GENETICS OF POLYSACCHARIDE BIOSYNTHESIS[1]

BY P. HELENA MÄKELÄ AND B. A. D. STOCKER
State Serum Institute, Helsinki, Finland, and Department of Medical Microbiology, Stanford University, Stanford, California

INTRODUCTION

When the structure of deoxyribonucleic acid and the nature of the genetic code were unknown, it was not unreasonably surmised that a DNA molecule which effected type-transformation of a *Pneumococcus* determined the structure of the type-specific polysaccharide concerned by serving as a template for its synthesis. In recent years it has become apparent that sugar units are added to various macromolecules, including polysaccharides, by transfer from small compounds, such as UDP-glucose, under the influence of specific glycosyl-transferring enzymes. One might therefore suppose that two kinds of gene would be needed for synthesis of a given polysaccharide —one sort specifying enzymes involved in the synthesis of the donor forms of its sugar components, the other sort specifying the various enzymes needed to take each sugar component from its donor and to attach it, by the correct linkage, to the appropriate site on the growing polymer. Investigation of the structure, biosynthesis, and genetics of two classes of bacterial polysaccharide have shown that these two sorts of gene are indeed key elements in the genetic determination of these polysaccharides. They have also revealed additional elements—enzymes, and therefore genes, concerned with the formation of a linear polymer from preformed oligosaccharide units, others for the transfer of this polymer from a lipid carrier to its final attachment site on a quite different polysaccharide, and, perhaps, genes regulating the activity of other genes concerned in polysaccharide synthesis. We here review what is known of the genetics of *Pneumococcus* capsular polysaccharide and *Salmonella* somatic lipopolysaccharide, dealing in more detail with the latter since it is the more extensively investigated field and that with which we are familiar. We also make a cursory survey of available information on the genetics of some other bacterial polysaccharides and of human blood-group-active polysaccharide, which is virtually the only polysaccharide of higher organisms about which both structural and genetic information is available.

PNEUMOCOCCAL CAPSULAR POLYSACCHARIDE

The type-specific polysaccharide of a wild-type pneumococcal strain constitutes the capsule which surrounds every bacterium. These polysaccharides

[1] Abbreviations used in this review are: LPS (lipopolysaccharide), KDO (2-keto 3-deoxyoctonate), ACL (antigen carrier lipid, a C_{55} isoprenoid alcohol).

are interesting as determinants of pathogenicity (for their presence protects the bacteria against phagocytosis, unless anticapsular antibody is present), as the subject-matter of classical investigations in immunochemistry, and because of their role in type-transformation, whose discovery by Griffith (1) in 1928 made possible the identification of the chemical constitution of the gene. The inoculation of capsulated pneumococci into mammals evokes anticapsular antibodies. Some eighty serological types, conventionally designated type I, type II, etc., are thus recognized within this otherwise rather homogeneous taxonomic group. The type-specific substances are polysaccharides, made up of different combinations of various monosaccharide components, including some uncommon sugars [see (2) for a review]. Type III polysaccharide, the simplest, is a linear polymer of [(→ 3) —β-D-glucuronic acid (1 → 4)—β-D-glucose (1 →)] units; in one sample the molecular weight corresponded to some 500 repeats. Other type-specific polysaccharides are probably also linear polymers of oligosaccharide units, which in some types are made up of five or more sugar units.

Biosynthesis

Investigations of the biosynthesis and genetic determination of pneumococcal capsular polysaccharides have revealed principles which seem to apply to many other polysaccharides. Studies on biosynthesis have been reviewed (3) by Mills & Smith, the main contributors to this field. Particulate fractions from broken cells catalyze the incorporation into serologically active material of isotopically labeled sugars from appropriate nucleotide sugar compounds—for instance, from UDP-glucose and UDP-glucuronic acid in the case of type III preparations. Some such compounds (UDP-glucose, UDP-galactose, and UDP-N-acetylglucosamine) and the enzymes needed for their synthesis have been found in cells of all types examined, even those in which one or more of these sugars are absent from the type-specific polysaccharide—presumably these nucleotides have roles other than as precursors of capsular polysaccharide. Other nucleotides, such as UDP-glucuronic acid and UDP-galacturonic acid, and the special enzymes for their synthesis, have been found only in cells of types in which the relevant sugars (or their biosynthetic derivatives) are components of the capsular polysaccharide; such nucleotides and enzymes may be termed "type-specific."

It appears that the steps involved in capsular biosynthesis are: (*a*) the synthesis of the activated forms of the sugar components of the polysaccharide by the action of enzymes, some of general occurrence and others "type-specific"; and (*b*) the transfer of the sugars from these donor molecules into polysaccharide by type-specific transferases. It is not known whether the polymer is lengthened by addition of sugars at the reducing or the nonreducing end, nor whether a "physiological" repeating unit is synthesized as such, and then polymerized. An enhancement of the rate of *in vitro* incorporation of labeled sugars into polysaccharide suggests that a

short polysaccharide chain may function as a "starter" (3), but the phenomenon of type transformation indicates that specific polysaccharide or oligosaccharide is not an indispensable requirement for the synthesis of a new polysaccharide by whole cells.

GENETICS

Pneumococcal capsular types are stable, in that a strain of one type never alters into another type (except by transformation reaction, i.e., genetic recombination). However, it is easy to isolate noncapsulate variants from capsulate strains, e.g., by growth in broth containing anticapsular serum, the antibody probably serving merely to select, not to induce, them. Some noncapsulate mutants make no capsular polysaccharide; others make a small amount and may be termed "leaky" mutants. Some noncapsulate mutants, most of them making no polysaccharide, never revert, even when tested by methods which would detect a proportion of 10^{-9} capsulate cell (1, 4). Others, including many leaky strains, produce capsulate revertants, always of the ancestral type. The biochemical defects causing absence of capsule have been determined in some mutants (3). Extracts of several derived from type III parents had no or greatly reduced UDP-glucose dehydrogenase activity, this being the type-specific enzyme which converts UDP-glucose to UDP-glucuronic acid. An extract of one of these mutants synthesized polysaccharide when provided with UDP-glucuronic acid and UDP-glucose. This showed that lack of the dehydrogenase was the sole cause of the absence of capsules. Of three type I mutants tested, one was deficient of UDP-glucose dehydrogenase and two of UDP-glucuronic-epimerase, these being the two type-specific enzymes which convert UDP-glucose to the nucleotide which is the donor of the galacturonic acid component of type I polysaccharide. Some noncapsulate mutants synthesize all the sugar nucleotides required for synthesis of their polysaccharide, and by exclusion must be deficient of one or more sugar-transferring (or polymerizing) enzymes (4, 5).

One may assume that a leaky reverting mutant arises by a missense mutation in the structural gene specifying the affected enzyme; that is, from a base change causing an amino acid substitution such that the protein produced has a low specific activity. Some base changes might instead cause amino acid substitutions such that all enzymic activity was lost; these mutants would be nonleaky but reverting. Base changes producing nonsense (chain-termination) codons and base insertions or deletions causing frameshifts would also result in nonleaky but revertible defects. Nonreverting nonleaky mutants presumably result from longer deletions. One such mutant, derived from type III, was inferred from genetic evidence to have a mutation deleting part of the structural gene for UDP-glucose-dehydrogenase and also some unidentified adjacent gene or genes concerned with assembly of type III polysaccharide (6).

DNA-mediated transformation is the only available method for obtain-

ing recombination in *Pneumococcus*. A molecule of transforming DNA as ordinarily prepared carries only a very small fraction (0.01 to 0.001) of the DNA of a whole genome. If a single particle of DNA effects transformation in respect to two genes the loci must be closely linked. But at saturating DNA concentrations the fraction of competent cells singly transformed may be 0.01 and double transformation by coincidence is then not very rare. Proof of linkage can, however, be obtained by measurements of ratios of double to single transformation at limiting DNA concentrations. In the earlier work on *in vitro* type transformation, noncapsulate recipient strains were transformed to encapsulation during growth in broth containing normal serum or the like; the agglutination of the noncapsulate bacteria served both to facilitate induction of "competence" (i.e., phenotypic transformability) and to select capsulate transformants (or revertants). The relation between DNA concentration and number of transformations to encapsulation is difficult to measure in such experiments.

Intra-type transformation.—The simplest sort of capsular transformation is that of a noncapsulate strain back to its ancestral capsular type by treatment with DNA from a capsulate strain of that same type. DNA from some other noncapsulate mutant derived from the same type may also effect this kind of transformation (4–11). This shows that the sites of mutation of the two mutant strains concerned are nonidentical and do not overlap. Various observations on such "intra-type" transformation suggested that many different mutations causing diminished production of type III polysaccharide were linked (8). Ravin (10, 11) was able to infer the order of several such mutations from the rarity of fully capsulate (i.e., wild-type) transformants in certain three-point crosses in which one partner had both a leaky and a nonleaky defect. In experiments on transformation of noncapsulate mutants of type-I origin capsulate transformants were obtained by the interaction of mutants each lacking a different type-specific enzyme needed for synthesis of UDP-galacturonic acid; and also when both strains concerned lacked the same enzyme (3). In the latter case, crossing-over within the structural gene for the enzyme is presumably required. Bernheimer and her colleagues (6) tested 21 noncapsulate mutants of type III, all inferred to be defective of UDP-glucose dehydrogenase. All pair-wise combinations of 20 strains yielded capsulate transformants; the remaining strain, inferred on other genetic evidence to lack also some enzyme concerned with polysaccharide assembly, gave no wild-type recombinants with 7 of the other 20. The overlapping of the deletion in this strain with the sites of seven other mutations provides the beginning of a fine-structure map of the gene for UDP-glucose dehydrogenase in type III.

Ravin (9) showed that capsulate strains could be transformed to nonencapsulation by DNA from noncapsulate mutants of the same type, though the yield of transformants was low when the recipient was fully capsulate, perhaps because it was difficult to obtain competence of such bacteria.

Inter-type transformation.—Griffith transformed a noncapsulated type II derivative to type I encapsulation by the simultaneous inoculation of mice with live noncapsulated cells and heat-killed type I cells. Such "intertype" transformations have been extensively studied since. The direct transformation of a capsulated strain from one type to another type has also been achieved (12)—but with more difficulty, presumably because the presence of the capsule on the recipient cells makes it more difficult to achieve competence and to detect rare transformants. In many inter-type transformations the new polysaccharide contains several sugars absent from the polysaccharide of the original type, so that the transformation must involve the simultaneous acquisition of several genes. Though the proof (linear relation of proportion of transformants to DNA concentration) is lacking in most systems, it seems probable that such transformations result from single events, rather than from two or more coincident events. If so, then the genes concerned, that is all the "type-specific" genes of the donor type, must be clustered in the genome of the pneumococcus. Conversely, transformation of type often results in the demonstrable loss of one or several enzymes, and, by implication, of one or several genes. For instance, a mutant no longer able to make type I polysaccharide (because of loss of the type-specific enzyme for synthesis of UDP-glucuronic acid), retained the now useless type-specific enzyme normally used to epimerize it; on transformation to type III this enzymic activity was lost (13).

It thus seems that typical inter-type transformation consists in the replacement of one cluster of type-specific genes by another cluster, sometimes containing quite different genes. It is surmised that in such transformations the synapses which permit replacement of one gene-cluster by another "nonhomologous" cluster, affect regions of homology on each side of the clusters.

Some noncapsulate strains [including one used by Griffith; see a recent reconsideration of his experiments by Hayes (14)] when treated with DNA from a capsulate strain of some other type yield some capsulate transformants of the ancestral (recipient) type, as well as others, of the donor type. Presumably the capsular-polysaccharide gene-cluster of the donor strain includes a gene homologous with that mutated in the recipient, so that the incorporation of part (or the whole) of this donor gene, by one cross-over on each side of the site of mutation, completes the set of functional genes required for synthesis of the polysaccharide of the recipient type. Several noncapsulate derivatives of type II (but not one mutant suspected to be deficient of more than one gene) produced some type II capsulate transformants when treated with DNA from types I, VII, VIII, XIV, or XVIII, but not when the donor was type III (5, 15, 16). If the structures of the polysaccharides were known, it might be possible to infer the enzymic deficiency of the recipient strain by considering what enzymic functions would be required for synthesis of the polysaccharide of both the re-

cipient and the effective donor types, but not for synthesis of the polysaccharide of the noneffective donor types.

Binary transformants.—Though as a rule transformation of capsular character results from substitution of new genetic information for old, one exception has been extensively investigated (13, 17, 18). When (any of several) noncapsulate mutants of type III, deficient only of UDP-glucose dehydrogenase, are exposed to DNA from a capsulate strain of type I (or of any of several other types whose polysaccharide contains a hexuronic acid) the majority of encapsulated transformants obtained are of the donor type, and presumably result from the usual replacement of the whole of the type III gene-cluster by the type I gene-cluster. However, a minority (0.02 to 0.05) are of "binary type" and react with both type III serum and, though less strongly, with type I serum. Each cell of a binary transformant strain synthesizes two distinct polysaccharides, not a single polysaccharide with two different specificities (17). Genetical analysis suggests that a binary transformant possesses the whole of its parental (mutated) type III gene-cluster together with the whole of the wild-type type I gene-cluster of the donor, which includes a gene specifying a UDP-glucose dehydrogenase. The UDP-glucuronic acid synthesized by this enzyme is then utilized for synthesis of both type I polysaccharide and type III polysaccharide (in larger amount). Transformants making both type III and type I polysaccharide have also been obtained by treatment of an encapsulated type III recipient with DNA from a type I strain: they are inferred to have gained a type I gene-cluster but to retain their ancestral, wild-type, type III gene-cluster. The types of transformation effected by DNA from such binary transformants suggest that the supernumerary (e.g., type I) gene-cluster has been inserted in the "chromosome" of the strain somewhere other than the usual capsular-polysaccharide-determining region: DNA derived from the type III gene-cluster (wild-type or mutant) produces the same kinds of type III transformants as DNA extracted from the ancestral type III strain, capsulate or noncapsulate; DNA derived from the supernumerary type I gene-cluster effects transformations which can be attributed to addition of a type I gene-cluster to the genome of the recipient strains, without loss of their own type-specific gene-clusters (18). When DNA from a III–I binary strain is applied to a noncapsulate recipient strain derived from some type other than III or I some of the capsulate transformants obtained are of the III–I binary type—but so few that it is supposed that they result from coincidence, rather than from DNA molecules containing both gene-clusters. Thus the supernumerary gene-cluster is not close to the normal capsular-polysaccharide-determining gene-cluster. As typical binary strains are stable, the supernumerary gene-cluster has presumably been incorporated into the "chromosome" by nonhomologous crossing-over, either as an insertion or by recombination in place of genes whose loss is not detectable. The type III UDP-glucose dehydrogenase gene seems to lack homology with the

type I dehydrogenase gene—for type I DNA applied to nonreverting noncapsulate type III mutants never evokes typical type III transformants, such as would result from pairing and appropriate recombination of the donor and recipient dehydrogenase genes (18).

Mutants making an unusual kind of capsule have been isolated from noncapsulate strains of several types; the constituent polysaccharide is antigenically very similar to the C polysaccharide, believed to be a cell-wall component, which can be extracted from strains of any type, or from their noncapsulate mutants (19). The mutant ability to make this sort of capsule can be transferred to other strains by DNA transformation, and is then expressed simultaneously with the ability to make the ordinary type-specific polysaccharide in the case of capsulate recipient strains. Presumably the mutant locus is unrelated to the ordinary type-specific-polysaccharide gene-cluster. It may be a regulator locus, whose alteration results in excessive production of a polysaccharide normally made in amounts insufficient to produce a capsule.

SALMONELLA SOMATIC LIPOPOLYSACCHARIDE

The *Salmonella* are a large, fairly homogeneous, group of the Enterobacteriaceae, found as parasites of vertebrate hosts. Many hundreds of species or serotypes are grouped into some fifty O groups, each defined by possession of a characteristic O (i.e., somatic) antigenic factor—for instance, every strain of group B (and no strain of any other group) possesses O factor 4. Strains of a given O group vary in respect to other O factors, some of which, e.g., factor 1, occur in more than one group. All the O serological specificity of a strain is present in an extractable macromolecular material, termed somatic lipopolysaccharide (LPS), complete O antigen or endotoxin. All or nearly all specificity is also found in a lipid-free polysaccharide fraction which can be obtained from LPS by partial degradation. Two experimental approaches led to the inference that each O antigenic factor or specificity is determined by a particular chemical grouping in the polysaccharide part of LPS. Westphal and his collaborators (for review see reference 20) found that the sugar components of LPS were the same for all strains of a given O group, but varied greatly between groups. LPS from every O group contained a "basic set" of sugars, comprising glucose, galactose, heptose, 2-keto 3-deoxyoctonate (KDO) and N-acetylglucosamine; LPS from a given group contained in addition a characteristic set of sugars therefore termed O-specific—for instance, LPS from *Salmonella* of group B contains rhamnose, mannose, and abequose, as well as the "basic set." Staub and her collaborators [for review see (20, 21)] obtained more direct evidence by a combination of immunochemical and analytical methods and showed, for instance, that O factor 1 reflects the presence in LPS polysaccharide of an α-glucose (1-6) galactose group (22). The genetical approach also provided evidence for this conclusion, and showed that LPS

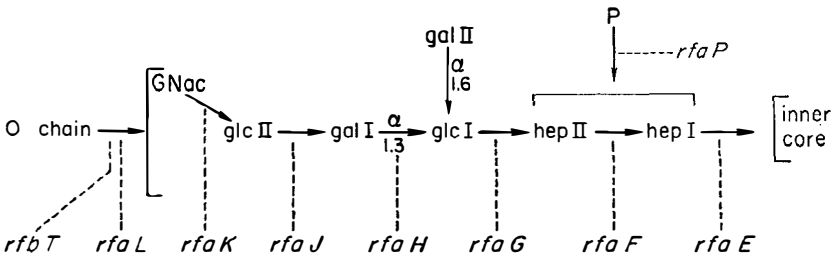

FIG. 1. Supposed structure of the side-chain of *Salmonella* LPS core, and gene symbols for the transferases known or assumed to form the indicated bonds. Inner core contains KDO, ethanolamine, phosphate, and lipid A; square brackets indicate that sites of attachment of P and of O chains are not precisely known.

glc = glucose, gal = galactose, GNac = N-acetylglucosamine, hep = heptose, P = phosphate.

contains two parts. It had long been known that wild-type ("smooth") *Salmonella* (of any O group) produced variants termed "rough" having a characteristic combination of new characters (rough colonial morphology, spontaneous agglutination in saline, loss of O antigenic specificity and of virulence, etc.). The LPS of rough forms was found to contain only the basic sugars, and to lack all the O-specific sugars of the smooth parent strain (23). Westphal and his colleagues therefore proposed that *Salmonella* LPS comprised a core, containing the basic sugars and invariant between O groups (Fig. 1) and outer O-specific side-chains. Subbaiah & Stocker (24) found that the sites of mutation of 18 rough mutants of *S. typhimurium* divided them into two groups, which they termed *rouA* (now *rfa*) and *rouB* (now *rfb*); studies by Beckmann (25) and Nikaido (26) on the biochemistry and physiology of these mutants, described below, suggested that *rfb* mutants were deficient in synthesis of O side-chains, *rfa* mutants in synthesis of the core (26).

GENETIC DETERMINATION OF LPS CORE

Conjugational crosses, in which colicine factors were used to induce mating, showed that in 12 of 18 rough mutants of *S. typhimurium* tested (24) the site of mutation was in the *str-xyl-metA* region (i.e., the top left quadrant of the conventional map (Fig. 2) (27). Beckmann (25) found that phenol extracts of several of these mutants yielded typical rough LPS (i.e., lacking O-specific sugars) but that the extracts contained also "nonsedimentable" O-specific polysaccharide, unattached to LPS. It is thought that this material consists of O side-chains still attached to the lipid carrier molecules on which they are synthesized, as described below. As rabbits inoculated with bacteria containing such material generally make little or no O-specific antibody the material is commonly termed O hapten. It was sur-

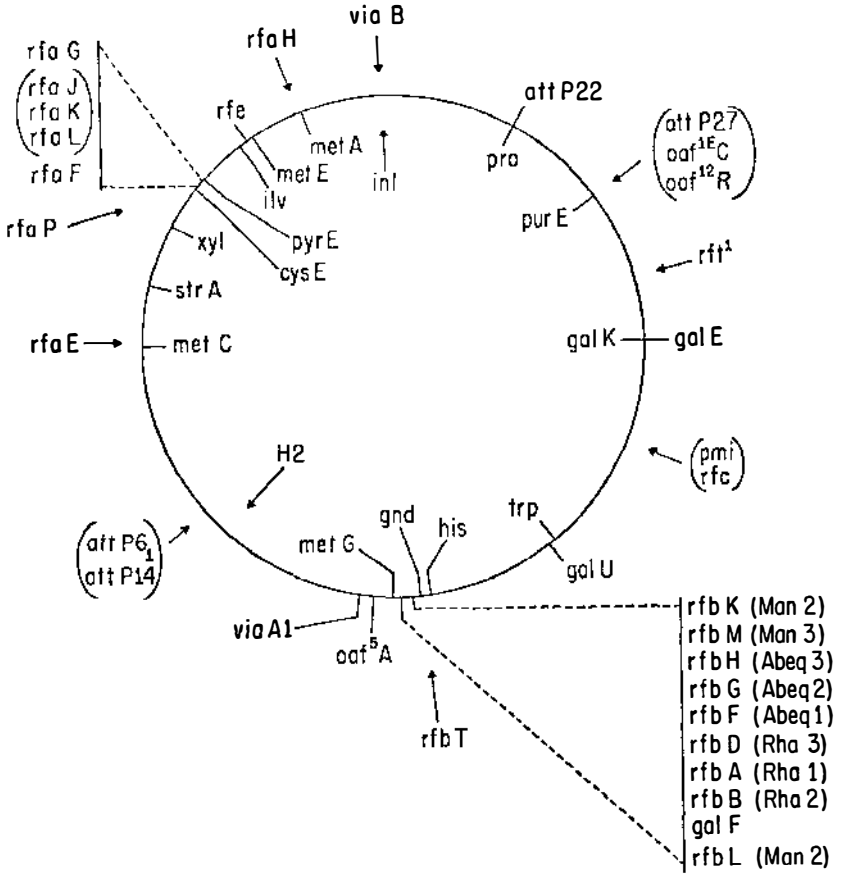

FIG. 2. Linkage map of *Salmonella,* based on the *S. typhimurium* map (27) but no gross differences between species are known. Distances approximately to scale; positions not accurately known are indicated by arrows. Loci whose order is not known are bracketed. Genes named outside circle affect polysaccharide synthesis. Gene symbols (symbols previously used added in parentheses): *att*—prophage attachment site for phages 6_1, 14, P22 and 27; *cys*—cystine biosynthesis; *gal*—galactose metabolism *galE* (*galD*) for UDP-galactose epimerase, *galF* (*galE*) for UDP-glucose pyrophosphorylase II, *galK* for galactose kinase, *galU* for UDP-glucose pyrophosphorylase I); *gnd*—gluconate-6-phosphate dehydrogenase; *H2*—phase 2 flagellar protein; *his*—histidine biosynthesis; *ilv*—isoleucine and valine biosynthesis; *inl*—inositol fermentation; *met*—methionine biosynthesis; *oaf*—O antigen factors determined by modification of repeating unit (the factor concerned is indicated by an optional superscript: $oaf^{5}A$ (*0–5, ofi, fiv*) for factor 5, $oaf^{1E}C$ (*one*) for factor 1 in group E, $oaf^{12}R$ (*R-12₂, xii*) for factor 12_2); *pmi*—phosphomannoisomerase; *pro*—proline biosynthesis; *purE*—purine biosynthesis; *pyrE*—pyrimidine biosynthesis; *rf.* (*rou*)—LPS biosynthesis (*rfa* (*rouA*), of core; *rfb* (*rouB, O*) and *rfe* of O-repeating unit;[a] *rfc* (*rouc, SR*), polymerization of repeating unit; rft^{1} (superscript optional), T1 side-chain synthesis); *strA*—streptomycin resistance *trp*—tryptophan biosynthesis; *via* (*Vi*)—Vi antigen synthesis; *xyl*—xylose fermentation

[a] The functions of biosynthetic enzymes are indicated in parentheses, e.g. (Man 3), for the third enzyme on the path to GDP-mannose.

mised that in such *rfa* mutants O side-chains were synthesized normally but could not be attached to LPS because the LPS core lacked the normal site for their attachment, being uncompleted in consequence of mutation of a gene or genes for its biosynthesis.

The "complete core" of *Salmonella* LPS (i.e., the structure to which the O chains are normally attached) has an inner part containing KDO, ethanolamine, phosphate, and lipid; nothing is known about the genes which determine the structure of this inner part. To it are attached short side-chains, believed to have the structure shown in Fig. 1. Experiments on broken-cell preparations indicate that the glucose, galactose, and N-acetylglucosamine units of the core side-chains are built up by the successive addition of single sugar units, transferred from the corresponding UDP compounds. It may be surmised that the heptose units are first added by similar transfer from some unidentified donor compound (or perhaps two different donor compounds, since according to one report (28) LPS contains two different heptoses). The enzymic transfer of phosphate from ATP to the nonphosphorylated heptose of a mutant LPS has also been demonstrated *in vitro* (29a).

On the assumption that the addition of each unit is effected by a specific transferase symbols have been assigned to the corresponding structural genes, as shown in Fig. 1. Many different phenotypic classes are found amongst *rfa* mutants, and two of them have been proven by *in vitro* assay to result from loss of such transferases—those specified by genes *rfaG* and *rfaH*. The enzyme defects of various other classes can, within limits, be inferred from the properties of their LPS and from their cultural characters.

Though the enzymic defects of complete mutants can, in theory, be unequivocally determined there are many *rfa* mutants, termed "leaky" or "part-rough," which are intermediate in phenotype between smooth and rough (to be distinguished from SR or "semi-rough" mutants, discussed below, which lack O polymerase, by mutation at *rfc*). The LPS of leaky *rfa* mutants contains some O-specific sugars, though less than the wild-type amount, and these mutants are sensitive to both "smooth-specific" and to "rough-specific" phages (30–33, 35, 36). Such a mutant is believed to have greatly reduced activity of some *rfa* transferase—so that many core side-chains are left uncompleted, terminating in the unit proximal to that whose addition is defective. Presumably when the low-activity transferase does succeed in adding the relevant sugar, the distal units are immediately added by the other, normal transferases, and the completed core side-chain capped by attachment of an O side-chain. Thus, the LPS of a leaky *rfa* mutant will bear two sorts of side-chain, some normal, with a long O-specific distal part, and others ending at a particular unit in the core portion. This interpretation, though unproven, accounts for the known properties of various phenotypic classes of partly rough mutant. The corresponding complete class (and enzyme defect) can in some cases be inferred from phenotypic

characters, e.g., serological cross-reaction with LPS of a class of nonleaky mutant.

The *rfa* genes have been mapped both by conjugational and transductional crosses. The existence of an *rfa* locus or region was first inferred from crosses in which conjugation was obtained by the use of colicine factors (24). Crosses using Hfr lines of *S. typhimurium* showed that genes determining several additional phenotypic classes, including some leaky *rfa* classes, were also located in the *str—xyl—metA* region (30–33). Phage-mediated transduction has recently been used for a more precise mapping. Sanderson & Saeed (36) used the smooth-specific phage P22 propagated on leaky *rfa* mutants to test for co-transducibility of their *rfa* loci with various nutritional-requirement loci located in the *str—metA* region; and found co-transduction (c. 0.2) of *rfa* with *cysE,* and also, at about the same frequency, with *pyrE*. As the frequency of co-transduction of *pyrE* with *cysE* was only about 0.01, this showed that the *rfa* loci concerned lie between *cysE* and *pyrE* (Fig. 2). As phage P22 does not adsorb to bacteria whose LPS is devoid of O polymer, it cannot be used for transductional mapping of the *rfa* genes affected in nonleaky (and therefore P22-resistant) mutants. However, another phage, ES18, which attacks both smooth and rough forms of (some sub-lines of) *S. typhimurium* strain LT2, proved to be a general transducing phage, and Kuo & Stocker (37) found that it co-transduced some *rfa* genes with *cysE,* or with *pyrE* (and *cysE* and *pyrE*) at about the same frequencies as previously found for phage P22 in the case of leaky mutants.

rfaL.—Four of the original set of 12 *S. typhimurium* mutants assigned to class *rfa* because of the general location of their mutant genes differed from the rest by their sensitivity to Felix O phage (24). Their pattern of phage sensitivity is identical with that characteristic of *rfb* mutants and their LPS resembles the "complete core" LPS of *rfb* mutants (see below) by the presence of the terminal acetylglucosamine of the core side-chain and in other respects (32). However, these mutants, like other *rfa* mutants and unlike typical *rfb* mutants, accumulate hapten and it was previously surmised that their LPS lacked the attachment site for O side-chains, perhaps because of absence of some hypothetical unit (or modification) "X." The gene symbol *rfaL* was tentatively assigned to the gene determining the hypothetical feature. However, recent experiments by Cynkin & Osborn (personal communication) show that the LPS of several *rfaL* mutants accepts O-specific material when incubated with wild-type cell extracts, and that *rfaL* cell extracts do not catalyze this O-translocase or O-ligase reaction. (As noted below, under *rfb,* one representative of the class of *his*-linked but hapten-positive rough mutant apparently lacks this activity—presumably by deficiency of some translocase component other than that determined at *rfaL*. Logically, perhaps, these two classes of mutants, deficient neither in synthesis of core nor of O side-chain, are neither

rfa nor *rfb,* but for the moment it is convenient to use *rfaL* and *rfbT* for the two genes affecting translocase activity.) Phage ES18 co-transduced two *rfaL* sites with *pyrE,* and also with *cysE* (37); as the co-transduction rates were about 0.25 for each nutritional marker, *rfaL* must map about midway between *cysE* and *pyrE.*

rfaK and rfaJ.—Eight of the *rfa* mutants of Subbaiah & Stocker made LPS whose polysaccharide portion contained glucose and galactose but lacked acetylglucosamine. It is supposed, partly because of their similarities to more fully investigated mutants in other species, that these mutants are deficient either in attachment of acetylglucosamine or in attachment of glucose to form the glucose II unit. Two phenotypic classes can be distinguished by their phage resistance patterns (R-res-1 and R-res-2) and LPS serological character (32, 33), and they perhaps correspond to the two transferase defects and are therefore mutated at *rfaK* and *rfaJ.* Phage ES18 co-transduced genes of two mutants of each class with both *cysE* and *pyrE* (37). As the linkage was in all cases about 0.2, these two genes also map about midway between *cysE* and *pyrE.*

rfaH.—Mutants lacking the transferase which forms the α 1-3-linked galactose I unit are termed *rfaH.* Their characteristic phage-sensitivity pattern, Epi-1, is the same as that of *galE* mutants, which lack UDP-galactose-epimerase and therefore cannot make UDP-galactose from UDP-glucose nor metabolize exogenous galactose (34). The LPS of *rfaH* mutants, unlike that of *galE* mutants, contains some galactose, made up of the α 1-6-linked galactose II branch units attached to glucose I. Both sorts of galactose-deficient LPS accept labelled galactose from UDP-galactose when incubated with wild-type cell extracts; cell extracts of *rfaH* mutants are unable to effect this transfer (38). *Hfr* crosses suggested that two *rfaH* sites mapped in the *str—xyl—metA* region (34). Kuo & Stocker (37) found that three *rfaH* sites were not co-transduced with either *cysE* or with *pyrE.* The precise location of *rfaH* remains to be determined.

rfaG.—Mutants lacking the transferase which forms the glucose I unit have a distinctive phage-sensitivity pattern, termed "Epi-2" (34); in this respect and in their LPS constitution they resemble mutants unable to make UDP-glucose, because of lack of UDP-glucose pyrophosphorylase. Their lack of transferase has been demonstrated by *in vitro* assay (38). *Hfr* crosses of three *rfaG* mutants showed that this locus was linked to the *str—xyl—metA* region (34). ES18 co-transduction (37) showed that *rfaG* maps between *cysE* and *pyrE,* probably nearer the latter (co-transduction rate about 0.3) than to *cysE* (rate about 0.1).

rfaF.—Some rough mutants of *S. typhimurium* fail to grow on media containing bile-salts, a property first observed (33) in an *S. minnesota* mutant whose LPS lacks heptose, and all the sugars distal to it (39). Chemical and serological studies of bile-salt-sensitive mutants of *S. typhimurium* have, however, shown that several of them correspond phenotypically to another class first defined in *S. minnesota,* viz., those whose LPS contains the

proximal but lacks the distal of the two heptose units of the core side-chain (40). Presumably these mutants lack a transferase for the addition of the second heptose unit by transfer from some (unidentified) heptosyl donor substance, and on this presumption they are termed *rfaF*. Conjugal crosses showed that *rfaF* mapped in the top left quadrant of the map (33); and transduction by ES18 of complete (and by phage P22 of leaky) *rfaF* alleles (36, 37) shows that it lies between *cysE* and *pyrE*, nearer to *cysE* (co-transduction rate about 0.36) than to *pyrE* (rate about 0.1).

rfaE.—One nonleaky bile-salt-sensitive *rfa* mutant of *S. typhimurium* LT2 makes LPS which lacks both heptose units (33); other bile-salt-sensitive mutants are inferred to have incomplete defects in the formation of the proximal heptose unit. Though there is as yet nothing to disprove the hypothesis that these mutants are defective in the synthesis of a heptosyl donor rather than in respect of a transferase for formation of the heptose I unit, they will here be assumed to be transferase mutants and termed *rfaE*. Phage ES18 does not co-transduce *rfaE* with *cysE* or with *pyrE* and Hfr crosses (41) indicate that *rfaE* maps somewhere near *metC* (Fig. 2).

rfaP.—Some rough mutants of *S. minnesota* do not effect the phosphorylation of the heptose of the core sidechain; the glucose I unit is added to the abnormal LPS, but none of the more distal sugars (*29*, *29*a). The absence of LPS-phosphorylating activity has been proven by *in vitro* assays, and we propose *rfaP* as a symbol for the locus affected, presumably the structural gene for the phosphate-transferring enzyme. F-factor-mediated crosses indicate that *rfaP* of *S. minnesota* is closely linked to *xyl*, and very closely linked to a *rfa* site, probably *rfaF* (42).

There are indications of the existence of additional classes of mutants, with undetermined defects in core synthesis. For instance, some *S. typhimurium* mutants resistant to Felix O phage, but otherwise of "smooth" phage pattern, arise by mutation at sites co-transducible with *pyrE;* the diminished mouse-virulence of some such mutants suggests that their LPS is abnormal, even though they are "smooth" in respect of O agglutinability and colonial morphology (43).

Nonsense, missense, and insertion rfa mutants.—Some rough mutants of *S. typhimurium* appear to be nonleaky, in that they give completely negative results with anti-O sera. When an ochre-suppressor gene was introduced into such mutants some of them became weakly agglutinable by anti-O serum and partly sensitive to transduction (of nutritional character) by the smooth-specific phage P22 (44). Thus the partial restoration of transferase activity by the nonsense-suppressor gene gave them a slightly leaky phenotype, such as is very common among *rfa* mutants. Presumably both slightly leaky *rfa* mutants and the more obviously leaky class termed "part-rough" result from missense mutations, causing production of transferase enzymes with different degrees of functional defect. Nonleaky *rfa* mutants which are not affected by nonsense suppressor genes may be frameshift (base insertion or deletion) mutants. Long deletions of the *rfa* region, which might be

very useful for determining the order of the *rfa* genes in the *cysE—pyrE* segment, have not been recognized. However, Sanderson and his co-workers (45) have encountered variants of smooth F+ *S. typhimurium* which have become both Hfr and rough, apparently by insertion of the F plasmid between *cysE* and *pyrE*, in some of them between *rfaF* and *rfaG*.

In summary, seven loci concerned with biosynthesis of LPS core are recognized, all assumed (and three proven) to determine sugar or phosphate transferases. All are in the top left quadrant of the map, four at least (and an O translocase gene) in the short *cysE—pyrE* interval and two at least outside it. It is not known whether the genes of the main cluster adjoin each other and form an operon. As *galU* and *galE* mutants are unable to make UDP-glucose or UDP-galactose they are *rfa*-like in LPS character, but these loci are not closely linked to any known *rfa* locus. Many genes for steps in assembling of the inner part of LPS core remain to be discovered.

The O-Specific Side-Chains

In groups B and E the side-chains are known to be composed of repeating units (Fig. 3); the structure in other O groups is insufficiently known but there seems little reason to doubt the generality of this composition (46, 47). The repeating unit is synthesized on a lipid molecule called ACL or antigen carrier lipid (48, 49). ACL is a C_{55} isoprenoid alcohol (50), similar or identical with the ACL involved in the biosynthesis of the peptidoglycan which forms the rigid layer of the bacterial cell wall (51). The sugar constituents of the repeating unit are transferred to the lipid one by one, each, it is supposed, by a specific transferase which determines the linkage

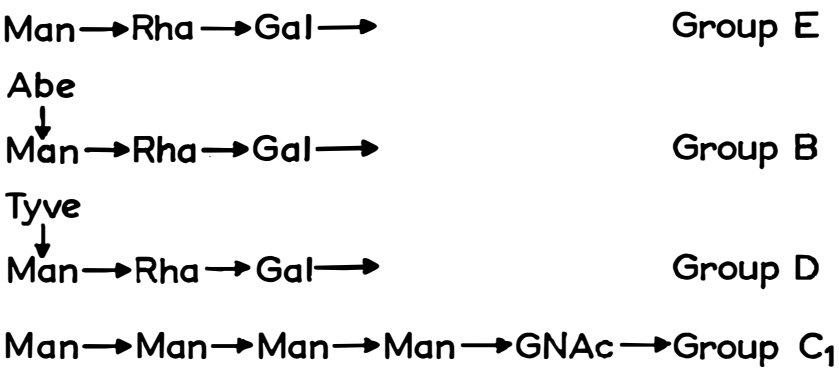

Fig. 3. Examples of the structure of O side-chains in different *Salmonella* O groups (46,47,90). In groups B, D, and E the structure shown is known to be a repeating unit; in group C_1 this is uncertain. Abe = abequose, Gal = galactose, GNAc = N-acetylglucosamine, Man = mannose, Rha = rhamnose, Tyve = tyvelose.

formed. The substrates for each transferase are diphosphonucleotides of the monosaccharides. The next step is the polymerization of the repeating units into a linear polymer, still attached to the ACL (52). Finally, the polymerized O side-chains are "translocated" from the ACL to the lipopolysaccharide "complete core" (53). The O side-chain of LPS contained an average of 30 repeating units in a group E organism (54), and of six to ten in *Salmonella typhimurium* of group B (55), but these may be averages of very unequal lengths.

This mode of biosynthesis is in many ways different from that of the LPS core. The repeating-unit structure seems useful for the synthesis of very long chains of complex structure. The use of a lipid intermediate and the elongation of the O side-chain polymer at its reducing end (56) may be important for the synthesis of the side-chains, which are finally located outside the cell membrane.

The rfb cluster.—Mutants defective in the synthesis of O side-chains are expected to have the complete core LPS, but no O-specific structures either in the LPS or as hapten. Many mutations causing such a phenotype map in one tight cluster close to *his,* an operon containing the genes for histidine biosynthesis (Fig. 2). This cluster, first called *rouB* (24), is now termed *rfb* (57). One of the first six *rfb* mutants of *Salmonella typhimurium* investigated turned out to be almost completely unable to synthesize TDP-rhamnose (26), the precursor of the rhamnose of the repeating unit of group B, to which *S. typhimurium* belongs. No defects in the synthesis of the O-specific sugars were found in five other mutants, and they were therefore assumed to lack some transferase needed for assembly.

The *rfb* cluster thus seemed to contain information for the synthesis of the O side-chains. Whether it contained all the information needed was tested by a different approach. Two *Salmonella* species of different O antigen groups, B and C_1, were crossed (58). In one cross the *Hfr* donor was of group C_1 and *his*$^+$, and the recipient of group B and *his*$^-$; recombinants in which the recipient *his*$^-$ had been replaced by the donor *his*$^+$ were selected. Most of these recombinants had the O antigens of the donor group, C_1. As usual in bacterial crosses, these recombinants derived the greater part of their chromosome from the recipient parent. It was concluded that most of the *his*$^+$ recombinants had received the donor *rfb*^{C1} region together with the donor *his*$^+$ allele. This had given them all the information necessary for the synthesis of the donor-type O side-chains. The reverse cross gave similar results; recombinants inherited the donor *rfb*^{B} region with the donor *his*$^+$ allele, and synthesized O side-chains of group B specificity (but see the section on *rfc* genes, below).

The amounts (activities) of various enzymes participating in the synthesis of the O-specific sugars were measured in the parent strains and representative recombinants (59). The result was as expected; the group B parental strain had all the enzymes for the synthesis of CDP-abequose, TDP-rhamnose, and GDP-mannose, while the group C_1 parent strain had

only the enzymes for GDP-mannose synthesis (compare the composition of the O side-chains in these groups, Fig. 3). The recombinants had the same enzymes (and the same levels of activity) as the parent with the same O antigen. Thus the *rfb* region seemed to determine both the presence or absence and the amount of these enzymes. As the recombinants could synthesize LPS with the donor O specificity they must also have acquired the several donor transferases for assembling the repeating units, and therefore the genes for these transferases must reside in the *rfb* cluster.

Some recombinants would have been expected to have acquired only part of the donor *rfb* cluster and thus to contain *rfb* genes from both parents. Such new combinations of genes might have produced new antigenic structures, or, more probably, resulted in an inability to synthesize any repeating units at all and therefore in the *rfb* phenotype. The crosses mentioned produced 90 per cent donor-like and 10 per cent recipient-like recombinants among the 1755 recombinants analyzed, plus three with the *rfb* phenotype. These three had gross deficiencies in several enzymes of nucleotide-sugar synthesis, and may have resulted from such rare cross-over cases within the *rfb* cluster. Their rarity may be a consequence of extensive nonhomology of the *rfb* regions of the parents.

The order of many of the *rfb* genes could be determined from studies of mutants of *Salmonella typhimurium* with long deletions, from *his* into *rfb* (57). The activities of the enzymes of nucleotide-sugar synthesis were altered in these mutants, and the pattern of the changes was such that a linear order of the corresponding genes could be inferred (see map of *rfb* in Fig. 2). All three genes needed for synthesis of CDP-abequose are together, though not in the sequence of the biosynthetic steps they determine —so also are the three genes for synthesis of TDP-rhamnose, and two of the three for GDP-mannose (Fig. 2). This conforms with the situation in many other bacterial systems (*27*).

Phosphomannoisomerase, the first enzyme in the GDP-mannose pathway, was present in similar amounts in all the deletion mutants examined, as also were several enzymes of the glucose, galactose, and glucosamine pathways. The corresponding genes are thus not contained in the *rfb* cluster; the gene *pmi* for phosphomannoisomerase in *Salmonella typhimurium* seems to be between *gal* and *trp* (31), and *galE*, the gene for UDP-galactose epimerase, is in the *gal* cluster (60). None of these sugars except mannose is specific to the O antigen; and phosphomannoisomerase is used in the fermentation of exogeneous mannose as well as in synthesis of GDP-mannose.

Ten genes concerned in the synthesis of the three O-specific sugars of *Salmonella typhimurium* have thus been identified in the *rfb* cluster. The presence of two further sites, deletions of which effected a derepression of other *rfb* genes, was also inferred. The cluster is thought to contain also a minimum of four structural genes for the enzymes transferring the four

monosaccharides of the repeating unit into position. Among the 21 *rfb* point mutants so far tested [(26), unpublished observations of Nikaido & Levinthal] four have been defective in TDP-rhamnose synthesis, one in CDP-abequose synthesis, one in rhamnose transfer, and 15 in the transfer of galactose to ACL. The cause of the preponderance of the last class is not known.

The typical *rfb* mutants so far discussed have no O-specific hapten. A few hapten-positive *his*-linked (thus *rfb*) rough mutants have been found (33, 35). Because of this phenotype they are believed to lack O-translocase activity. The locus concerned may be termed *rfbT* (for translocase), but it is not known whether it is part of the main *rfb* cluster.

The nucleotide-sugar-synthesizing *rfb*-determined enzymes have always shown the same levels of activity[2] in a given strain whatever the growth conditions (61). In the mutants with deletions joining the "right" half of the *his* operon (which is transcribed from right to left) to the left part of the *rfb* cluster the activities of the *rfb* enzymes were unaltered, and insensitive to repression or derepression of the *his* operon (57). This indicates that the *rfb* cluster is read in the opposite direction to *his*, that is, from left to right or counter-clockwise. Only the *rfb* gene closest to the deletion was affected on derepression of *his*, an effect attributed to the collision of transcription in opposite directions, presumably on opposite strands of the DNA (62). Studies on polar *rfb* mutants have led to a similar conclusion: at least the part of the *rfb* cluster from *rfbD* to *rfbM* belongs to one operon, read from D to M, i.e., from left to right (61).

Many of the *rfb* point mutations are somewhat leaky, just as was the case with *rfa* mutations. Their phenotype combines features of the nonleaky (e.g., deletion) mutants (sensitivity to rough-specific phages, rough cultural characteristics, lack of virulence) with those of smooth strains (presence of O-specific sugars in LPS, reactivity with anti-O serum) (33, 35, 132). This phenotype suggests that the leaky *rfb* mutants have long side-chains reduced in numbers rather than shortened ones (63). Many *pmi* mutants are likewise leaky, with a similar phenotype (unpublished observations). A

[2] Though there is no evidence for regulation of *rfb* genes, some of the biosynthetic enzymes they determine have been shown to be susceptible to feedback control (136). In *S. typhimurium*, in which GDP-mannose serves only as a precursor of LPS mannose, the *rfbM*-determined pyrophosphorylase for its synthesis is inhibited by GDP-mannose. In *S. urbana* (of group N) GDP-mannose serves only as a precursor (via GDP-fucose) of LPS fucose, and the GDP-mannose pyrophosphorylase of this bacterium (presumably determined by a *rfb* gene) was inhibited by GDP-fucose, which had little or no effect on the *S. typhimurium* enzyme. The observed intracellular concentration of GDP-fucose in several strains and the failure of a rough mutant of *S. champaign* (whose O chains contain both fucose and mannose) to accumulate GDP-mannose or GDP-fucose suggests that feedback inhibition is effective *in vivo*.

class of *his*-linked mutants termed "*rfb,* culturally smooth" (33, 35) presumably have even leakier *rfb* defects.

The genes determining the O specificity of several other O antigen groups (D, E, G, L, R, U) have been shown to map in the *rfb* region [(31, 64–66) and unpublished observations]. In *Escherichia coli* also a locus or gene cluster near *his* is the determinant of O specificity (67).

The position of the *rfb* cluster on the bacterial chromosome is well defined. It lies between the *his* and *metG* loci, which are separated by 2 min transfer time or 2/138 of the whole chromosome (27). The *rfb* cluster is co-transducible with *his* but not with *metG* (31, 64). An apparently unrelated gene, *gnd,* determining gluconate-6-phosphate dehydrogenase, lies between *his* and *rfb* (57, 68).

The rfc genes.—The polymerization of the repeating units of the O side-chain has not yet been accounted for. There are mutants in both groups B and E which cannot perform this function (30, 69). Their LPS contains only one repeating unit per side-chain (69, 70). This must mean that the translocase, which normally transfers long O side-chains from ACL to LPS, also accepts these short side-chains. The gene(s) affected in group B mutants, of phenotype termed semirough or SR, are called *rfc,* and map far from *rfb,* between the *trp* and *gal* clusters (31, 58). Recombinants that have the rfb^{B} cluster of their group B parent in an otherwise group C_1 genome are also of semirough phenotype; they become smooth, with long O sidechains, if they acquire the rfc^{B} region of the group B parent in a second cross (58). When the rfb^{O1} region was brought into a group B genome in a reverse cross, the recombinants had long, smooth-type O side-chains. A plausible interpretation is that the polymerase in group B is determined by one or more genes in the *rfc* region, but that the polymerase of group C_1 is determined by a gene in the *rfb* cluster. No SR mutants have been found in group C_1, and there is no proof that a polymerase or repeating unit structure exists in this group (see Fig. 3). The polymerase gene in group E has not been mapped.

The O repeating units of groups B and D are very similar (Fig. 3). Recombinants with the genotypes rfb^{B}, rfc^{D} and rfb^{D}, rfc^{B} were synthesized by appropriate crosses; the phenotypes were smooth, with the O-antigenic specificity determined by the *rfb* allele (65). From these and other crosses it appears that the *rfc* of group D maps between *gal* and *trp,* as does rfc^{B}. The two rfc^{+} genes may be identical or both the polymerases may fail to discriminate between the two kinds of repeating units. However, a repeating unit which is normal except that it lacks the dideoxyhexose (made by a mutant of *Salmonella typhimurium* deficient in CDP-abequose synthesis) is not accepted by the rfc^{B} polymerase, for this mutant is phenotypically rough (61).

rfe mutants.—All the mutations in *Salmonella typhimurium* and most of those in *S. montevideo* (group C_1) and *S. minnesota* (group L) that lead to

a phenotype with complete LPS core and no hapten map at *rfb* (or *pmi*). However, some such mutations in the two last mentioned organisms map at a completely different site, *rfe,* near *ilv* (42). Thus they are close to *rfa,* which is between *xyl* and *ilv.* However, the *rfe* mutations were easily separated from *rfa* and seemed to map on the opposite side of *ilv,* between *ilv* and *metE* (42). In *S. montevideo* it has previously been shown that the *rfb* cluster contained all the information required for the synthesis of the group C_1 side-chain and not shared with organisms of group B. Since *rfe* mutants occur in *S. montevideo* and lead to absence of all O-specific material it seems that the *rfe* mutation may involve information which is needed for the synthesis of O chains and is common to groups B and C_1. ACL seems the most likely candidate; if ACL was absent no repeating units could be made and the phenotype expected would be that found. However, a similar ACL is necessary for peptidoglycan synthesis, and its loss would be lethal, at least under ordinary cultural conditions. The possibility remains that the ACL in the two polysaccharide systems may not be quite the same. Another possibility, not yet tested, is that *rfe* is a regulator gene for the *rfb* cluster.

LPS Modifications

The O side-chain structures so far discussed are the simplest encountered in the O groups concerned. In many *Salmonella* species and strains the structures contain also glucosyl side-branches or O-acetyl groups or both. The simple structures appear to be those determined by the basic set of *rfb* genes. Any mutations in these cause inability to synthesize O repeating units and therefore a rough phenotype. By contrast the presence or absence of these modifications does not affect smooth LPS synthesis.

One may ask at what stage in LPS synthesis the modifications occur, whether before or during the synthesis of a repeating unit, or on the polymerized chain attached to ACL, or after its transfer to the LPS core; and whether the genes reponsible for these modifications occur in the same clusters as other LPS synthesis genes, in separate modification clusters, or randomly distributed.

Many modifications depend on bacteriophages that infect the bacterium —a phenomenon known as antigenic conversion by phage. Conversion is observed both when the phage multiplies vegetatively and when it is in the prophage state (71, 72). Two ways of effecting the conversion seem possible a priori. Either the phage carries the structural gene for the enzyme(s) responsible for the modification of the LPS structure, or it specifies a regulator molecule, which derepresses the relevant bacterial gene. Recent results in the $\varepsilon 15$ phage conversion system (see below) strongly support the first possibility. In the prophage state most phage genes are kept inactive by the action of the phage repressor, whose synthesis is directed by a prophage gene. The phage gene(s) participating in the conversion, like the repressor gene, continue to function in the prophage. The enzymes they determine

may become subject to fluctuations in activity in the "form variation" which affects many conversion antigens (see below), but this seems a different thing.

O-acetyl groups.—In many group B species, strains with and others without factor 5 occur (73). Mutants lacking factor 5 have been obtained in strains originally possessing it (74). Factor 5 is determined by a bacterial gene *oaf*^{5}*A,* which maps close to the *rfb* cluster but is separated from it by at least one unrelated gene, *metG* (75). The gene is expressed in smooth and SR forms of group B, but not if the *rfb* region comes from groups D or C_1 (58). It was formerly thought that the acetyl group was attached to the galactose of the repeating unit (76), but it now appears that the O-acetyl group is attached to abequose, which does not exist in groups D or C_1 (77).

Factor 10 in group E is likewise an O-acetyl group. The transacetylase responsible for it can be assayed *in vitro,* where it transfers O-acetyl groups to both LPS and oligosaccharides (78). Which is the natural acceptor is not clear.

Glucosyl branches.—Antigenic factors 1 and 12_2, in both groups B and D, are determined by glucosyl side-branches on galactose. The linkage is α 1-6 for factor 1, and α 1–4 for factor 12_2 (22, 79). Factor 1 depends on conversion by P22 or related phages (80–82). The prophage site of P22 (*attP22*) maps close to *pro,* and is thus not closely linked to any *rf.* gene (83, 84). There is a chemically similar factor 1 in group E, which does not appear to depend on phage (22). A gene *oaf*^{1E}*C,* near *purE,* is necessary for its synthesis (85).

Factor 12_2, like many other "modification" antigens, is subject to "form variation" (86). This means that in any culture of a strain able to make the antigen there are some cells in which the antigen is well developed and others in which it is absent (or nearly so). There is a rapid reversible change from one state to the other, at rates of about 10^{-2} to 10^{-3} per cell generation. The rate differs between strains, and is usually higher from + to − than vice versa. A locus mapping between *pro* and *gal* (very near to *purE*) has been found to determine both the "state" (expressed or not) of factor 12_2, and its rate of variation (87). This gene may be a regulator, and it is therefore called *oaf*^{12}*R.* Or it may be the structural gene for the glucose transferase, with a built-in control (switch) mechanism. Its behaviour resembles that of the *H2* locus, which controls a flagellar protein and the phase variation of flagella (88).

The conversion, by phage 14, of group C_1 bacteria from 6_2, 7 to 6_2, 7, 14 (89) probably involves a shift of a glucose side-branch from one sugar in the O-side-chain to another (90), and thus a change in the glucose-transferase. Another phage, $P6_1$, converts the same 6_2,7 bacteria to 6_1,7 (91). The chemical basis of this conversion is not known. The prophage sites of these two phages appear to be the same, close to *H2* but not to any of the *rf.* or *oaf* loci (92).

Polymerase modifications.—A third sort of modification alters the linkage between repeating units. In all known cases it is determined by a phage. In groups A, B, and D phage 27 is reported to bring about such a change (93, 94). The 27 prophage site is close to *purE,* and thus near *oaf*^{1E}*C* and *oaf*^{12}*R* (95).

A similar modification has been studied in detail in group E. Phage ε15 has at least three functions in the conversion: it prevents O-acetylation (factor 10) by repressing the synthesis of a transacetylase (78), it inhibits the cellular polymerase that normally forms α-linkages between the repeating units, and it causes the appearance of a new β-polymerase (46, 96). Phage ε34 adds glucose to galactose as α 1–4 linked side-branches, but only after the ε15 conversion has taken place (46). Cells lysogenized by a mutant of ε15 which cannot effect β-polymerization become phenotypically semirough (96). This indicates that the bacterial polymerase is still inhibited and supports the idea that the reaction affected in the conversion is indeed polymerization. The bacterial α-polymerase is apparently inhibited by the phage, since after ε15 infection its activity disappears more rapidly than would be expected only from repression of new synthesis. Most significant was the finding that in cells infected with a temperature-sensitive phage mutant the β-polymerase was active at 20° but inactive at 40°. This means that a phage-determined protein, presumably the polymerase, participated in the reaction. Losick & Robbins (69) studied the effect of ε15 on a bacterial mutant, whose α-polymerase was inactive at a high temperature (39°), leading to an SR phenotype in cells grown at this temperature. The phage made this mutant smooth even at 39°, which indicates that the phage had provided a temperature-insensitive polymerase.

The phage-determined modifications take place rapidly. The transacetylase of group E is repressed 5 to 10 min after phage infection (78). The new antigen factors 1 and 15 become serologically detectable 7 to 10 min after infection (71, 97). Factor 15, dependent on a new type of polymerization, could probably arise only in newly synthesized LPS. The fact that antigen 34 only appears after the ε15 conversion suggests that this modification occurs on the polymerized O side-chains.

T Forms as Lipopolysaccharide Variants

T (transient) forms are sometimes isolated during *Salmonella* epidemics (98). They have no O specificity, but have a new, T, specificity (T1, T2, etc.) unrelated to the O antigen of the species. They differ from R forms in growth characteristics, probably in virulence, and in the possession of the T specificity.

T1 has been studied in some detail. The LPS of the T1 strain of *Salmonella paratyphi B,* of group B, lacked the O-specific sugars of group B, but contained much ribose and galactose (39, 99), probably in long T1-specific side-chains attached to LPS. The "complete core" is apparently required for

the attachment of T1 side-chains (100). The introduction from a T1 strain of a gene or cluster *rft*[1], close to *gal,* conferred T1 specificity on bacteria of groups of B, C_1, C_2, and E, whether these were smooth, semirough or rough because of a *rfb* mutation, but not if they had a *rfa* or *rfe* mutation [(100), unpublished observations]. A natural T1 strain was shown to lack O specificity because of an *rfb* mutation (101). It is not known whether *rft*[1] contains the structural genes which direct synthesis of T1-specific structures, or a regulator gene that derepresses appropriate bacterial genes.

Recombinant strains possessing both *rfb*[+] and *rft*[1+] synthesize LPS having both T1 and O specificities, but the amount of the latter is greatly reduced (63, 100). This reduction might result from competition between the two structures for an intermediate (ACL?) or synthesis site. It does not appear to be due to competition for available attachment sites on the LPS core, because there is no competition between T1 and the short O chains of SR forms (63).

The T1 antigen is yet another variable structure in the *Salmonella* LPS. T1-positive cultures produce T1-negative cells at fairly high rates. The opposite change could not be observed by Sarvas (100) although reported by Schlosshardt (102). The type of change thus differs from form variation, which occurs at higher rates and is reversible. Three T1-negative variants of a T1 strain were examined genetically, and the altered site mapped at *rft*[1] (100).

Summary

In summary, both biosynthesis of *Salmonella* LPS and its genetic determinants are broken up into blocks, which perhaps facilitates synthesis of this large, complex molecule. Synthesis of core is directed by genes termed *rfa,* most of them clustered, that of O repeating units by the clustered *rfb* genes, and their polymerization by a separate *rfc* gene. Translocation of polymer to core apparently involves one gene in the *rfa* cluster and another in (or near) the *rfb* cluster. Modifications of the O side-chains are specified by *oaf* genes, not linked to *rf.* genes and often part of bacteriophage genomes. Polar effects show that many *rfb* genes belong to one operon and many *oaf* genes alternate rapidly between expression and nonexpression ("form-variation"), but otherwise we know nothing about regulation of the genes mentioned.

OTHER POLYSACCHARIDES OF ENTERIC BACTERIA

Most of the capsules, which in *Escherichia coli* seem to surround the LPS layer, are acidic polysaccharides, and probably not connected to a lipid (103). A locus near *his* and *rfb* determines the structure of several capsular antigens (67).

An apparently similar substance is the Vi antigen of *Salmonella typhi,*

which is an important determinant of virulence in this organism (104). It is a polymer of galactosamine-uronic-acid units, both N- and O-acetylated (105). Johnson and his colleagues studied the antigen in crosses between *S. typhi,* with or without Vi, and *S. typhimurium,* which never has Vi (64, 75). The introduction of an *S. typhi* gene, *viaB* (formerly *ViB*), linked to *inl,* induced the synthesis of Vi antigen in *S. typhimurium.* The Vi-negative *S. typhi* strain had an intact *viaB,* but was defective at another locus, *viaA. S. typhimurium* had a functional *viaA* allele, mapping close to oaf^5A, which determines O-acetylation of LPS. Possibly the *viaA* gene, like oaf^5A, is concerned with acetylation reactions.

Many enteric bacteria, including *Escherichia coli* and *Salmonella* strains, sometimes synthesize a slimy, mucoid substance. This always has the same antigenic specificity, M (106), and contains glucose, galactose, fucose, and uronic acid (107–110). The synthesis of the M substance is influenced by growth conditions: low temperatures, simple defined media with excess fermentable sugar, and, at least in *Salmonella,* a high salt concentration favour its production (109–111). Mucoid mutants produce M substance even at 37° or on ordinary media or both (110, 112). In *E. coli* mutations leading to the mucoid phenotype have been found to map at a locus *capR,* near *pro,* or at *capS,* near *trp* (110, 113, 114). The locus designation *cap* seems an unfortunate choice, since it seems to imply capsular substance; yet the M substance can be found together with both LPS and capsular polysaccharides (103). Both *capR* and *capS* are believed to be regulator genes, which normally repress other genes concerned with the synthesis of the M substance. In the *capR* mutants several such nucleotide-sugar-synthesizing enzymes show increased activity. Most *capR* mutations are recessive, which supports the notion of its regulatory function. Growth in the presence of fluorophenylalanine leads to a mucoid phenotype, presumably because an inactive repressor is made (115). However, some *capR* mutations are dominant, producing the mucoid state, when they are in a F′ episome, although they are recessive in the chromosome (113, 116). This was explained rather plausibly (116) by assuming that the repressor is composed of several monomeric units; as genes in the episome produce two or three times as much of their product as do the same genes in the chromosome, the relative abundance of the mutated monomers might lead to inactivity of most of the repressor molecules. The regulatory function of the *capS* gene was not so well established. The *capS* mutants had normal levels of the nucleotide-sugar-synthesizing enzymes tested, except of GDP-mannose pyrophosporylase, which was depressed tenfold (117). This gene was not studied in a *capS* diploid. Mucoid mutants might be expected to lose their ability to synthesize the M substance by mutation at any gene determining a relevant nucleotide-sugar-synthesizing or transferring enzyme. Several *pmi* mutants of mucoid strains were examined and found to be nonmucoid, except when their growth medium was supplemented with mannose, which can be converted to GDP-mannose, a precursor of the fucose of the M substance (114).

THE ABO AND LEWIS HUMAN BLOOD GROUP SYSTEMS

Although the determinants of several mammalian blood group specificities are polysaccharides, detailed knowledge of the structural basis is available only for the human ABO and Lewis systems. For the inheritance of the antigenic specificities in these systems the reader is referred to the monograph of Race & Sanger (118), and for a recent discussion of the biochemical genetics to a review of Watkins (119). The antigenic specificities of ABO and Lewis systems are carried by the same polysaccharide, which is part of a glycoprotein in secretions such as saliva, or of a glycolipid, on the red cells. The following discussion is based on the glycoprotein, for which detailed biochemical data are available.

Three polymorphic, unlinked loci are known to affect the structure of this polysaccharide. These loci are called *Le* (Lewis), *H*, and *A* or *B*, the two last being two different functional alleles of one locus. At each locus there is an allele, called *le*, *h*, or *O*, which has no apparent effect on the polysaccharide. In addition, two alleles (*Se* and *se*) of a fourth, likewise unlinked, locus play a controlling role: the *se se* genotype prevents the action of *H* in glycoprotein-secreting cells but not in red-cell precursors. Probably the *Le*, *H*, *A* and *B* genes determine proteins that act as specific transferases for the monosaccharide unit responsible for the specificity concerned (119).

In the blood group substance (Fig. 4) the specific groups are attached to a "precursor" molecule as nonreducing end groups. All individuals studied

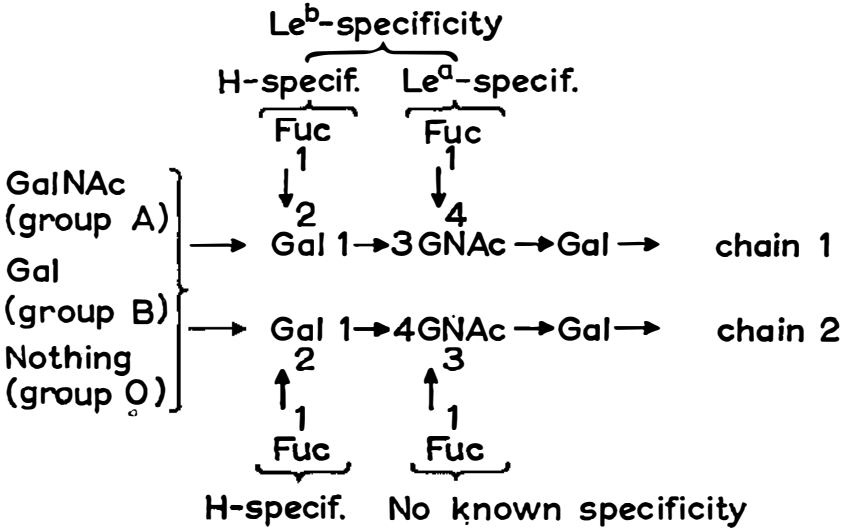

FIG. 4. ABO-Lewis blood group substance: the nonreducing terminals of the two chains (120, 121). Fuc = fucose, Gal = galactose, GalNAc = N-acetylgalactosamine, GNAc = acetylglucosamine.

have the precursor substance, therefore, nothing is known of its genetic determination. The precursor has two kinds of "side-chain"; chain 1 with a 1–3 and chain 2 with a 1–4 linkage between galactose and N-acetylglucosamine (120). The two chains are probably two branches of the same polysaccharide (121). The interrelationships of the ABO and Lewis specificities and rare blood types lacking some of these indicate sequential transfer of monosaccharides by specific transferases determined by the corresponding genes (119).

The *Le* gene determines the addition of fucose (the Le-fucose unit) in α 1-4 linkage to the acetyl-glucosamine of the precursor, but only in chain 1, for in chain 2 position 4 is already occupied. In chain 2, fucose is apparently present on the carbon 3 of this acetyl-glucosamine but the grouping has no Le specificity and the transferase for its formation is not known (121). The *H* gene acts independently of *Le*, to add fucose (the H-fucose unit) in an α 1–2 linkage to the galactoses which are terminal in both chains of the assumed precursor molecule. In secretions of *se se* individuals this fucose is absent.

The Le-fucose is the main determinant of Le^a specificity, the H-fucose of H specificity, and the presence of both fucoses on the same chain determines Le^b specificity. Thus the Le^b structure can be formed only if the *H* gene (as well as *Le*) has been active, and is absent in the rare *h h* (Bombay) persons and in the secretions (for red cells see below) of *se se* individuals (122). The Lewis antigens in the saliva accord with this theory: *se se Le* individuals have much Le^a and no Le^b activity, *Se Le* individuals have much Le^b and little Le^a activity (123), presumably because the H-transferase has not had time to transfer the H-fucose to all the chains in the precursor.

For red blood cells the picture is much less simple. This is mainly due to the odd circumstance that the Le antigens of red cells are determined by the environment (blood plasma) surrounding the cells (124). The factor in the plasma responsible for the Le reactions is stable on heating (100°, 2 min) and nondialyzable (125). This seems to exclude both the possibility that it is a fucosyltransferase or a fucosyl donor in the plasma, that would alter the blood group substance on the red cells. It seems more probable that Le activity is adsorbed from the plasma onto the red cells. Le activity can be demonstrated in the plasma in small amounts. Le transformation can also be brought about by oligosaccharides isolated from urine (126). The Le factor in the plasma might come from salivary blood group substances, since it has Le^a specificity only in *se se Le* individuals (whose salivary glycoprotein is devoid of H-fucose, and therefore of Le^b specificity), making their cells Le (a+b−). However, it differs from the salivary substances by absence of A or B specificity (125). Perhaps fragments of the salivary glycoprotein carrying A or B specificity do not reach the plasma, although those with Le specificity do.

A further complication is that the Le^b antigen is weak or undetectable in

A_1 red blood cells of *Se Le* persons. Race & Sanger (118) suggested that the amount of the precursor substance secreted may be a limiting factor. When much of it carries A specificity, as would be the case in A_1 persons (see below), the transfer of the Le-fucose may be largely inhibited. This would result in there being too little Le^b-specific substance to transform cells effectively. This theory is supported by the finding that A_1 cells are readily transformed by the Le^b-active plasma of O persons (*Se Le*) (127).

The A or B specificities are determined by the nonreducing terminals, respectively N-acetylgalactosamine or galactose, attached to the galactoses of both chains of the precursor substance. The H-fucose, whose presence is a prerequisite for the action of *A* or *B*, is covered by the new end-groups, so that H specificity is not usually detected in the red cells of A or B individuals. Heterozygotic *A B* individuals are expected to possess both the galactosamine- and the galactose-transferases specified respectively by the *A* and *B* genes; both A and B specificities are detected in their blood group substance. *A O* and *B O* heterozygotes are not distinguishable from *A A* and *B B* homozygotes; this indicates that a single gene dose can produce enough transferase to convert nearly all H-active precursor into A- or B-specific substance.

Weak variants of either *A* or *B* are determined by further alleles at the *ABO* locus. The normal alleles are called A_1 and *B*, and the weak variants A_2, A_3, etc., and B_v. They result in less A- or B- specific material in saliva and on red cells, leaving more H specificity detectable even on the red cells. At present it seems likely that in the weaker variants the specific transferase is less active, so that fewer precursor chains acquire the A- or B-specific end-groups (128). This situation would be comparable to the leaky *rf.* mutants of *Salmonella.* The red cells would have fewer specific groupings than normal A_1 or B cells, and this could account for the different reactivities of, for instance, A_1 and A_2 cells towards certain antibodies (129).

The gene causing the simultaneous inheritance of both A and B specificities may be a rare allele at the *ABO* locus (130, 131). Such an allele might have arisen by unequal crossing-over, bringing the *A* and the *B* genes into the same chromosome. However, in all reported cases both the A and B specificities were weak, and an alternative interpretation seems more probable. According to this (130) the allele might be a slightly altered form of either the *A* or the *B* gene, determining a transferase with a slightly altered specificity so that it now transfers both galactosamine and galactose more or less indiscriminately.

The theory that the sugar transferases are the immediate products of the blood group genes has been tested in one case (137). The test concerned the *H* gene, which is expected to be active in the secretory cells of *Se* (secretor) but not of *se se* (nonsecretor) individuals. The source of the enzyme was human milk. The milk of secretors could promote the transfer of fucose from GDP-fucose to certain oligosaccharides including lactose. Three different kinds of linkage were obtained: α 1–3 to glucose, α 1–4 to

N-acetylglucosamine, and α 1–2 to galactose. The last linkage was produced by the milk of secretors, but not by that of nonsecretors. The second linkage might be directed by the *Le* gene, but this could not be tested with the milk donors available.

The ABO-Lewis blood group system can thus be interpreted in simple biochemical terms. The reactions postulated are the most reasonable ones, and accord with what has been learned from the bacterial polysaccharides. One of the postulated transfer reactions has already been demonstrated *in vitro*. It it not clear why mutations of the genes directing the synthesis of the precursor substance have never been found. The *A-B* alleles are interesting from the point of view of evolution. The two alleles differ only in that one directs an enzyme whose substrate is galactosamine, and the other an enzyme whose substrate is galactose. The difference in enzyme structure may be slight, and the evolution of one gene from the other seems feasible.

CONCLUSIONS

The O-specific polysaccharide of *Salmonella* LPS and the capsular polysaccharide of *Pneumococcus* are the major components of the external surface of these bacteria; in each group these polysaccharides are chemically diverse. Bacteria of both groups are parasites of vertebrates, and therefore in danger of engulfment and destruction by host phagocytic cells. The presence at the bacterial surface of the normal amount of O polymer or capsular polysaccharide confers protection (except in the presence of homologous antibody) against phagocytosis, and, in the case of *Salmonella,* against the bactericidal action of normal serum. Rough and partly rough *Salmonella* mutants and noncapsulate *Pneumococcus* mutants are nonvirulent, presumably because they lack this protection. It therefore seems that natural selection will ensure the maintenance of these polysaccharides in some form. A vertebrate host which has previously been infected by bacteria of a given type thereafter makes anti-O or anti-capsular antibody which annuls the protection against phagocytosis conferred by the corresponding form of polysaccharide, but leaves unaltered that conferred by antigenically unrelated (i.e., chemically dissimilar) forms. This suggests that selection may favor the evolution and maintenance of chemical diversity of these protective polysaccharides. Antibodies directed against deeper bacterial components, such as LPS core and pneumococcal C polysaccharide, do not make wild-type bacteria susceptible to phagocytosis. So far as is known these polysaccharides are of uniform composition in each genus. We do not know what selective pressures, if any, account for the polymorphisms of human blood-group polysaccharide. The reported differences in susceptibility to various diseases of persons of different ABO groups (118) may be relevant.

In both *Salmonella* and *Pneumococcus,* alternative forms of a polysaccharide structure are inherited as though each form was determined by a different allele at a single locus, but it turns out that the segregating unit is not one gene but several closely linked genes—probably at least 15 in the

case of the *rfb* cluster of group B *Salmonella.* It is not clear to what extent any individual gene in one "allelic cluster" can be considered genetically homologous with any gene (even one determining a functionally similar enzyme) in some other allelic cluster. There seems to be no comparable clustering of the genes determining the human blood-group polysaccharide, as the four loci so far identified are unlinked.

Several possible causes for the clustering of polysaccharide genes in bacteria may be envisaged. Clustering of genes of related function is common in bacteria. The grouping of the genes of a metabolic pathway into an operon is perhaps advantageous since it facilitates their simultaneous regulation in response to environmental conditions. However, it seems unlikely that joint regulation is concerned in the case of the *rfb, rfa,* and capsular-polysaccharide gene-clusters, since so far as is known these genes function at about the same rate (in proportion to rate of growth) regardless of environmental conditions. Perhaps each cluster arose by local gene reduplication, followed by differentiation; the close similarity of the *E. coli* and *S. typhimurium* maps (*27*) shows that few major translocations or inversions have occurred during evolution of the *Salmonella* group. Many, at least, of the enzymes determined at the *rfb* cluster are membrane-bound and it may be that they are associated in the membrane in some arrangement necessary for their normal functioning—disorganization of such arrangement by extraction might account for the diminished specificity (polymerization and transfer to LPS core of incomplete O repeat units) of cell-free preparations, as compared with *in vivo* synthesis (49, 70). The grouping of the genes into an operon, so that all the enzymes are synthesized close together under the direction of a single messenger molecule, might facilitate the necessary association of the enzymes.

In the bacterial systems discussed, most of the genes so far identified are of the expected kinds, specifying either enzymes for synthesis of the immediate precursors of monosaccharide constituents or enzymes for polysaccharide assembly, but at least one regulatory gene is known. The four loci known to affect human blood-group polysaccharide comprise three genes which probably specify transferases and one which is probably regulatory. A few genes affecting other polysaccharides of higher organisms are known, but it is not clear whether they are enzyme-specifying or regulatory genes: see (133) for two genetically determined forms of glycogen deposition disease in man, which perhaps result from deficiencies of glycogen "branching" and "debranching" enzymic activities; and (134) for the "cryptocephal" phenotype in *Drosophila,* in which the integument is abnormally rigid, apparently as a result of increased content of glucosamine (in the polysaccharide chitin), determined either genetically or by the feeding of glucosamine hydrochloride. One may surmise that differentiation in multicellular animals will be found to involve a variety of regulatory genes, affecting the activity of polysaccharide-determining genes at the transcription, translation, and later stages. One newly discovered method of "regula-

tion" of a sugar transferase concerns an enzyme which can be extracted from cow's milk; it transfers galactose from UDP-galactose to N-acetylglucosamine, but α-lactalbumin inhibits this reaction and galactose is instead attached to glucose, to produce lactose (135). The acceptor specificity of a galactose-transferring enzyme from rat liver was similarly changed by α-lactalbumin. Though these reactions synthesize oligosaccharides, it is evident that an alteration of the acceptor specificity of a transferase by a genetically determined "specifier" protein might be involved in the genetic determination of a polysaccharide.

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