl IS)*[c]	*Rabbit 397*[b] *(μg Ab/30 μl IS)*[c]
A [46]	4.6[d]	5.6
		6.1
D [27]	10.8	8.6
	10.6	8.6
A [46] and D [27]	10.8	8.6
	11.2	8.6

[a]The anti-*S. senftenberg* antiserum from rabbit 398 contained neither anti-27 nor anti-46 antibodies. [b]The antiserum 397, saturated with polysaccharide extracted from *S. strasbourg* (9, 46), contained neither anti-27 nor anti-46 antibodies. [c]Abbreviations: Ab, antibodies; IS, immune serum. [d]Maximum of antibodies precipitable from the antiserum.

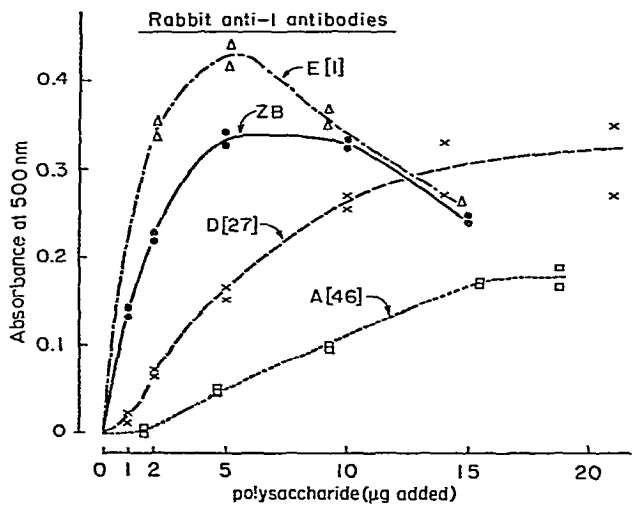

Fig. 8. Precipitation of rabbit (397) anti-1 antibodies (70 μl) by polysaccharides "specifically" extracted from ZB: ZB (●); D [27] (×); A [46] (□), and E [1] (△). Anti-1 antibodies were obtained after saturation of an anti-*S. senftenberg* (1, 3, 19) immune serum by O polysaccharides extracted from *S. strasbourg* (9, 46), which slightly cross-react with this serum.

results presented in Fig. 8. In addition, since the mixture of polysaccharides A [46] and D [27] did not precipitate more anti-1 antibodies than did polysaccharide D [27] alone, it can be concluded that some anti-1 antibodies can recognize the factor 1 present in both molecules (1, 27) and [1, (46)], whereas other anti-1 antibodies cannot recognize factor 1 when it is present in the same molecule as factor 46. On the basis of the chemical constitution of the factor determinants (Fig. 1), these data suggest that the reaction of anti-1 antibodies with D-glucose groups (factor 1) is more strongly inhibited by the proximal tyvelose groups (steric hindrance), when the latter are linked to a β-D-mannosyl residue of the main-chain (factor 46) than when they are linked to an α-D-mannosyl residue (factor 27). This steric hindrance might be reciprocal, according to the observation that polysaccharide E [1] partly precipitated anti-27 antibodies (Fig. 6), and did not precipitate alone the anti-46 antibodies (Fig. 7), though it is coprecipitable.

CONCLUSION

In conclusion, immunochemical analyses have shown that the polysaccharide extracted from *S. zuerich* carries, as the *Salmonella* cell, specificities 1, 27, and 46. The specificity 27 of *S. zuerich* is very similar to the O factor 27 present on the polysaccharide extracted from *S. typhi* T_2 1^- 27^+ (D_1 group, converted by phage φ 27). The specificity 1 of *S. zuerich* is close to the O factor 1 present on the polysaccharide extracted from *S. senftenberg* (E_4 group, wild strain), but the cross-reaction observed with the polysaccharide extracted from *S. strasbourg* (D_2 group), which carries O factor 46, is very weak. These results are in perfect agreement with the results of agglutination of the cells, which detected also a very weak factor 46 on *S. zuerich*[6].

The polysaccharide extract contained a very small proportion of an impure polysaccharide ZA that carries factor 1 but reacted only very poorly with anti-27 and anti-46 antibodies.

Polysaccharide ZB, the main O specific polysaccharide fraction of *S. zuerich*, is composed of two fractions, ZB 1^+ and ZB 1^-. Polysaccharide ZB 1^+, perhaps present in polysaccharide ZA, is a mixture of at least two kinds of precipitable polysaccharides: (a) a polysaccharide 1, (46) precipitable by some anti-1 antibodies, and coprecipitable but not precipitable by anti-46 antibodies; and (b) a polysaccharide (1, 27) precipitable by both anti-1 and some anti-27 antibodies. A small fraction containing factors [1, 27, (46)] may exist, but has not been detected in this study. The polysaccharide containing factors (1, 27) precipitated in general two families of anti-1 antibodies: one precipitated by [1, (46)], the other only by (1, 27). Polysaccharide ZB 1^- contains mainly a structure that carries the O specificities 46 and 27 on the same molecule. Molecules carrying only one specificity (27 or 46) have not been detected in this study.

From our present knowledge of the chemical structure of the O factors, the specificity 1, (46) is related to α-D-glucosyl side-groups and to the β configuration of the D-mannosyl residues of the main chain, whereas the specificity (1, 27) is related

to an α-D-glucosyl side-group attached to a main chain composed of α-D-Man→L-Rha→D-Gal repeating units (Fig. 9)[29]. Our results suggest that some steric hindrance occurs between factors 1 and 46 and, at a lower degree, between factors 1 and 27, when they are present on the same polysaccharide molecule.

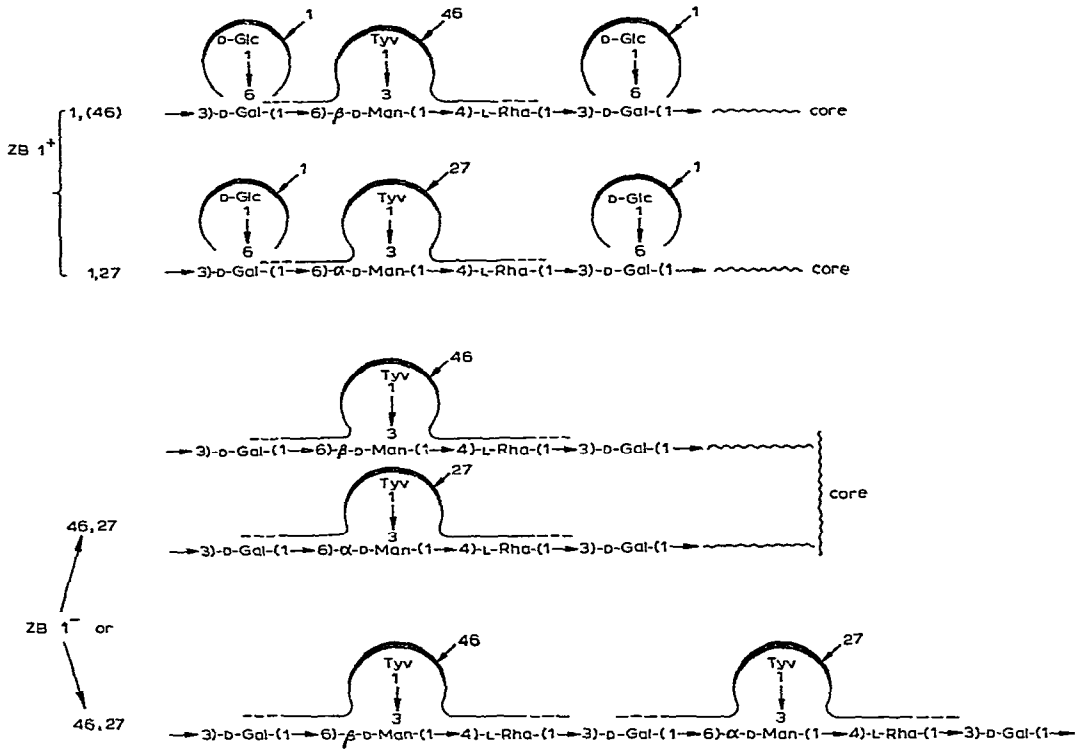

Fig. 9. Hypothetical structures of *S. zuerich* cell-wall O polysaccharide ZB. For abbreviations, see Fig. 1.

The structure carrying factor (46, 27) and present in polysaccharide ZB 1^- is characterized by a main chain containing both α- and β-D-mannosyl residues or by two main-chains; one with only α- and the other with only β-D-mannosyl residues, both linked to the core polysaccharide (Fig. 9)[29].

Three different polysaccharides containing factors [1, (46)], (1, 27), and (46, 27), respectively, have been isolated from a preparation obtained from *S. zuerich* cells by 0.1M acetic acid hydrolysis. This observation disagrees with previous suggestions that all the polysaccharide O specific chains on a *Salmonella* bacteria are identical and carry all the factors related to the *Salmonella* strain[1,28]; thus raising the problem of the biosynthetic mechanism of the carbohydrate chains in such a bacteria.

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