

# Immunochemistry of *Shigella flexneri* O-Antigens: a Study of Structural and Genetic Aspects of the Biosynthesis of Cell-Surface Antigens

D. A. R. SIMMONS

*Department of Bacteriology and Immunology, University of Glasgow, Glasgow, Scotland*

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## INTRODUCTION

*Shigella flexneri* are a serologically heterogeneous group of dysentery bacilli whose O-antigens are polysaccharide-lipid A-protein-lipid B complexes comparable in their gross structural and biological properties to the analogous substances first characterized in *S. shigae* (65) and

now thought to be present in all gram-negative bacilli of the family *Enterobacteriaceae* (11, 54, 57). Although much is known of the physical, chemical, and biological properties of O-antigens, their importance to the bacterium remains an enigma. Many speculations as to their function in the metabolic economy and natural history

of the cell are unsubstantiated and their role in the pathogenesis of bacillary dysentery is virtually unknown. By contrast, their importance to man is both considerable and clearly discernible as they determine (among other properties) the serological behavior, immunological specificity, colicin activity, and endotoxicity that are of prime interest to the medical laboratory worker involved in the recognition, prevention, and prophylaxis of bacillary dysentery. In addition to their unequivocal importance in medicine, these O-antigens are also of special relevance to the biology of gram-negative bacilli in that comparative structural analyses with other genera are beginning to reveal differences of the kind that will help to elucidate the taxonomy, biosynthesis, genetics, and evolutionary history of this important family of polymers. One such difference is seen in the relative ease with which it has been possible to classify *Salmonella* serologically, whereas *Shigella flexneri* have proved more difficult despite the fact that they are a smaller and more compact group of organisms. Weil, Black, and Farsetta (112, 113), referring to these taxonomic problems, expressed the view that "our present approach is by necessity a purely empirical one" and "a deeper understanding cannot be expected as long as our knowledge of the chemistry of the somatic antigens of the Flexner species is at its present embryonic state." Recent chemical studies (96) support this view in that the *S. flexneri* O-specific side chains have proved to be very closely related structures comprised of identical hexose constituents in which antigenic differences are much less distinct than in *Salmonella*, so that their serotyping is that much more difficult in consequence.

However, the relevance of structural studies extends beyond taxonomy to include wider biological problems such as the biochemical basis of the well-known phenomenon of smooth to rough (S → R) mutation. In a detailed study of this problem (40), it has been shown that *S. flexneri* R-lipopolysaccharides of different chemotype form a structurally related sequence of polymers (Re → Rd → Rc → Rb → Ra → S) which are cryptic antigens in the smooth situation. These R-polymers, which are the consequence of single enzyme defects interrupting the biosynthetic pathway to the complete smooth form, were quite analogous to those of salmonellae, but the fine structures of the higher chemotypes proved different in both genera. Comparative studies of the extent of these similarities and differences are of general interest and may ultimately be of value in tracing the evolutionary history of the different genera in the family *Enterobacteriaceae*.

More recently, complete analysis of the *S. flexneri* O-specific side chains (96) has made it possible to extend the biosynthetic pathway from the preliminary rough stages shown above to include the more complex pathways for the various smooth serotypes. Variants X and Y are intermediates in the biosynthesis of these smooth serotypes from the rough precursor substances. From comparisons of the *Salmonella* and *Shigella* pathways, it is apparent that the general mechanisms of O-antigen synthesis are closely similar in both genera, although the actual structures of the resulting end products are, of course, quite different.

Finally, structural analyses of lipopolysaccharides from *S. flexneri*-*E. coli* hybrids (61) have defined the precise antigenic changes that occur as a direct consequence of genetic recombination by bacterial conjugation and lysogenic conversion. These studies provided information about the normal genetic control of the biosynthesis of cell surface O-antigens and showed how the specificity of these antigens is altered when the bacterial genome is modified by random mutation, bacterial conjugation, and lysogenic conversion.

From these general considerations, there is every reason to believe that a knowledge of the chemistry of the *S. flexneri* O-antigens will elucidate many medical and biological aspects of the world-wide problem of bacillary dysentery. In this review, the immunochemistry of these O-antigens will be summarized and an attempt will be made to correlate the results with structural and genetic aspects of the biosynthesis of these important cell-surface components.

#### GENERAL NATURE AND PROPERTIES OF O-ANTIGENS

In the past decade, the O-antigens of *Salmonella* and of some related *Enterobacteriaceae* have been the subject of much research which has been summarized in a number of excellent reviews (11, 54, 57). As these have dealt rather exhaustively with the isolation, purification, and general properties of O-antigens, including those of *Shigella*, these topics need not be reconsidered here at length. However, a brief summary is necessary to appreciate the origin and nature of the particular *S. flexneri* antigenic components discussed in this report.

#### Gross Composition of O-Antigens

From the reviews cited above (11, 54, 57), there is much evidence that all O-antigens of *Enterobacteriaceae* are polysaccharide-lipid A-protein-lipid B macromolecular complexes which are bound by

physical (noncovalent) forces to the cell wall mucopeptide (117). More recently, a third lipid (usually referred to as third lipid or lipid B-like material) has been described as playing an important role as a lipid cofactor in O-antigen biosynthesis (114, 115, 119). The general properties of these different moieties may be summarized as follows.

**Lipid A component.** Although the exact structure of this lipid has yet to be characterized, it is generally accepted that it contains a backbone of N- $\beta$ -hydroxymyristoyl-glucosamine phosphate in which all available hydroxyl groups in the hydroxy acid and glucosamine are esterified with long-chain fatty acids (8, 70). Despite much research, the precise relationship of lipid A to endotoxin remains unclear. Earlier studies suggested that endotoxin and lipid A were virtually synonymous because antigens or antigen fragments containing this component were usually potent endotoxins, whereas their lipid A-free analogues were devoid of such activity. However, the isolation of highly toxic, lipid A-poor lipopolysaccharides (79) has cast some doubt on this conclusion by showing that there is in fact no precise quantitative relationship between lipid A and endotoxic activity.

Active preparations of endotoxin cause a wide variety of biological effects including pyrexia, leucopenia followed by leucocytosis, a rise in non-specific immunity, the Shwartzman reaction, adjuvant activity, enhancement of hormonal activity, and tumor necrosis (52). Although definite proof is lacking, it seems that lipid A is identical throughout *Enterobacteriaceae*.

**Polysaccharide component.** The polysaccharides of gram-negative bacilli are complex heteropolymers whose structures determine the serological specificity of the O-antigens that are used extensively in the classification of *Salmonella*, *Shigella*, and *Escherichia*. Their structural analyses have thrown much light on the molecular basis of antigenic specificity and cross-reactivity and also on structural and genetic aspects of O-antigen biosynthesis. Degraded and purified polysaccharide components are nonantigenic, though they react specifically in vitro with antibody prepared against whole antigen. They are therefore to be regarded as classic haptens. When pure they are nontoxic, but if bound with lipid A as in lipopolysaccharides they display the biological properties of endotoxin. Thus, the properties of the polysaccharide are those relating to serological specificity and, in some instances at least, they act as specific bacteriophage receptor sites (39, 111). In this review, the (polysaccharide-dependent) type specific and group factors are designated according to the serological classifica-

TABLE 1. *Internationally accepted designations for the subserotypes of Shigella flexneri*<sup>a</sup>

| Serotype  | Subserotype | Abbreviated antigenic formula |
|-----------|-------------|-------------------------------|
| 1         | 1a          | I:4                           |
|           | 1b          | I:6                           |
| 2         | 2a          | II:3,4                        |
|           | 2b          | II:7,8                        |
| 3         | 3a          | III:6:7,8                     |
|           | 3b          | III:3,4:6                     |
|           | 3c          | III:6                         |
| 4         | 4a          | IV:3,4                        |
|           | 4b          | IV:6                          |
| 5         | 5           | V:7,8                         |
| 6         | 6           | VI:-                          |
| Variant X |             | -:7,8                         |
| Variant Y |             | -:3,4                         |

<sup>a</sup> This serological classification, which is that of Ewing and Carpenter (18), does not subdivide serotype 5. In the present studies two distinct subserotypes could be recognized on the basis of structural chemistry and serology. These two subtypes, which had the antigenic formulas V:3,4 and V:7,8, were designated 5a and 5b, respectively. It should be stressed, however, that these are not internationally recognized designations. Reproduced from the *International Journal of Systematic Bacteriology*.

tion of Ewing and Carpenter (18) shown in Table 1.

**Protein component.** In terms of structural chemistry, little is known of the protein moiety but it determines two important biological properties of O-antigen. First, it confers antigenicity on the macromolecular complex, although the specificity of the antibody response is directed against limited structural sequences in the polysaccharide O-specific side chains. Second, it determines the colicin (bacteriocin) activity of the organism. These activities have been most extensively studied in the case of colicins K (28) and V (35).

**Lipid B component.** This lipid component which has been found to consist mainly of palmitic and oleic acids, is a cephalin type of phospholipid. Evidence has been produced (82) that lipid B or lipid B-like material acts as a cofactor in the biosynthesis of the innermost part (the basal structure) of the lipopolysaccharide component of O-antigen. This finding, together with the fact that the lipid B-like material is associated with the protein moiety, has given rise to the suggestion that the protein component of the O-antigenic complex probably represents the enzymes involved in the biosynthesis of the polysaccharide component (54).

### Isolation of *S. flexneri* O-Specific Substances

Much of our knowledge of the extraction and purification of gram-negative O-antigens stems directly or indirectly from the pioneer studies of Morgan and his colleagues (65, 66) who, by treating *S. shigae* with diethyleneglycol at normal temperatures and pH, first showed that the extracted O-antigen was a polysaccharide-lipid-protein complex indistinguishable from the whole antigen obtained by the trichloroacetic acid method of Boivin and Mesrobian (4, 5). Hydrolysis of this antigenic complex with acetic acid at 100 C gave a lipid corresponding to lipid B, a degraded polysaccharide derived from the polysaccharide moiety described above, and a conjugated protein which is presumed to be a lipid A-protein complex. Upon treatment with phenol, the conjugated protein yielded a simple amphoteric protein. Similar treatment of the whole antigenic complex yielded a polysaccharide (presumably lipid A-polysaccharide) and protein. Formamide treatment of the antigenic complex in neutral conditions yielded a lipid corresponding to lipid B and an antigenic polysaccharide-protein complex. Similar treatment in acid conditions gave complete dissociation of the complex. Thus, these studies showed for the first time that bacterial O-antigens could be extracted in near normal conditions and that their various components could be recovered singly or in combination by the judicious use of phenol and formamide.

Unfortunately, when Goebel and his colleagues (3, 29, 76) carried out the first detailed study of *S. flexneri*, diethyleneglycol was found to be too specific for general use as a solvent for bacterial O-antigens. Although good yields were obtained with *S. shigae* and *S. flexneri* type Z (serotype 3a), all other serotypes extracted gave poor results, and with serotype 6 no serologically active material was recovered at all. However, all *S. flexneri* serotypes treated with pyridine yielded O-antigens which were identical in every respect with the complexes obtained with diethyleneglycol. The yields with pyridine were high but the products contained much contaminating nucleic acid which could be separated to some extent by acetone fractionation.

In an attempt to find the most satisfactory solvent for *S. flexneri*, the trichloroacetic acid (4, 5), pyridine (29) and phenol-water (49, 116) methods were compared by extracting eight representative serotypes (91, 93, 94) by all three procedures. The trichloroacetic acid method was included because it is now generally accepted that antigenic preparations can be obtained in this way from the majority of gram-negative bacilli (4, 5, 9, 59) although it fails to remove the analo-

gous substances from *Pasteurella pestis* (27) and some strains of *Escherichia coli* (60). Such extracts are not homogeneous, however, but contain two main components, the one being antigenic complex and the other free, serologically active polysaccharide (12). Although the yields are not so high, these preparations are less contaminated with other cell components such as the nucleic acids that are present in phenol-water and pyridine extracts. With the trichloroacetic acid method, the gross chemical and biological properties of the extracted *S. flexneri* antigens and antigenic components (91, 92, 94) closely paralleled those of the analogous pyridine extracts described by Goebel and his colleagues (3, 29, 76). The O-antigenic complex was a potent toxin. Upon acid hydrolysis, it dissociated into a nontoxic, nonantigenic polysaccharide hapten, a phospholipid (lipid B), and a toxic antigenic protein (a lipid A-protein presumed to be identical with the conjugated protein of Morgan). Ultraviolet irradiation rendered the trichloroacetic acid-extracted antigenic complex and its toxic protein component nontoxic and nonantigenic.

The marked similarity in the bacterial fractions recovered by the pyridine and trichloroacetic acid methods contrasts sharply with the results obtained when gram-negative bacilli are extracted with phenol-water. With this solvent the *S. flexneri* (93, 95) yielded a toxic lipopolysaccharide (lipid A-polysaccharide) that is quite analogous to the lipopolysaccharides recovered in a high state of purity from many types of *Enterobacteriaceae* (47-49). Such preparations are protein free and nonantigenic in contrast to the undegraded pyridine and trichloroacetic acid extracts which are highly toxic and antigenic in the rabbit.

Other methods that have been used for the extraction of *S. flexneri* have involved treatment with strong acid (24, 64, 104) or alkali (72, 73). Such procedures have not been widely adopted, however, because the products are highly degraded compared with the serologically active fractions obtained by milder extraction methods.

In my experience, the choice of solvent for extracting *S. flexneri* O-antigens will always be governed to some extent by the purpose for which the extract is required. In the great majority of cases, however, lipopolysaccharides extracted by phenol-water and purified in the ultracentrifuge are to be preferred as they can be obtained in large amounts in a high degree of purity from almost all smooth and rough gram-negative bacilli. Such polysaccharides are nonantigenic, however, and in experiments in which antigenicity is a required biological property I prefer to use undegraded complexes obtained by the trichloroacetic acid method.

### Hexose Constituents of *S. flexneri* Polysaccharides

A necessary prerequisite to polysaccharide structural analysis is qualitative and quantitative analysis of the sugar constituents of the polymer under study. The first serious attempt to identify the hexoses in partially purified *S. flexneri* polysaccharides was undertaken by Goebel et al. (29), who noted the presence of a hexosamine (estimated as glucosamine) in serotypes 1a, 2a, 3, and 6. Other reducing sugars (estimated as glucose) were also detected after acid hydrolysis, but no attempt was made to characterize these. After the introduction of carbohydrate paper chromatography, Slein and Schnell (101-103) identified the major constituents of serotype-3 polysaccharide as glucose, glucosamine, and rhamnose. These authors also noted the presence of small amounts of an aldohexose which proved to be L-glycero-D-mannoheptose phosphate.

In a later, more comprehensive series of qualitative and quantitative analyses of the eight main serotypes (95), it was shown that the percentage composition of most smooth *S. flexneri* lipopolysaccharides lay in the following range: rhamnose (17.6-22.2), *N*-acetylglucosamine (14.4-17.9), glucose (11.0-13.1), galactose (2.0-2.4), aldohexose phosphate (4.4-6.0), 3-deoxy-2-oxo-octonate (1.4-3.1), and *O*-phosphorylethanolamine (2.4-8.7). These findings confirmed the observations of Slein and Schnell (101-103) that glucose, *N*-acetylglucosamine, and rhamnose were the main constituents of serotype-3a polysaccharide. They also confirmed earlier reports of small amounts of galactose in the polysaccharides of serotypes 6 (16), 2a (83), and variant Y (84); of aldohexose phosphate in serotype 3a (103); and of *O*-phosphorylethanolamine in an unspecified *S. flexneri* species (30). The discovery of 3-deoxy-2-oxo-octonate in this genus, though interesting, was hardly surprising as this substance, which was first described in *E. coli* O-111 (14, 31), was known to be a common constituent of *Salmonella* lipopolysaccharides (57). However, previous reports of mannose and xylose (84) and uronic acids (16) could not be confirmed despite careful attempts to detect these monosaccharides.

From quantitative analyses (95), 58.9 to 74.4% of these lipopolysaccharides could be accounted for as carbohydrate. The precise amount of the lipid moiety was undetermined, but from unpublished observations made during the ether extraction of acetic acid-degraded antigens (91), it was believed to constitute about 30% of the complex. Thus, making no allowance for experimental losses, at least 89 to 100% of the *S. flexneri* lipopolysaccharides could be accounted for in terms of lipid A and the hexoses defined above.

It seems unlikely, therefore, that major components of the lipopolysaccharides remain to be detected. Moreover, as will be shown, the serological behavior of these polymers can be explained entirely in terms of structural sequences comprised of the above sugars only. This fact again suggests that the major components of serological importance have probably all been identified. On the other hand, the possible presence of unrecognized minor components cannot be excluded.

In addition to comparative paper chromatography in a number of solvents with authentic samples as reference compounds, the nature and anomeric form of the above constituents were determined as follows.

***N*-acetylglucosamine.** The nature of the hexosamine in polysaccharide hydrolysates was confirmed by three methods. The first method, described by Slein (101), depends on the specific phosphorylation of D-glucosamine with hexokinase, whereas the second depends on the conversion of 2-aminohexoses to their corresponding parent pentoses by ninhydrin in pyridine buffer as described by Stoffyn and Jeanloz (107). The third method (56), which depends on the specificity of yeast hexokinase for D-glucosamine and of yeast *N*-acetylase for the resulting D-glucosamine-6-phosphate, provides a highly sensitive and highly specific method of determining D-glucosamine even in the presence of other 2-aminohexoses. From the formation of glucosamine-6-phosphate in the first method, arabinose in the second, and *N*-acetyl-D-glucosamine-6-phosphate in the third, it was shown that all *S. flexneri* lipopolysaccharides contained D-glucosamine. Moreover, quantitative studies (93, 95) showed that the total hexosamine content of these polymers was present as this sugar and that all of it was *N*-acetylated in the native polysaccharides. Thus, the hexosamine of this family of polymers is *N*-acetyl-D-glucosamine.

**Rhamnose.** When compared on a weight-for-weight basis, the color intensity and spectrum of the 6-deoxyhexose isolated from the *S. flexneri* polysaccharides were indistinguishable from those of an authentic sample of L-rhamnose on testing in the cysteine-sulfuric acid reaction of Dische and Shettles (95). Moreover, the *S. flexneri* 6-deoxysugar reacted quantitatively in the L-rhamnose-isomerase reaction, which indicates that all of it is present in the form of L-rhamnose.

**Galactose and glucose.** From quantitative analysis with galactose and glucose oxidases which are specific for the D-forms, it was shown that both these sugars were present in *S. flexneri* polysaccharides in the D-configuration.

**Aldohexose.** When compared weight-for-weight

with an authentic sample of glyceromannoheptose, the *S. flexneri* aldoheptose gave the same color intensity and spectrum in the Dische reaction. Although these reactions do not discriminate between the different optical isomeric forms, there is good evidence that this heptose is probably present in all polysaccharides of *Enterobacteriaceae* as L-glycero-D-manno-heptose phosphate (57). However, D-glycero-D-manno-heptose has also been demonstrated recently in a number of gram-negative bacilli, including an *S. flexneri* strain of unspecified serotype (1). The isolation of the phosphorylated form of heptose free and bound in oligosaccharides (95) indicated that this residue is also phosphorylated in the *S. flexneri* polysaccharides.

Thus, early studies (91, 93, 95) established that the *S. flexneri* polysaccharides all contained O-phosphorylethanolamine, 3-deoxy-2-oxo-octonate, L-glycero-D-manno-heptose phosphate, D-galactose, D-glucose, N-acetyl-D-glucosamine, and L-rhamnose. They belong, therefore, to a single chemotype (a family of polymers with the same qualitative sugar composition) which was indistinguishable from *Salmonella* chemotype VII (49).

#### Gross Structure of *S. flexneri* Polysaccharides

There is now much evidence (57) to support the view that the *Salmonella* lipopolysaccharides share a common basal structure or core to which side chains carrying the different O-specific determinants are attached. The basal sugars—aldoheptose phosphate, 3-deoxy-2-oxo-octonate, O-phosphorylethanolamine, galactose, glucose, and N-acetylglucosamine—are therefore found in all *Salmonella* lipopolysaccharides, whereas the "special" (side chain) sugars associated with O-specificity differ from chemotype to chemotype. The presence of the same six basal components in *E. coli* (47), *Arizona* (48) and *S. flexneri* (95) indicates that a similar core probably exists in all *Enterobacteriaceae* and there is now much evidence that this region of the molecule contains the cryptic (hidden) rough determinants of the above genera (57, 96). Although the basal structure is probably identical or at least very similar throughout a given genus, the smooth O-specific side chains almost certainly have a structure that is unique to each serotype. With the *S. flexneri*, in which the O-specific side chains contain the same hexose constituents—namely, D-glucose, N-acetyl-D-glucosamine, and L-rhamnose—type specificity is determined by unique linkages between these residues and cross-reactivity by the sharing of common structural sequences (40, 92, 93, 96). As these side chains comprise the greater bulk of the total molecule, they were assumed to contain a

number of identical repeating units (95) analogous to those proposed for *Salmonella* groups B, D, E, G, N, and U (55, 81, 99, 100, 106). As will be shown, there is now much structural evidence that this assumption was correct. From the quantitative analyses presented above (95), the molar ratios of the hexoses in the *S. flexneri* repeating units were calculated. Serotypes 1a, 2a, 3a, 4a, and variant X contained tetrasaccharide repeating units of glucose, N-acetylglucosamine, and rhamnose in the proportions 1:1:2, respectively. Serotype 5 repeating unit contained an additional glucose residue, whereas that of variant Y contained no glucose. With serotype 6 polysaccharide, which has a high galactose content and was therefore thought to contain this sugar in its O-specific side chains (95), it has now been shown that all the galactose is basal in origin (96) and that the side chains of this serotype contain N-acetylglucosamine and rhamnose only.

At the present moment, the O-specific side-chain length is not known precisely but estimates of its size have been derived by two independent methods. The first of these, based on the molar ratio of the side-chain rhamnose to the basal galactose (95), gave a value of six repeating units whereas the second method, based on analytical ultracentrifugal studies (93), gave a value of eight repeating units on the assumption that the degraded polysaccharide with a molecular weight of 26,000 had four side chains of equal length. In both methods it was also assumed that all the core side chains carried O-chains. Although these methods are not very accurate, they do indicate that the average O-specific side-chain length is probably six to eight repeating units.

The gross structure of a typical smooth *S. flexneri* polysaccharide can therefore be represented diagrammatically (Fig. 1).

#### STRUCTURE AND BIOLOGY OF THE BASAL REGION OF *S. FLEXNERI* POLYSACCHARIDES

##### Basal Constituents and Sugar Sequences

From the above summary of the gross structure of the polysaccharides of *Enterobacteriaceae*, it is apparent that these complex polymers possess two distinct regions. The first of these is the basal structure which contains the rough antigenic determinants, and the second is comprised of the long O-specific side chains which determine the specificity and cross-reactivity of the various smooth serotypes. Moreover, smooth (S)-to-rough (R) mutation results when single enzyme defects in the biosynthetic pathway for the S-lipopolysaccharide prevent the incorporation of the O-specific side chains, with the consequent

## SCHEMATIC DIAGRAM OF SMOOTH SHIGELLA FLEXNERI O-POLYSACCHARIDES

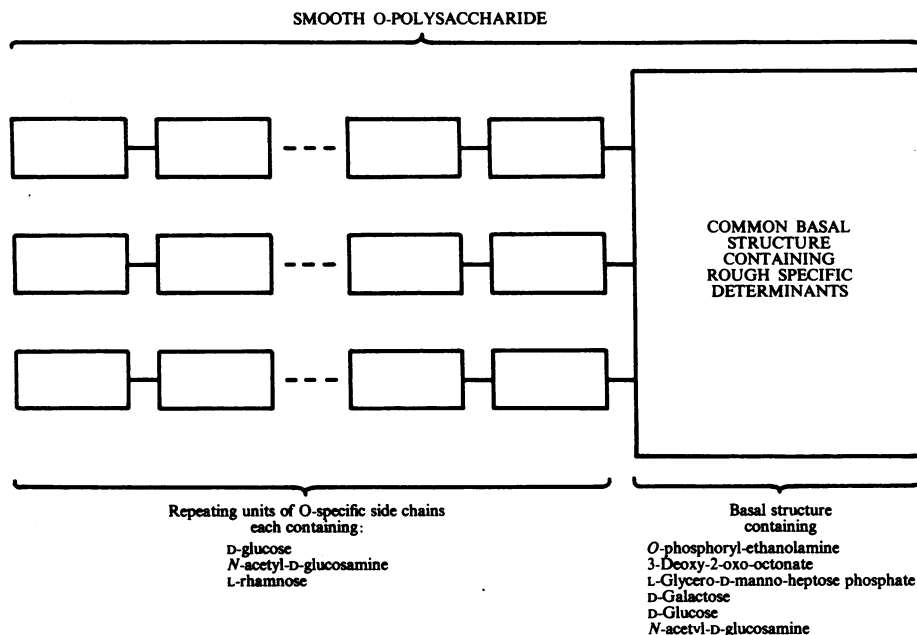


FIG. 1. Gross structure of *Shigella flexneri* polysaccharides showing constituents of the common basal structure and O-specific side-chain regions.

exposure of the underlying R-specific determinants in the basal region. Though a comparatively large number of enzyme lesions may give rise to R-mutants, two distinct mechanisms of smooth-to-rough mutation can be recognized. In the first type, which results from the loss of a side-chain synthetase or transferase, there is a total absence of O-specific side chains but the basal structure is completed normally. In the second type, which results from the loss of a basal synthetase or transferase, the biosynthesis of the O-specific side chains proceeds normally but these cannot be transferred to the incomplete basal structure which lacks the appropriate acceptor. Comparative studies (40-42) of serially related lipopolysaccharides from *S. flexneri* R-mutants of both the above classes have not only clarified the biochemical basis of smooth-to-rough mutation in this genus but have also elucidated the molecular structure of the common basal region.

In these studies (40), 10 rough mutants were isolated from a representative selection of 10 smooth serotypes. (The rough mutants were designated by adding the letter R to the parent serotype so that the symbol 1a-R, for example, denotes a rough mutant derived from smooth serotype 1a and not a rough mutant with 1a serological specificities.) The extracted smooth and rough lipopolysaccharides were then com-

pared by quantitative microanalysis of their hexose constituents. The 10 *S. flexneri* S-lipopolysaccharides were so similar that only 1 representative type is included in Table 2 for comparison with the R-lipopolysaccharides. By contrast, the 10 R-lipopolysaccharides could be divided into 4 distinct groups or chemotypes on the basis of their qualitative sugar composition. As all the lipopolysaccharides of any one chemotype were found to have the same quantitative composition, only one representative member from each R-chemotype is shown in Table 2. Compared with the S-lipopolysaccharides from the parent smooth forms, those from the rough mutants were characterized by the loss of rhamnose but many also lacked one or more of the basal sugars. Compared with serotype 4b-R lipopolysaccharide which contained all the basal sugars (aldoheptose phosphate, galactose, glucose, and N-acetylglucosamine), type 4a-R lacked N-acetylglucosamine; type 1a-R lacked N-acetylglucosamine and galactose; and type 3a-R lacked N-acetylglucosamine, galactose, and glucose. Thus the *S. flexneri* R-lipopolysaccharides shown in Table 3 belong to four chemotypes that are qualitatively indistinguishable from *Salmonella* R-chemotypes Ra, Rb, Rc, and Rd (57) in that they contain the following sugars: Rd, aldoheptose; Rc, aldoheptose + glucose; Rb, aldoheptose + glucose + galac-

TABLE 2. Quantitative microanalysis of the sugar components of some representative *Shigella flexneri* smooth (S)- and rough (R)-lipopolysaccharides<sup>a</sup>

| Serotype | Percentage of sugar in lipopolysaccharide |                           |                       |           |             |                                     |                |
|----------|-------------------------------------------|---------------------------|-----------------------|-----------|-------------|-------------------------------------|----------------|
|          | 3-Deoxy-2-oxo-octonate                    | O-phosphoryl-ethanolamine | Aldoheptose phosphate | D-Glucose | D-Galactose | N-acetyl-D-glucosamine <sup>b</sup> | L-rhamnose     |
| 4b-S     | 2.4                                       | 1.3                       | 6.0                   | 14.7      | 1.0         | 15.8                                | 20.1           |
| 4b-R     | 8.6                                       | 8.0                       | 13.5                  | 10.6      | 3.8         | 5.8                                 | — <sup>c</sup> |
| 4a-R     | 8.4                                       | 7.1                       | 24.6                  | 6.9       | 7.4         | —                                   | —              |
| 1a-R     | 8.7                                       | 6.5                       | 20.3                  | 6.8       | —           | —                                   | —              |
| 3a-R     | 7.8                                       | 5.0                       | 23.4                  | —         | —           | —                                   | —              |

<sup>a</sup> Reproduced from *Biochemical Journal*.

<sup>b</sup> Analysis of lipid A-free polysaccharide.

<sup>c</sup> Sugar not detected on chromatographic or quantitative analysis.

TABLE 3. Molar ratios of sugar components in a representative selection of *Shigella flexneri* rough (R)-lipopolysaccharides<sup>a</sup>

| Sero-type | Aldoheptose phosphate | Glucose | Galactose | N-acetylglucosamine | Rhamnose       | R-chemotype <sup>b</sup> |
|-----------|-----------------------|---------|-----------|---------------------|----------------|--------------------------|
| 4b-R      | 2.0                   | 2.7     | 1.0       | 0.9                 | — <sup>c</sup> | Ra                       |
| 4a-R      | 2.0                   | 0.9     | 1.0       | —                   | —              | Rb                       |
| 1a-R      | 2.0                   | 1.1     | —         | —                   | —              | Rc                       |
| 3a-R      | 2.0                   | —       | —         | —                   | —              | Rd                       |

<sup>a</sup> Reproduced from *Biochemical Journal*.

<sup>b</sup> According to the classification of Lüderitz et al. (57). Molar ratios were derived from the analytical data in Table 1. Aldoheptose phosphate was taken as 2.0. The R-lipopolysaccharides not included in this table were assigned to the following chemotypes: 6-R to chemotype Ra; variant Y-R to chemotype Rb; 2a-R, 5-R, and variant X-R to chemotype Rc; 1b-R to chemotype Rd.

<sup>c</sup> Sugar not detected on chromatographic or quantitative analyses.

tose; Ra, aldoheptose + glucose + galactose + N-acetylglucosamine. These four chemotypes formed a series in which each member differed from its precursor by the addition of a single sugar type; namely, glucose, galactose, and N-acetylglucosamine, respectively. These findings suggested that the biosynthesis of the basal structure commenced with the polyheptose phosphate "backbone" of chemotype Rd and proceeded by the sequential addition of glucose, galactose, and N-acetylglucosamine in that order to the growing side chain of the basal structure. However, the finding of 3 moles of glucose per 2 moles of aldoheptose phosphate in chemotype Ra lipopolysaccharides (Table 3) revealed the presence of glucose residues which were subsequently shown to be bound to the above sequence as secondary side chains. These lipopolysaccharides also contained

3-deoxy-2-oxo-octonate and O-phosphoryl-ethanolamine. In *Salmonella* lipopolysaccharides, the former is believed to be a "bridge" between the lipid A and polysaccharide components of the antigenic complex (74), and the O-phosphoryl-ethanolamine may be bound to the heptose residues of the backbone (30). Although experimental proof is lacking, it may be presumed that the structural relationships of these residues within the *S. flexneri* lipopolysaccharides are closely analogous to those in *Salmonella*.

#### Fine Structure of the Basal Region

The recovery of identical R-lipopolysaccharides from R-mutants isolated from different smooth serotypes provided strong evidence that the basal structure is common to all *S. flexneri* strains (40). This common basal structure was thought to be identical with the R-lipopolysaccharide of serotype 4b-R, as this polymer contained all the basal sugars and the mutant from which it was extracted contained an O-specific side chain defect which was presumed to not interfere with the synthesis of the basal structure. This polymer therefore provided an ideal model for the analysis of the *S. flexneri* basal region. Its structure was elucidated by characterizing seven electrophoretically neutral oligosaccharides isolated from the partial acid hydrolysate of this substance. The structures of the seven oligosaccharides were as shown in Fig. 2.

From this figure it is evident that the isolated oligosaccharides are degradation products of a single oligosaccharide unit that is identical with C1. This pentasaccharide probably represents the basal structure side chain because it is the highest oligosaccharide unit isolated and the molar ratios of its three constituents are the same as those in the undegraded *S. flexneri* 4b-R lipopolysaccharide (Table 3).



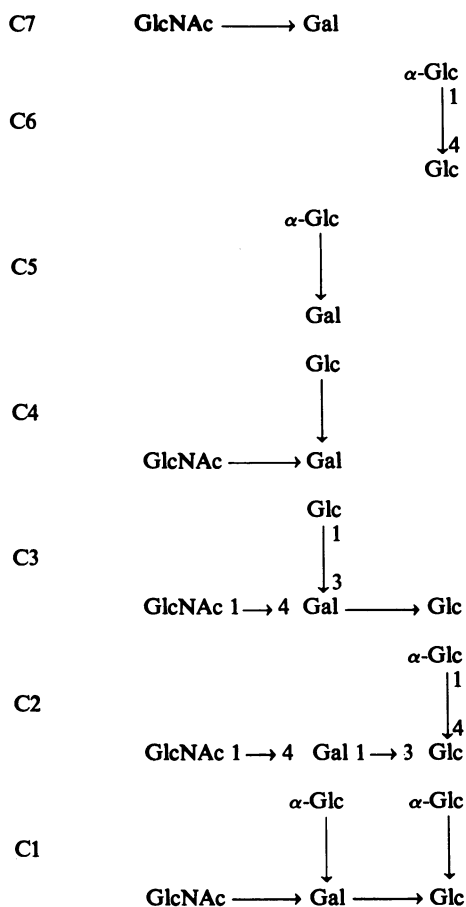


FIG. 2. Structural relationship of neutral oligosaccharides isolated from *Shigella flexneri* serotype 4b-R lipopolysaccharide which is identical to the common basal structure. From *Biochemical Journal*, vol. 101, no. 1.

Confirmatory evidence in favor of this branched structure was provided by periodate oxidation of intact (unhydrolyzed) serotype 4b-R lipopolysaccharide. Analysis of its hexose composition before and after such oxidation showed that all the galactose and one-third of the glucose could be recovered. As one-third of the glucose is equivalent to one residue (there being three glucose residues in the side chain), these results provided good evidence that the galactose and glucose residues in the primary chains of this lipopolysaccharide were protected and, therefore, most probably 3-*O*-substituted.

Still further evidence in support of the proposed basal structure emerged from a study of chemotype Rb (serotype 4a-R) lipopolysaccharide in which an unsuccessful attempt was made to isolate the acid-labile galactosylglucose sequence.

Hydrolysis of this lipopolysaccharide with weak acid (0.1 *N*-sulphuric acid) gave 100% of the galactose as free galactose within 10 min, by which time only 2.5% of the glucose had been liberated. These results demonstrated the extreme acid lability of the galactosylglucose sequence and confirmed that the galactose is the nonreducing end sugar, as it could be selectively split from the molecule without any accompanying release of glucose or oligosaccharides. Quantitative analysis of the hexose constituents of a sample of 4a-R lipopolysaccharide before and after periodate oxidation showed that the galactose was totally destroyed, whereas the bulk of the glucose was recovered. This finding confirmed that the glucose residue in the primary chain was protected and, therefore, 3-*O*-substituted. Thus, the *S. flexneri* 4a-R lipopolysaccharide consisted of galactosyl-(1 → 3)-glucose side chains attached to the "polyheptose phosphate backbone." This result, which indicated the existence of a single glucose residue between the galactose unit and the heptose backbone, provided strong evidence that the second glucose residue in oligosaccharide C<sub>2</sub> (Fig. 2) was present as a secondary side chain and that the branched structure assigned to that oligosaccharide on the basis of periodate-oxidation studies was most probably correct. It is interesting to note that none of the Rc or Rd mutants used in these studies carried the secondary glucose side chains present in the complete (Ra) basal structure. This may mean that the secondary glucose chains are not incorporated into the growing molecule until the primary side chains of the core are capped with *N*-acetylglucosamine. However, there is no experimental proof of this view from the limited number of Rc and Rb polymers studied.

From a consideration of the oligosaccharide structures of Fig. 2, the periodate oxidation studies on unhydrolyzed serotype 4b-R and 4a-R lipopolysaccharides and the observation that *N*-acetyl- $\alpha$ -D-glucosaminyl residues play an immunodominant role in chemotype Ra lipopolysaccharide (40), the basal structure side chain was believed to be an *N*-acetyl- $\alpha$ -D-glucosaminyl-(1 → 4)-galactosyl-(1 → 3)-glucose sequence with  $\alpha$ -glucosyl residues substituted on the 3- and 4-positions of the galactose and glucose, respectively. The structure of this unit and its relationship to the rest of the *S. flexneri* lipopolysaccharide molecule are shown in Fig. 3.

#### Nature of Basal Enzyme Defects Causing Smooth-to-Rough Mutation

Attention has already been drawn to the two main mechanisms of smooth-to-rough mutation.

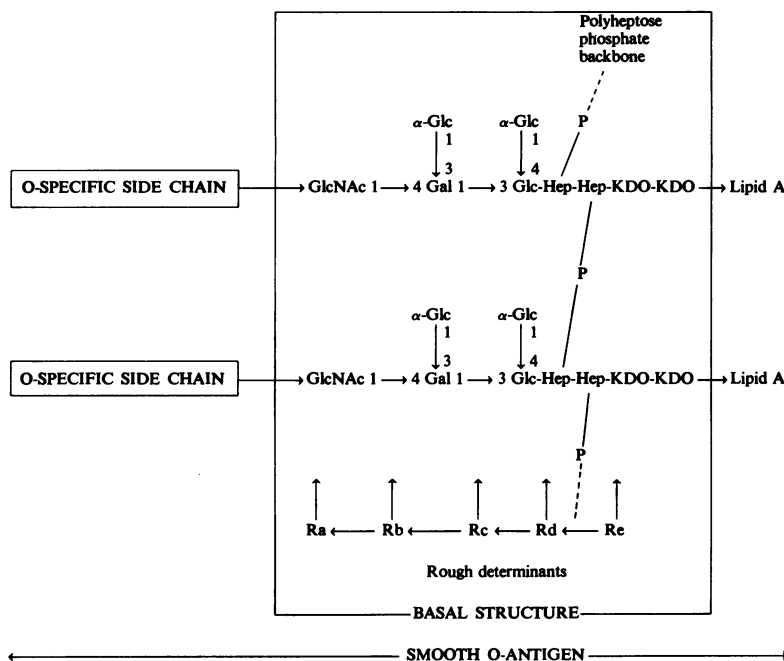


FIG. 3. *Shigella flexneri* basal structure and its relationship to the rest of the lipopolysaccharide molecule. The points at which enzyme defects interrupt biosynthesis with the consequent production of incomplete (rough) lipopolysaccharides are also shown by arrows. Key: KDO, 3-deoxy-2-oxo-octonate; HepP, aldoheptose phosphate; Glc, glucose; Gal, galactose; GlcNAc = N-acetylglucosamine.

In the first of these, the enzyme defect blocks the biosynthesis of the O-specific side chains which, therefore, are totally absent. In the second mechanism of S → R mutation, the enzyme defect blocks the completion of the basal region so that the O-specific side chains (which continue to be synthesized) cannot be attached to the deficient basal structure for lack of the appropriate acceptor site. In such circumstances, the intact O-specific side chains can be found free in the ultracentrifuge supernatant fluids of the extracted rough lipopolysaccharides. The presence or absence of specific side chains in these supernatant fluids therefore gives some indication of the site of action (basal or O-specific side chain) of the enzyme defect responsible for S → R mutation. In a detailed analysis of the supernatant fluids of *S. flexneri* R-lipopolysaccharides (42), those of serotypes 1a-R, 2a-R, 3a-R, 4a-R<sub>1</sub>, 4a-R<sub>2</sub>, 5-R, and variant X-R contained the side-chain sugars (rhamnose, N-acetylglucosamine, and glucose) bound together as in the O-specific side chains, as they precipitated with homologous smooth *S. flexneri* antiserum. These mutants, therefore, have no defects in the synthetases or transferases required for O-specific side-chain synthesis. This finding, together with the observation that all rough mutants given above lacked one or more

or the basal components, pointed to a lesion in one of the basal synthetases or transferases of these mutants. By contrast, the absence of side-chain sugars in the supernatant fluids of 1b-R, 4b-R, 6-R, and variant Y-R indicated the loss of a side-chain synthetase or transferase in these particular mutants. The 1b-R and variant Y-R mutants proved to be exceptional in that they were deficient in O-specific side chains and basal structure. It would appear, therefore, that their defects involved an enzyme that was required in the synthesis of both regions of the molecule, but the results did not exclude the possibility that these mutants had a double lesion.

The probable nature of the enzyme defects of the different R-chemotypes was defined more precisely as follows.

**Rd mutants.** These mutants elaborated a lipopolysaccharide identical to the polyheptose phosphate backbone but did not incorporate the first glucose residue into the basal structure side chain. This failure might have been due to their inability to synthesize uridine diphosphate (UDP)-glucose or to their inability to transfer glucose from this sugar nucleotide to the heptose backbone. With *S. flexneri* serotype 3a-R, the former possibility was excluded by the finding of glucose in the O-specific material in the ultracentrifuge supernatant

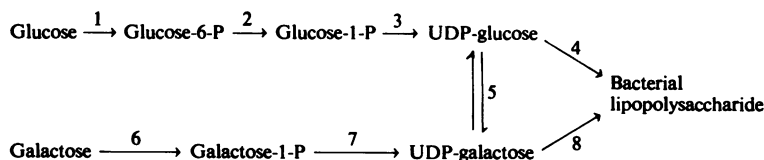


FIG. 4. Biosynthetic pathways for the incorporation of exogenous glucose and galactose into bacterial lipopolysaccharide. From Osborn *et al.* (75). 1, glucokinase; 2, phosphoglucomutase; 3, UDP-glucose pyrophosphorylase; 4, UDP-glucose transferase; 5, UDP-galactose-4-epimerase; 6, galactokinase; 7, galactose-1-P uridyltransferase; 8, UDP-galactose transferase.

fluid, so that this mutant probably lacked a UDP-glucose transferase analogous to UDP-glucose transferase I of *Salmonella* (75). With *S. flexneri* serotype 1b-R, the absence of O-specific side chains from the ultracentrifugal supernatant fluids indicated that this mutant was unable to synthesize UDP-glucose. Figure 4 shows that such a failure could arise from a defect in glucokinase, phosphoglucomutase, or UDP-glucose pyrophosphorylase, but because this mutant synthesized nonspecific polyglucosan in which the first two enzymes are necessary for transglucosylation (90) the defective enzyme is probably UDP-glucose pyrophosphorylase.

**Rc mutants.** The Rc lipopolysaccharides from *S. flexneri* serotypes 1a-R, 2a-R, 4a-R<sub>1</sub>, 5-R, and variant X-R resembled those of *Salmonella* in that all contained heptose phosphate and glucose and all were unable to incorporate galactose into the growing side chains of the basal structure when grown in the presence of exogenous glucose. This defect in galactose incorporation was bypassed when two representative Rc mutants (serotype 1a-R and variant X-R) were mass cultured in the presence of exogenous galactose because these mutants then synthesized lipopolysaccharides containing all the sugars normally present in the smooth form, including rhamnose. The formation of smooth lipopolysaccharide by these two mutants does not appear to be due to reversion, because subculture on galactose-deficient media at the end of mass culture showed that they were still rough as judged by colonial appearance, sedimentation in broth culture, and electrolyte agglutinability (42). Because Rc mutants contain glucose in their lipopolysaccharide, they must possess all the enzymes necessary to synthesize UDP-glucose (Fig. 4). Their failure to produce smooth lipopolysaccharide from exogenous glucose could be due to a defect in UDP-galactose-4-epimerase or UDP-galactose transferase. As the production of smooth lipopolysaccharide from exogenous galactose ruled out the possibility of a defect in the latter enzyme, these Rc mutants are probably deficient in UDP-galactose-4-epimerase. However, these mutants

differed from the classic M-mutants (chemotype Rc) of Nikaido (69) in their ability to ferment galactose and in the absence of bacteriolysis when tested for galactose sensitivity by the method of Nikaido (25). In this test, classic M-mutants are almost completely lysed 2 to 3 hr after the addition of galactose to the cultures due to the rapid accumulation of UDP-galactose. By contrast, the *S. flexneri* Rc mutants showed a more rapid rate of growth so that at 24 hr the optical density of the galactose cultures was two to three times greater than that of the controls. The ability of the *S. flexneri* Rc mutants to metabolize galactose probably saved them from bacteriolysis by preventing the rapid accumulation of intracellular UDP-galactose, which is believed to divert nucleotide bases from essential cell-wall synthesis in classic M-mutants.

A further interesting difference between *S. flexneri* Rc mutants and their analogues from *Salmonella* serogroups B, D, and E, is the ability of the former to synthesize O-specific side chains. This difference is due to the fact that galactose is both a basal and side-chain constituent of these *Salmonella* lipopolysaccharides (69), whereas in *S. flexneri* it is found in the basal region only. Thus, a defect in UDP-galactose-4-epimerase blocks the biosynthesis of both basal structure and side chains in *Salmonella* but only the basal structure in *S. flexneri*. The existence of such Rc mutants with the ability to synthesize O-specific material was predicted by Lüderitz *et al.* (57).

**Rb mutants.** The two mutants of this class (serotype 4a-R<sub>2</sub> and variant Y-R) contained heptose phosphate, glucose, and galactose in their polysaccharides and *N*-acetylglucosamine in their lipid-A component. They were, therefore, able to synthesize all the basal sugars. However, they could not incorporate the *N*-acetylglucosamine residue that completes the basal structure and were assumed by analogy with the corresponding *Salmonella* mutants (75) to lack the appropriate UDP-*N*-acetylglucosamine transferase. (Serotype 4a-R<sub>2</sub> is the same mutant as 4a-R above. Redesignation became necessary when further rough mutants were isolated in later studies. The serotype

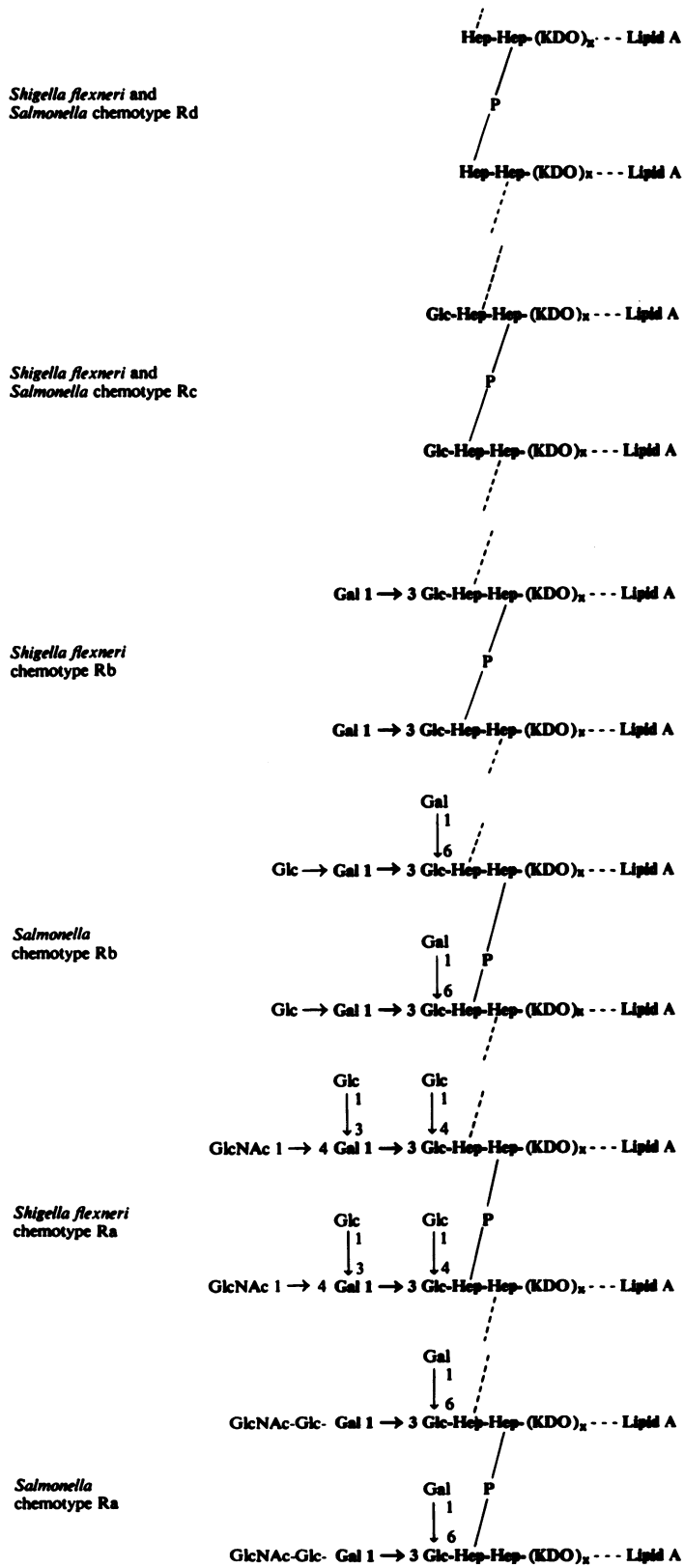


FIG. 5

4a-R<sub>2</sub> and variant Y-R polymers both lacked secondary glucose side chains.)

**Ra mutants.** Mutants of this class completed the basal structure but lacked O-specific side chains which contain glucose, *N*-acetylglucosamine, and rhamnose. Because these mutants could synthesize *N*-acetylglucosamine and glucose, as evidenced by the presence of these sugars in the basal structure, they presumably lacked a rhamnose synthetase or an O-specific side-chain transferase.

**Semirough (SR) mutant.** From its quantitative microanalysis and its behavior in a quantitative complement fixation system containing *S. flexneri* serotype 4a antiserum, a third rough lipopolysaccharide from serotype 4a (4a-R<sub>3</sub>) was shown to consist of the basal structure and the first repeating unit of the O-specific side chain. As judged by its structure, this lipopolysaccharide is an intermediate in the biosynthesis of smooth polysaccharide from its rough (chemotype Ra) precursor. Mutants of this class which were first described by Naide et al. (67) were assumed to possess transferase I, which transfers the first repeating unit of the O-specific side chain to the basal structure, but to not possess transferase II, which transfers the second repeating unit to the first. It was suggested that the *S. flexneri* SR mutant also lacked the analogous transferase II. Structural studies of the oligosaccharide sequences of this polymer would be of special interest in that they might define the biological as opposed to the chemical repeating unit of this O-specific side chain. An attempt was made to do this but, unfortunately, the results were inconclusive with the limited amounts of material available.

It should be noted that chemotype Ra and SR mutants have enzyme defects involving their O-specific side chains and not their common basal structure. Strictly speaking, they should not have been included in this section, which deals with basal structure biology, but it was convenient to do so at this stage to complete this summary of the enzymic basis of smooth-to-rough mutation in *S. flexneri*.

#### Comparison of *S. flexneri* and Other Enterobacteriaceae Basal Structures

**Salmonella basal structure.** The studies described above have shown that the *S. flexneri* and *Salmonella* basal structures are similar in a num-

ber of important respects. They contain the same basal constituents (95), have some internal structural sequences in common, and yield the same series of R-chemotypes when enzyme defects interrupt their biosynthesis during smooth-to-rough mutation (42). Despite these close similarities, two independent lines of evidence have indicated that the basal structures of these two genera are probably not identical. First, quantitative analysis has shown that the *Salmonella* basal side chain contains two glucose and two galactose units (108), whereas the *S. flexneri* analogue has three of glucose and one of galactose. Second, the higher R-chemotypes of these genera did not cross-react to any significant extent, which indicated that they are structurally different. The precise nature of this difference was clarified when it was shown that the *S. flexneri* basal side chain consisted of an *N*-acetyl- $\alpha$ -D-glucosaminyl-(1  $\rightarrow$  4)- $\alpha$ -D-galactosyl-(1  $\rightarrow$  3)-D-glucose sequence with  $\alpha$ -glucosyl residues substituted on the 3- and 4-positions of the galactose and glucose, respectively (40), whereas the *Salmonella* analogue had an *N*-acetyl- $\alpha$ -D-glucosaminyl- $\alpha$ -D-glucosyl- $\alpha$ -D-galactosyl-(1  $\rightarrow$  3)-D-glucose sequence with a further galactose residue  $\alpha$ -(1  $\rightarrow$  6)-linked to the reducing glucose unit (75, 108). These structures, together with those of the other R-chemotypes shown in Fig. 5, are particularly interesting in that they account for the observed similarities and differences in the biological properties of the *Salmonella* and *S. flexneri* basal regions. In particular, comparison of the analogous R-lipopolysaccharides explains why defects that block the synthesis of S-lipopolysaccharides produce the same R-chemotypes from both genera. Because a chemotype is a family of polymers with the same qualitative sugar composition, it follows that the chemotype of a polysaccharide under synthesis changes with the incorporation of each different sugar type but not with the addition of residues already present in the molecule. From Fig. 5, it is apparent that the four sugar types in *Salmonella* and *S. flexneri* basal structures are incorporated into the growing molecule in the same order in both genera; namely, aldoheptose phosphate, glucose, galactose, and *N*-acetylglucosamine. Thus, the biosynthesis of *Salmonella* and *S. flexneri* lipopolysaccharides proceeds through the same series of R-chemotypes to the complete

FIG. 5. Comparison of the structures of the analogous *Shigella flexneri* and *Salmonella* R-chemotypes. These chemotypes are cryptic situations in the complete basal structure which is identical with chemotype Ra. Structures shown in bold type are shared by both genera. Structural differences between the two genera are shown in normal type. These molecular similarities and differences explain the similarities and differences, respectively, in the biology of *Salmonella* and *Shigella* R-chemotypes. For key to symbols, see Fig. 3. In *S. flexneri* polymers,  $x = 2$ . In *Salmonella* polymers,  $x = 2$  or 3.

smooth form (Rd → Rc → Rb → Ra → S) and enzyme defects that interrupt this pathway will produce R-lipopolysaccharides of these chemotypes from both genera.

The structures of the various R-chemotypes (Fig. 5) also account for the presence or absence of cross-reaction between *S. flexneri* and *Salmonella* R-lipopolysaccharides in complement fixation tests (*personal observation*) and in passive haemagglutination studies (Lüderitz, *personal communication*; 87). These cross-reactions may be summarized briefly as follows.

(i) Chemotype Rd. *S. flexneri* and *Salmonella* Rd lipopolysaccharides all reacted very markedly when tested against *S. flexneri* or *Salmonella* Rd antiserum in quantitative complement fixation systems. Indeed, the curves obtained when these lipopolysaccharides were titrated over a wide weight range were indistinguishable throughout the regions of antibody excess, equivalence, and antigen excess. In the hemagglutination system, the *S. flexneri* Rd polymers (serotypes 1b-R and 3a-R) were also found to be powerful inhibitors of a homologous *Salmonella* Rd (*Salmonella Minnesota* mR7) system. These observations show that *S. flexneri* and *Salmonella* Rd lipopolysaccharides are indistinguishable in complement fixation and hemagglutination-inhibition tests and are therefore inferred to be structurally identical (Fig. 5). (The chemotype Rd structures have been simplified in the interests of brevity and clarity. In the genus *Salmonella*, chemotype Rd can be subdivided structurally and serologically into Rd<sub>1</sub> and Rd<sub>2</sub>, the former having both heptose residues and the latter the proximal residue only. All the *S. flexneri* Rd polymers studied to date have been of pattern Rd<sub>1</sub>. Rd<sub>2</sub> patterns presumably exist but have not yet been encountered.)

(ii) Chemotype Rc. *Shigella flexneri* Rc (serotype 4a-R<sub>1</sub>) lipopolysaccharide was indistinguishable from *Salmonella* Rc lipopolysaccharides (*S. typhimurium* tmM and *S. minnesota* mR5 polymers) when tested with *Shigella flexneri* or *Salmonella* Rc antiserum by quantitative complement fixation. Once again the curves obtained were identical throughout the regions of antibody excess, equivalence, and antigen excess. This result was confirmed by hemagglutination-inhibition as the *S. flexneri* Rc polymers (serotypes 2a-R and 4a-R<sub>1</sub>) were powerful inhibitors of a homologous *Salmonella* Rc (*Salmonella typhimurium* tmM) system. The observation that *S. flexneri* and *Salmonella* Rc lipopolysaccharides are serologically indistinguishable is readily explained by the fact that these polymers are structurally identical (Fig. 5).

(iii) Chemotype Rb. No cross-reaction was noted between *S. flexneri* Rb polymers and *Salmo-*

*nella* Rb antiserum or between *Salmonella* Rb polymers and *S. flexneri* Rb antiserum in complement fixation or hemagglutination-inhibition studies. These results may be explained by the finding that these two polymers (Fig. 5) are structurally different despite the fact that both contain the sequence  $\alpha$ -galactosyl-(1 → 3)-glucose. In the *S. flexneri* polymer, this unit is exposed as a terminal sequence (Fig. 5), whereas in *Salmonella* Rb polymers it is doubly masked by 6-O-substitution of the glucose with a further galactose residue and by substitution of the galactose residue with a glucose unit (Fig. 5). Although there is no serological cross-reaction between *S. flexneri* and *Salmonella* chemotype Rb mutants, it has been proposed (86) that the common sequence  $\alpha$ -galactosyl-(1 → 3)-glucose forms the receptor site for phage RFFm which lyses chemotype Ra and Rb mutants of both genera.

(iv) Chemotype Ra. In detailed quantitative complement fixation studies (40), the activity of *S. flexneri*-*Salmonella* cross-reacting Ra systems amounted to less than 5% of that obtained in the homologous Ra systems. These findings are consistent with the different structures assigned to *S. flexneri* and *Salmonella* Ra chemotypes (Fig. 5). Weak hemagglutination-inhibition reactions obtained with *S. flexneri* Ra lipopolysaccharide (serotype 4b-R) in homologous chemotype Ra *Salmonella minnesota* mR60 (Lüderitz, *personal communication*) and *S. paratyphi* B (87) systems are probably due to the presence of common terminal *N*-acetyl- $\alpha$ -D-glucosaminyl residues in these polymers (53).

Thus, there is much analytical and serological evidence that the inner part of the *Shigella flexneri* and *Salmonella* basal regions (represented by chemotypes Rd and Rc) are very similar if not actually identical. By contrast, the outer regions (represented by the higher chemotypes Rb and Ra) contain different sugar sequences so that the complete *Shigella flexneri* and *Salmonella* basal regions are structurally and serologically distinct.

***S. flexneri* serotype-6 basal structure.** Structural and serological analysis of many R-lipopolysaccharides have shown that the *S. flexneri* basal structure is probably common to all serotypes in this subgenus (40-42). However, the observation that serotype-6 had a much higher basal galactose content than other *S. flexneri* suggested that this serotype might be an exception to the general rule (96). Subsequent structural analysis of a rough serotype-6 lipopolysaccharide (44) showed that this polymer was indeed quite different from the *S. flexneri* basal structure in that it contained *O*-acetylated pentasaccharide side chains of the following structure:  $\alpha$ -D-Gal-(1 → 4)-D-Gal- $\beta$ -D-Glc-(1 → 3)- $\alpha$ -D-Glc-(1 → 3)-D-Glc. Because this

sequence has no structural features in common with either the *S. flexneri* or *Salmonella* basal regions, it is difficult to account for the observed cross-reactions of this polymer with antisera to *S. flexneri* (41) and *Salmonella* (Lüderitz, *personal communication*). A possible explanation is that one of the glucose residues in serotype-6 lipopolysaccharide is 2-*O*-acetylated so that it cross-reacts with the structurally similar terminal *N*-acetylglucosamine of the Ra structures. Although these structural studies are not yet sufficiently detailed to explain the serological behavior of this polymer, they do establish that the *S. flexneri* serotype-6 basal structure is quite different from its *Salmonella* and other *S. flexneri* analogues. Further evidence will be presented below showing that the O-specific side chains of this serotype-6 strain (a Manchester variant) are also atypical of the Flexner group.

**Basal structure of other *Shigella* subgroups.** The basal sugars have now been demonstrated in the lipopolysaccharides of *S. sonnei* (38, 77, 78, 91), *S. schmitzii* (9, 85, 91), *S. alkalescens* (9, 91), *S. dispar* (9), and *S. boydii* (85). *S. dysenteriae* (shiga) lipopolysaccharide probably also contains these constituents except that there is some dubiety in respect to glucose. This sugar was not detected in early studies (13, 65, 91) but has now been described as a minor component of both smooth (85) and rough (10) *S. dysenteriae* polysaccharides. The presence of the basal sugars in most (if not all) *Shigella* species raises the interesting and important questions of whether the basal structure is the same throughout the entire genus and what the relationship of this structure is to the basal regions of other genera in the family *Enterobacteriaceae*. Unfortunately, these questions must remain unanswered at the moment because detailed structural analysis of the basal regions of the above shigellae has not yet been attempted.

**Basal structure of *E. coli*.** Many *E. coli* O-specific lipopolysaccharides have now been analyzed and the majority have been found to contain the so-called basal sugars (54). Although the basal structure of this genus has not yet been completely elucidated, considerable progress has been made in this direction by Heath and his colleagues (14, 15, 32, 63), who showed that galactose, glucose, and *N*-acetylglucosamine were transferred sequentially to the incomplete (chemotype Rc) lipopolysaccharide of an *E. coli* O-111:B4 mutant in the same order as in *Salmonella*. These findings demonstrated the presence of similar sugar sequences in *Salmonella* and *Escherichia*. However, comparative analysis of oligosaccharides obtained from *E. coli* O-111:B4 and various *Salmonella* lipopolysaccharides has shown that the *N*-acetylglucosaminyl-glucosyl-galactose se-

quences of these two genera are not identical. In particular, the  $\alpha$ -glucosyl-(1  $\rightarrow$  4)-galactose disaccharide isolated from *E. coli* was quite distinct from the corresponding disaccharide of *Salmonella minnesota* R60 (108) and the *N*-acetylglucosamine in *E. coli* was  $\beta$ -linked, whereas the analogous *Salmonella* residue was  $\alpha$ -linked. There is good structural evidence, therefore, that the *Salmonella* and *Escherichia* basal regions differ from one another and from that of *Shigella flexneri* (Fig. 5). Although these results do not exclude the possibility that the inner zone of the *Escherichia* basal region (corresponding to chemotype Rc) is identical with its *Salmonella* and *Shigella* analogues, the serological evidence does not support this view. Indeed, the finding that the lipopolysaccharides of the *E. coli* O-111B4 mutant (chemotype Rc) and a lysine-dependent *E. coli* mutant of chemotype Rb (118) do not cross-react with *Salmonella* Rc and Rb antisera in hemagglutination-inhibition tests (Lüderitz, *personal communication*) or with *Shigella flexneri* Rc and Rb antisera in quantitative complement fixation reactions (*personal observation*) suggests that the inner *E. coli* basal region is also different from its *Salmonella* and *Shigella* analogues.

A further interesting feature of a well-defined group of chemotypes XVII and XIX strains, including *E. coli* O-17, O-44, O-59 and O-77, is the absence of the basal sugar galactose (37, 47). Thus, the basal structures of these *E. coli* serotypes must differ from that of *E. coli* O-111:B4, which implies that the basal region of *Escherichia* is almost certainly not homogeneous throughout the genus.

The importance of the basal region in relation to structural, taxonomic, genetic, and evolutionary aspects of the biology of the *Enterobacteriaceae* has been stressed in earlier studies (40, 57, 96). At the moment, most of these problems remain unsolved and further studies are required to extend our knowledge to other serotypes and genera so that comparisons within this family may be as broadly based as possible. Nevertheless, the rather limited results reviewed above are beginning to reveal some of the structural and biological characteristics of the different basal regions. These may be summarized as follows. First, with a few well-defined exceptions, the lipopolysaccharides of most gram-negative genera contain the basal constituents *O*-phosphorylethanolamine, 3-deoxy-2-oxo-octonate, aldoheptose phosphate, glucose, galactose, and *N*-acetylglucosamine. The presence of the same constituents and similar sequences in these different genera is probably not the product of chance but represents fundamental relationships between different bacterial groups within the family *Enterobacteriaceae*

(40). Second, despite many features in common, the *Salmonella*, *Shigella*, and *Escherichia* basal regions are structurally and serologically distinct. From the limited number of polysaccharides studied, it would appear that each genus contains one or, at most, a very limited number of basal structural patterns. In particular, the *Salmonella* basal region seems to be shared by all serotypes of this genus. Similarly, with the exception of the atypical serotype 6, all *Shigella flexneri* serotypes possess a common basal region, but it is not yet known whether this structure occurs in other *Shigella* subgroups. By contrast, structural and gross analytical results indicate the presence of at least two basal patterns in *Escherichia*. In general, however, the basal structure may be regarded as an expression of genus in the same way that the O-specific side chains are an expression of serotype. Third, because the biosynthesis of the basal region is under the genetic control of the bacterial nucleus, it can be assumed that structural similarities and differences between the various basal regions reflect corresponding similarities and differences in the genetic apparatus (and therefore in the evolutionary history) of the various genera. For example, the finding that the inner regions of the *Salmonella* and *Shigella flexneri* basal structures (Fig. 5) are identical, whereas the *Escherichia* analogue is quite different, implies that the evolutionary relationship between *Salmonella* and *Shigella flexneri* may be close compared with the relationship between these two genera and *Escherichia*. Similarly, the finding that *S. flexneri* serotype-6 basal region is totally different from those of the other *S. flexneri* and *Salmonella* serotypes implies that the genetic relationship between the atypical serotype-6 and the other *S. flexneri* is more remote than that between *Salmonella* and typical *Shigella flexneri*. Despite these interesting exceptions, our present understanding of genetic relationships between *S. flexneri* and other *Enterobacteriaceae* is still limited. However, it would seem reasonable to predict that the structural elucidation of the basal regions of further genera will increase our knowledge of intergeneric relationships in the family tree of *Enterobacteriaceae* and may even be of value to taxonomists in the generic classification of atypical serotypes such as *S. flexneri* serotype-6.

#### STRUCTURE AND BIOLOGY OF O-SPECIFIC SIDE-CHAIN REGION OF *S. FLEXNERI* LIPOPOLYSACCHARIDES

In the preceding sections, structural and biological aspects of the *S. flexneri* basal region were correlated. In particular, it was shown that fine structural studies have elucidated the biochemical basis of smooth-to-rough mutation and defined

the biosynthetic pathways for this region of the molecule. Such studies are also beginning to reveal evolutionary relationships between different genera in the *Enterobacteriaceae*. In the following sections, an attempt will be made to correlate the fine structures of the O-specific side chains with the biological problems associated with this region of the molecule; namely, the molecular basis of type specificity and cross-reactivity, the biosynthesis of the O-specific determinants, and taxonomic and genetic relationships within the subgroup *S. flexneri*.

#### Fine Structure of *S. flexneri* O-specific Side Chains

The structures of a representative selection of *S. flexneri* O-specific side chains have been determined by methods described in detail (96). In brief, oligosaccharides were isolated by partial acid hydrolysis and purified by chromatography and high voltage electrophoresis in pyridine-acetic acid and borate buffers; their hexose constituents (D-glucose, N-acetyl-D-glucosamine, and L-rhamnose) were quantitatively analyzed before and after borohydride reduction; and their behavior in Morgan-Elson, periodate-oxidation, and enzymic reactions were studied quantitatively. From the elucidated structures of overlapping oligosaccharide sequences obtained from *S. flexneri* serotype 1a, the fine structure of the whole 1a O-specific side chain was determined (Fig. 6).

In the same way, the structures of the *S. flexneri* serotype 2a, 3a, 4a, 5b, 6, and variants X and Y analogues were also elucidated (96). These are shown in Fig. 7 and 8, together with the structures of *S. flexneri* serotype 5a and the original variant Y of Hiss and Russell (34) which have been determined more recently by the same methods (*personal observation*).

These structures confirmed the results of an earlier study (95) in which quantitative analysis of the monosaccharide constituents of the *S. flexneri* lipopolysaccharides indicated that the O-specific side chains of serotypes 1a, 2a, 3a, 4a, and variant X contained tetrasaccharide repeating units of glucose, N-acetylglucosamine, and rhamnose in the proportions 1:1:2, respectively. The same study showed that serotype 5b repeating unit contained an additional glucose residue and that of variant Y no glucose. These conclusions were also confirmed by the structures proposed for these serotypes. The atypical serotype-6 O-specific side chains, which were found to be free of glucose and galactose, were comprised of N-acetyl- $\alpha$ -D-glucosaminyl-(1  $\rightarrow$  3)- $\alpha$ -L-rhamnosyl-(1  $\rightarrow$  2)-L-rhamnose repeating units linked together by  $\alpha$ -(1  $\rightarrow$  3)-bonds. The proposed O-specific side-chain structures also offered explanations for the results obtained in another



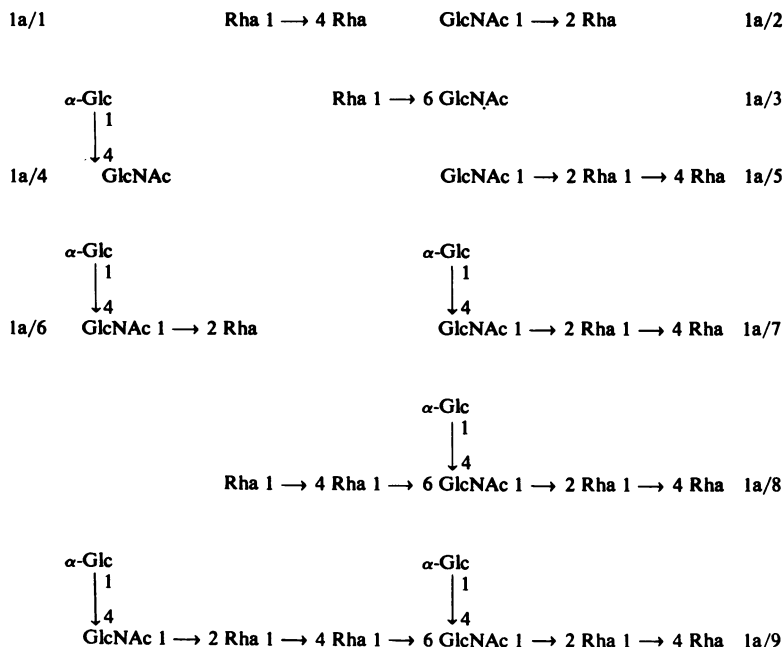


FIG. 6. Structural relationship of oligosaccharides from *Shigella flexneri* serotype 1a lipopolysaccharide. Key: Glc, glucose; GlcNAc, N-acetylglucosamine; Rha, rhamnose.

earlier study (43) in which the inhibiting effect of different monosaccharides was tested in homologous *S. flexneri* complement fixation systems to detect which sugar was immunodominant and, therefore, most probably the end-group determinant. With the serotype 1a, 2a, 4a, 5b, and variant X systems, glucose inhibited, and in each instance this sugar has proved to be the terminal nonreducing residue by structural analytical methods. The 3a system was not inhibited by glucose, which is in agreement with the observation that its terminal *O*-acetylglucose remained fully acetylated during extraction. With the variant Y system, rhamnose was the immunodominant sugar, which is in agreement with the structural findings that this polymer contains no glucosyl side chains and consists predominantly of rhamnose. The proposed *O*-specific side chain structures, therefore, were consistent with earlier conclusions as to the composition of their repeating units and the nature of the sugar residues that comprise their end-group determinants.

From the structures shown in Fig. 7 and 8, it is apparent that the *O*-specific side chains of this subgenus are comprised of a primary chain of *N*-acetylglucosamine and rhamnose to which secondary side chains of  $\alpha$ -glucosyl or *O*-acetylated  $\alpha$ -glucosyl residues are attached. Moreover, with the exception of serotype 6 which will be discussed below in detail, the *S. flexneri* *O*-antigens can be

divided into two groups according to primary chain structure. The first of these groups (Fig. 7), which contains serotypes 1a, 2a, 5a, and variant Y, has a primary chain structure that is identical with the *O*-specific structure of variant Y. It consists of *N*-acetyl- $\alpha$ -D-glucosaminyl-(1 → 2)- $\alpha$ -L-rhamnosyl-(1 → 4)-L-rhamnose repeating units linked together by  $\alpha$ -(1 → 6)-bonds. Primary chains of this structure have been designated  $Y_1$  to distinguish them from a second primary chain structure ( $Y_2$ ) which will be described below. The finding that the  $Y_1$  structure is a cryptic situation in serotypes 1a, 2a, and 5a suggests that Y variants arise as a result of defects in the specific UDP-glucose transferases that are required to transfer the terminal  $\alpha$ -glucosyl units to the *N*-acetylglucosamine and rhamnose residues of the  $Y_1$  primary chains. The second group (Fig. 8) contains serotypes 4a, 3a, 5b, and variant X. The primary side chains in these *O*-antigens were also identical, but different from those of the group  $Y_1$  polymers. They consisted of *N*-acetyl- $\alpha$ -D-glucosaminyl-(1 → 3)- $\alpha$ -L-rhamnosyl-(1 → 4)-L-rhamnose repeating units linked together by  $\alpha$ -(1 → 4)-bonds. Primary chains of this structure were designated  $Y_2$  to distinguish them from their  $Y_1$  analogues. Attempts to isolate the  $Y_2$  structure as a separate chemical entity have not been successful, but mutants with this structure might yet be found in nature as a consequence of defects

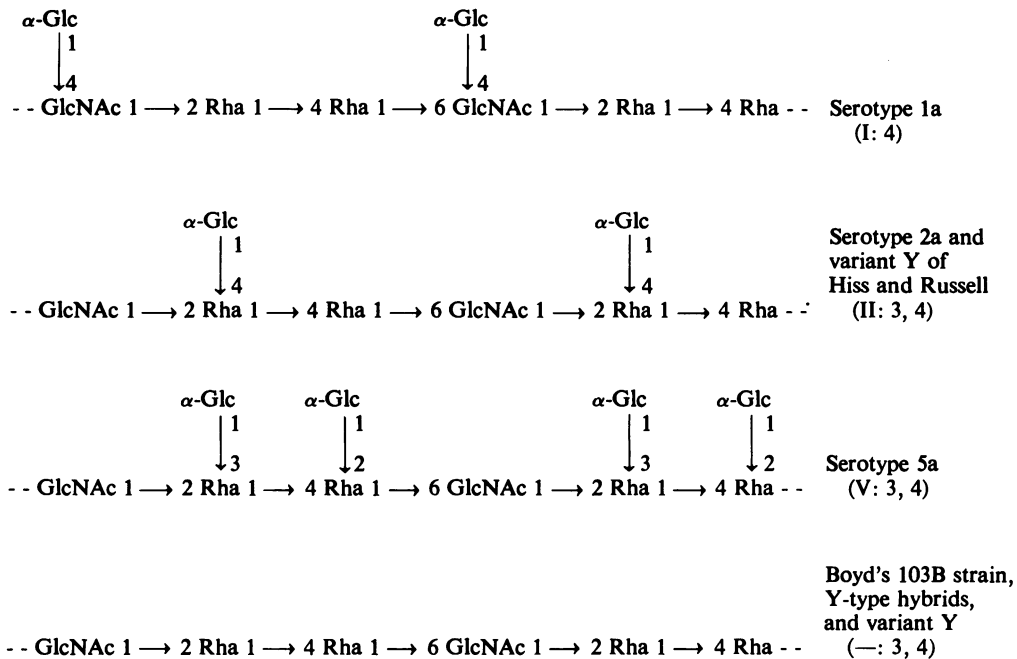


FIG. 7. Comparison of the O-specific side chains from *Shigella flexneri* serotypes 1a, 2a, 5a, variant Y of Hiss and Russell, Boyd's 103B strain, variant Y-type hybrids, and a classical variant Y showing that the Y<sub>1</sub> structure (which is identical with the primary chain of variant Y) is a cryptic situation in these smooth serotypes. Throughout this report, the antigenic formulas for the different serotypes are given in brackets according to the classification of Ewing and Carpenter (18), in which type-specific factors are designated by roman capitals and group factors by arabic numerals (see Table 1). For key to symbols, see Fig. 6. From *European Journal of Biochemistry*.

in the specific UDP-glucose transferases required to complete the biosynthesis of the four serotypes in this group. Furthermore, the variant X structure, which is a cryptic situation in serotypes 3a and 5b, is simply a deacetylated form of the former and differs from the latter in having one  $\alpha$ -glucosyl residue less per repeating unit. These findings suggested that X variants were the result of defects in the specific transacetylase and UDP-glucose transferase systems that complete the biosynthesis of serotype 3a and 5b antigens, respectively, from the precursor variant X structure. Thus, there is very good evidence that the *S. flexneri* O-antigens are a closely related family of polymers containing an underlying Y-type structure to which  $\alpha$ -glucosyl or O-acetylated  $\alpha$ -glucosyl secondary side chains are attached in a manner that is specific to each serotype and that X-variants are intermediates in the biosynthesis of serotypes 3a and 5b from precursor Y-substance.

With *S. flexneri* serotype 6, the structure of the O-specific side chain is unusual in that the rhamnosyl-(1  $\rightarrow$  2)-rhamnose and rhamnosyl-(1  $\rightarrow$  3)-N-acetylglucosamine sequences are not present in any other *S. flexneri* serotype studied to date.

Moreover, the basal structure of this serotype has also been shown to be quite different from that of classic *S. flexneri* (44). In addition to these structural differences, the serotype-6 strain used in all the above studies is a well-recognized biochemical (Manchester) variant which ferments glucose and mannite with the production of acid and gas, whereas other *S. flexneri* species produce acid only from these sugars. In view of these gross structural and biochemical differences, we no longer regard this particular strain as a true *S. flexneri* species.

#### Analysis of *S. flexneri* Group-Factor Determinants

In earlier attempts to elucidate the molecular basis of the serological behavior of *S. flexneri*, it was observed that the O-antigens of the different serotypes all contained the same hexose constituents, which suggested that type specificity was due to a unique difference in the linkage or sequence of the sugar residues in each polysaccharide and cross-reactivity to the sharing of common sequences (91, 92, 95). This view was substantiated by the elucidation of the O-specific side chain structures (96) in which it was known

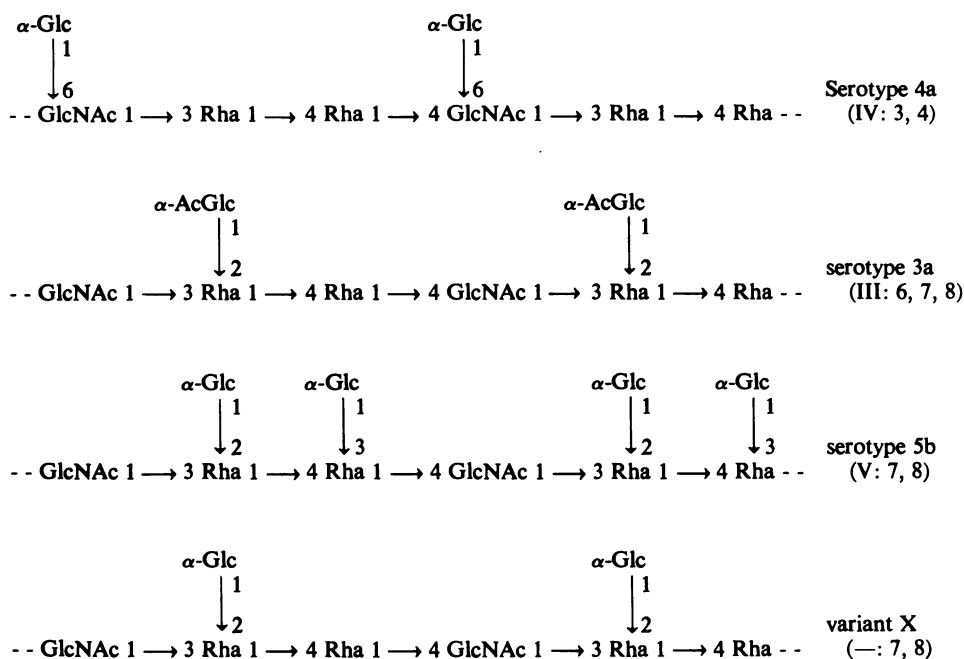


FIG. 8. Comparison of the O-specific side chains from *Shigella flexneri* serotypes 4a, 3a, 5b, and variant X showing that the Y<sub>2</sub> structure (which is identical with the primary chains of this group of polymers) is a cryptic situation in these smooth serotypes and that the variant X structure is a cryptic situation in serotypes 3a and 5b. For key to symbols, see Fig. 6.  $\alpha\text{-AcGlc}$  = *N*-acetylated  $\alpha$ -glucosyl radical. From *European Journal of Biochemistry*.

that the primary chains shared identical sequences, whereas the secondary chains differed from serotype to serotype. Therefore, the role of the common structural sequences in determining cross-reactivity was investigated (23) by inhibiting homologous and heterologous *S. flexneri* group-factor 4 and 3,4 complement fixation systems with oligosaccharides from *S. flexneri* variant-Y lipopolysaccharide. Because these oligosaccharides had been completely characterized, the precise structures of the common sequences involved in the group antigen could be defined. From the observation that rhamnose was the immunodominant sugar in group-factor serological systems (43), it was expected that these determinants would center around the sequence rhamnosyl-(1 → 4)-rhamnose. Indeed, this structure proved to be the determinant of group factor 4. Group factor 3,4 was found to be rhamnosyl-(1 → 4)-rhamnosyl-(1 → 6)-*N*-acetylglucosamine. Furthermore, from the inhibitory strengths of the various oligosaccharides tested, it was clear that the nonterminal rhamnose of the 3,4 determinant was the immunodominant sugar. The acidity of the 3,4 antibody was not only directed against this residue but also against the (1 → 6)-linkage to the adjacent *N*-acetylglucosamine and less so to the (1 → 4)-linkage of the rhamnosyl-

rhamnose sequence. Thus, complement fixation inhibition studies have characterized the 4 and 3,4 determinants very precisely and have shown that they are structurally related.

Although these studies indicated a close correlation between common internal sequences and group-antigen specificity, they also raised two apparent anomalies. The first of these concerned serotype 4a, which does not contain the precise structural sequence of the 3,4 determinant but which nevertheless has been assigned group factors 3,4 on serological grounds. This is due to true cross-reaction between the rhamnosyl-(1 → 4)-rhamnosyl-(1 → 4)-*N*-acetylglucosamine sequence of serotype 4a and antibody to the sequence rhamnosyl-(1 → 4)-rhamnosyl-(1 → 6)-*N*-acetylglucosamine of factor 3,4. It is interesting to note, however, that serological classifications which include minor antigenic factors do discriminate between such subtle differences. In particular, Boyd (7) assigns group-antigen factors 1,2,4 to serotypes 1a, 2a, and variant Y, all of which contain the rhamnosyl-(1 → 4)-rhamnosyl-(1 → 6)-*N*-acetylglucosamine sequence, and group factors 1,2,3 to serotype 4a which contains rhamnosyl-(1 → 4)-rhamnosyl-(1 → 4)-*N*-acetylglucosamine. The second apparent anomaly is the structural evidence for the presence of the

3,4 determinant in serotype 1a which taxonomists have characterized as containing group factor 4 only. In our view, 4-O-substitution of the *N*-acetylglucosamine in the sequence rhamnosyl-(1 → 4)-rhamnosyl-(1 → 6)-*N*-acetylglucosamine with the type-specific determinant of antigen factor I (97) produces steric hindrance over the rhamnosyl-(1 → 6)-*N*-acetylglucosamine linkage that is essential to the 3,4 determinant. In consequence, only antibody to the rhamnosyl-(1 → 4)-rhamnose sequence (determinant of factor 4) is able to combine with serotype 1a. This finding lends support to the view (96) that, in some instances at least, unexplained discrepancies in group factors associated with identical structural sequences are probably the result of modification of the stereochemistry of the internal determinants by their substitution in different positions with the secondary side-chain end-group determinants associated with type specificity.

#### Analysis of *S. flexneri* Type-Specific Determinants

From comparative structural analysis of the *S. flexneri* O-specific side chains (96) and from the observation that glucose is the immunodominant sugar in serotypes 1a, 2a, 4a, 5b, and variant X (43), it was expected that the determinant groups of the type-specific factors of these O-antigens would center around the glucose residues of the secondary side chains. Direct evidence in support of this view was provided by a detailed study (97) in which homologous type-specific complement fixation systems were inhibited with chemically characterized oligosaccharides of the same serotype. In each of the antigen-antibody systems of the above serotypes, the disaccharide sequence containing the terminal  $\alpha$ -glucosyl (secondary side chain) unit and the primary-chain residue to which it was linked was always a powerful inhibitor. Higher saccharides containing this disaccharide were equally potent inhibitors, but never more so. These findings provided strong evidence that the various type-specific factors were determined by the unique linkage of the  $\alpha$ -glucosyl secondary side chains to the primary side chains.

With the serotype 3a determinant, which appears to involve a highly labile *O*-acetyl group (96), oligosaccharide inhibitions were not possible because acetylated oligosaccharides could not be obtained by the chemical methods of hydrolysis employed in this study. However, from the structural studies with serotype 3a lipopolysaccharide (96) and from the observation that *N*-acetylglucosamine cross-inhibits homologous complement fixation systems of this serotype (43), the 3a determinant appeared to be

TABLE 4. Molecular structures of *Shigella flexneri* antigenic determinants<sup>a</sup>

| <i>S. flexneri</i> factor | Determinant                                                  |
|---------------------------|--------------------------------------------------------------|
| I . . . . .               | $\alpha$ -Glucosyl-(→4)- <i>N</i> -acetylglucosamine         |
| II . . . . .              | $\alpha$ -Glucosyl-(1→4)-rhamnose                            |
| III . . . . .             | 2- <i>O</i> -acetyl- $\alpha$ -glucosyl-(1→2)-rhamnose       |
| IV . . . . .              | $\alpha$ -Glucosyl-(1→6)- <i>N</i> -acetylglucosamine        |
| V . . . . .               | $\alpha$ -Glucosyl-(1→3)-rhamnose                            |
| 7,8 . . . . .             | $\alpha$ -Glucosyl-(1→2)-rhamnose                            |
| 4 . . . . .               | Rhamnosyl-(1→4)-rhamnose                                     |
| 3,4 . . . . .             | Rhamnosyl-(1→4)-rhamnosyl-(1→6)- <i>N</i> -acetylglucosamine |

<sup>a</sup> Reproduced from *Immunology*.

2-*O*-acetyl- $\alpha$ -D-glucosyl-(1 → 2)-L-rhamnose. The finding that a labile 2-*O*-acetyl group is involved in antigenic specificity has also been noted in *Salmonella* factor 0-5 in which *N*-acetylgalactosamine cross-reacts with 2-*O*-acetylgalactose in complement fixation tests (50). If one accepts the indirect serological evidence in respect to the structure of 3a type-specific antigen, the *S. flexneri* determinants that have now been characterized are as shown in Table 4.

The observation that the disaccharide structures assigned to antigen factors I, II, III, IV, and V (Table 4) specifically inhibit the homologous monospecific serological systems confirms that they are the type-specific determinants. This view is further supported by the absence of cross-inhibition of the homologous systems when tested with the type-specific determinant disaccharides of other serotypes. These findings are in agreement with the earlier results (23) in which it was shown that cross-reactivity in serotypes 1a, 2a, and variant Y depends on the common rhamnosyl-(1 → 4)-rhamnosyl-(1 → 6)-*N*-acetylglucosamine sequence in the primary chains of these serotypes. Cross-reactivity in serotypes 3a, 5b, and variant X (due to group factor 7,8) is determined by the common sequence  $\alpha$ -glucosyl-(1 → 2)-rhamnose in the lipopolysaccharides of these three serotypes.

#### Stereochemical Aspects of Antigenic Specificity in *S. flexneri* Polysaccharide Determinants

**Gross stereochemistry.** The importance of stereochemical factors in serological specificity has been stressed in the previous section in which it was noted that secondary side chains sometimes modified the stereochemistry of the group-factor

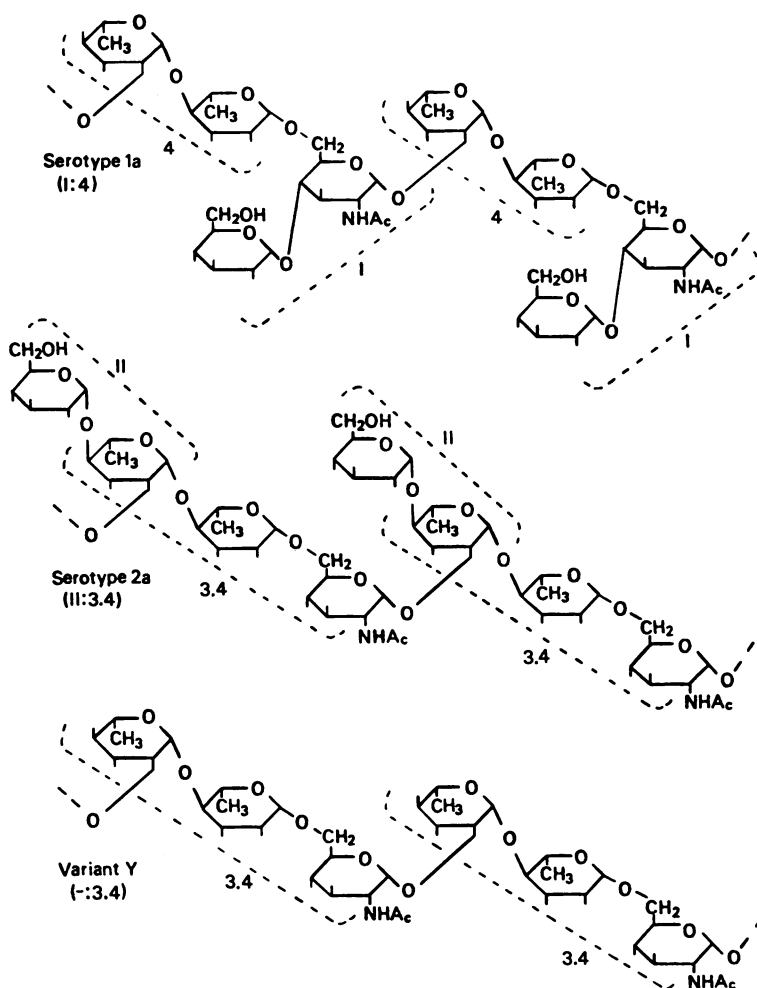


FIG. 9. Structures of *Shigella flexneri* group  $Y_1$  O-specific side chains showing the stereochemistry of the various specific and group determinants. The primary chains of this family of polymers are structurally identical. Stereochemical features of note are (i) the marked buckling of the molecules due to (1  $\rightarrow$  2)-linkages which split up the primary chains into rhamnosyl-(1  $\rightarrow$  4)-rhamnosyl-(1  $\rightarrow$  6)-N-acetylglucosamine sequences that comprise the group factor 3,4 determinant; (ii) the terminal position of the type-specific determinants and the nonterminal position of the group-factor determinants. Sugar residues can be identified rapidly as follows: rhamnose, by the methyl group at C6; N-acetylglucosamine, by the N-acetyl radical at C2; glucose, by its position as secondary side chains. For key to antigen factor designations, see Fig. 7. From *European Journal of Biochemistry*.

determinants that are associated with the primary-chain sequences. To obtain a deeper understanding of stereochemical aspects of antigenic specificity and cross-reactivity in *S. flexneri*, a detailed study of the shape of these molecules was undertaken (98), using molecular models of a representative selection of O-specific side chains constructed from the above structural information. In view of the well-known difficulties in representing such three-dimensional models on a plane surface, the spatial configurations of these models are presented in Fig. 9 and 10 in the form of

simple "projection formulas" which show two adjacent serological repeating units from each side chain.

It should be noted that the serological repeating units of Fig. 9 and 10 do not correspond with the structural sequences of the chemical repeating units of Fig. 7 and 8 (so called because they were isolated by chemical methods). Such differences in the chemical and serological units of a given polymer are due to acid-labile glycosidic linkages in these molecules. For example, a polysaccharide chain—ABCABCABC—comprised of ABC sero-

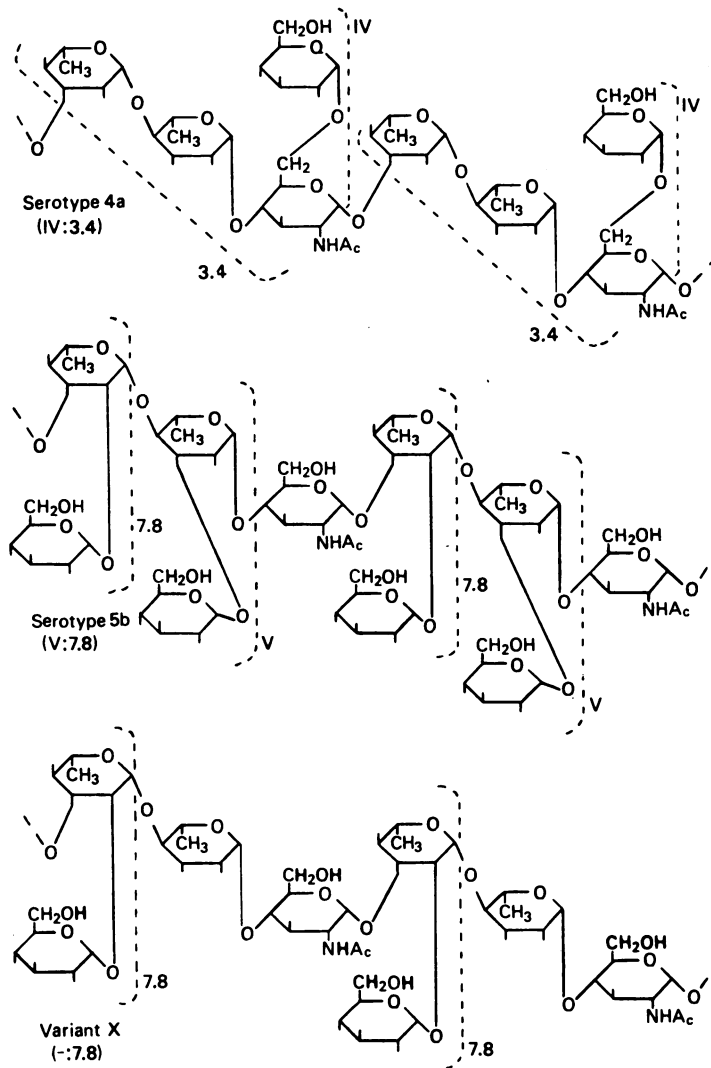


FIG. 10. Structures of *Shigella flexneri* group  $Y_2$  O-specific side chains showing the stereochemistry of the various specific and group determinants. The primary chains of this family of polymers are structurally identical, but are different from those of group  $Y_1$ . The stereochemical features of note are (i) the marked buckling of the molecules due to (1  $\rightarrow$  3)-linkages which split up the primary chains into rhamnosyl-(1  $\rightarrow$  4)-rhamnosyl-(1  $\rightarrow$  4)-N-acetylglucosamine sequences that comprise the group 3,4 determinant; (ii) the terminal position of the type-specific and 7,8 factors and the nonterminal position of the group factor 3,4 determinant. Sugar residues can be identified rapidly as follows: rhamnose, by the methyl group at C6; N-acetylglucosamine, by the N-acetyl radical at C2; glucose, by its position as secondary side chains. For key to antigen factor designations, see Fig. 7. From *European Journal of Biochemistry*.

logical repeating units with acid-labile AB linkages will yield BCA chemical repeating units upon partial acid hydrolysis. With the *S. flexneri* polysaccharides, the chemical repeating units are based on N-acetylglucosaminyl-rhamnosyl-rhamnose sequences (96), whereas the serological repeating units are based on rhamnosyl-rhamnosyl-N-acetylglucosamine sequences (23, 97).

It should also be noted that these projection formulas do not show the precise relationship between residues and radicals within these molecules. Nevertheless, they do illustrate the gross steric features that are of serological importance in this particular genus; therefore, they are adequate for the purposes of this discussion. The most striking of these features proved to be

the marked buckling produced by the (1 → 2)- and (1 → 3)-bonds in  $Y_1$  (Fig. 9) and  $Y_2$  (Fig. 10) primary chains, respectively. This buckling bent the primary chains into spatially discrete, sterically accessible repeating units that were structurally identical with the 3,4 group determinants—namely, rhamnosyl-(1 → 4)-rhamnosyl-(1 → 6)-*N*-acetylglucosamine in  $Y_1$  polymers and rhamnosyl-(1 → 4)-rhamnosyl-(1 → 4)-*N*-acetylglucosamine in  $Y_2$  polymers. Thus, the serological repeating units differed from the chemical repeating units described above but corresponded precisely with the “steric repeating units” of the molecular models. These findings provided strong evidence that the (1 → 2)- and (1 → 3)-bonds predetermine the sugar sequences involved in the group-factor determinants. However, comparative studies of complement-fixation inhibition have shown that these particular (1 → 2)- and (1 → 3)-bonds which define the group-factor determinants are not involved in combination with the homologous antibody-combining site (97). It was proposed, therefore, that determinants of this type should be called apodeterminants (Greek *apo*—away from or distant) because, by conferring a distinctive shape on the molecule, they determine the sequence and steric accessibility of the more distant structures that comprise the antigenic determinant without themselves forming an integral part of it.

A further stereochemical feature of the *S. flexneri* polysaccharides, apparent in the molecular models and the projection formulas of Fig. 9 and 10, is that the antigenic determinants fall into two spatially distinct families. The first family (containing the type-specific and group-factor 7,8 determinants) is associated with the  $\alpha$ -glucosyl secondary side chains which are classic end-group determinants of the type described by Kabat (45). They project from the primary chains and therefore occupy a stereochemically advantageous position for combining with homologous antibody. The second family of antigenic determinants contains group factors 4 and 3,4 which are determined by the sequences rhamnosyl-(1 → 4)-rhamnose and rhamnosyl-(1 → 4)-rhamnosyl-(1 → 6)-*N*-acetylglucosamine, respectively. They are “internal determinants” of the type first described in *Salmonella* group E by Robbins and his colleagues (110). At first sight, these determinants might seem to be at a disadvantage for combining with the appropriate antibody in consequence of their internal (non-terminal) situation in the main polysaccharide chain. However, the molecular models show that this is not the case, as such determinants occupy a sterically superficial position in consequence of the primary chain buckling described above.

These findings supported the view that immunodominant radicals, terminal and internal alike, probably occupy superficial positions that are sterically accessible to the homologous antibody-combining sites. It was proposed, therefore, that the term nonterminal determinant would be a more accurate description of those determinants previously referred to as internal and would have the added advantage of not prejudging whether such determinants are internal in a spatial sense.

**Fine stereochemistry.** From the evidence of the *S. flexneri* O-specific side-chain models (98), it was apparent that there was almost free rotation around the (1 → 2)- and (1 → 3)-bonds in the  $Y_1$  and  $Y_2$  primary chains, whereas by contrast the intervening rhamnosyl-rhamnosyl-*N*-acetylglucosamine sequences had a relatively rigid, almost rectilinear structure which was determined by a number of factors including hydrogen bonding, repulsion between like polar groups, and steric hindrance from adjacent repeating units and secondary side chains. In consequence of this relative rigidity, the repeating units of the primary chains had rather well-defined spatial configurations that could be accurately represented by the projection formulas of Fig. 11.

From a detailed consideration of the distribution of the polar groups in these  $Y_1$  and  $Y_2$  sequences, it was shown (98) that the two structures were sterically very similar. This finding offered an explanation for the experimentally observed cross-reaction between serotype 4a polysaccharide (which has  $Y_2$  basic sequences) and antiserum to variant Y (which has  $Y_1$  basic sequences). From comparative complement-fixation inhibition studies with a series of serologically active trisaccharides, it was shown that 3,4 antibody combined with the radicals that constituted the upper surface of the basic sequences, as shown by broken arrows in Fig. 11. This conclusion was in agreement with the evidence of the molecular models that this surface of the molecule is sterically accessible as the result of primary chain buckling, whereas the other faces are protected to a greater or lesser extent by steric hindrance from adjacent repeating units.

However, the most striking confirmation that 3,4 antibody combines along the upper face of the molecule came from the substitution of the basic sequences with  $\alpha$ -glucosyl side chains in positions indicated by heavy arrows in Fig. 11. Substitution from below during the biosynthesis of specific factors II, IV, and V (of V:3,4) does not mask the 3,4 determinant so that such polysaccharides carry antigen 3,4. Substitution from above during the biosynthesis of specific factors I, III, 7,8, and V (of V:7,8) does mask the spatial configuration of the 3,4 determinant so that such

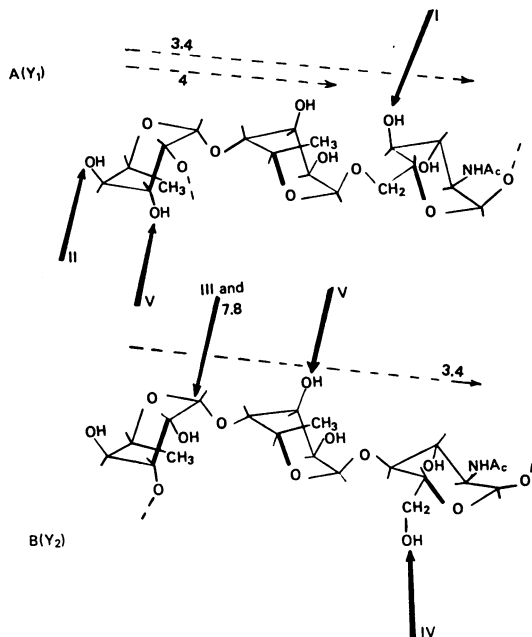


FIG. 11. Fine stereochemical structures of the *Shigella flexneri*  $Y_1$  and  $Y_2$  serological repeating units showing (i) the close steric relationship between the serological repeating units of  $Y_1$  (Fig. 11A) and  $Y_2$  (Fig. 11B), which accounts for the cross-reaction between serotype 4a polysaccharide and antiserum to variant  $Y$ ; (ii) the upper surfaces of the sequences that determine the 4 and 3,4 determinants; (iii) the serological consequences of the biosynthesis of the type-specific factors from precursor group antigen. Serotypes formed by incorporating  $\alpha$ -glucosyl residues below the basic sequences do not mask the 3,4 determinant and therefore possess 3,4 specificity. Serotypes formed by incorporating  $\alpha$ -glucosyl residues above the basic sequences do mask the 3,4 determinant and therefore do not possess 3,4 specificity. Broken arrows indicate the 4 and 3,4 determinants. Solid arrows indicate the type-specific determinants formed by incorporating  $\alpha$ -glucosyl side chains. From *European Journal of Biochemistry*.

polysaccharides do not display 3,4 specificity. Nevertheless, the 3,4 determinant is a cryptic situation in these antigens. With type-specific factor I however, the masking is not complete and antibody is still able to combine with the rhamnosyl-(1  $\rightarrow$  4)-rhamnose sequence of group factor 4. The antigenic formula of serotype 1a, therefore, is I:4. Thus, these detailed stereochemical considerations offer explanations for the serological factors assigned to the various serotypes by taxonomists. They also provide good experimental evidence for the view—first put forward by Kabat (46) with respect to blood group A determinant and later by Staub and her

colleagues (68, 105) with respect to *Salmonella strasbourg* determinants—that antibody to a polysaccharide determinant is sometimes directed against one face or aspect of the molecular surface.

### BIOSYNTHESIS OF *S. FLEXNERI* O-ANTIGENS

In an earlier study of smooth-to-rough mutation in this genus (40), it was shown that the isolated R-lipopolysaccharides were structurally related polymers that could be arranged in a sequence (Re  $\rightarrow$  Rd  $\rightarrow$  Rc  $\rightarrow$  Rb  $\rightarrow$  Ra  $\rightarrow$  S) that represented both their structural relationships and biosynthetic pathways. In this scheme, the biosynthesis of *S. flexneri* lipopolysaccharide commences with chemotype Re (containing O-phosphorylethanolamine and 3-deoxy-2-oxo-octonate) and proceeds by the sequential addition of aldoheptose, glucose, galactose, and N-acetylglucosamine in that order to give chemotypes Rd, Rc, Rb, and Ra, respectively, which were analogous to their *Salmonella* counterparts described by Lüderitz et al. (57). The synthesis of the smooth lipopolysaccharide is then completed by the incorporation of the O-specific side chains to the chemotype Ra structure. The structural analysis of these O-specific side chains has made it possible to extend this scheme to show the more complex relationships and biosynthetic pathways for the various smooth serotypes (96) (Fig. 12). This scheme also shows the sugar involved in the biosynthetic block and the presumed nature of the enzyme defect in each chemotype or serotype. In a previous report (42), evidence was presented that the defective enzyme in chemotype Rc was UDP-galactose-4-epimerase, and in two chemotype Rd strains the enzyme defects seemed to be UDP-glucose transferase in one case and UDP-glucose-pyrophosphorylase in the other. In all other instances, the nature of the enzyme defect has been presumed from the circumstantial evidence presented by the structural analytical results.

Although the biosynthesis of the basal structure proceeds by the sequential addition of single-sugar residues, the precise mechanism of synthesis of the *S. flexneri* O-specific side chains is not so well understood. In a few *Salmonella* species (114, 115, 119), there is evidence that the O-repeating units are synthesized and polymerized on an antigen-carrier lipid and, in some instances at least, incorporation of the secondary side chains then follows. With the *S. flexneri* O-specific side chains, the isolation of the glucose-deficient Y structure suggests that its repeating units are polymerized before the incorporation of the  $\alpha$ -glucosyl secondary side chains that charac-



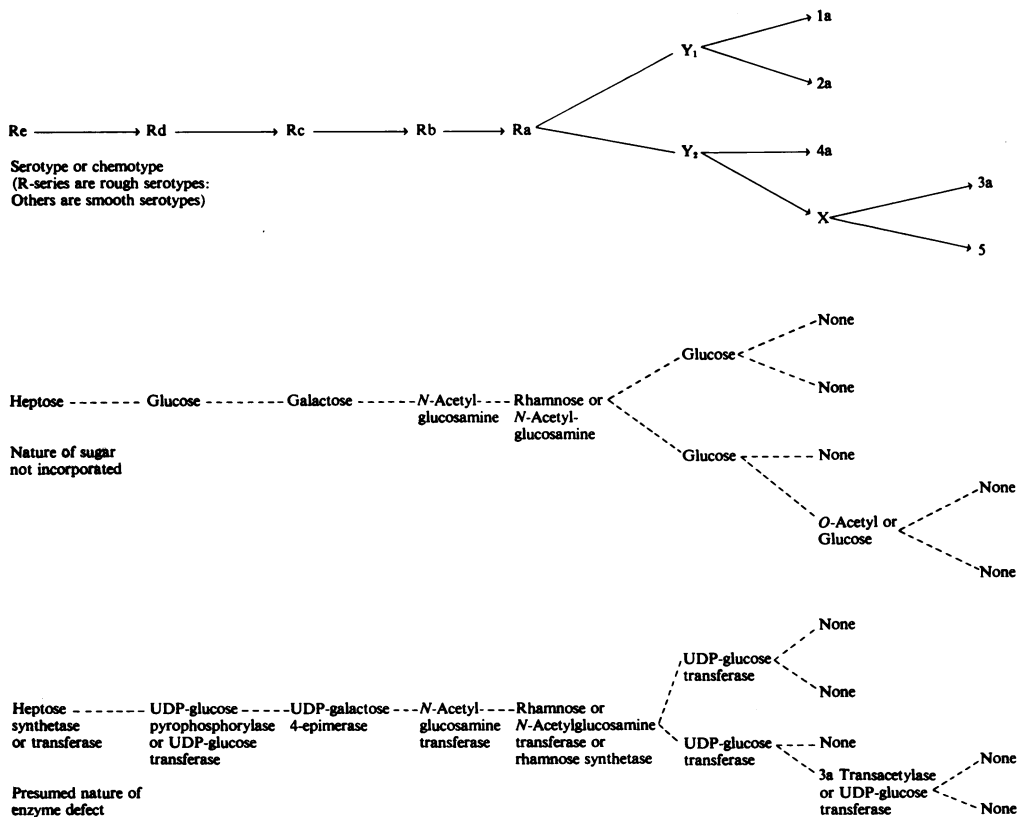


FIG. 12. Structural relationships and biosynthetic pathways of *Shigella flexneri* smooth and rough antigens showing the probable nature of the sugars and enzymes that are involved in the biosynthetic blocks that give rise to the different serotypes. From *European Journal of Biochemistry*.

terize serotypes 1a and 2a. This situation is strictly analogous to that in *Salmonella* factor 34 as described by Robbins in a personal communication to Lüderitz et al. (57). Similarly, the finding that the variant X structure is simply a deacetylated form of serotype 3a suggests, but does not conclusively prove, that transacetylation takes place after the rest of the side chain has been synthesized. This situation is strictly analogous to that in *Salmonella* factor 10 (80). From the above findings, it seems reasonable to conclude that the biosynthesis of the *S. flexneri* O-antigens takes place in three distinct stages. In the first, the basal structure is completed by the sequential addition of single-sugar residues to the primitive chemotype Re structure. In the second, the repeating units of the primary side chain probably polymerize in a manner analogous to that in *Salmonella* to give a variant Y-type structure, and in the third stage the biosynthesis is completed by the incorporation of the  $\alpha$ -glucosyl secondary side chains in a linkage that is specific for each serotype. *S. flexneri* variant X represents an

intermediate stage in the biosynthesis of serotypes 3a and 5b from the precursor Y structure.

#### TAXONOMIC ASPECTS OF *S. FLEXNERI* O-ANTIGENS

A necessary prerequisite to the correlation of serological specificity and molecular structure is a reliable serological classification. With salmonellae, the Kauffmann-White schema has provided a classification that has stood the test of time and has made the assignment of molecular structures to the various *Salmonella* O-factors a comparatively easy matter. With *S. flexneri*, the situation is not so simple in that the numerous schemes proposed have rarely been accepted without alteration for more than a few years and even the *Shigella Commission Reports* of 1951 (88) and 1958 (89) have been subject to modification. The explanation for this basic difference between the two genera is apparent from the chemical structures of their O-antigens. With *Salmonella*, the immunodominant sugars of the various O-factors are frequently different—for

example, glucose, paratose, mannose, abequose, acetylabequose, tyvelose, rhamnose, and colitose in factors 1, 2, 3, 4, 5, 9, 12, and 35, respectively (57). Even in *Salmonella* groups of the same chemotype (for example, serogroups G, N, and U of chemotype VI which all have the same constituents), the distribution of the hexoses between the basal structure and side chains is such that the immunodominant sugar of the O-specific determinant is different in each case (55). By contrast, the *S. flexneri* O-antigens all consist of a Y-type structure substituted with an immunodominant  $\alpha$ -glucosyl or O-acetylated  $\alpha$ -glucosyl residue. With such closely related cross-reacting structures, the antigenic differences between the various *S. flexneri* serotypes are much less distinct than with salmonellae, and their serotyping and taxonomy are that much more difficult in consequence.

However, these taxonomic difficulties were due not to poor reproducibility of experimental results but to "considerable uncertainty as to the interpretation of these data and the related problem of nomenclature" (112). In particular, the taxonomic relationship of certain serotypes and the serological status of variants X and Y have been the subject of much debate that has led to the introduction of some 20 classifications. Most of these, however, were based on one of two concepts. The first of these was put forward by Andrewes and Inman (2), who suggested that the serological behavior of the *S. flexneri* could best be explained by postulating four main races which were designated V, W, X, and Z. Each serotype was a mixture of the four factors, but with one in quantitative predominance. The Y race was thought to be a more even mixture of the four factors, but the inability of suspensions of the other four races to absorb Y antiserum led these authors to conclude that they had "failed fully to solve the problem of the antigenic structure of the Y races." This view of the *S. flexneri* antigens was widely held until the second concept was introduced by Boyd (7) who proposed that there were six main serotypes, each of which possessed a type-specific antigen which never occurred in the other serotypes and a group antigen which was shared with the other types. Strains X and Y were regarded as group variants, Y being a degraded form of a number of types and X a degraded form of Z (serotype 3a). Furthermore, Boyd believed that A  $\rightarrow$  B variation represented the loss of the type-specific antigen with the production of a pure-group variant which had been isolated on three occasions—namely, in strains 103B, 119B, and the original variant Y of Hiss and Russell. This concept of the antigenic structure of *S. flexneri* has formed the basis of all

subsequent classifications, including those of the *Shigella Commission Reports* (88, 89) and that of the currently accepted schema of Ewing and Carpenter (18).

In my view, the structural studies reviewed above are particularly interesting in relation to these concepts in a number of ways. First, they show that the O-antigenic structure of a given *S. flexneri* serotype is not a mixture of type-specific antigens or of type-specific and group antigens but a single molecular entity carrying different antigenic determinants. Proof of this concept is found in the isolation of small oligosaccharide fragments of undoubted purity which carry both type and group specificity as, for example, in serotype 1a (I:4) in which the tetrasaccharide  $\alpha$ -glucosyl-(1  $\rightarrow$  4)-*N*-acetylglucosaminyl-(1  $\rightarrow$  2)-rhamnosyl-(1  $\rightarrow$  4)-rhamnose has type factor I activity due to the sequence  $\alpha$ -glucosyl-(1  $\rightarrow$  4)-*N*-acetylglucosamine and group factor 4 activity due to the sequence rhamnosyl-(1  $\rightarrow$  4)-rhamnose. Second, Boyd's view of one type-specific factor and one or more group factors is borne out by the structural analysis of all serotypes studied to date in that type specificity is due to the unique linkage of the terminal  $\alpha$ -glucosyl secondary side chains in each serotype and group specificity is due to the sharing of common structural sequences in the primary chains of all serotypes. The presence of these terminal immunodominant secondary side chain glucose residues in all *S. flexneri* species except variant Y explains the observation of Andrewes and Inman (2) that *S. flexneri* suspensions were unable to completely absorb antiserum to variant Y in which the immunodominant sugar is non-terminal primary-chain rhamnose. Third, the suggestion that group antigen was present in most serotypes has been substantiated by the above observation that the structures of Boyd's original 103B strain and all Y-specific *S. flexneri*-*E. coli* hybrids (61) are identical with that of classical variant Y strains. However, the original variant Y of Hiss and Russell, which many observers have thought closer to W (serotype 2a) than any other *S. flexneri*, has proved to be structurally and serologically identical with serotype 2a. Fourth, Boyd's view that variant X is a degraded Z (serotype 3a) is confirmed by the finding that the former is a deacetylated form of the latter. The present studies also show, moreover, that variant X could arise as a degraded form of serotype 5b. Fifth, the analysis of the *S. flexneri* determinants seems to be relevant to the debate that has always surrounded the serological status and biological significance of the X and Y variants. The Y variants clearly have incomplete antigens that have arisen as the result of the loss or repres-

sion of the genetic locus controlling the UDP-glucose transferases necessary to incorporate the  $\alpha$ -glucosyl secondary side chains that are responsible for type specificity. The structural and biosynthetic evidence that they are incomplete antigens, therefore, agrees with the taxonomists' view that they are degraded forms of the type-specific antigens. With the X variants, the evidence as to their nature is conflicting in some respects. Their determinant belongs to the same family as those of the type-specific serotypes, and the gene controlling its UDP-glucose transferase maps genetically with the type specific or "T-locus" near the *lac* region (109). On the other hand X variants, which represent an intermediate stage in the biosynthesis of serotypes 3a and 5b from the precursor Y structure, are the consequence of a loss or repression of the genes controlling 3a transacetylase and factor V-specific UDP-glucose transferase, respectively (96). They may be regarded, therefore, as degraded forms of serotypes 3a and 5b with which they hold common determinants. Thus, structurally and genetically variant X antigens belong to the type-specific class, but from the point of view of biosynthesis and serology they behave as group antigens. These findings appear to offer an explanation for the difficulties that have arisen in the past in deciding the serological status of X variants.

One of the primary objects of the present structural studies was the elucidation of the molecular basis of serological specificity and cross-reactivity with a view to improving our understanding of taxonomic problems. This aim has been achieved to some extent in that the general structural pattern of the *S. flexneri* O-antigens has been established, the precise sequences of specific and group antigens have been resolved, the relationship of serotype 3a and variant X strain has been determined, and the nature and significance of X and Y variants have been clarified.

#### GENETIC ASPECTS OF *S. FLEXNERI* O-ANTIGENS

From the preceding evidence, the *S. flexneri* O-antigens are a closely related family of polymers with primary side chains of pattern  $Y_1$  or  $Y_2$  to which the  $\alpha$ -glucosyl secondary side chains that determine serological specificity are attached. If this concept is correct, it should be possible to recover Y-type variants from all *S. flexneri* serotypes as a result of defects in the genes controlling the synthesis of the appropriate specific UDP-glucose transferases. One or two naturally occurring examples of this kind of mutation can be found in early literature (33),

but detailed studies of this phenomenon have been described on three occasions only. In the first of these, Kruse et al. (51) showed that the Wilz B strain derived from Wilz A (serotype 1a) was serologically indistinguishable from Strong B (a classical variant Y strain). In the second detailed study of A  $\rightarrow$  B variation, Boyd (6) noted that *S. flexneri* type 103A frequently mutated to a stable form, type 103B, which was closely related but not quite identical to variant Y. Our earlier interpretation of this result (96) was that 103A (serotype 4a) could be presumed upon structural evidence to have an underlying  $Y_2$  structure and that 103B polysaccharide would be of pattern  $Y_2$ , thus accounting for the observed serological differences. On subsequent analysis however, 103B proved to have a classic Y structure of pattern  $Y_1$  which implied that a change  $Y_2 \rightarrow Y_1$  had occurred during A  $\rightarrow$  B mutation. As will be shown below, there is evidence that this change is not an uncommon event. In the third study of A  $\rightarrow$  B mutation, Ewing (17) described the isolation of a classic Y variant from serotype 2a, which is consistent with the structures proposed for these serotypes in Fig. 7. These experiments show that Y-type variants can arise in nature as a result of A  $\rightarrow$  B mutation.

More recently, Y-type variants have been obtained artificially in the laboratory by conjugating *S. flexneri* serotypes with *E. coli* Hfr strains. In the first experiments of this kind with *S. flexneri*, Luria and Burrous (58) isolated recombinants of this class from serotypes 2a, 2b, and 4a and showed that the genes controlling type specificity mapped near the *lac* locus. Even more recently, Petrovskaya and her colleagues (109) have shown that Y-type hybrids can be obtained rather readily from most *S. flexneri* serotypes, using *E. coli* HfrC as the donor strain, and that the type specific or T-locus for antigens I, II, IV, V, and 7, 8 (but not III or VI) also maps near the *lac* locus. These genetic studies and the present structural investigations complement one another in two interesting and important ways. First, the isolation of Y-type hybrids from so many serotypes supports the chemical evidence of a cryptic Y situation in all *S. flexneri* serotypes. Second, the structural studies show that the type-specific locus controls precisely those determinants in which glucose is the immunodominant sugar, whereas in antigens III and VI which map elsewhere the immunodominant structures are *O*-acetyl and rhamnose residues, respectively. These findings, together with the observation that loss of the type-specific locus leads to the synthesis of glucose-deficient Y-type variants, provides strong evidence that the T-locus is a gene or cluster of genes controlling the

synthesis of the specific UDP-glucose transferases that are required to complete the biosynthesis of the various *S. flexneri* serotypes from the precursors  $Y_1$  and  $Y_2$  structures.

In an attempt to define the relationship of *S. flexneri*-*E. coli* hybrids to the underlying  $Y_1$  and  $Y_2$  cryptic structures, a detailed comparative structural analysis of *S. flexneri* parent and hybrid polysaccharides was undertaken (61). All hybrid polysaccharides, including those from *S. flexneri* serotypes in which the O-specific side chains were of pattern  $Y_2$ , proved to be structurally and serologically identical to one another and to the classic variant Y structure of pattern  $Y_1$ . These findings defined the precise changes that occurred in *S. flexneri* O-antigens as a result of genetic recombination by conjugation with *E. coli*. With group  $Y_1$  polymers, the change is a simple loss of the  $\alpha$ -glucosyl side chains (Fig. 7). With group  $Y_2$  polymers (Fig. 8), the  $\alpha$ -glucosyl secondary side chains are again lost, but there is also a change in the primary chain structure from pattern  $Y_2$  to pattern  $Y_1$ . Thus, the questions that were raised as to (i) the nature of the antigenic changes in *S. flexneri* after genetic recombination and (ii) the relationship of the Y-type hybrids to the primary chain patterns  $Y_1$  and  $Y_2$  have been answered in terms of structural chemistry. By contrast, the mechanism of these changes and the way in which they are regulated by the genome of the cell are not so precisely understood but, as will be seen from the following studies, there is good reason to believe that phages are involved.

In recent years certain changes in *S. flexneri* O-specificity have been shown to depend on phage or lysogenic conversion. Matsui (62) converted antigens I and III of serotypes 1a and 3a, respectively, to IV with a phage from serotype 4c. Later workers, using the appropriate phages, converted the analogous antigens of 1a, 1b, 2a, 3b, and variant Y to IV (36) and those of 2a, 2b, 4a, variant X, and variant Y to I (71). Even more recently, a variant Y was converted to variant X and then to serotype 2b by Giammanco (26), using two phages (f-7,8 and f-II) sequentially. Comparisons of the relevant O-specific side chain structures in Fig. 7 and 8 show that many of these conversions can be accounted for as simple changes in the glucose secondary side chains. Indeed, some of them could be accomplished by single enzyme changes. However, in those lysogenic conversions involving a change from group  $Y_1$  to group  $Y_2$  [as, for example, with I  $\rightarrow$  IV (62), II  $\rightarrow$  IV (36) and variant Y to variant X (26)], the primary chain pattern is also modified at the same time as the glucose side chains, which suggests that the same phage determines the

changes in both areas of the molecule. Such a gross change would probably involve a number of enzyme systems so that the earlier suggestion (96) that lysogenic conversions can be explained as single enzyme changes is probably not true in every instance. However, these studies show that *S. flexneri* type specificity is determined by prophages and that primary chain conversion from  $Y_1 \rightarrow Y_2$  may be similarly controlled.

The results of these lysogenic and conjugation studies are particularly interesting in that they elucidate the mechanisms involved in the genetic control of *S. flexneri* O-antigen biosynthesis. In our view, the biosynthesis of these O-antigens proceeds through the rough precursor antigens to the group antigen variant  $Y_1$  form under the influence of the bacterial chromosomal genes as detailed in Fig. 12. Type specificity is then conferred on the growing molecule with the incorporation of uniquely linked terminal  $\alpha$ -glucosyl secondary side chains by specific UDP-glucose transferases under the control of the appropriate phages of the T-locus which also change the primary chain from  $Y_1$  to  $Y_2$  when this is required to give the appropriate acceptor group. On genetic recombination by conjugation with *E. coli* HfrC, the *lac* region with its T-locus phage-attachment site(s) is lost so that biosynthesis of antigen is halted at the end of the bacterial chromosomal or variant  $Y_1$  stage. These findings offer an attractive explanation for the experimental observation that conjugation of the various *S. flexneri* serotypes of groups  $Y_1$  and  $Y_2$  with *E. coli* HfrC commonly yields Y-type hybrids which are always of pattern  $Y_1$ .

A further interesting observation relevant to the present review is found in the work of Formal et al. (19), who transferred *S. flexneri* antigens to *E. coli* K-12 by using an Hfr strain of *S. flexneri* serotype 2a as the donor. In their experiments, it was found that the *S. flexneri* group antigen linked to *his* locus, the type-specific antigen to the *pro* locus and that the expression of the type-specific antigen II depends on the presence of the group antigen. The latter observation is explained by the structure of 2a lipopolysaccharide which shows that the  $\alpha$ -glucosyl secondary side chains involved in specific antigen II cannot be transferred into the lipopolysaccharide before completion of the biosynthesis of the primary chain which carries the group antigenic determinant in this serotype. Furthermore, from the limited number of examples studied, it seems reasonable to predict that the expression of type-specific antigens will depend, as a general rule, on the presence of the group factors because the former are always end-group determinants which cannot

be incorporated into the growing molecule before biosynthesis of the more central regions which determine group antigenicity.

In this review an attempt has been made to correlate the chemistry and biology of *S. flexneri* O-antigens. These studies are of intrinsic value in that they have solved the molecular basis of serological specificity and cross-reactivity, elucidated the biochemical and enzymic basis of S → R and A → B variation, defined the structural relationship and biosynthetic pathways of the smooth and rough serotypes, and indicated the general mechanisms involved in the genetic control of *S. flexneri* O-antigen biosynthesis. At the same time, they are of practical importance in taxonomy and in the production of special vaccine strains for immunization against bacillary dysentery. An interesting example of the latter application is found in the work of Formal and his colleagues (20–22) who, by “genetic engineering” have “tailor-made” *S. flexneri*-*E. coli* recombinants which retain their immunological specificity while lacking pathogenicity. Such hybrids have been used already as live oral vaccines to give good immunity against bacillary dysentery in monkeys.

In my view, however, one of the most interesting aspects of this study is the way in which bacterial somatic mutation has provided an ideal model for investigating the role of the bacterial nucleus in controlling the biosynthesis of cell-surface antigens through a coordinated sequence of regulating enzyme systems. Moreover, these studies have also shown how the genetic changes that occur in spontaneous mutation, bacterial conjugation, and lysogenic conversion modify the “normal” biosynthetic pathways to produce precisely characterized changes in the chemical structure, and hence in the serological specificity, of O-antigens. Similar findings have, of course, been obtained with other *Enterobacteriaceae* (54, 57) so that it is now possible to make comparisons that are beginning to show intergeneric relationships within this family. It seems reasonable to predict that further studies of the type described above will continue to reveal genetic relationships between the different genera that will ultimately clarify the evolutionary history of *Enterobacteriaceae*.

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