O-10 TRANSACETYLASE: CONTROL OF SYNTHESIS BY BACTERIOPHAGE ϵ^{15} AND SUBSTRATE SPECIFICITY OF THE ENZYME

by

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ABSTRACT

0-10 Transacetylase: Control of Synthesis by Bacteriophage ϵ^{15} and Substrate Specificity of the Enzyme

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Submitted to the Department of Biology, Division of Biochemistry, on August 1, 1966, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The enzyme 0-10 transacetylase, which is involved in the biosynthesis of Salmonella O-antigen, has been studied. The enzyme is present in particulate material prepared from Salmonella group E_1 cells. The enzyme catalyzes the transfer of acetyl groups from acetyl-coenzyme A to lipopolysaccharide that is present in the enzyme particles. Various oligosaccharide 1-phenylflavazole derivatives, which were prepared and partially characterized, are active acetyl acceptors.

Acetyl acceptors, which are not present in the enzyme particles, were divided into two classes on the basis of their behavior as substrates. The first class is composed of acceptors that contain the structure L-rhamnosyl-(1→3)-D-galactose. Acceptors in the second class contain terminal non-reducing D-galactosyl residues. The presence of the L-rhamnosyl residue results in approximately a 10-fold increase in the rate of acetyl transfer. Each class was subdivided on the basis of the anomeric configuration of the D-galactosyl bond. All substrates with α-D-galactosyl linkages were acetylated at a higher maximum velocity than the corresponding beta isomer.

Attempts were made to determine the stage in lipopolysaccharide biosynthesis at which acetylation occurs. The intermediate L-rhamnosyl-D-galactose phospholipid does not appear to be an acetyl acceptor. Particles prepared from an R_I mutant of Salmonella anatum A_1 incorporate acetyl groups. Thus, acetylation presumably occurs at either the level of polymerized trisaccharide repeating units or lipopolysaccharide.

The disaccharide L-rhamnosyl-(1→3)-D-galactose was prepared from S. newington lipopolysaccharide and partially characterized by standard procedures. The results of structural

studies on the enzymatically acetylated disaccharide suggest that the acetyl group is attached to position 6 of the D-galactose unit. This O-acetyl position assignment is tenuous, however, since alkaline-catalyzed acyl migration may have taken place.

The phage ϵ^{15} converts Salmonella O-antigen 10 to O-antigen 15. Synthesis of the enzyme O-10 transacetylase appears to stop very soon after phage infection. It is suggested that enzyme synthesis is repressed by the product of a phage gene. The existence of a deacetylase or an inhibitor of O-10 transacetylase was partially excluded.

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BIOGRAPHICAL NOTE

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LIST OF ABBREVIATIONS

A_1	*Salmonella anatum* A_1
$A_1(\epsilon^{15})$	*S. anatum* $A_1(\epsilon^{15})$ = *S. newington*
Abe	Abequose
CMP-KDO	Cytidine monophosphate KDO
CoA	Coenzyme A
DEAE-cellulose	Diethylaminoethyl-cellulose
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetate
Flavazole	1-Phenylflavazole (see Materials and Methods)
Gal	Galactose
GDPMan	Guanosine diphosphate mannose
Glc	Glucose
GlcNAc	N-Acetyl glucosamine
Heptose	L-*glycero*-D-*manno*-Heptose
KDO	a 2-keto-3-deoxy octonic acid
LPS	Lipopolysaccharide
Man	Mannose
Rha	Rhamnose
RNase	Ribonuclease
TDPRha	Thymidine diphosphate rhamnose

Tris	Tris(hydroxymethyl)aminomethane
UDPG	Uridine diphosphate glucose
UDPGal	Uridine diphosphate galactose

I. INTRODUCTION

A. Phage Conversion

The phage-mediated transfer of bacterial genes is known as transduction. Changes in bacterial phenotype caused by phage genes are termed phage conversions (1). The question of whether conversion represents an extreme example of transduction is unanswered (2). A probable manifestation of conversion was observed as early as 1893 when it was reported that a non-toxin-producing strain of Clostridium tetanii acquired the hereditary ability to produce toxin after growth in the presence of metabolic products of virulent bacilli (3). The required presence of the phage genome, however, was first indicated by the study of diphtheria toxin production by virulent strains of Corynebacterium diphtheriae (4). Phage prepared from virulent strains were shown to confer toxicity to avirulent C. diphtheriae. These newly toxic strains were able to produce the converting phage.

The conversion of Salmonella O-antigen, a strongly antigenic component of the bacterial cell wall, was first reported by Bruner and Edwards (5). Group E_2 Salmonellae were isolated from a culture of group E_1 cells that had been grown in the presence

of anti-10 serum. This antiserum was specific for the group E_1 cells, which carry O-antigens 3 and 10. The group E_2 organisms were not agglutinated by the anti-10 serum, since these cells carry O-antigens 3 and 15. In the presence of anti-15 serum, however, the converted strain was shown to revert to its original identity. The mechanisms of these conversions were not understood. Iseki and Sakai (6) postulated that a phage could have been responsible. Since the anti-10 serum had been prepared by absorption of anti-3, 10 serum with S. newington, an E_2 strain, it was possible that the serum had become contaminated with phage particles that had been released from S. newington. If the phage carried genes that caused antigen 15 to appear in place of antigen 10, the E_1 to E_2 conversion could take place. The reversion to the original E_1 type was presumably due to the loss of phage and subsequent reappearance of antigen 10. The Japanese workers characterized the active agent in the culture as follows. The agent was isolated from the culture filtrate of group E_2 organisms. It was inactivated by treatment at 70° for 15 minutes or by exposure to a pH lower than 3.0 or higher than 10.0. Furthermore, it would not pass through a dialysis membrane and was resistant to attack by DNase, RNase and trypsin. From these results, the conclusion was drawn that the change in O-antigen was the

result of the presence of a phage, ϵ^{15}. This conversion was subsequently found to be independent of the state of the phage (7). Thus, the conversion occurred whether the phage was present as a prophage or vegetative particle. In addition, the conversion could be detected with serological methods within five minutes after infection.

The chemical changes in the O-antigen which are caused by the phage ϵ^{15} were elucidated by Robbins and Uchida (8, 9). The O-antigen present in the non-lysogenic E_1 strains contains a heteropolysaccharide with the structure $\{$3)-α-D-galactosyl-(1→6)-α-D-mannosyl-(1→4)-L-rhamnosyl-(1$\}_n$, where n = 1, 2, ... In addition, there is an O-acetyl group esterified to the primary hydroxyl group of the D-galactosyl residues. The heteropolysaccharide of the E_1 strains lysogenic for ϵ^{15} differs in two respects from that of the non-lysogenic strain. The D-galactosyl linkages are all beta and the O-acetyl groups are absent.

The work presented in this thesis was undertaken in order to elucidate the mechanism of O-antigen conversion. The loss of the O-acetyl group, which forms the major part of O-antigen 10 in the E_1 strains, was used as a model system of conversion. The lack of knowledge concerning the biosynthesis of the O-antigen, however, necessitated a study of this biosynthetic problem. As

an introduction to the experimental portion of this thesis, some of the physical, chemical and biological properties of the Salmonella O-antigen will be described. Since these and other properties have been the subject of several extensive reviews (10, 11, 12), a comprehensive literature survey is not presented.

B. O-Antigen

The O-antigen is a macromolecular complex composed of lipid, carbohydrate and protein found in the cell wall of many gram-negative bacteria. The classical Boivin preparations of this material were obtained by extraction of whole bacterial cells with cold trichloroacetic acid (13). The more recent use of hot phenol-water mixtures has allowed the isolation of a substance free from protein but containing the other components of the O-antigen (14). This material has been called lipopolysaccharide (LPS). Nothing is known concerning the nature of the protein of the Boivin antigen or whether it is covalently bonded to the rest of the LPS. The possibility that this protein is derived from membrane protein, however, is something that probably should be examined.

Lipopolysaccharide manifests a number of biological activities although its physiological function is unknown. It may cause

fever and death when injected into experimental animals, and it carries the receptor sites for several bacteriophages (10). For example, the phage ϵ^{15} that causes the group E_1 to E_2 conversion does not attach to converted strains because of the structural changes in the LPS (7). The injection of heat-killed Salmonella cells into numerous hosts results in the production of antibodies. These antibodies have been shown to be specific for the polysaccharide portion of the LPS molecule. Detailed serological studies have demonstrated that there are degrees of cross-reaction between individual strains of Salmonellae. The Kauffmann-White scheme of Salmonella classification assigns numbers to the serological reactions, which are used to divide the genus into about 60 groups. Each of these groups is characterized by a particular major antigen. For example, all strains that have O-antigen 3 belong to group E. On the other hand, other serological markers such as O-antigen 1 occur in many different groups (15, 16).

The relationship between the LPS and the other components of the cell wall is not understood. Since LPS contains phage receptor sites and is highly antigenic, it should be readily available at the exterior of the cell wall. Electron microscopic studies have led to the suggestion that the LPS forms a layer in the cell wall between the murein, which is believed to be adjacent

to the cell membrane, and a lipoprotein layer that forms the outermost layer of the cell wall (17, 18). The use of ferritin labeled anti-O serum has indicated, however, that the LPS can extend at least 150 mμ beyond the cell wall (19). The discrepancy between these two observations may be attributable to the selectivity of the staining techniques (17). A stain that labeled protein, but not polysaccharides, was used in the former studies. The ferritin stain, on the other hand, was specific for polysaccharides. In addition, embedding techniques that involve dehydration in organic solvents were used in both studies. The effects of these solvents on the lipid portions of the cell wall are not known. Thus, the structure of the cell wall remains ambiguous. The studies with ferritin-labeled serum, however, suggest that partial intercalation of wall components does occur.

The distribution within the cell wall of newly synthesized LPS has been extensively studied. Using the immunofluorescent staining technique, the production of new LPS was shown to occur over the entire surface of several Salmonella strains (20, 21). The cell wallsof growing cells were first saturated with the appropriate anti-O serum coupled to fluorescein. Growth of the cells was then allowed to continue. Samples of the culture were removed at various times and observed in a microscope under ultra-

violet irradiation. After short periods of growth, the cells were coated with an intense fluorescent layer. Samples taken at later times were also found to be coated with a uniform, but less intense, fluorescent layer. Analogous experiments with the E_1 to E_2 converting system gave similar results (22). It was thus apparent that the newly formed LPS was intercalated randomly over the surface of the cell (20, 21, 22). This pattern of growth was in contradistinction to that observed in the case of E. coli LPS and Streptococcal cell-wall polysaccharide (23, 24). The explanation for the pattern obtained with the E. coli strain is not known, since uniform labeling has been observed in similar experiments with another E. coli strain (25). It is possible, however, that the strain used was rough and lacked the smooth O-antigen. As will be discussed later, the synthesis of the smooth O-antigen and the rough antigen occur independently.

C. Lipopolysaccharide

A structure which appears to agree with most of the published information concerning the LPS from S. typhimurium is presented in Figure 1. This structure consists of three basic parts. The smooth O-antigen represents that part of the molecule which contains the long chain composed of acetylated pentasaccharide repeating units. Lipid A is thought to be a fully acylated di-

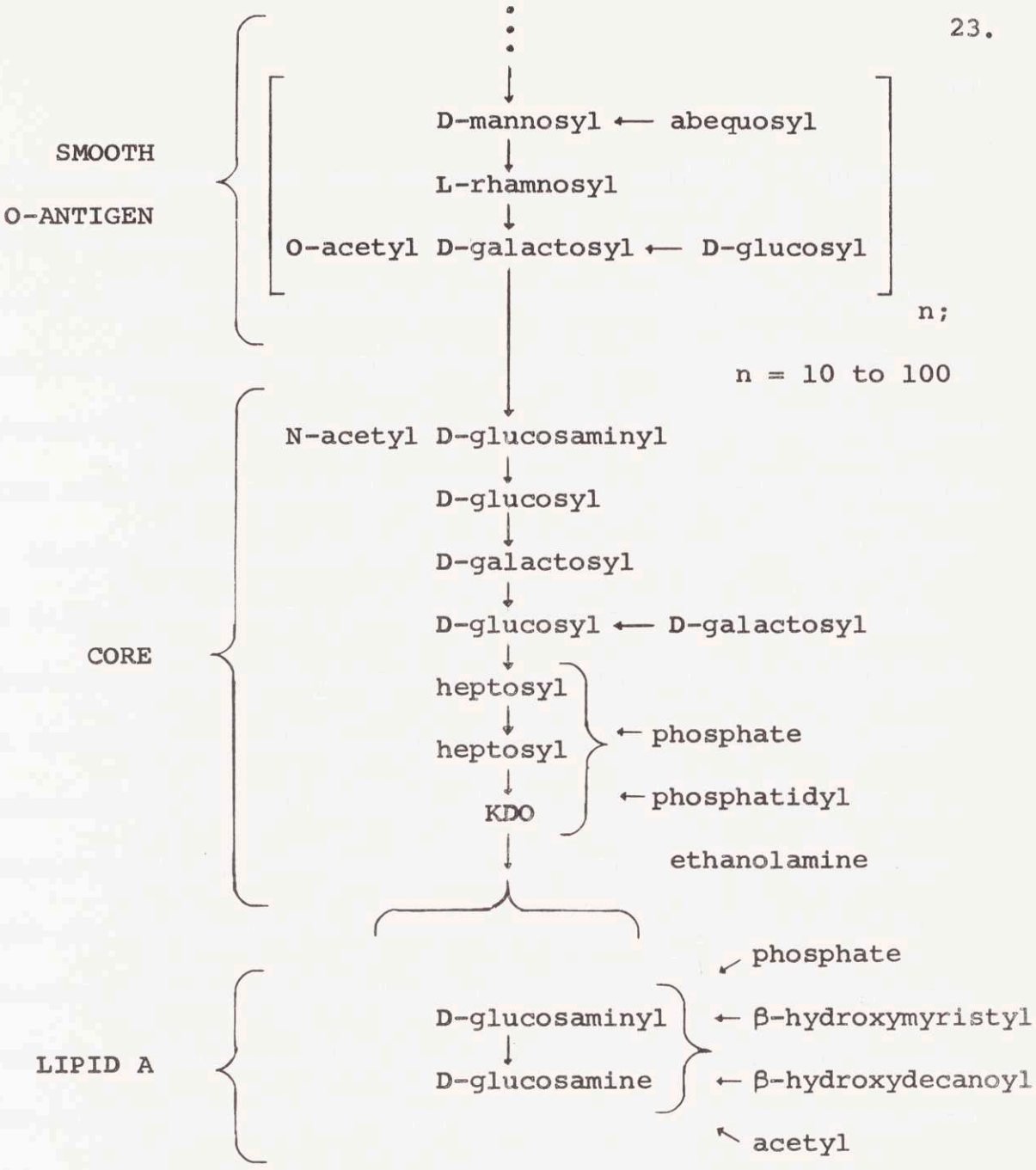


Figure 1. Proposed Structure of Salmonella typhimurium LT-2 LPS

saccharide composed of two D-glucosamine residues. The core forms the linkage between the smooth O-antigen and lipid A. Current evidence suggests that the smooth O-antigen is synthesized independently of the rest of the molecule. Some of the evidence, which has been used to determine this structure as well as its mode of biosynthesis, will now be discussed in more detail.

Highly purified LPS has a molecular weight of 1 to 20 million (10). Viscosity and light scattering measurements have indicated that the LPS isolated with cold trichloroacetic acid is a flexible coil with a molecular weight of 6 million (26). Treatment of purified LPS with mild alkali causes the release of fatty acids and the formation of material with a molecular weight of 200,000 (10). These latter units are believed to represent a single deacylated molecule of LPS. The very large material mentioned earlier is believed to be formed by van der Waals forces between the alkyl portions of the long chain fatty acids contained in the small units.

LPS is easily hydrolyzed to water soluble and insoluble components by mild acid. The water-insoluble material, lipid A, is soluble in organic solvents and does not contain the phage receptor sites or antigenicity mentioned earlier (27). Rather, these biological properties remain with the water soluble com-

ponents. The composition of this latter fraction has been the subject of extensive study. The analyses of the products released by complete acid hydrolysis from the 60 or more serological groups of Salmonellae have demonstrated that the large number of distinct antigenic groups is built from various combinations of 14 monosaccharides (10).

As an aid to understanding the structure of LPS, the Salmonellae were reclassified into 16 chemotypes that were distinguished by their constituent sugars (28). In spite of the differences, all chemotypes were found to contain a group of five sugars: D-glucosamine, a 2-keto-3-deoxyoctonate (KDO), L-glycero-D-manno-heptose (heptose), D-galactose and D-glucose. This result suggested that the water-soluble portion of LPS contained two distinct components (10). One of these, composed of the sugars mentioned above, was probably common to LPS from all Salmonellae. The other portion was responsible for the vast array of distinct serological reactions. Thus, for example, although group E is in chemotype XIII and group B is in chemotype XIV, their monosaccharide composition differs only by the presence of abequose in group B.

1. Lipid A

Lipid A is the chloroform-extractable material that is hydrolyzed from purified LPS in 0.1 N aqueous mineral acid for 30 minutes (27). The lipid A isolated from the LPS of E. coli 0111:B4 is believed to have a molecular weight of about 1700 (29). Based on this molecular weight, each molecule contains two D-glucosaminyl residues and one phosphate, as well as acetyl, β-hydroxymyristyl and β-hydroxydecanoyl groups. As a result of various chemical treatments, a structure composed of two glycosidically linked fully acylated glucosaminyl residues has been suggested (29). The aggregation that LPS is known to undergo is probably the result of the interaction of the long chain fatty acids present in this portion of the molecule.

2. Core

The core polysaccharide is that portion of LPS that contains the five monosaccharides common to all Salmonella chemotypes (10). This core is the polysaccharide that is synthesized by most rough mutants lacking the smooth O-antigen. These rough mutants, after closer analysis, have been divided into at least four groups based on antigens that are not found in the parent smooth strains (10). Chemical analyses have indicated that there are differences in both the qualitative and quantitative sugar com-

position in the LPS of different rough serotypes. In addition to the sugars previously mentioned, the core contains phosphate (10) and ethanolamine (30). Chemical, biochemical and immunological studies of the core have resulted in a clearer understanding of its general structure.

The observation that Salmonella strains that lack the enzyme UDPGal-4-epimerase also lack smooth O-antigen was the starting point for the recently gained knowledge concerning the core structure (31, 32, 33, 34). The LPS of these mutants contains only KDO, heptose and D-glucose. However, if galactose is added to the growth medium, the mutant cells make the smooth O-antigen characteristic of the parental strain. Rough mutants lacking either phosphomannose isomerase (35) or phosphoglucose isomerase (36) have also recently been isolated. These mutants have properties similar to the mutant that lacks UDPGal-4-epimerase. Neither mutant will make complete LPS unless the growth medium is supplemented with the sugar it is incapable of making: D-mannose in the first instance and D-glucose in the latter. The sugar composition of the LPS isolated from these and other mutants is presented in Table I.

The enzymatic properties of these mutants were studied concomitantly with their chemical properties (Table I). Crude

TABLE I

Sugar Composition of the LPS Isolated from S. typhimurium and Rough Mutants Derived from S. typhimurium[a]

Enzyme missing	Substrate deficiency	Sugar components							
		KDO	Heptose	Glc	Gal	GlcNAc	Man	Rha	Abe
None (wild type)	None	+	+	+	+	+	+	+	+
Phosphomannose isomerase	GDPMan	+	+	+	+	+	-	(+)	-
Transfer of GlcNAc		+	+	+	+	-	-	-	-
UDPGal-4-epimerase	UDPGal	+	+	+	-	-	-	-	-
Phosphoglucose isomerase	UDPG	+	+	-	-	-	-	-	-

[a]Table adapted from reference 10.

extracts of the UDPGal-4-epimerase deficient mutants were able to incorporate galactose from UDPGal into LPS (33). Similar preparations from the mutant lacking phosphoglucose isomerase were able to incorporate glucose from UDPG. However, they would not incorporate galactose from UDPGal unless UDPG was also present in the incubation mixture (37). It became evident from these and similar studies that the various properties of the rough mutants could be the result of a simple core structure (11); and this has subsequently been established by structural analysis (38). This common core consists of a pentasaccharide linked to a structure composed of KDO, heptose, phosphate and ethanolamine (Figure 1).

The organization of the other core components is less clear. The transfer of KDO from CMP-KDO to saponified lipid A by cell-free extracts prepared from E. coli demonstrated that KDO is probably linked to the lipid portion of LPS (39). The linkage between KDO and lipid A was assumed to be glycosidic on the basis of its resistance to alkali and susceptibility to acid, and because the carbonyl group of KDO could be reduced only after acid hydrolysis. The identification of KDO as the only reducing group in the polysaccharide formed during the hydrolytic cleavage of lipid A from LPS suggested that KDO linked the polysaccharide

to lipid A (40). The analyses of the LPS from the rough mutants already described indicated that KDO, heptose, phosphate and ethanolamine are always present. Thus, these compounds are assumed to form a bridge that links the rough pentasaccharide to lipid A (40). The available analytical data indicate that each bridge consists of 2 heptosyl residues, 2 phosphates and 1 ethanolamine per residue of KDO. Recent structural studies have shown that this part of the core consists of the trisaccharide heptosyl-heptosyl-KDO (41). The ethanolamine and phosphates are linked to this trisaccharide in an unidentified manner. The pentasaccharide described above is glycosically linked to the non-reducing terminal heptose (41). The results of these studies have been summarized in the scheme presented in Figure 1.

3. Smooth O-Antigen

The Salmonella smooth O-antigens are heteropolysaccharide chains, which are believed to be glycosidically linked to the N-acetyl D-glucosaminyl residue in the core portion of the LPS (11). From the results of chemical and serological studies, Staub originally concluded that each O-antigenic activity was related to a specific sugar. Since at this time antigenic specificity in carbohydrates was considered to be due only to non-reducing terminal sugar residues, the LPS molecule was

schematically represented as a globule with a variety of short O-antigen chains extending from it. The contribution of non-terminal sugars to the antigenicity of O-antigen polysaccharides was demonstrated in detailed studies of the structure of LPS from group E organisms before and after antigenic conversion by phages ϵ^{15} and ϵ^{34} (8, 9, 42). These studies strongly suggested that the O-antigens were long chains composed of repeating trisaccharide units. A generalization of this idea to the LPS structure of other Salmonella groups was suggested on the basis of the isolation of similar units by Staub from the LPS of S. typhimurium, a group B organism. The lack of serological cross-reactions between groups B and E prompted the further suggestion that the two sequences contained different linkages. The structural information now available on the smooth antigen from the two strains substantiates these ideas (8, 10).

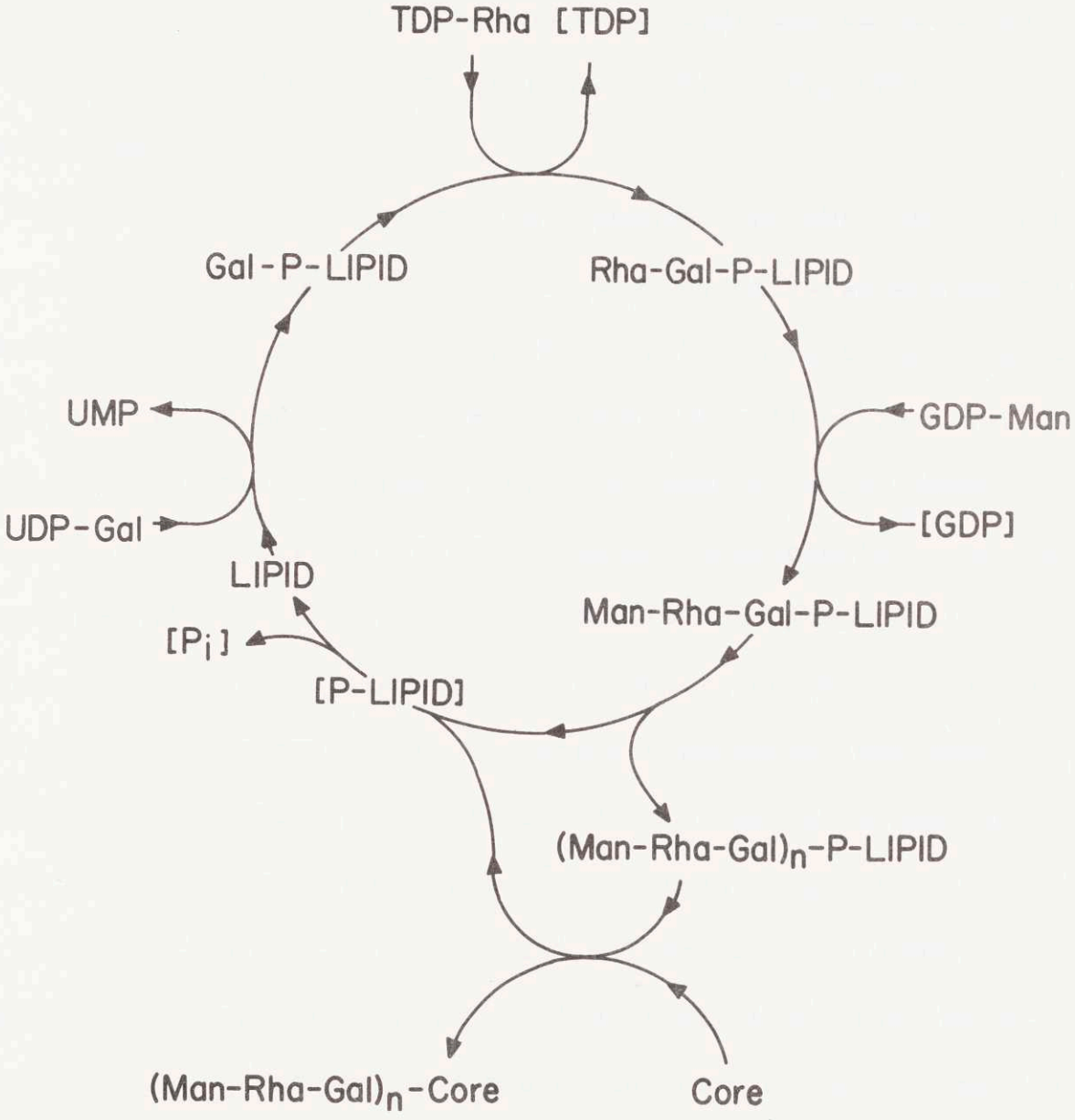
The length of the smooth O-antigen chains is not as yet known. The low number of non-reducing terminal sugars in the group E smooth O-antigens led to the suggestion of a chain length of at least 10 to 20 monosaccharides. More recent analyses have suggested that the chains contain as many as 200 to 300 monosaccharides (Dr. P. W. Robbins, unpublished observations). On the assumption that a glycopyranoside has an overall length of

about 3 Å, a single extended smooth O-antigen chain would have a length on the order of 600 to 900 Å. The presence in the bacterial cell wall of these long chains, which are composed of identical trisaccharide subunits, poses at least two fundamental questions. The first of these concerns the specification of sequence. The second question is how the chains are positioned in the cell wall. The problem concerning chain positioning has not yet been considered experimentally. However, recent studies on the biosynthesis of the O-antigen have elucidated some of the details concerning the specification of sequence (43, 44).

The biosynthesis of the trisaccharide repeating unit occurs by the sequential transfer of monosaccharides from activated sugar donors to a phospholipid (43, 44) (Figure 2). The structure of the phospholipid is unknown. The nucleoside diphosphate sugar derivatives UDPGal, TDPRha and GDPMan act as sugar donors. The order of sugar incorporation is of particular interest. Thus, the sequential addition of D-galactose, L-rhamnose and D-mannose results in the formation of a repeating unit with the sequence D-mannosyl-L-rhamnosyl-D-galactose attached to the phospholipid. These repeating units are believed to be synthesized individually.

Figure 2. Postulated Scheme for the Biosynthesis of the *S. newington* Smooth O-Antigen

This scheme was taken from reference 44.


TDP-Rha [TDP]
Rha-Gal-P-LIPID
GDP-Man
[GDP]
Man-Rha-Gal-P-LIPID
(Man-Rha-Gal)n-P-LIPID
Core
(Man-Rha-Gal)n-Core
[P-LIPID]
[Pi]
LIPID
UDP-Gal
UMP
Gal-P-LIPID

The formation of the long chains then presumably occurs by a polymerization reaction. At least some of the trisaccharide units are transferred to a growing chain that may be attached to the lipid mentioned above (41; Mr. D. Bray, unpublished observations).

The relationship between the O-antigen and the rest of the LPS molecule is being elucidated by a study of various Salmonella mutants that have abnormal LPS. The rough A mutants are characterized as rough mutants by the criteria of cell morphology, serological activity, sensitivity to a rough-specific phage and the absence in purified LPS of the sugars characteristic of the smooth O-antigen. However, serologically active material and polysaccharides containing the sugars characteristic of smooth O-antigens are present in crude extracts prepared from rough A mutants (45, 46). The existence of this type of mutant indicates that the formation of smooth O-antigen occurs independently of the formation of the rest of the molecule. Furthermore, it implies that a specific enzyme, which is distinct from the polymerizing enzyme, transfers the chain to the rest of the LPS molecule either during the polymerization step or after it has been completed. Further evidence for the existence of two enzymes, a polymerase and a transferase, has been obtained from studies of

the semirough mutants of Salmonella (47). These mutants have properties intermediate between the smooth and rough strains. For instance, low levels of the smooth O-antigen specific sugars are found in the purified LPS from these mutants. These low levels are believed to result from the presence of only a single repeating unit attached to the core polysaccharide. The loss of the polymerizing enzyme and the independent functioning of the transferring enzyme would be expected to produce such structures.

II. MATERIALS AND METHODS

A. Microorganisms

The Salmonella anatum strain A_1 and various lysogenic derivatives of A_1 have been described (8, 48). All cells were grown in LB medium, which consisted of 1 per cent (w/v) Bacto-tryptone, 0.5 per cent (w/v) yeast extract, 0.5 per cent (w/v) sodium chloride and water adjusted to pH 7.0 with 1 N sodium hydroxide. Growth was followed by measurement of the optical density at 650 mμ of 1 ml samples diluted into 2 ml of 1 N hydrochloric acid. An optical density of 0.1 represents about 10^8 viable cells per ml under these conditions. The various phages used were originally isolated and described by Uetake and Uchida (49). Phage stocks were prepared from the filtrate of infected cells and assayed by the standard agar layer method (50).

The rough mutants L-8 and L-14 were obtained by treatment of S. anatum A_1 with ethyl methanesulfonate. An overnight culture of A_1 cells was diluted 1:20 into LB medium and allowed to grow into the exponential growth phase. Ethyl methanesulfonate (0.03 ml) was added to 2 ml of the culture and growth was continued two hours at 37°. An aliquot was then diluted 1:20 into fresh medium and allowed to grow overnight. The culture was infected with ϵ^{15} vir

at a multiplicity of about 5. After lysis, droplets of the culture were streaked on LB agar plates, which were incubated overnight at 37°. Rough colonies were picked and restreaked several times. The rough mutants finally obtained underwent spontaneous agglutination when grown in LB medium and were resistant to attack by ϵ^{15} vir. The 0-10 transacetylase particles prepared in the standard manner from L-8 had no endogenous acetyl acceptor. Neither rhamnose nor mannose was detected in acid hydrolysates of purified L-8 LPS. Further characterization of L-14 is presented elsewhere in this thesis.

B. Antiserum

Anti-3, 10 serum was prepared and assayed by standard methods (8, 48).

C. Chemicals-Sources

All of the chemicals used, unless otherwise stated, were obtained from the Office of Laboratory Supplies, at the Massachusetts Institute of Technology, or commercial supply houses and were used without further purification. D-Threitol was the gift of Dr. N. K. Richtmyer of the National Institutes of Health. The following materials were prepared and supplied by collaborators: UDPGal, containing uniformly ^{14}C-labeled galactose (51);

TDPRha (52); TDPRha, containing uniformly ^{14}C-labeled rhamnose (53); α-galactosidase (8), D-galactosyl-(1→6)-D-mannose (8); α-D-glucosyl-(1→4)-D-galactose (8); UDPGal-4-epimerase (51); ^{14}C-phosphatidyl glycerol (44) and ^{14}C-cardiolipin (44).

D. Electrophoresis

All electrophoretic separations were performed in a hanging strip chamber at 200 to 250 volts and run for 14 to 20 hours unless otherwise stated. The electrophoretic mobilities, which are not corrected for endosmosis, of some sugars relative to glucose in 0.05 M sodium borate, pH 9.2, on Whatman No. 1 paper are presented in Table II (54). Sugars were detected by the lead tetraacetate-p-rosaniline method (55). This method was modified by the use of methanol in place of chloroform. Lead tetraacetate was prepared by a standard method (56) and stored in glacial acetic acid.

E. Paper Chromatography

The solvents used and the retardation factors of important compounds are presented in Tables III and IV. All paper chromatograms were developed by the descending technique. Reducing sugars were detected with the silver nitrate-sodium hydroxide method (59). Methyl glycosides were detected by dipping the paper in 0.05 M

TABLE II

Electrophoretic Mobilities of Sugars Relative to Glucose in 0.05 M Sodium Borate, pH 9.2

Compound	$R_{Glucose}$[a]
D-Galactose	0.92
D-Glucose	1.00
D-Mannose	0.70
L-Rhamnose	0.54
L-Rhamnosyl-(1→3)-D-galactose	0.66
α-D-Mannosyl-(1→4)-L-rhamnose	0.53
β-D-Galactosyl-(1→6)-D-mannose	0.68

[a] These values are not corrected for endosmosis.

TABLE III

Composition of Solvents Used for Paper Chromatography

Solvent No.	Composition (reference)
1	n-Butanol-pyridine-water, 6:4:3
2	n-Butanol-pyridine-morpholinium tetraborate 0.05 M, pH 8.6, 7:5:2 (57)
3	Water-saturated n-butanol
4	t-Amyl alcohol-n-propanol-water, 4:1:1.5 (58)

TABLE IV

R_F Values of Sugars on Paper Chromatography

Compound	Solvent No.			
	1	2	3	4
Dulcitol	0.31	0.04		
Erythritol	0.46	0.46		
D-Galactose	0.31	0.28		0.24
β-D-Galactosyl-(1→6)-D-mannose	0.13			
D-Glucose	0.35			
Glycerol	0.54	0.56		
D-Lyxitol	0.39	0.23		
D-Lyxose	0.44	0.41		
D-Mannitol	0.38	0.12		
D-Mannose	0.39	0.33		
α-D-Mannosyl-(1→4)-L-rhamnose	0.27			
Methyl β-D-galactoside	0.42		0.55	0.37
Methyl α-D-glucoside	0.49			
Methyl L-rhamnoside				0.70
Methyl L-rhamnosyl-(1→3)-D-galactoside	0.50		0.50	0.22
L-Rhamnitol	0.54	0.33		
L-Rhamnose	0.55	0.53		0.47
L-Rhamnosyl-(1→3)-D-(6-OAc)galactose[a]	0.46			
L-Rhamnosyl-(1→3)-D-galactose	0.27			0.16
D-Threitol	0.48	0.35		

[a] Identification of acetyl position is not complete.

sodium borate and then following the procedure described for detection of sugars after borate electrophoresis. A periodate spray was used as an alternative method to detect polyhydroxylated compounds (60).

F. Determination of Radioactivity

Radioactivity was measured with a Nuclear Chicago gas flow counter equipped with a Micromil window unless otherwise specified. Self-absorption was assumed to be negligible. Radioactivity on paper chromatograms was detected with either a Packard Radiochromatogram Scanner equipped with a Disc integrator or Ansco X-ray film, which was subsequently developed by standard procedures. The radioactivity in the double-labeling experiment was determined in a Nuclear Chicago scintillation spectrometer with 0.5 or 1.0 ml samples in 5 or 10 ml of Bray's scintillation fluid (61). The values obtained were corrected for channel overlap and variations in the amount of salt present. The absolute efficiencies of the ^{3}H counting procedures were determined with a standard kindly supplied by Mr. John B. Mathis. The absolute efficiency of the ^{14}C counting methods were determined by Dr. A. Wright.

G. Preparation of Acetyl-Coenzyme A

Acetyl-coenzyme A was prepared from acetic anhydride and coenzyme A (62). The non-radioactive derivative was prepared in the presence of a ten-fold excess of the acid anhydride, and the radioactive forms were prepared from equimolar amounts of labeled acetic anhydride and coenzyme A (CoA). The radioisotopes were purchased from New England Nuclear Corporation as solutions in benzene. The appropriately labeled anhydride solution was transferred by pipette to the surface of the standard reaction mixture. The benzene was then removed by evaporation under a stream of nitrogen. After 10 minutes, the mixture was neutralized to pH 6.8 (pH paper) and stored frozen. Portions were removed when needed and purified by paper electrophoresis on Whatman No. 31 chromatography paper in 0.1 M ammonium acetate buffer, pH 5.5. This buffer was prepared by the addition of one part of 0.1 M acetic acid to seven parts of 0.1 M ammonium acetate. The radioactive acetyl-CoA was identified on a radioautograph as the slower of two strongly UV-absorbing spots. It was eluted with distilled water, concentrated and stored frozen at pH 6. In the radioactive preparations greater than 92 per cent of the radioactivity behaved as acetyl-CoA after a second electrophoresis.

H. Preparation of Lipopolysaccharide and Related Products

1. Lipopolysaccharide

Lipopolysaccharide was prepared from cells grown in LB medium by Westphal's hot phenol method as described by Robbins and Uchida (8). Uniformly ^{14}C-labeled LPS was prepared from cells grown in LB supplemented with 1.5 per cent (w/v) agar and 1 per cent (w/v) ^{14}C-labeled glucose (8). Purified LPS preparations were stored in chloroform-saturated aqueous solutions at 3°. If stored frozen, the LPS precipitated from solution and was difficult to redissolve. Deacetylated LPS was prepared from the purified material by treatment with 0.01 N sodium hydroxide for 30 minutes at room temperature followed by neutralization with 0.01 N hydrochloric acid (9).

2. Oligosaccharides by Acetolysis

Acetolysis was performed essentially by the method reported by Aspinall (63). In a typical reaction, 333 mg of LPS were slowly added to a stirred mixture of 31 ml of acetic anhydride and 0.93 ml of concentrated sulfuric acid cooled in an ice bath. After 90 minutes the reaction mixture was placed at room temperature and stirred overnight. The mixture was then poured into 62 ml of ice water. The acetylated products were removed 4 hours later by extraction with chloroform (4 x 28 ml). The chloroform

extracts were pooled and washed once with 50 ml of water, four times with 50 ml volumes of 1 M sodium bicarbonate, and once again with 50 ml of water. The final solution was dried with sodium sulfate and concentrated under reduced pressure to a yellow syrup. This was stored overnight in a desiccator to remove the last traces of water. The syrup was dissolved in a mixture of 5 ml of methanol and 3 ml of sodium methoxide, which was prepared by the addition of a small piece of sodium metal to 10 ml of methanol, and was kept overnight at 3°. After the methanol was removed under reduced pressure, the syrup remaining was dissolved in 10 ml of water and neutralized (pH paper) with acetic acid. The resultant mixture of deacetylated oligosaccharides was concentrated and then applied to a 1 x 25 cm charcoal-Celite column, which was prepared by a standard method (8). The column was eluted with an ethanol gradient prepared as described elsewhere (51). Fractions (2.5 ml) were collected every 15 minutes and analyzed for carbohydrate (Figure 17). The characterization of the products is described elsewhere in this thesis.

3. 1-Phenylflavazole Derivatives

The 1-phenylflavazole (≡ flavazole) derivatives of oligosaccharides were prepared by a modification of the method des-

cribed by Nordin and French (64). In a typical preparation, 370 mg of LPS were dissolved in 37 ml of 0.2 N sulfuric acid and heated for 20 minutes in a boiling-water bath. The acid lability of the L-rhamnosyl-D-galactose linkage resulted in the release of oligosaccharides composed primarily of the structure $\{$D-galactosyl-D-mannosyl-L-rhamnose$\}_n$, where n = 1, 2 .... The solution was cooled and neutralized with barium hydroxide. The barium sulfate precipitate was removed by centrifugation and the solution was concentrated to dryness. The residue was dissolved in a solution consisting of 3.1 ml of water, 0.414 ml of phenylhydrazine, 0.165 mg of o-phenylenediamine dihydrochloride and 0.41 ml of glacial acetic acid. The mixture was placed in a sealed tube with a nitrogen atmosphere and heated for 6 hours in a boiling-water bath to form the flavazole derivatives (Figure 3). The reaction mixture was cooled and fractionated by partition chromatography on a 2.6 x 29 cm column of Celite 535 prepared in n-butanol-saturated water (65) and eluted with water-saturated n-butanol at a rate of 0.27 ml per minute. The optical density of the 8.2 ml fractions was measured at 410 mμ (Figure 4). The fractions representing the major components were pooled, concentrated, and purified chromatographically in solvent 1 on EDTA-washed Whatman 3MM or 3 HR paper (66).

Figure 3. Formation of 1-Phenylflavazole Derivatives

These derivatives can be prepared only from reducing sugars that contain no substituents on carbons 1, 2 and 3 (67).

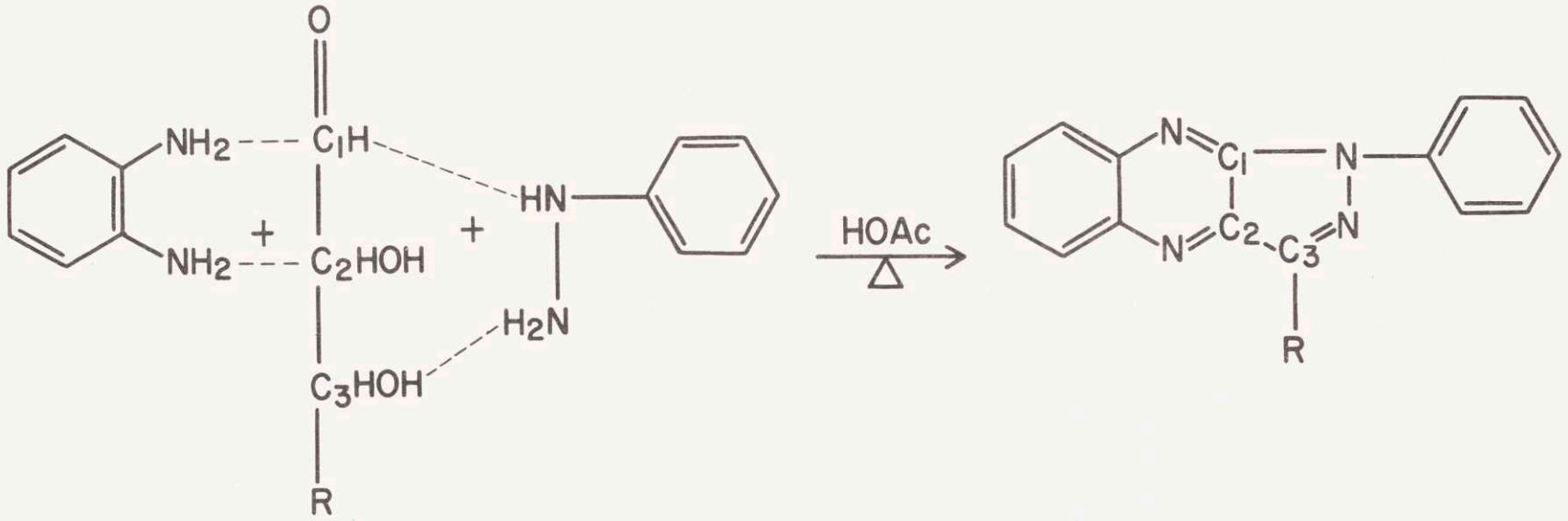
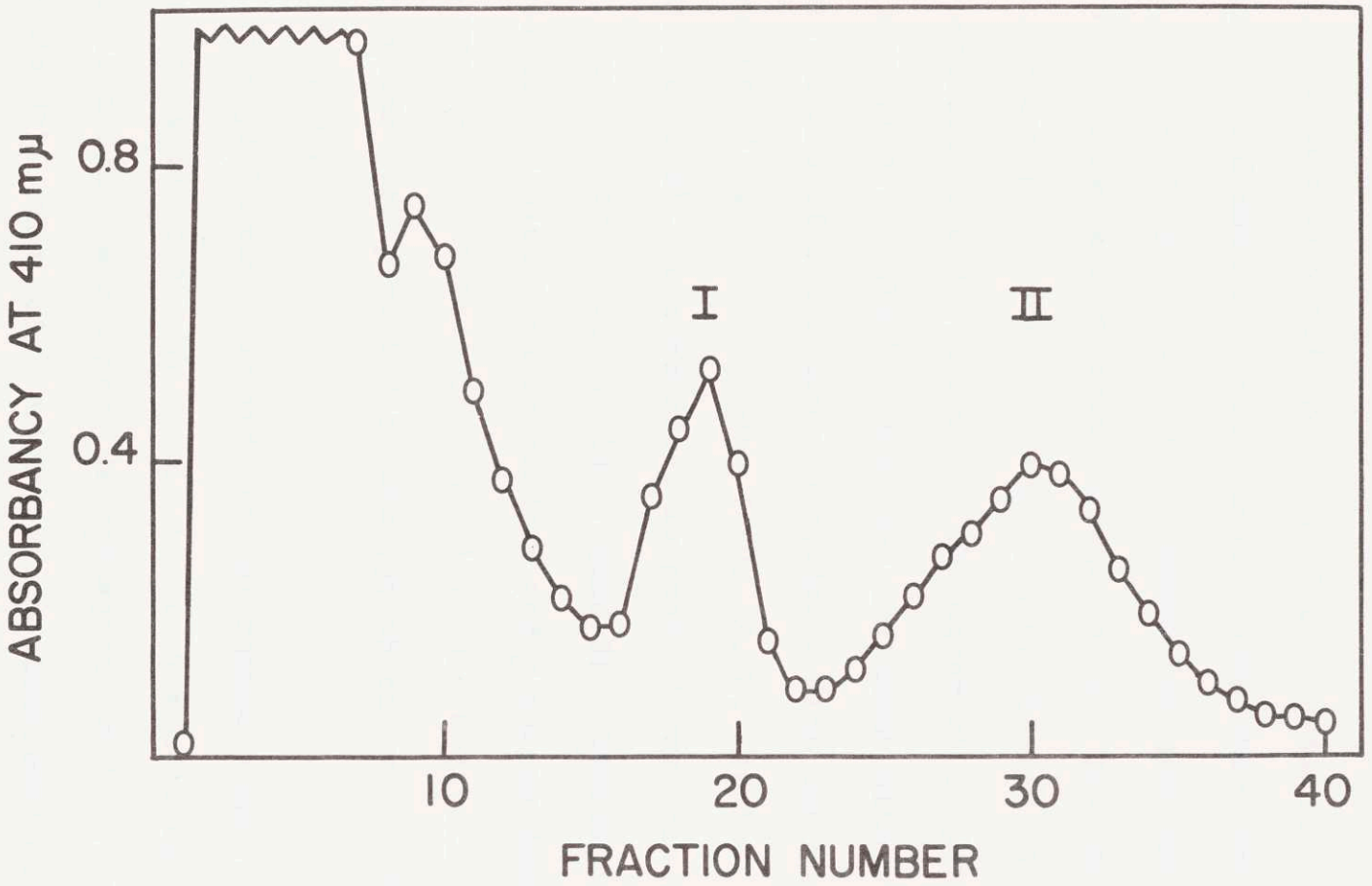

O
NH2
C1H
HN
NH2
C2HOH
H2N
C3HOH
R
HOAc
N
C1
N
N
C2
C3
N
R

Figure 4. Purification of Oligosaccharide 1-Phenylflavazole Derivatives by Partition Chromatography on Celite 535

The preparation of the column and the elution of the flavazole derivatives are described in the text. The absorbancy at 410 mμ was determined in a Zeiss spectrophotometer.


ABSORBANCY AT 410 mμ
0.8
0.4
I
II
10
20
30
40
FRACTION NUMBER

The material from peak I was assumed to be D-galactosyl-D-mannosyl-L-rhamnose flavazole because of the appearance of only free galactose and mannose after complete acid hydrolysis. Treatment with α- or β-galactosidase, depending upon the origin of the LPS, produced a flavazole derivative that yielded only mannose and rhamnose flavazole after acid hydrolysis (Dr. P. W. Robbins, unpublished observations). The material in peak II was also sensitive to galactosidase and treatment with mild acid caused the release of a flavazole derivative with the properties of the compound isolated from peak I (Dr. P. W. Robbins, unpublished observations). The material in peak II was thus assumed to be the hexasaccharide flavazole (Figure 5). Degradation with β-galactosidase or with β-galactosidase and α-mannosidase produced the penta- and tetrasaccharide flavazole derivatives respectively, which were purified chromatographically as already described (Table V).

The 1-phenylflavazole derivatives of rhamnose and lactose were prepared as described by Nordin (67). The melting points were taken in a hot-stage apparatus and agreed with the published values (Table VI). The molar extinction coefficients of various flavazole derivatives, calculated from the absorption at 335 mμ of solutions in 70 per cent n-propanol, had previously been shown to be independent of the carbohydrate portion of the molecule (69).

Figure 5. Presumed Structure of the β-Hexasaccharide 1-Phenylflavazole Derivative Prepared from *S. newington* LPS

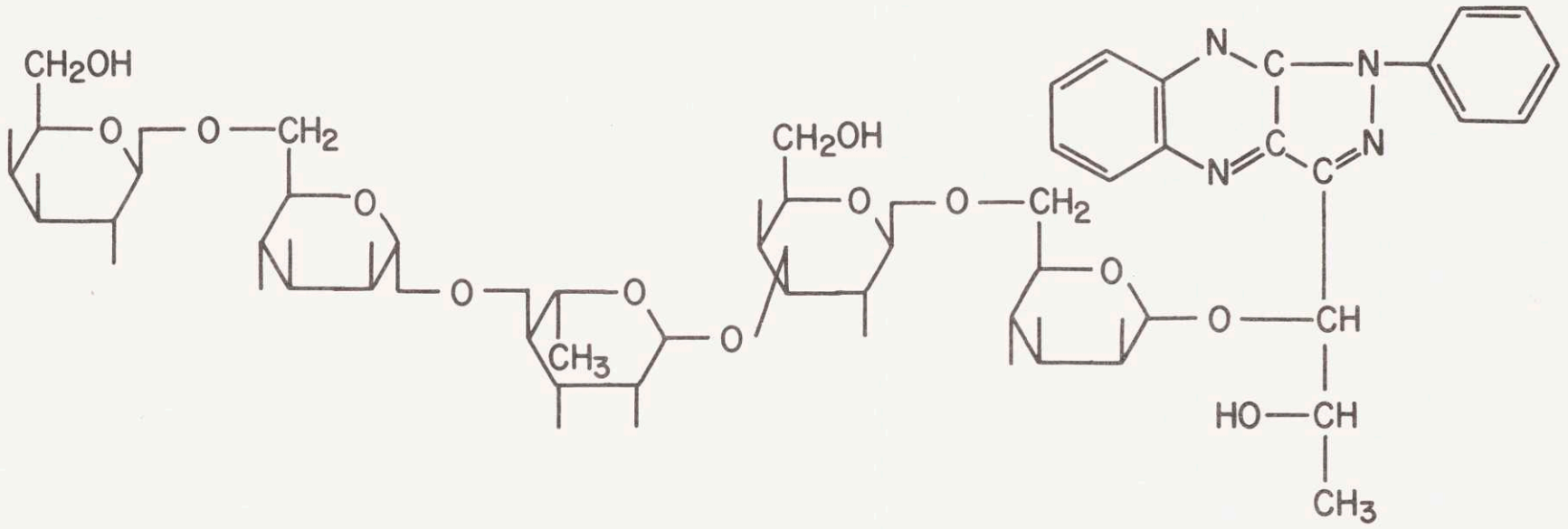

CH2OH
O
O
CH2
O
O
O
CH3
O
CH2OH
O
O
CH2
O
O
CH
HO
CH
CH3
N
C
N
N
C
C
N

TABLE V

R_F Values of Flavazole Derivatives on Paper Chromatography

Flavazole	R_F (solvent 1)
Hexasaccharide	0.21
Pentasaccharide	0.43
Tetrasaccharide	0.66
Trisaccharide	0.74
Disaccharide	0.90

TABLE VI

Melting Points of 1-Phenylflavazole Derivatives

Compound	m. p.	
	Experimental	Literature
Rhamnose flavazole	214-215°	214-216° (68); 211° (67)
Lactose flavazole	258-262°	272-274° (67)
β-Trisaccharide flavazole	214-215°	-

The β-trisaccharide flavazole was found to have the same extinction coefficient of 1.01×10^4. The concentrations of all flavazoles were determined in aqueous solutions from the absorption peak at 335 mμ.

4. L-Rhamnosyl-(1→3)-D-Galactose Phospholipid

The L-rhamnosyl-D-galactose phospholipid was prepared in EDTA-treated cells and extracted with n-butanol by the standard method (44). The preparation of the disaccharide-phospholipid in 0-10 transacetylase particles was performed as follows. A reaction mixture containing 50 μmoles of phosphate buffer, pH 7.4; 0.049 μmole of ^{14}C-TDPRha; 5 μmoles of UDPG; 0.1 ml of an E. coli K 12 extract containing UDPGal-4-epimerase (51) and 40 μmoles of magnesium chloride in a total volume of 1.9 ml was incubated for 20 min at 30°. A 0.1 ml aliquot of 0-10 transacetylase particles (11.9 mg protein/ml) was then added and the incubation was continued. The addition of n-butanol stopped the reaction and extracted the disaccharide-phospholipid as described for the incubations with EDTA-treated cells.

5. Methyl D-Galactoside

Methyl D-galactoside, uniformly ^{14}C-labeled in the galactosyl residue, was synthesized from ^{14}C-UDPGal. The UDPGal was refluxed for 30 minutes in freshly prepared 1 per cent (w/v) hydrogen

chloride in methanol. The methyl D-galactoside was purified chromatographically on Whatman No. 1 paper in solvent 1.

6. Methyl L-Rhamnosyl-(1→3)-D-Galactoside

Methyl L-rhamnosyl-D-galactoside, uniformly ^{14}C-labeled in the galactosyl residue, was prepared from L-rhamnosyl-D-galactose phospholipid by a procedure suggested by Dr. A. Wright. A sealed tube containing the starting material suspended in 0.01 N hydrogen chloride in methanol was heated for 10 minutes in a boiling-water bath. Purification was performed chromatographically on Whatman No. 1 papers in solvents 4 and 1.

7. O-Acetyl L-Rhamnosyl-(1→3)-D-Galactose, Containing Uniformly ^{14}C-labeled Galactose

The acetylated L-rhamnosyl-D-galactose containing uniformly ^{14}C-labeled galactose was prepared as follows. A 0.1 ml reaction mixture consisting of 10 μmoles of phosphate buffer, pH 7.4; 0.11 μmole of acetyl-CoA; 50,000 cpm of L-rhamnosyl-D-galactose (uniformly ^{14}C-labeled in the galactosyl residue); 0.11 mg of O-10 transacetylase particles from L-8 and 10 μmoles of Tris buffer, pH 7.8, (final pH about 7.2) was incubated overnight at 37°. The reaction mixture was streaked onto Whatman No. 1 chromatography paper and developed in solvent 3. The separation between the disaccharide and the acetylated disaccharide

was achieved by use of two overnight developments with the same solvent. The paper was allowed to dry between runs. The product was eluted with glass-distilled water and stored frozen. Based on the disaccharide initially present, the yield was about 3 per cent.

8. O-Acetyl L-Rhamnosyl-(1→3)-D-Galactose

The acetylated disaccharide that contained no radioactivity in the saccharide portion of the molecule was prepared as follows. A 5.15 ml reaction mixture containing 25 μmoles of ^{14}C-acetyl-CoA (about 0.01 μC per μmole); 15 μmoles of L-rhamnosyl-D-galactose; 0.40 mmole of phosphate buffer, pH 7.4, and 17.5 mg of O-10 transacetylase particles prepared from L-8 (final pH about 6.8) was incubated for two days at 37°. The transacetylase particles were precipitated by centrifugation at 30,000 x g for 30 minutes. The particles were washed one time with water. The combined supernatant liquids were then passed through a 1 x 15 cm column of Dowex-AG-1 (acetate form), which was subsequently washed with three column volumes of water. The combined effluents were concentrated and then applied to a 1 x 23 cm charcoal-Celite column, which was prepared and eluted with an ethanol gradient as described in the acetolysis procedure. After the ethanol gradient was depleted, a second ethanol gradient,

prepared from 100 ml of 30 per cent ethanol and 100 ml of 50 per cent ethanol, was used to elute the acetylated product (Figure 6). The fractions containing the acetylated product were pooled, concentrated and stored frozen at pH 5. About 30 per cent of the disaccharide was recovered as the acetylated product.

9. Polyalcohols

L-Rhamnitol, D-lyxitol and D-mannitol were synthesized from the parent aldoses by reduction with excess sodium borohydride. The reactions were terminated by the addition of Dowex-AG-50 (hydrogen form) to destroy excess borohydride. The solutions were then passed through small columns of Dowex-AG-50 (hydrogen form) to remove sodium ions. Effluents were concentrated to dryness under reduced pressure and the boric acid was then removed by evaporation with anhydrous methanol.

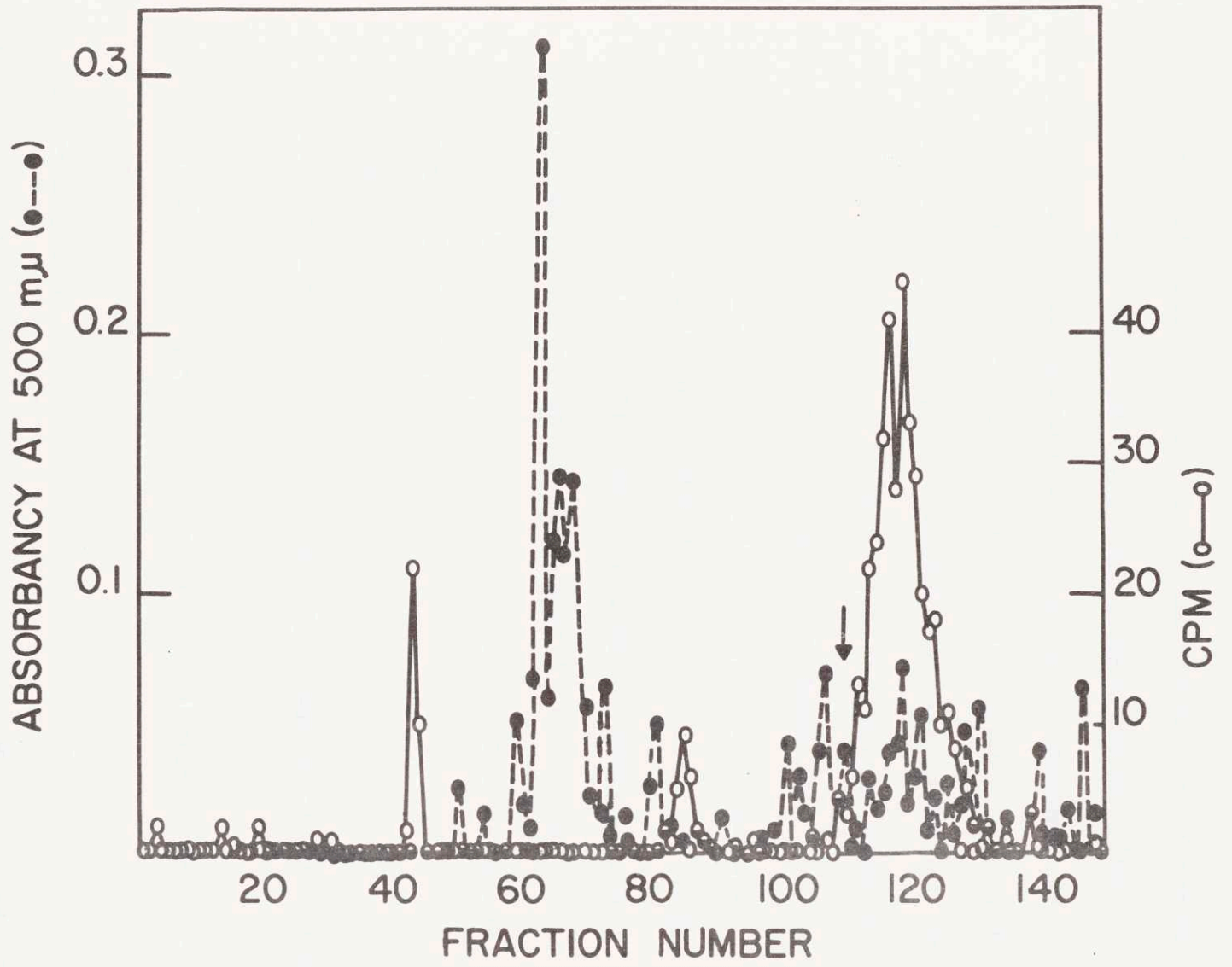
I. Enzyme Preparations

1. 0-10 Transacetylase Particles

After the initial studies, 0-10 transacetylase particles were prepared in a standard fashion. Bacteria were poured over ice, harvested by centrifugation and washed once with cold 0.9 per cent (w/v) sodium chloride. The final pellet was frozen with

Figure 6. Purification of O-^{14}C-Acetyl L-Rhamnosyl-D-Galactose by Chromatography on a Charcoal-Celite Column

The column preparation and elution procedure are described in the text. The arrow in the figure indicates the point at which the second ethanol gradient was started. Aliquots (0.01 ml) of each fraction were placed onto planchets, evaporated to dryness and then analyzed for radioactivity in a gas flow counter. Carbohydrate was determined by the cysteine-sulfuric acid method with a 0.03 ml aliquot from each fraction. The carbohydrate material in fractions 58 through 78 is unacetylated disaccharide. The solutions in fractions 108 through 131 were pooled, concentrated and stored frozen. O—O, ^{14}C; ●--●, carbohydrate.


ABSORBANCY AT 500 mμ (●--●)
0.3
0.2
0.1
CPM (o—o)
40
30
20
10
20
40
60
80
100
120
140
FRACTION NUMBER

either liquid nitrogen or a mixture of dry ice and acetone and crushed in a Hughes press. The crushed material was suspended in a standard buffer, composed of 10 mM Tris-hydrochloride, pH 7.75, and 10 mM 2-mercaptoethanol. Deoxyribonuclease (2 to 5 μg per ml) was added and the preparation was allowed to stand at 3° until it could be easily transferred with a pipette. Whole cells were precipitated by two centrifugations for 10 minutes at 4,000 x g. The enzyme particles were isolated from the supernatant liquid by centrifugation at 30,000 x g for 30 minutes. The pellet was washed once with standard buffer and then resuspended in standard buffer. Enzyme particles, prepared from a 500 ml culture harvested at an optical density of 1, had a protein concentration of about 4.5 mg per ml after suspension in 2 ml of standard buffer.

2. α-Mannosidase

α-Mannosidase was prepared from rat epididymis tissue (70).

3. β-Galactosidase

β-Galactosidase was prepared from a constitutive mutant of E. coli K 12 (8).

4. EDTA-Treated Cells

EDTA-treated cells were prepared by the standard method (51).

5. Thiogalactoside Transacetylase

Thiogalactoside transacetylase was prepared from E. coli K 12 (71). The purification was carried through the step that involved treatment with heat.

J. Assay Procedures for Specific Chemical Components

1. Carbohydrate

Carbohydrate was determined by the phenol-sulfuric acid method (72). A 1 ml solution containing 0.025 μmole of glucose had an optical density at 500 mμ of about 0.102 after the additions of 0.05 ml of phenol and 2.5 ml of concentrated sulfuric acid. Under these conditions, 1 mg of LPS produced 2.21 times the absorption given by 1 μmole of glucose (Dr. P. W. Robbins, unpublished observation).

2. Rhamnose

Rhamnose was determined by the Dische cysteine-sulfuric acid method (73). A 2.25 ml aliquot of the sulfuric acid reagent was added slowly to a 0.5 ml sample cooled in an ice bath. The solution was mixed and then heated for 10 minutes in a boiling-water bath. After the solution had cooled to room temperature (10 to 15 minutes), 0.05 ml of 3 per cent (w/v) cysteine-hydrochloride was added. The optical densities at 396 and 427 were

measured 2 hours later. Under these conditions, the optical density difference produced by 0.03 μmole of rhamnose was about 0.180.

3. Galactose

Galactose was measured with the Galactostat reagent (Worthington Biochemical Corp., Freehold, N. J.). The procedure described by the manufacturer was altered because of the instability of the reagent during storage. The chromogen was dissolved in 0.5 ml of methanol and then brought to a final volume of 25 ml with water (solution A). The contents of the Galactostat vial were dissolved in 25 ml of water (solution B). These solutions were stable for several weeks at -20°. Before each assay, equal volumes of solutions A and B were mixed in order to prepare fresh reagent. The assay was performed on one-tenth of the scale suggested by the manufacturer. Under the conditions described above, 0.025 μmole of galactose produced an optical density of 0.180 at 425 mμ.

4. Ester-Linked Acetyl Groups

Ester-linked acetyl groups were measured by a modification of the standard method (74, 75). A 0.17 ml aliquot of freshly prepared hydroxylamine solution was added to a 0.33 ml sample.

After 10 minutes, 0.5 ml of freshly prepared ferric chloride reagent was added and the optical density at 540 mμ was measured. An optical density of about 0.132 was produced by 0.5 μmole of succinyl hydroxamate, prepared by the standard method (74). The hydroxylamine solution consisted of equal volumes of 3.5 N sodium hydroxide and 2 M or 4 M hydroxylamine-hydrochloride. The former alkaline mixture was used to measure total ester while the latter neutral mixture was used to measure only reactive esters such as acetyl-CoA. The ferric chloride solution consisted of equal portions of 5 per cent (w/v) ferric chloride in 0.1 N hydrochloric acid, 3 N hydrochloric acid and 12 per cent (w/v) trichloroacetic acid. A correction for turbidity in the LPS samples was made with controls to which the ferric chloride reagent had been added before the hydroxylamine solution. The turbidity of LPS samples was occasionally so great that the acetyl content could not be measured. The reason for this variation is unknown.

The acetyl concentration of the ^{3}H-labeled acetyl-CoA preparation was measured with a spectrophotometric assay adapted from Stadtman (62). Samples were saponified with 0.5 N sodium hydroxide for 1 hour at room temperature and then adjusted to pH 7.0 by the addition of a large excess of 0.1 M phosphate

buffer, pH 7.0. The optical densities of the saponified sample and the non-saponified control were measured at 232 mμ. Saponification caused a change in optical density equivalent to 4.5×10^3 (liter per mole per cm) at pH 7.0. The values obtained were 72 per cent of those obtained with the ferric chloride assay as judged by samples measured by both methods.

5. Protein

Protein concentrations were determined by the Lowry procedure (76). The concentration of the bovine serum albumin standard was determined by use of an extinction coefficient at 280 mμ of 6.6 (77). A 0.5 ml sample containing 0.025 mg of bovine serum albumin had an optical density at 540 mμ of about 0.121 under the conditions of assay.

6. Formaldehyde

Formaldehyde formed during periodate oxidations was determined by the acetylacetone-ammonia method (78). The periodate oxidation was performed in a total volume of 0.125 ml. After one hour, 0.25 ml of water and 0.062 ml of 5 N sulfuric acid were added and the solution was carefully mixed. The metaperiodate and iodate were then reduced to iodide by the addition of 0.063 ml of 4 M sodium arsenite. The solution was mixed and allowed to stand until the yellow-brown color had appeared and

then vanished, an indication that the reduction to iodide had taken place. An equal volume of the acetylacetone reagent was then added and the mixture was heated at 60° for 10 minutes. The reaction mixture was cooled, and the optical density at 412 mμ was measured. A sample containing 0.05 μmole of formaldehyde prepared by periodate oxidation of erythritol gave an optical density of about 0.378 under the conditions described. When the whole periodate oxidation reaction mixture was analyzed for formaldehyde, a control tube containing no carbohydrate material was carried through the complete procedure.

K. Assay of 0-10 Transacetylase

The enzyme 0-10 transacetylase was assayed in several ways. A unit of enzyme activity was defined as the transfer of 1 mμmole of acetyl per minute from acetyl-CoA to the β-hexasaccharide flavazole under the conditions described for the charcoal assay.

1. Particle Assay

The membrane filter assay of particles depends upon the presence of endogenous acceptors. The standard assay mixture contained 2.5 μmoles of phosphate buffer, pH 7.4; 52 mμmoles of ^{14}C-acetyl-CoA and 20 to 50 μg of enzyme in a total volume of 0.1 ml and was incubated at 30°. Aliquots (25 μl) were removed at 4.5 minute intervals and added to 0.1 ml of neutral

2 M hydroxylamine. After 5 minutes, 0.1 ml of 2 M acetic acid was added. The particles were collected on a membrane filter (type B-6: Schleicher and Schuell, Keene, N. H.) and washed three times with water. The filter was then pasted onto a planchet and counted in a Nuclear Chicago gas flow counter. The same results were obtained if the 25 µl aliquots were added to 0.175 ml of 50 per cent (v/v) aqueous ethanol, instead of the hydroxylamine and acetic acid, and then filtered as described above.

2. Qualitative Paper Assay

The standard reaction mixture contained 5 µmoles of phosphate buffer, pH 7.4; 14 mµmoles of ^{14}C-acetyl-CoA; 37 mµmoles of β-hexasaccharide flavazole and 20 to 50 µg of enzyme in a total volume of 0.1 ml and was incubated at 30°. The reaction was stopped by the addition of 0.01 ml of neutral 2 M hydroxylamine. The samples were spotted onto Whatman No. 1 chromatography paper, which was then developed overnight in solvent 1. The paper was dried and cut into squares, which were 1 cm on a side. These were placed onto planchets and counted in a gas flow counter.

3. Charcoal Assay

In the charcoal assay, 25 µl aliquots of the reaction mixture described in the previous paragraph were removed at 4-minute

intervals and added to 0.175 ml of 50 per cent (v/v) aqueous ethanol. Each sample was then applied to a 0.4 x 2 cm column of Dowex-AG-1 (acetate form) and eluted with 1 ml of water. The effluent was treated with 15 µl of a 5 per cent (w/v) suspension of acid-washed Norite A in water. After a 30-minute adsorption period, the charcoal was collected on a membrane filter, which was pasted onto a planchet and counted in a gas flow counter.

4. Quantitative Paper Assay

The quantitative paper assay used in the substrate specificity studies involved the following modifications of the charcoal assay. The effluent of the Dowex-AG-1 (acetate form) column was concentrated to a small volume and spotted onto a strip of Whatman No. 1 chromatography paper, which was then developed overnight in solvent 3. The radioactivity on the dried strip was determined with a Packard strip scanner equipped with a Disc integrator. The factor for the conversion of radioactivity as measured by the strip scanner to comparable values measured in the gas flow counter was determined from radioactivity measurements of a ^{14}C-standard under both conditions.

5. Comparison of Assay Procedures

The charcoal assay is the easiest and most accurate method to measure O-10 transacetylase. The particle assay is useful in the measurement of endogenous O-acetyl acceptor. The quantitative paper assay has to be used when substrates that do not adsorb to charcoal are being tested. If the minor contaminant in the acetyl-CoA preparations could be removed, the paper chromatography step could be eliminated. Then, the column effluents could be collected directly on planchets and counted. This would be the simplest assay procedure. The qualitative paper assay was used only before the binding of flavazole derivatives to charcoal was examined. It is of little value unless there is interest in the acetyl-CoA hydrolyzing activity discussed elsewhere in this thesis.

L. Chemical Techniques

1. Acid Hydrolysis

Complete acid hydrolysis of compounds was performed in sealed tubes with sulfuric acid in an oven at 105° for at least 4 hours. LPS was hydrolyzed with 1 N acid, and oligosaccharides were hydrolyzed with 0.4 to 1 N acid. The solutions were then passed through columns of Dowex-AG-1 (acetate form) and Dowex-AG-50 (hydrogen form). The column effluents were taken to dryness to remove acetic acid.

2. Smith Degradation

Smith degradations (79) were performed as follows. Aliquots of the appropriate samples were concentrated to dryness and then dissolved in 0.03 ml of 0.1 M sodium metaperiodate. After 1 hour, 0.03 ml of 0.88 M ethylene glycol was added to destroy excess periodate and the solution was allowed to stand for 40 minutes. The oxidized products were reduced by treatment for 4 hours with 0.06 ml of 1 M aqueous sodium borohydride. The reduction was terminated by the addition of Dowex-AG-50 (hydrogen form). Borate was then removed by evaporation with methanol in the standard way. The residue was dissolved in 0.02 ml of 0.1 N sulfuric acid and allowed to stand overnight at room temperature. Samples were then spotted onto Whatman No. 1 chromatography paper, which was then developed twice for 10 to 14 hours in solvent 4.

3. Sodium Borohydride Reduction in Methanol

Reduction with sodium borohydride in methanol was performed in reagent grade methanol. The sample to be reduced was first concentrated to dryness under a stream of nitrogen. A 40 µl aliquot of freshly prepared 25 mM sodium borohydride in methanol was then added. After 30 minutes, the methanol was removed by evaporation under a stream of nitrogen. The residue was dissolved in 62 µl of 0.05 M sodium acetate, pH 4.5, and 63 µl of 0.2 M

sodium metaperiodate (final pH about 5.2 by paper). After one hour, samples were analyzed for formaldehyde. As determined by the formaldehyde assay, maltose was completely reduced within 5 minutes under the conditions described.

III. RESULTS

A. Enzymatic Properties of O-10 Transacetylase

The infection of Salmonella E_1 strains by the phage ϵ^{15} results in two well-defined structural changes in the O-antigen. These changes are the loss of O-acetyl groups and the change from an α- to a β-D-galactosyl linkage (see Introduction). In order to understand the mechanism(s) by which the virus causes these changes, the enzymes responsible for these structural features were studied. No simple assay to distinguish between the enzymes that form the α- and β-D-galactosyl linkages was available. The presence or absence of the O-acetyl group, on the other hand, seemed to offer an easier experimental approach. Thus, experiments were undertaken in an attempt to find and characterize the acetylating enzyme.

1. Demonstration of O-10 Transacetylase Activity

The search for the acetylating activity necessitated some speculation about the possible nature and location of the substrates involved. The acetyl derivative of CoA was selected as the most likely acetyl donor. The choice of an acetyl acceptor,

however, was not obvious. One acceptor tried was the alkali-treated (deacetylated) LPS from an E_1 strain (S. anatum A_1). Attempts to demonstrate acetyl-transferring activity in the supernatant liquid isolated after high-speed centrifugation of disrupted S. anatum A_1 ($\equiv A_1$) cells were unsuccessful. Since the LPS is on the exterior of the cell, the possibility existed that the synthesis of the molecule might in some way be connected with the cell membrane. Thus, enzyme activity was sought in the cell wall-membrane complexes, which were isolated by differential centrifugation after the cells had been crushed in a Hughes press. The crushed material was centrifuged three times for 10 minutes at 4,000 x g in order to sediment any whole cells remaining. The supernatant liquid was then centrifuged for 30 minutes at 30,000 x g. The acetyl-incorporating activity of the particulate material collected during the 30,000 x g centrifugation was tested by incubation of the particles with ^{14}C-acetyl-CoA. After 30 minutes, the reaction was stopped by the addition of neutral hydroxylamine and the incubation mixtures were filtered through membrane filters. The radioactivity in the material retained by the filters was then determined (Table VII). Incorporation of radioactive acetyl groups occurred only in the

TABLE VII

Effect of Cytoplasmic Fractions
on 0-10 Transacetylase Activity

Additions	mμmole of acetyl incorporated		
	0 min	60 min	Δ
None	0.058	0.162	0.104
A_1 30,000 x g supernatant liquid	0.070	0.040	-0.030
$A_1(\epsilon^{15})$ 30,000 x g supernatant liquid	0.076	0.047	-0.029

Each incubation mixture contained 25 μmoles of phosphate buffer, pH 7.4; 14 mμmoles of ^{14}C-acetyl-CoA; 0.15 mg of deacetylated A_1 LPS; 0.25 mg of A_1 particles and further additions, as indicated, in a total volume of 0.5 ml. Reactions were stopped after 60 min by the addition of 0.05 ml of neutral 2 M hydroxylamine. Hydroxylamine was added to duplicate incubation mixtures before the A_1 particles to obtain the 0 min incorporation values. The acetyl incorporated was determined as described in the particle assay procedure.

presence of the 30,000 x g particles prepared from the A_1 cells. Addition of the 30,000 x g supernatant liquid from either the non-lysogenic or lysogenic strain, however, caused a loss of activity, which will be considered in detail in later experiments. The lack of a similar acetyl-incorporating activity in the 30,000 x g particles prepared from a lysogenic strain [$A_1(\epsilon^{15})$] was demonstrated in other experiments. The alkali-treated LPS was shown in similar experiments to have no effect on the total incorporation.

The occurrence of acetyl-incorporating activity in particles from the non-lysogenic strain but not in particles from the lysogenic strain provided a good indication that this activity was responsible for the acetylation of LPS. In order to prove this, however, the endogenous product was isolated and characterized. A large scale incubation of A_1 particles with ^{14}C-acetyl-CoA was performed. The reaction was stopped by the addition of neutral hydroxylamine. The particles were then separated from the radioactive acetohydroxamate by centrifugation for thirty minutes at 30,000 x g. The pellet was washed two times with 0.05 M phosphate buffer, pH 7.4, and then resuspended in a small volume of buffer. The LPS in this preparation was ex-

tracted by the standard hot phenol method. As shown in Table VIII, 91 per cent of the radioactivity originally present in the washed particles was recovered in the aqueous layer as non-dialyzable material. A portion of this partially purified radioactive material was sedimented at 100,000 x g in the presence of carrier A_1 LPS. After centrifugation, the supernatant liquid was fractionated by removal of small volumes from as close to the meniscus as possible with a Pasteur pipette. This was done in order to determine whether there was an even distribution of any soluble material present. Traces of radioactivity were found in the fluid from the upper portion of the tube, although most of the soluble radioactivity was recovered near the bottom. The pellet contained 57 per cent of the radioactive material initially added. Another portion of the non-dialyzable radioactive material was tested for precipitation with anti-3, 10 serum (Table VIII). The fact that 54 to 62 per cent of the added radioactive material was recovered in the antigen-antibody precipitate provided additional evidence that the radioactive material was LPS. This characterization of the radioactive material as LPS, in addition to the presence of acetyl-incorporating activity in only the non-lysogenic cells, demonstrates the coupling of the transferring activity with the known effects of the prophage.

TABLE VIII

Characterization of LPS as an Endogenous Acetyl Acceptor

Fraction	Per cent of radioactivity
Washed particles	(100)
Phenol extract	87
Dialyzed phenol extract	91
Precipitate after 100,000 x g centrifugation	57
Antigen-antibody precipitate	54-62

A reaction mixture containing 9.5 mg of A_1 particles; 50 μmoles of phosphate buffer, pH 7.4, and 0.21 μmoles of ^{14}C-acetyl-CoA in a total volume of 1.4 ml was incubated for 120 min at 37°. The reaction was stopped by the addition of 0.2 ml of neutral 2 M hydroxylamine, and 0.4 ml of 2 M acetic acid was added 10 min later. The particles were isolated and washed as described in the text. The radioactivity present in the washed particles was defined as 100 per cent. The other procedures are described in the text.

2. Assay of O-10 Transacetylase

The procedures utilized in the previous experiments depended upon endogenous substrates. If the absence of acetyl-transferring activity in the lysogenic strain was the result of a change in the structure of the endogenous acceptor, enzymatic activity would not have been detected. Therefore, it was necessary to develop an enzyme assay that would be independent of endogenous acceptor.

Purified LPS, as mentioned earlier, was inactive as an acceptor. Since the enzyme appeared to be within the cell wall-membrane complex, it seemed possible that only small substrates would be able to reach the catalytic sites. Thus, the use of oligosaccharides, which would hopefully still carry the specificity required by the enzyme, was investigated. A crude fraction of oligosaccharides formed during partial acid hydrolysis of LPS was found to contain active acceptors (Dr. P. W. Robbins, unpublished observations). The purification of these oligosaccharides, however, appeared to be a formidable task. The preparation and purification of the 1-phenylflavazole (≡ flavazole) derivatives of oligosaccharides was then brought to my attention. A detailed report on the synthesis, purification and characterization of these derivatives is included in the Materials and Methods section of this thesis.

The β-hexasaccharide flavazole was incubated with enzyme particles and ^{14}C-acetyl-CoA as described in Figure 7. The reaction was terminated with hydroxylamine and the resultant mixture was spotted onto Whatman 3 MM chromatography paper, which was developed overnight in solvent 1 (Figure 7 and Table IX). The position of the flavazole was easily determined by its fluorescence under exposure to ultraviolet irradiation. The chromatogram was then cut into small pieces, which were counted in a gas flow counter. The radioactivity at the origin (I) represented incorporation into endogenous acceptors. The radioactive material in area III was assumed to be acetohydroxamate. The radioactivity that chromatographed slightly ahead of the β-hexasaccharide flavazole (III) was in the area where the acetylated flavazole derivative would be expected to run. The isolation of this radioactive material from only those incubation mixtures containing A_1 particles demonstrated that the β-hexasaccharide flavazole was a substrate for the enzyme.

The addition of A_1 30,000 x g supernatant liquid after 90 minutes of incubation followed by a further 30-minute incubation period had no effect on the incorporation into the flavazole derivative (Figure 7 and Table IX). The loss of radioactivity from the acetohydroxamate area near the solvent front suggested

Figure 7. Acetyl-Accepting Activity of the β-Hexasaccharide Flavazole Derivative

Each reaction mixture contained 5 μmoles of phosphate buffer, pH 7.4, 14 mμmoles of ^{14}C-acetyl CoA and, as indicated, 37 μg of A_1 particles, and 88 mμmoles of β-hexasaccharide flavazole in a total volume of 0.1 ml, and was incubated to 30°. The reactions were stopped and analyzed by the procedures used in the standard qualitative paper assay. The closed circles represent fluorescence, and the open circles represent radioactivity. I, origin of chromatogram; II, position of the hexasaccharide flavazole; III, position of acetohydroxamate.

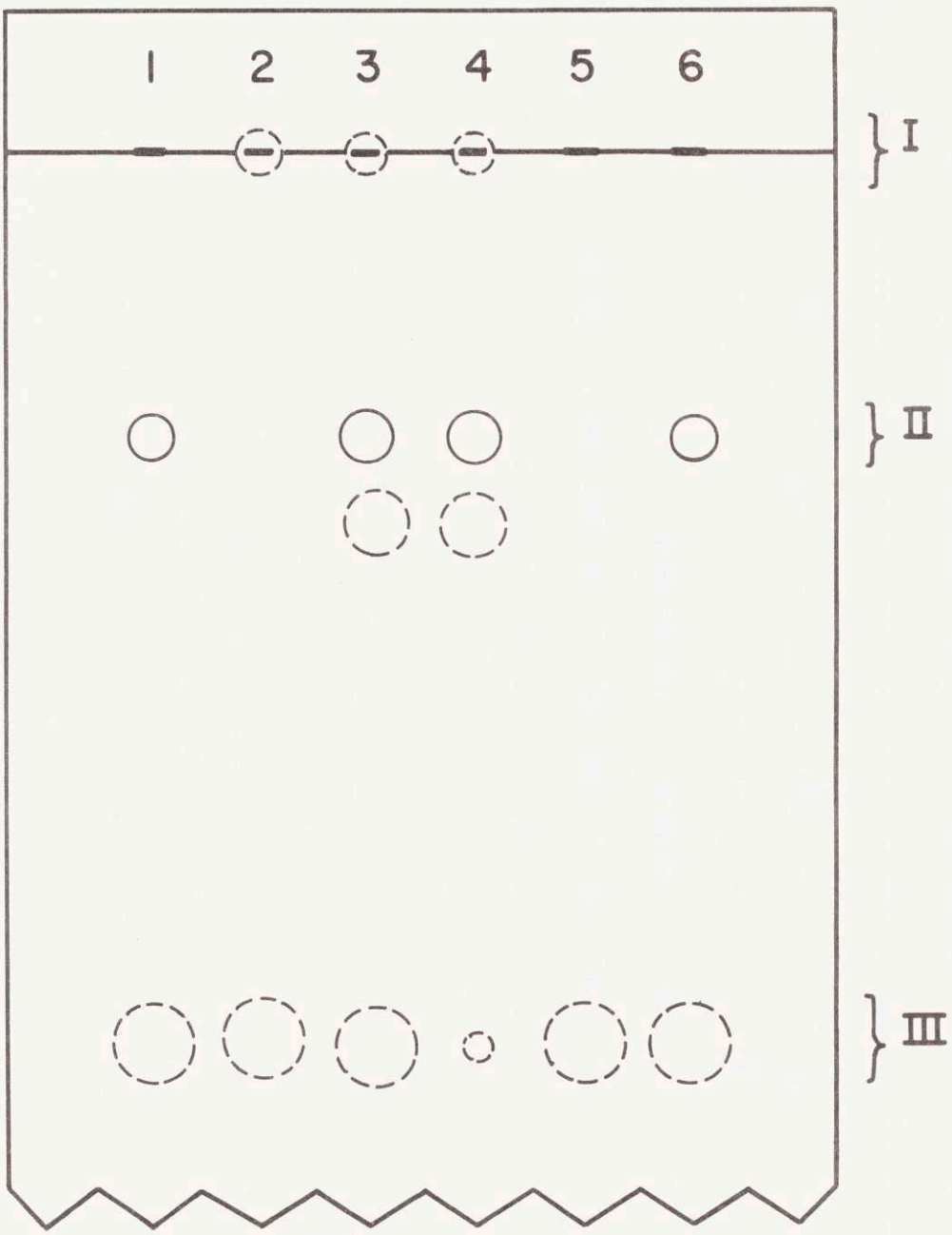

1
2
3
4
5
6
I
II
III

TABLE IX

Effect of the A_1 30,000 x g Supernatant Liquid on the ^{14}C-Acetylated Flavazole Derivative and ^{14}C-Acetyl-CoA

Sample No.	Additions; incubation period	Total cpm in region		
		I	II	III
1	A_1 particles, β-hexasaccharide flavazole; 0 min	26	40	2803
2	A_1 particles; 90 min	245	44	1207
3	A_1 particles, β-hexasaccharide flavazole; 90 min	186	1023	782
4	A_1 particles, β-hexasaccharide flavazole; 90 min then A_1 30,000 x g supernatant liquid; 30 min	137	1089	159
5	$A_1(\epsilon^{15})$ particles; 90 min	20	34	1828
6	$A_1(\epsilon^{15})$ particles, β-hexasaccharide flavazole; 90 min	22	28	2326

The details of this experiment are described in Figure 7.

that the supernatant liquid contained an acetyl-CoA hydrolase. A similar activity in E. coli has been reported by Zabin in his studies of the enzyme thiogalactoside transacetylase (80). The results with the β-hexasaccharide flavazole were surprising since this flavazole derivative was prepared from the LPS of the lysogenic strain. The α-hexasaccharide flavazole was demonstrated to be an acceptor in similar experiments. Thus, the enzyme did not appear to discriminate between the two possible anomeric configurations of the D-galactosyl linkage. This probably eliminates the loss of acetyl acceptor as the reason for the loss of acetyl-transferring activity after phage conversion.

The effects of cell fractions prepared from the lysogenic strain on the transacetylase activity with the β-hexasaccharide flavazole derivative were tested with the qualitative paper assay (Table X). The A_1 (ϵ^{15}) particles have no effect on the transfer of acetyl groups. Thus, the absence of acetyl-transferring activity in the lysogenic strain cannot be ascribed to the presence of a deacetylating enzyme. The loss of activity in the presence of the A_1 (ϵ^{15}) supernatant liquid is assumed to be caused by the same acetyl-CoA hydrolase as that believed to be present in the non-lysogenic strain.

TABLE X

Effect of Subcellular Fractions from A_1 and $A_1(\epsilon^{15})$ on 0-10 Transacetylase Activity

Additions; incubation period	Total cpm in region	
	I	II
A_1 particles, A_1 30,000 x g supernatant liquid; 0 min	37	73
A_1 particles; 90 min	197	693
A_1 30,000 x g supernatant liquid; 90 min	25	97
A_1 particles, A_1 30,000 x g supernatant liquid; 90 min	72	148
A_1 particles, $A_1(\epsilon^{15})$ particles; 90 min	74	574
A_1 particles, $A_1(\epsilon^{15})$ 30,000 x g supernatant liquid; 90 min	79	195

The incubation mixtures and assay procedures were identical to those described in Figure 7, except that only 44 mμmoles of β-hexasaccharide flavazole were used. The $A_1(\epsilon^{15})$ 30,000 x g supernatant liquid (42 μg) was added as indicated.

The value of the flavazoles as substrates is considerably enhanced by their high extinction coefficients and their tenacious binding to charcoal. The latter property was utilized to develop the charcoal assay for the enzyme 0-10 transacetylase in the presence of the β-hexasaccharide flavazole. The transfer of acetyl groups is linear with time in the presence of excess acetyl CoA and β-hexasaccharide flavazole (Figure 8 and Table XI). Under the conditions of assay, the transfer of acetyl groups to flavazole was proportional to added enzyme (Figure 9). The enzymatic activity of 0-10 transacetylase was not affected by the presence of 5.0 mM magnesium chloride, calcium chloride or manganese chloride. The presence of 2-mercaptoethanol did not stimulate enzyme activity, but did stabilize the enzyme so that it could be stored for eight to ten days at -20° with only a 10 to 20 per cent loss in activity. The optimum pH for the reaction was found to be between 6 and 8 (Figure 10). The optimum pH for stability of enzymatic activity during storage was not determined. A unit of enzymatic activity was defined as the transfer of 1 mμmole of acetyl per minute from acetyl-CoA to the β-hexasaccharide flavazole derivative under standard conditions as described in Materials and Methods. The activity of particle preparations was usually about two units per mg of protein.

Figure 8. Charcoal Assay of 0-10 Transacetylase

The procedure used is described in Table XI. O—O, sample 1; □—□, sample 2; Δ—Δ, sample 3; ▽—▽, sample 4; ◇—◇, sample 5.

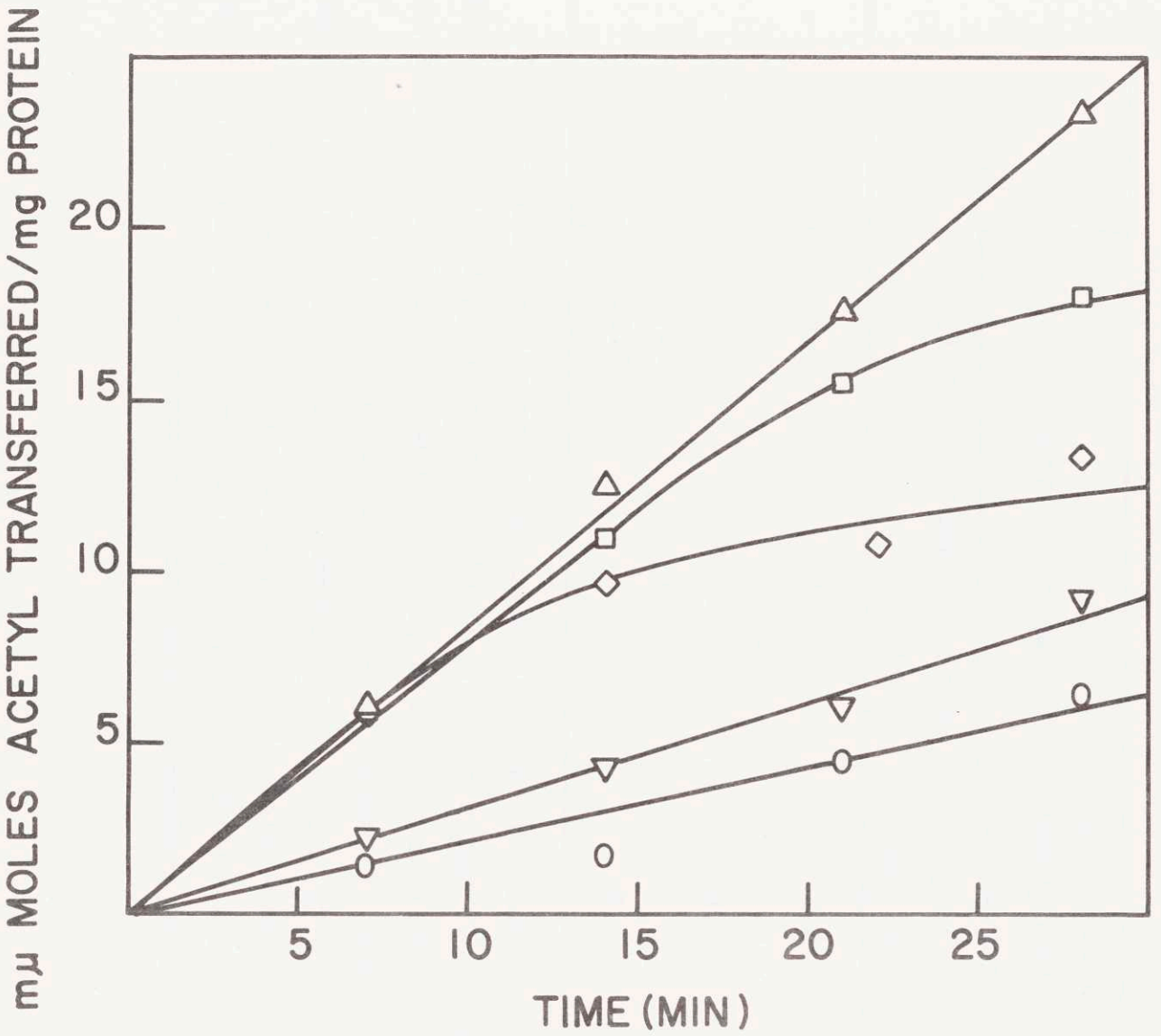

mμ MOLES ACETYL TRANSFERRED/mg PROTEIN
20
15
10
5
5
10
15
20
25
TIME (MIN)

TABLE XI

Effect of Substrate Concentration
on 0-10 Transacetylase Activity

Sample No.	Additions (mμmoles)		mμmole acetyl incorporated at 10 min/ mg protein (from Figure 8)
	Acetyl-CoA	β-Hexasaccharide flavazole	
1	14.3	18.7	2.2
2	"	37.4	7.9
3	"	56.1	8.3
4	7.15	18.7	3.1
5	28.6	"	8.3

Each incubation mixture contained 5 μmoles of phosphate buffer, pH 7.4, 48 μg of A_1 particles and other substrates, as indicated, in a total volume of 0.1 ml, and was incubated at 30°. Aliquots (25 μl) were removed at 7-min intervals and added to 0.1 ml of 1 M neutral hydroxylamine. After 10 min, 0.1 ml of 1 M acetic acid was added. The flavazoles were then adsorbed to charcoal, which was collected on membrane filters as described in the standard charcoal assay procedure (see Figure 8).

Figure 9. Effect of Protein Concentration on 0-10 Transacetylase Activity

The charcoal assay procedure as described for sample number 2 in Table XI was used with various amounts of the enzyme preparation.

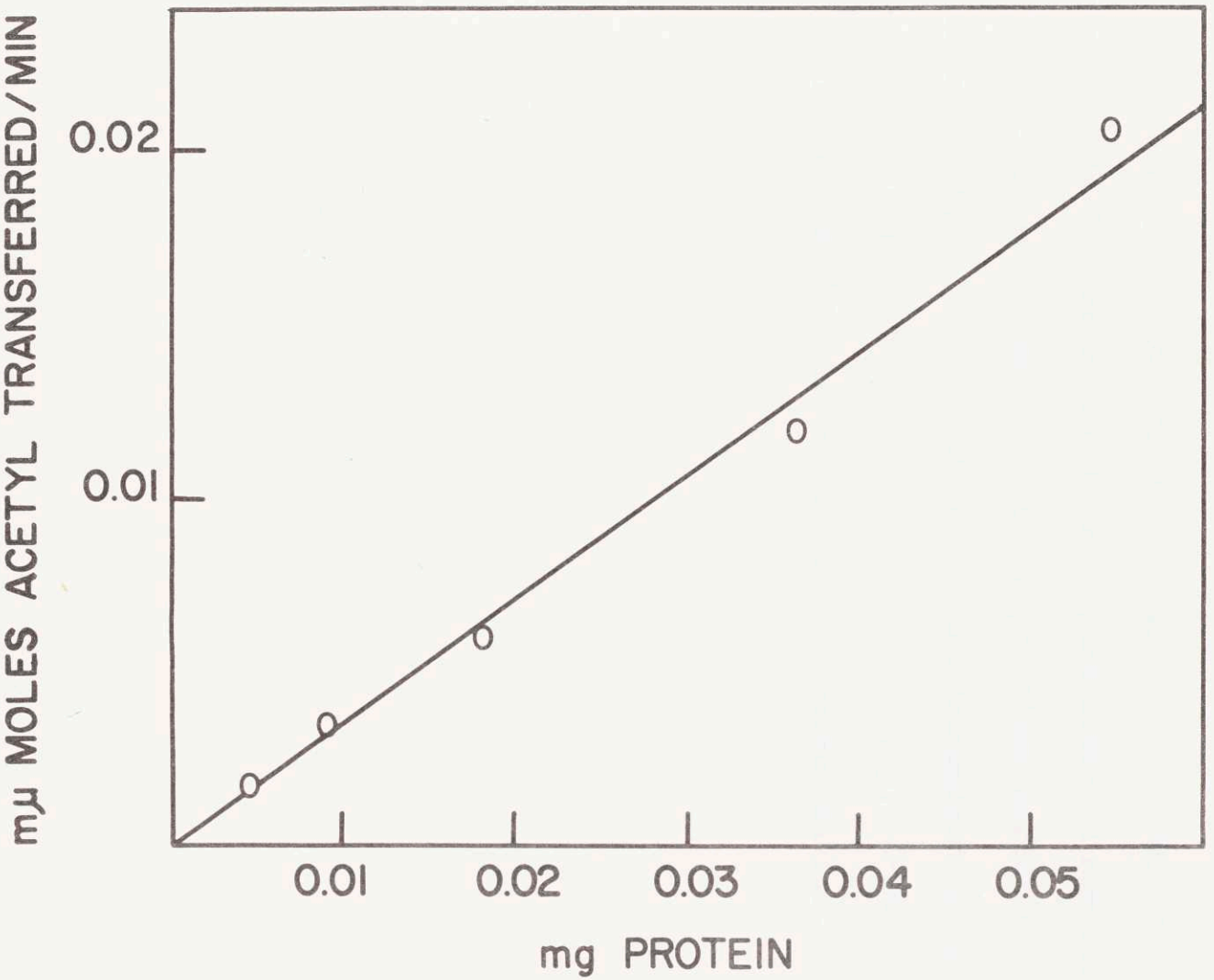
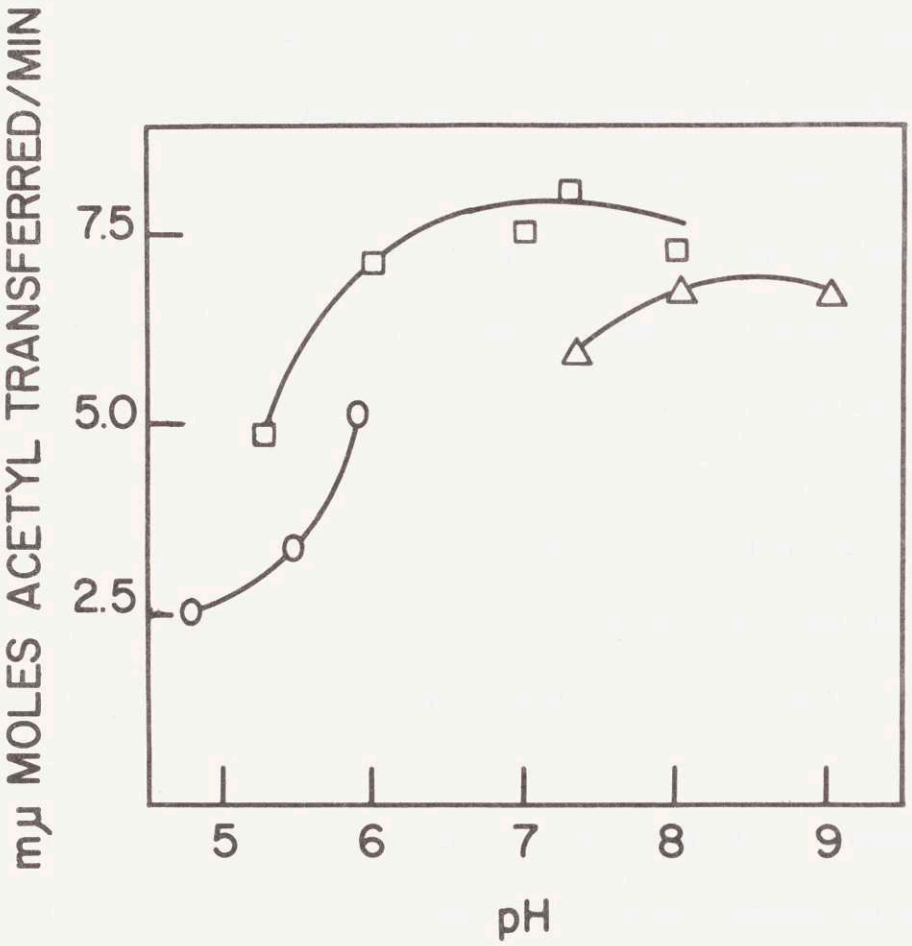

mμ MOLES ACETYL TRANSFERRED/MIN
0.02
0.01
0.01
0.02
0.03
0.04
0.05
mg PROTEIN

Figure 10. Effect of pH on 0-10 Transacetylase Activity

Each incubation mixture contained 13.8 mμmoles of β-hexasaccharide flavazole; 16 μg of A_1 particles; 52 mμmoles of ^{14}C-acetyl CoA and 5 μmoles of the appropriate buffer in a total volume of 0.1 ml. Initial rates were determined by the procedure described in the standard charcoal assay. □—□, phosphate buffer; Δ—Δ, Tris buffer; o—o, acetate buffer.


mμ MOLES ACETYL TRANSFERRED/MIN
7.5
5.0
2.5
5
6
7
8
9
pH

3. Substrate Specificity of O-10 Transacetylase

The transfer of acetyl groups by O-10 transacetylase to low molecular weight exogenous substrates, in addition to the transfer to high molecular weight LPS, suggested that it might be possible to define the substrate specificity of the enzyme. The specificities of the methylating enzymes that modify nucleic acids and proteins are unknown. Proteins and nucleic acids differ from the heteropolysaccharide chains of LPS in that neither is composed of identical small repeating units. However, they both contain highly ordered structural features that depend on non-covalent bonds in addition to the structure determined by covalent linkages. The enzymes responsible for the modification of these polymers may have specificity for structures determined by both covalent and non-covalent bonds. The presence of ordered non-covalent bonded structures has not been demonstrated in polysaccharides. It is thus not unreasonable to expect that enzymes that modify polysaccharides will also recognize low molecular weight compounds.

The standard O-10 transacetylase assay substrate, β-hexasaccharide flavazole, was prepared from the products resulting from partial acid hydrolysis of LPS. Because of the lability of the L-rhamnosyl-D-galactose bond, the acid treatment produces

a series of oligosaccharides consisting of D-galactosyl-D-mannosyl-L-rhamnose repeating units, which carry L-rhamnose on the reducing end and D-galactose on the non-reducing end. The two D-galactosyl residues in the hexasaccharide flavazole thus occupy the non-reducing terminal position and a site within the oligosaccharide.

The activity of various flavazoles, obtained by sequential degradation of the β-hexasaccharide flavazole as described in Materials and Methods, was tested in order to determine the relative contribution for acetyl-accepting activity of the other glycosyl residues in the chain. The penta- and tetrasaccharide derivatives, which both lack the terminal non-reducing D-galactosyl residue, were found to be active. The trisaccharide flavazole, which contains D-galactose only as a non-reducing terminus, was inactive as an acetyl-acceptor. It thus appeared that the presence of an L-rhamnosyl unit linked to D-galactose was necessary for acetyl-accepting activity. Possible differences in the specificity of the active flavazoles were also sought. The values of K_m and V_{max} were obtained from Lineweaver-Burk plots (Figure 11 and Table XII). The values obtained for the beta flavazole derivatives suggested that only one galactosyl residue of the β-hexasaccharide flavazole was acetylated under

Figure 11. Effect of β-Hexasaccharide Flavazole Concentration on 0-10 Transacetylase Activity

Each incubation mixture contained 18 μg of A_1 particles; 52 mμmoles of ^{14}C-acetyl-CoA; 2.5 μmoles of phosphate buffer, pH 7.4, and the indicated amount of the flavazole derivative in a total volume of 0.1 ml. Enzymatic activity was determined by the method described in the standard charcoal assay procedure.

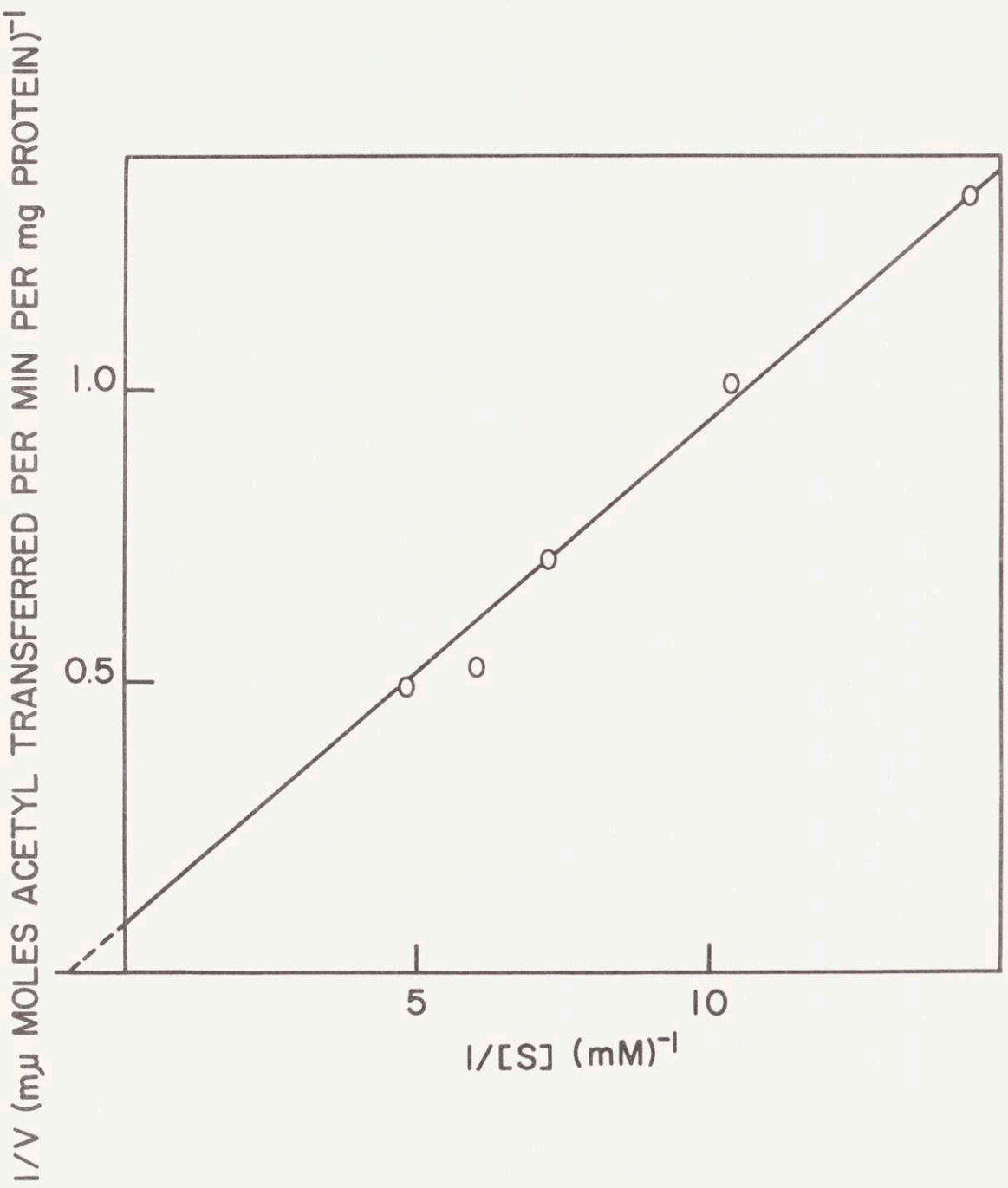

1/V (mμ MOLES ACETYL TRANSFERRED PER MIN PER mg PROTEIN)⁻¹
1.0
0.5
5
10
1/[S] (mM)⁻¹

TABLE XII

K_m and V_{max} Values of Flavazole Derivatives with 0-10 Transacetylase from A_1 and $A_1(\epsilon^{15a})$

Substrate (flavazole derivative)	A_1 particles		$A_1(\epsilon^{15a})$ particles	
	K_m	V_{max}	K_m	V_{max}
α-Hexasaccharide	7.7×10^{-3} M	25	4.0×10^{-3} M	9.1
β- " "	1.0 "	12	0.62 "	7.1
β-Penta "	0.93 "	15	0.49 "	7.2
β-Tetra "	1.2 "	13	-	-

These values were calculated from Lineweaver-Burk plots (see Figure 11). The V_{max} values are expressed as mμmoles of acetyl transferred per min per mg protein.

the assay conditions. The terminal non-reducing galactosyl residue was assumed to be inactive since the trisaccharide flavazole was inactive. The K_m and V_{max} values indicated that although the enzyme catalyzed the acetylation of both the alpha and beta linked galactosyl residues, some differences might exist. The constants obtained with the alpha derivative were higher, but these differences might have been caused by experimental error.

The inability of the trisaccharide flavazole to act as a substrate could be explained on the assumption that the acetyl group was transferred to the L-rhamnosyl residue. The evidence that the acetyl group is attached to galactose does not exclude other possibilities and will be discussed elsewhere in this thesis. In order to examine this point, a number of sugars and sugar derivatives were tested as substrates for O-10 transacetylase (Table XIII). Of the three monosaccharides found in the repeating unit of the smooth O-antigen, only D-galactose was acetylated by the enzyme. Thus, if O-10 transacetylase catalyzes the acetylation of the O-antigen, the acetyl group is probably linked to the D-galactosyl residue.

TABLE XIII

Acetyl-Accepting Activity of Sugar Derivatives

Substrate	Conc. (mM)	Assay[a]	Activity
D-Galactose	10	1	+
D-Mannose	10	1	-
L-Rhamnose	10	1	-
L-Rhamnosyl-D-galactose	8.5	1	+
α-D-Mannosyl-(1→4)-L-rhamnose	7.4	1	-
β-D-Galactosyl-(1→6)-D-mannose	4.6	1	+
Methyl β-D-galactoside	20	2	+
D-Glucosyl-(1→4)-D-galactose	4	2	-
D-Glucose	20	2	-
Methyl α-D-glucoside	40	2	-
Lactose	200	2	-
Melibiose	400	2	+
6-O-Methyl-D-galactose	10	3	-
D-Fucose	10	3	-
Isopropyl β-D-thiogalactoside	10	3	+

TABLE XIII (cont'd)

[a] Assay 1: The reaction mixture was identical to that described in Figure 11, except the flavazole derivative was replaced by the compound to be assayed. The reaction mixture was incubated at 37° for 16 hours; a 0.1 ml aliquot of 50 per cent ethanol was then added and the mixture was passed through a Dowex-AG-1 (acetate) column. The effluent was concentrated and then applied to Whatman No. 1 chromatography paper, which was developed in solvent 1. The distribution of radioactivity was determined by radioautography.

Assay 2: The reaction mixture was identical to that used in the standard quantitative paper assay, except the flavazole derivative was replaced by the compound to be assayed. The reaction mixture was incubated for 16 hours at 37° and then treated as described in assay 1, except solvent 3 was used for chromatography and the radioactivity was determined with a strip scanner.

Assay 3: The procedure was the same as described in assay 1, except the A_1 particles were replaced by 50 µl of EDTA-treated cells, solvent 3 was used for chromatography and radioactivity was detected with a strip scanner. The EDTA-treated cells transferred acetyl groups to the β-hexasaccharide flavazole under the assay conditions described.

All of the derivatives tested that contained a non-reducing terminal galactosyl residue, with the exception of lactose, were acetyl acceptors (Table XIII). The 1→3 linked disaccharide L-rhamnosyl-(1→3)-D-galactose was the only active derivative that carried a substituent linked to D-galactose at other than the reducing position. Modifications of D-galactose such as the loss of oxygen at position six (D-fucose) or epimerization of the hydroxyl at position four (D-glucose) resulted in the loss of acetyl-accepting activity. In cases where activity was found, a control with a particle preparation from $A_1(\epsilon^{15})$ cells was inactive, which eliminated the possibility of an artifact caused by non-enzymatic transfer of acetyl groups. The acetyl-accepting activity of isopropyl β-D-thiogalactoside, a known substrate of the enzyme thiogalactoside transacetylase, raised the possibility of a relationship between the two enzymes. However, thiogalactoside transacetylase prepared from E. coli K 12 was inactive with the flavazole derivatives. A close similarity between the enzymes is thus unlikely.

The relative importance of various structural features in active substrates was examined by comparisons of maximal velocities (V_{max}) and Michaelis constants (K_m). The substrates tested were divided into two classes according to enzymatic activity

(Figure 12). The first class contains β-hexasaccharide flavazole and L-rhamnosyl-D-galactose, which are the more active substrates. The feature common to these two compounds is the L-rhamnosyl-D-galactose structure. The second class, which is much less active, contains free D-galactose and derivatives that contain non-reducing terminal D-galactose. The small differences between members of the same class should be considered as tentative, however, since control experiments showed that excess acetyl-CoA may not have been present (Figures 12 and 13). The trisaccharide flavazole would be expected to be a member of this less active class of substrates. The failure to detect activity, however, probably resulted from its low solubility in aqueous solutions. Some of the details of the differences within the two substrate classes described above were investigated in the presence of 1.57 mM acetyl-CoA, a concentration that saturates the enzyme (Figures 13 and 14). The effect of the galactosyl linkage in the hexasaccharide flavazole is shown in Figure 14a. The V_{max} of the α-D-galactosyl derivative is higher than that of the beta isomer. The effect of the galactosyl linkage is also seen with the substrates of the poorer class (Figure 14b). The compound with an alpha linkage again gave the higher V_{max}. However, the K_m of methyl β-D-galactoside is higher than that of melibiose, which contains an α-D-galactosyl

Figure 12. Effect of Substrate Structure on 0-10 Transacetylase Activity

Each reaction mixture contained 2.5 μmoles of phosphate buffer, pH 7.4; 90 mμmoles of ^{14}C-acetyl-CoA; 18 μg of L-8 particles, which lack endogenous acetyl acceptors, and the appropriate substrate in a total volume of 0.1 ml. Enzymatic activity was determined by the standard quantitative paper assay procedure. The points represented by solid spots are from duplicate tubes containing twice the standard concentration of acetyl-CoA. ▽—▽, methyl β-D-galactoside; △—△, β-D-galactosyl-(1→6)-D-mannose; O—O, D-galactose; ◇—◇, melibiose; □—□, L-rhamnosyl-(1→3)-D-galactose; O---O, β-hexasaccharide flavazole (results for the flavazole derivative are from a separate experiment).

mμ MOLES ACETYL TRANSFERRED PER MIN PER mg PROTEIN
6
4
2
2.5
5.0
7.5
10.0
ACETYL ACCEPTOR CONCENTRATION (mM)

Figure 13. Effect of Acetyl-CoA Concentration on 0-10 Transacetylase Activity

Each reaction mixture contained 2.5 μmoles of phosphate buffer, pH 7.4; 21 μg of L-8 particles; 41 mμmoles of β-hexasaccharide flavazole and acetyl-CoA in a final volume of 0.1 ml. Enzymatic activity was determined as described in Figure 12.

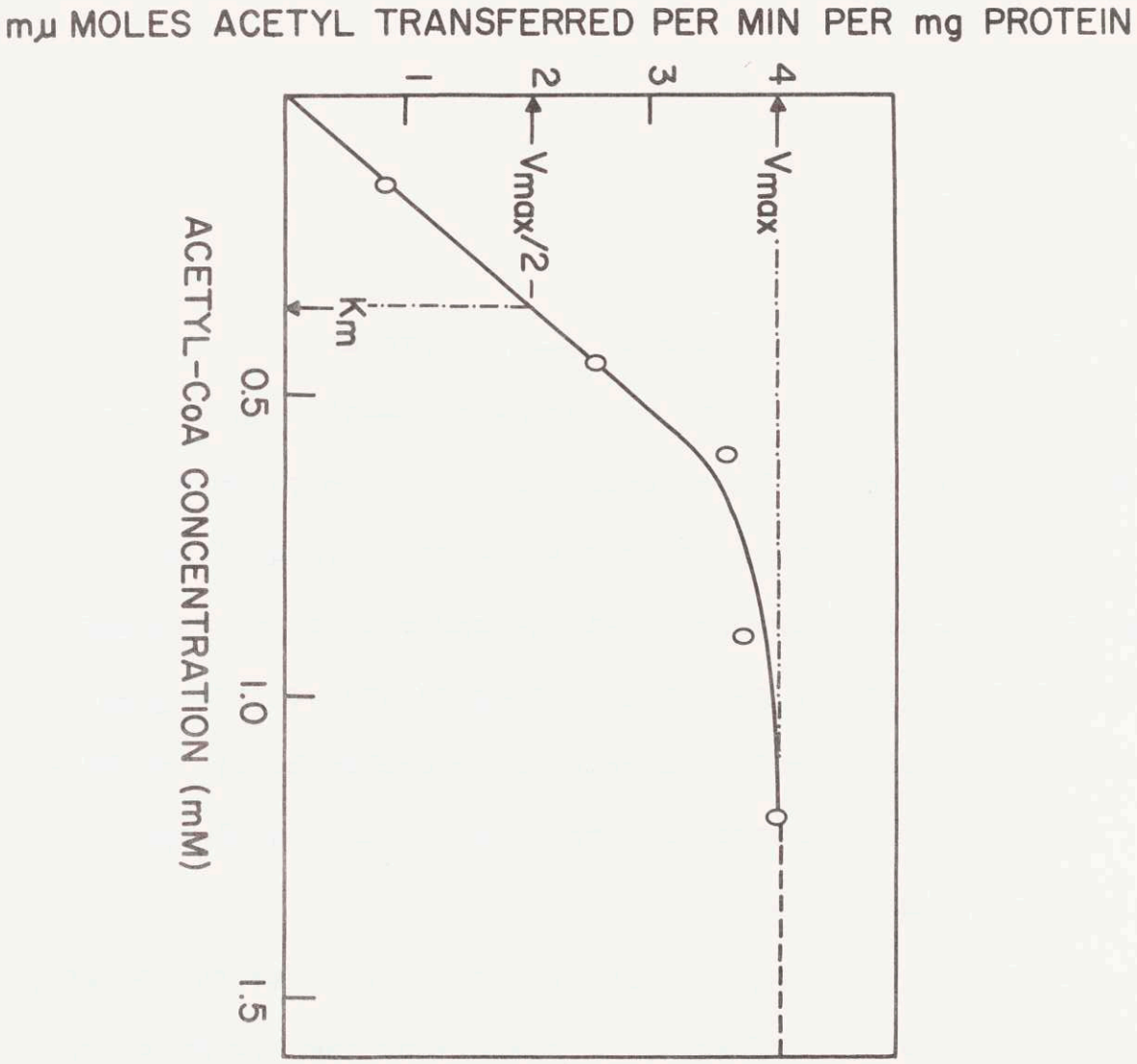
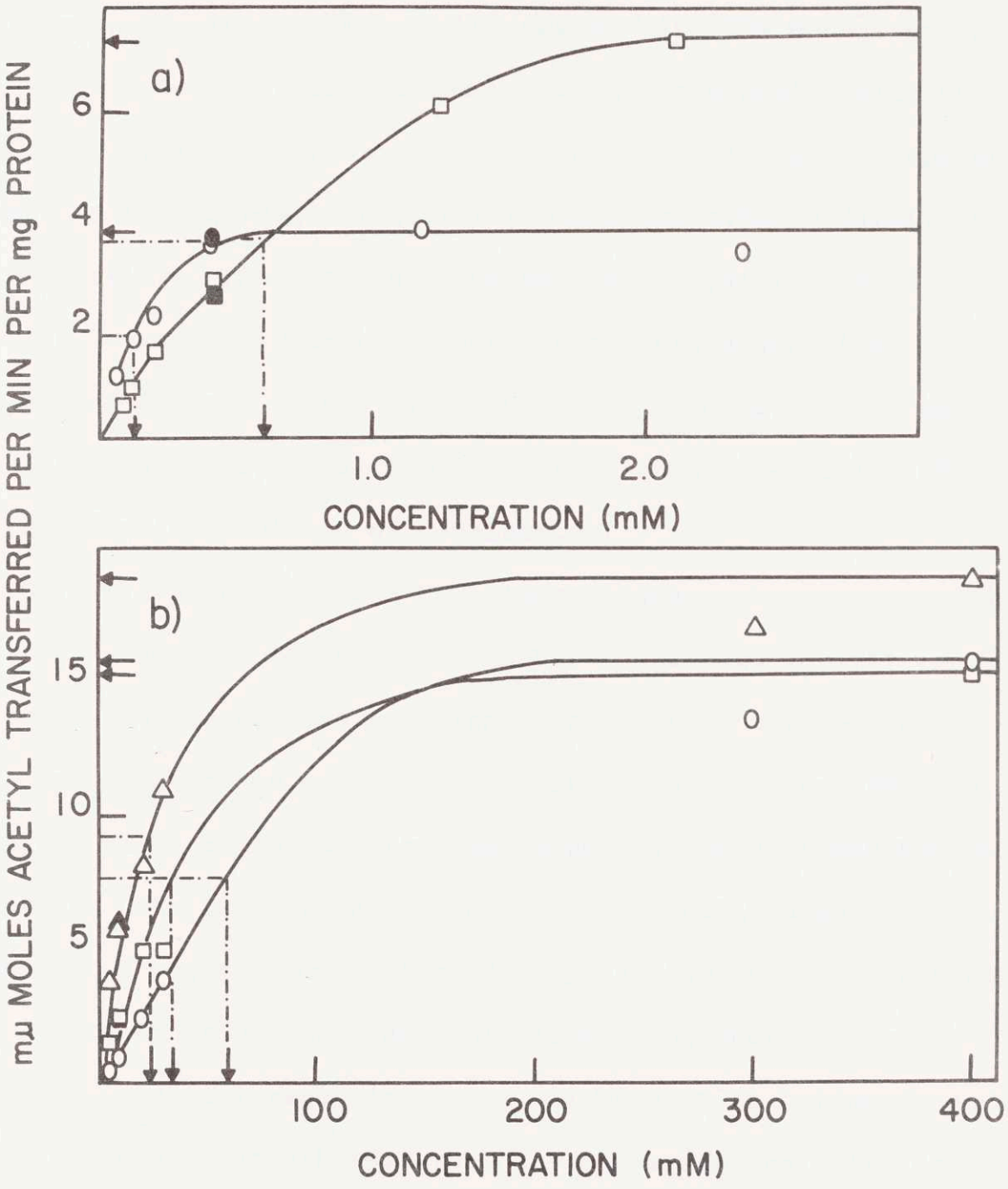

mμ MOLES ACETYL TRANSFERRED PER MIN PER mg PROTEIN
1
2
3
4
Vmax
Vmax/2
Km
0.5
1.0
1.5
ACETYL-CoA CONCENTRATION (mM)

Figure 14. Effect of the Galactosyl Linkage on O-10 Transacetylase Activity

The concentration of acetyl-CoA used in both sets of experiments was 1.57 mM. The solid spots represent controls as described in Figure 12.

a. Enzymatic activity was determined by the standard charcoal assay procedure, with enzyme particles prepared from the mutant L-8 as described in Figure 13. □—□, α-hexasaccharide flavazole; O—O, β-hexasaccharide flavazole.

b. Enzymatic activity was determined by the standard quantitative paper assay procedure, with enzyme particles prepared from the mutant L-8. O—O, methyl β-D-galactoside; □—□, D-galactose; Δ—Δ, melibiose.


a)
6
4
2
1.0
2.0
CONCENTRATION (mM)
b)
15
10
5
100
200
300
400
CONCENTRATION (mM)
mμ MOLES ACETYL TRANSFERRED PER MIN PER mg PROTEIN

linkage. This difference is presumably the result of the difference in the interaction of the enzyme with the methyl group of methyl β-D-galactoside compared to the interaction with the D-glucosyl unit in melibiose. Thus, although the enzyme appears to have a strong requirement for the L-rhamnosyl residue linked to D-galactose, the residue to which D-galactose is linked can be altered with only small changes in substrate activity. The failure of lactose to act as a substrate, however, indicates that there are structural limitations on substituents linked to the reducing position of D-galactose.

4. O-10 Transacetylase and LPS Biosynthesis

The study of LPS biosynthesis has progressed rapidly in the past few years. The reactions leading to the formation of the smooth O-antigen portion of the LPS have been demonstrated, although the details have remained obscure. Each trisaccharide repeating unit is first assembled on a phospholipid by the transfer of sugars from nucleoside diphosphate sugar derivatives. These trisaccharide units are then linked together to form a chain of identical repeating units, which also is believed to be bound to a phospholipid. The polysaccharide chain is then enzymatically transferred to the core of the LPS molecule, which is synthesized independently. At some stage in this process,

the enzyme O-10 transacetylase transfers an acetyl group from acetyl-CoA to the galactosyl residues of the smooth O-antigen. Results already presented have demonstrated that particle preparations that are presumably not synthesizing LPS are able to transfer acetyl groups to it. The experiments now to be described represent an attempt to determine the stage during normal O-antigen biosynthesis at which acetyl groups are added.

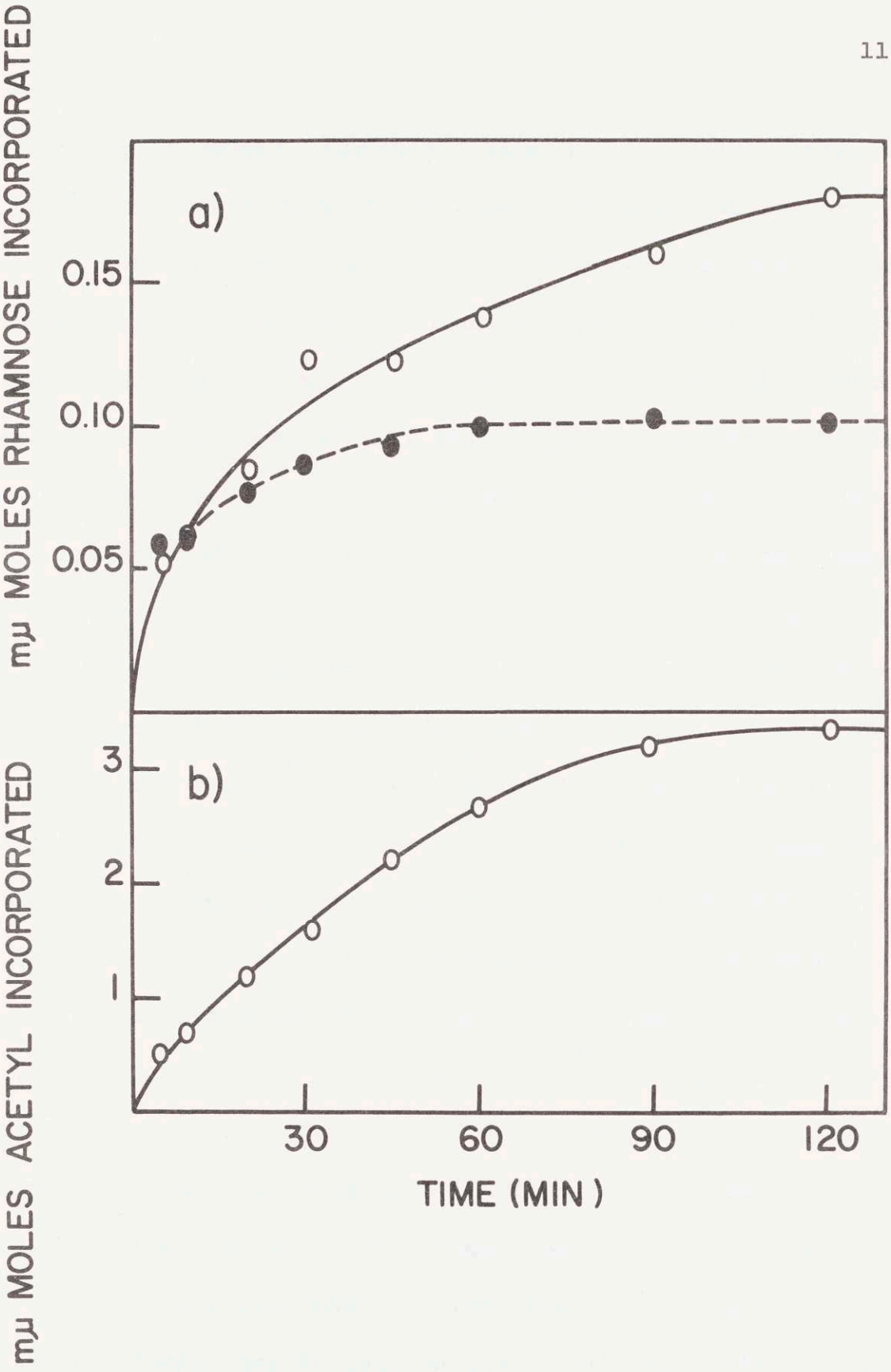
Results have indicated that the in vivo pool of intermediates in smooth O-antigen biosynthesis is small (53). Under appropriate conditions, however, EDTA-treated cells have been found to accumulate mono- and disaccharide-phospholipids (53). The O-10 transacetylase particle preparations accumulate L-rhamnosyl-D-galactose phospholipid under similar conditions. In the presence of saturating quantities of UDPGal and TDPRha, the formation of disaccharide-phospholipid, which is measured as butanol-extractable radioactivity, reaches a maximum after about two hours (Figure 15a). The maximal incorporation of acetyl groups by particles also is reached after two hours (Figure 15b). If disaccharide-phospholipid were a substrate for O-10 transacetylase, these conditions should be optimal for the synthesis of the acetylated derivative. The following experiment was carried out to determine whether O-10 transacetylase could acetylate this

Figure 15. Time Course of L-Rhamnose and Acetyl Incorporation by a Particle Preparation

a. A reaction mixture containing 50 μmoles of phosphate buffer, pH 7.4; 49 mμmoles of ^{14}C-TDPRha; 5 μmoles of UDPG; 0.1 ml of a crude UDPGal-4-epimerase preparation and 40 μmoles of magnesium chloride in a total volume of 1.9 ml was incubated for 20 min at 30°. A 0.1 ml aliquot of 0-10 transacetylase particles (11.9 mg/ml) prepared from A_1 cells was then added, and the incubation was continued at 30°. At the times indicated, 0.1 ml aliquots were removed from the reaction mixture and added to either 0.1 ml of 2 M acetic acid or 0.5 ml of 50 per cent (v/v) aqueous n-butanol. The particles in the former samples were collected on membrane filters, and radioactive rhamnose was determined in a gas flow counter. The butanol extracts were washed with 0.2 ml of water, placed onto ringed planchets and evaporated to dryness. The radioactive rhamnose was determined in a gas flow counter. O—O, total rhamnose incorporated; ●--●, rhamnose incorporated into butanol extractable material.

Figure 15 (cont'd)

b. A 1 ml reaction mixture containing 25 µmoles of phosphate buffer, pH 7.4, 1.35 µmoles of ^{14}C-acetyl-CoA and 0.60 mg of 0-10 transacetylase particles prepared from A_1 cells was incubated at 30°. Aliquots (0.1 ml) were removed at the indicated times and added to 0.1 ml of neutral 2 M hydroxylamine. Particles were collected on membrane filters and the radioactive acetyl was determined in a gas flow counter.


mμ MOLES RHAMNOSE INCORPORATED
a)
0.15
0.10
0.05
mμ MOLES ACETYL INCORPORATED
b)
3
2
1
30
60
90
120
TIME (MIN)

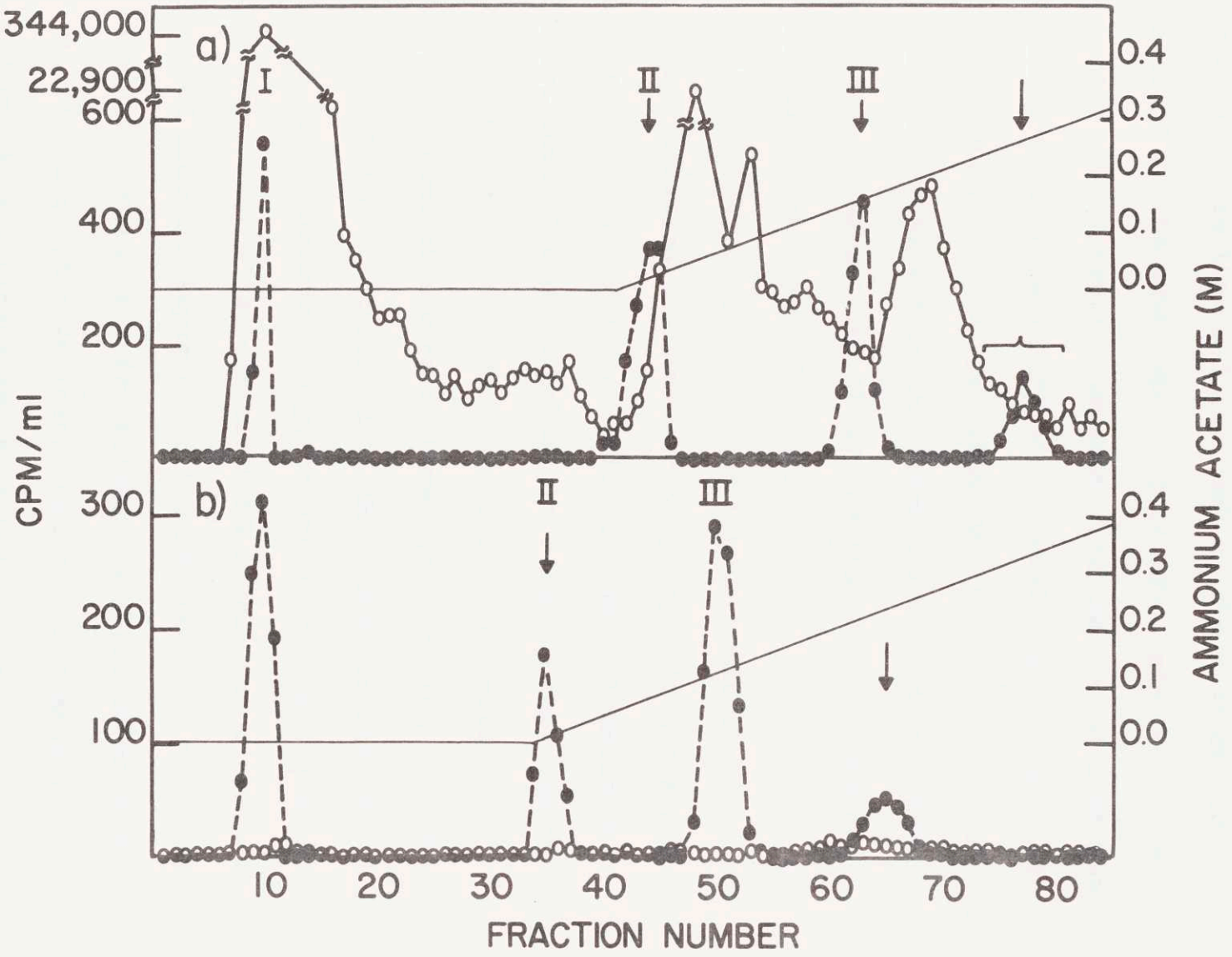
intermediate of smooth O-antigen biosynthesis. O-10 Transacetylase particles were incubated for 2 hours in the presence of UDPGal, ^{14}C-TDPRha and ^{3}H-acetyl-CoA. The reaction was stopped by the addition of n-butanol, which extracts the disaccharide-phospholipid. The butanol extract was washed three times with water and then was applied to a DEAE-cellulose column (acetate form) prepared in 99 per cent methanol. The column was eluted with a linear gradient of ammonium acetate in 99 per cent methanol (Figure 16a). The components in peak I were not analyzed. The ^{14}C-labeled material in peaks II and III were markers of phosphatidyl glycerol and cardiolipin, respectively. The radioactive material in the small ^{14}C-peak that follows peak III is disaccharide-phospholipid. Since the column separates compounds primarily by charge and since the mono- and disaccharide-phospholipids run at about the same position on this type of column, the acetylated disaccharide-phospholipid was assumed to elute at the same position as the unacetylated compound. Fractions 74 to 80 were pooled, concentrated and re-extracted with butanol. The washed butanol extracts were fractionated on a second DEAE-cellulose column in exactly the same fashion (Figure 16b). The results of calculations based on the specific activities of the radioactive TDPRha and acetyl-CoA used in the incubation mixture

Figure 16. Purification of Disaccharide-Phospholipid on DEAE-Cellulose Columns

The reaction mixture and the butanol extraction procedure are described in the text. In the procedure used to determine radioactivity, the activity of ^{3}H-acetyl was 251,000 cpm per mμmole and that of ^{14}C-rhamnose was 3830 cpm per mμmole. O—O, ^{3}H-acetyl; ●—●, ^{14}C-rhamnose and ^{14}C-labeled standards as described in the text; _____, approximate molarity of the ammonium acetate gradient.

a. Fractionation of the butanol soluble material extracted from the incubation mixture.

b. Fractionation of the material in fractions 74 through 80 from the column described above (a).


344,000
22,900
600
400
200
300
200
100
a)
b)
I
II
III
II
III
0.4
0.3
0.2
0.1
0.0
0.4
0.3
0.2
0.1
0.0
CPM/ml
AMMONIUM ACETATE (M)
10
20
30
40
50
60
70
80
FRACTION NUMBER

indicate that less than 0.5 per cent of the disaccharide-phospholipid in the peak tube (Figure 16b, fraction 65) was acetylated. These results suggest that the acetylation reaction occurs at a stage in LPS biosynthesis later than disaccharide-phospholipid. Since UDPGal was present in the experiment just described and is the precursor of the galactose in disaccharide-phospholipid, these results also eliminate acetylation at the level of UDPGal.

The reactions involved in the biosynthesis of LPS that follow the formation of disaccharide-phospholipid are less well characterized. There is evidence that polymerization of the trisaccharide units takes place before the transfer of chains to the heptose containing core. Rough mutants (R_I mutants) of Salmonella strains have been isolated that do not have the sugars characteristic of the O-antigen side chain in their LPS (46). However, polysaccharides containing the O-antigen immunological specificity and sugar composition can be isolated from the supernatant liquid after the purification of LPS by high-speed centrifugation. This polysaccharide has been called the soluble antigen. Whether this material is an intermediate in the biosynthesis of LPS or merely a side product has not been determined.

An analysis of the mutant L-14, obtained by treatment of S. anatum A_1 with ethyl methanesulfonate, suggested that this strain contained a lesion that prevented transfer of polymerized O-antigen to the core as in R_I mutants. Liquid cultures of L-14 spontaneously undergo the autoagglutination characteristic of strains that have no smooth O-antigen in LPS. In addition, the mutant is completely resistant to ϵ^{15} vir, which requires the smooth O-antigen for attachment to the bacterium, but is readily lysed by the phage Felix-O, which does not lyse the parent A_1 strain (Mr. D. Bray, unpublished observations). The sugars present after complete acid hydrolysis of purified L-14 LPS were analyzed by paper chromatography in solvent 1; glucose and galactose were found, but no mannose or rhamnose. Rhamnose was present, however, in the supernatant liquid remaining after the 100,000 x g centrifugation in the final step of LPS purification. The incorporation of galactose and rhamnose from their nucleotide precursors by EDTA-treated L-14 cells was similar to that reported for EDTA-treated S. anatum A_1 cells (53), when measured under the same conditions. Thus, at least some of the enzymes involved in O-antigen synthesis are present in L-14. Of particular interest, however, was the presence of endogenous acetyl-incorporating activity in L-14 particle prepara-

tions. This observation indicates that the membrane-bound form of soluble antigen is an active acceptor of acetyl groups. Since this membrane-bound antigen may be a normal precursor of the LPS-bound smooth O-antigen, it is possible that acetylation takes place at this stage of biosynthesis.

B. Structural Studies on the O-10 Transacetylase Substrate and Product

The importance of the L-rhamnosyl-D-galactose structure in the substrate specificity of O-10 transacetylase was suggested by the results of assays with various flavazole derivatives. In order to establish this specificity, it was of interest to isolate the disaccharide L-rhamnosyl-D-galactose. The lability of the L-rhamnosyl-D-galactose bond in mineral acids has already been discussed. However, disaccharides containing non-reducing 6-deoxyhexoses have been isolated from deoxyhexose-containing polysaccharides after acetolysis (63). Some of the products obtained by acetolysis of LPS have been isolated and are described below.

1. The Isolation and Characterization of L-Rhamnosyl-D-Galactose

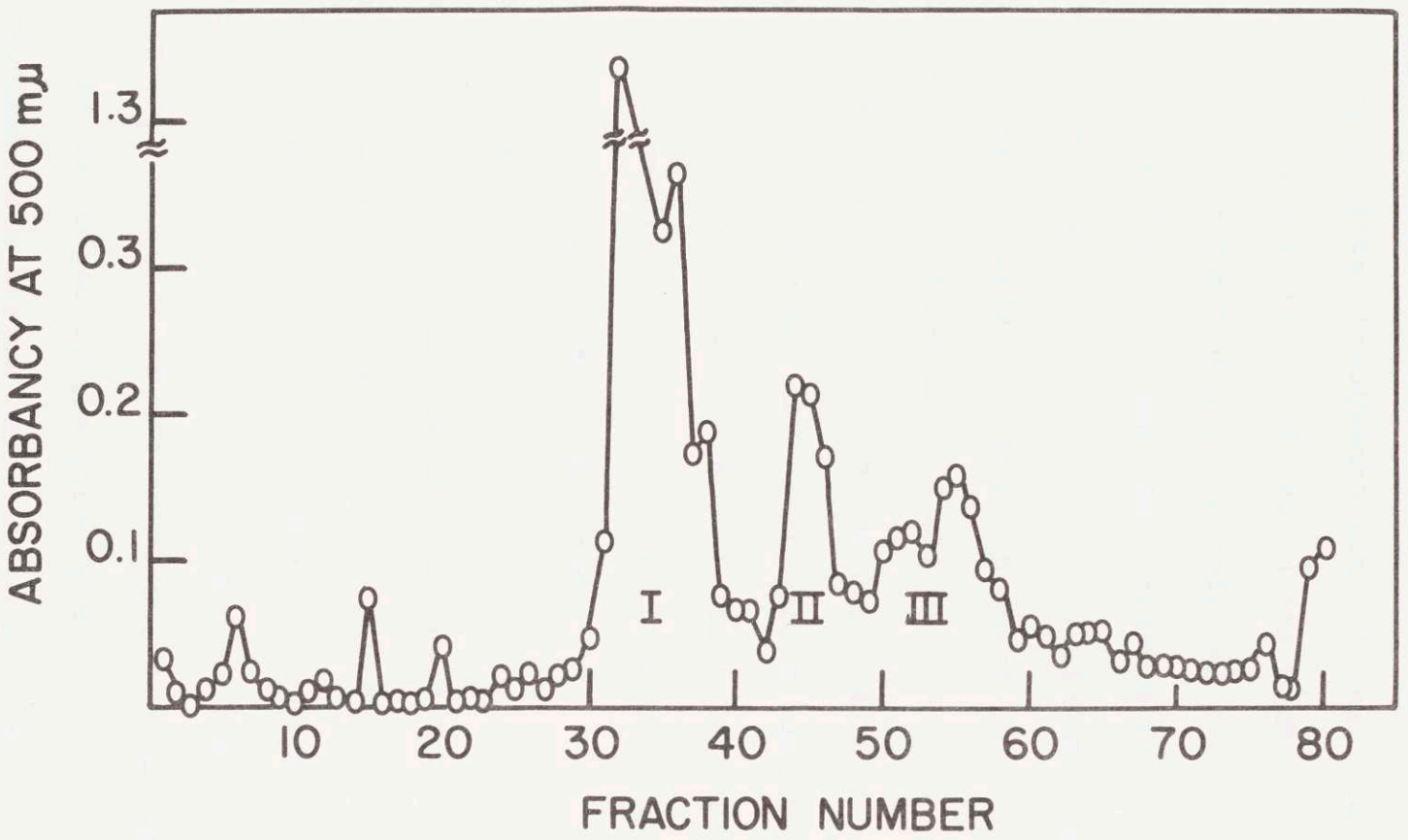
The cleavage of 6-deoxyhexose-containing polysaccharides by acetolysis has been shown by Aspinall to preserve the acid-labile glycosidic bond of the deoxyhexose (63). The deacetylated

products obtained from acetolysis of S. newington LPS were fractionated on a charcoal-Celite column with an ethanol gradient (Figure 17). The fractions were analyzed by paper chromatography before and after complete acid hydrolysis. Peak I was found to contain only free monosaccharides. Peak III was chromatographically identical to α-D-galactosyl-(1→6)-D-mannose and yielded mainly D-galactose and D-mannose after acid hydrolysis. Peak II was identical to α-D-mannosyl-(1→4)-L-rhamnose by paper chromatography, but after acid hydrolysis D-galactose, L-rhamnose and D-mannose were found. The unhydrolyzed material yielded two components after borate electrophoresis. The slower component had a mobility identical to that of α-D-mannosyl-(1→4)-L-rhamnose. The faster one yielded only L-rhamnose and D-galactose, in a ratio of 0.99:1.10, after complete acid hydrolysis. Since D-galactose but no L-rhamnose was reduced by sodium borohydride, the compound must be the disaccharide L-rhamnosyl-D-galactose.

The linkage position in the disaccharide was determined by Smith degradation of the methyl glycoside of the disaccharide, which was prepared from the disaccharide-phospholipid. The identity of the disaccharide L-rhamnosyl-D-galactose linked to the phospholipid with the disaccharide prepared by acetolysis

Figure 17. Column Fractionation of the Deacetylated Products Obtained from Acetolysis of S. newington LPS

The preparation of the column and the gradient elution are described in Materials and Methods.


ABSORBANCY AT 500 mμ
1.3
0.3
0.2
0.1
I
II
III
10
20
30
40
50
60
70
80
FRACTION NUMBER

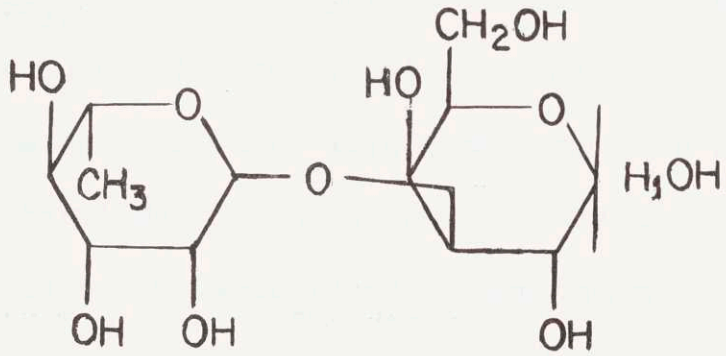
has been demonstrated (53). The single product isolated by paper chromatography after treatments with sodium metaperiodate, sodium borohydride and dilute acid was in the methyl D-galactoside region of the chromatogram. Degradation of the product with α-or β-galactosidase confirmed the presence of a mixture of methyl α- and β-D-galactosides. The only linkage position in the original disaccharide that would produce these results is L-rhamnosyl-(1→3)-D-galactose. In all other cases, periodate oxidation would have destroyed the galactosyl residue. This procedure was used to identify the disaccharide isolated from both the E_1 and E_2 group organisms as L-rhamnosyl-(1→3)-D-galactose. The anomeric configuration of the L-rhamnosyl-D-galactose bond has not yet been determined.

2. Position of the Acetyl Group

The major difficulties in the assignment of acetyl group position in polyhydroxylated compounds are migration of the acyl groups to neighboring hydroxyl residues and hydrolysis. The acetyl groups that partially determine O-antigen 10 are located on the D-galactosyl residues of the heteropolysaccharide side chains in the LPS as judged by the substrate specificity of O-10 transacetylase, the enzyme believed to be responsible for acetylation. These D-galactosyl residues occur as pyranosides,

carry L-rhamnosyl residues linked to position 3 and are glycosidically linked to mannosyl residues. As a result, the hydroxyl groups on carbons 1, 3 and 5 are blocked in the galactosyl residues of the polysaccharide. Thus, the acetyl residue is linked to one of the three remaining hydroxyl groups, which are present on carbons 2, 4 and 6. The standard methods used to determine substituent positions in polysaccharides cannot be applied because of the lability of the acetyl group and the absence of any adjacent hydroxyl groups. The isolation of the disaccharide L-rhamnosyl-D-galactose and its ability to serve as a substrate for O-10 transacetylase has made it possible to accumulate some evidence concerning the position of the acetyl group.

The structure of the disaccharide L-rhamnosyl-(1→3)-D-galactose is presented below.



No single procedure could be devised that would distinguish between the three possible acetylated products; therefore, a combination of two was selected. The first of these involved a study of the products released by periodate oxidation of the reduced acetylated disaccharide. The disaccharide was enzymatically acetylated at pH 6.8, isolated by passage of the reaction mixture through a column of Dowex-AG-1 (acetate form, previously washed until neutral) and purified on a charcoal-Celite column with an ethanol-water gradient. Samples of the acetylated or deacetylated disaccharide were concentrated to dryness and treated with a freshly prepared solution of sodium borohydride in methanol. After thirty minutes, the methanol was removed by evaporation under a stream of nitrogen. An excess of 0.05 M sodium acetate buffer, pH 4.5, was then added to neutralize the borate. The periodate oxidation was performed in 0.1 M sodium metaperiodate (final pH about 5.3). After one hour, the iodate and periodate were destroyed with sodium arsenite. The formaldehyde formed by the periodate oxidation was then measured by the acetylacetone-ammonia method. Any formaldehyde present could only have been formed by cleavage between carbons 1 and 2, and 5 and 6. Thus, the deacetylated disaccharide or the disaccharide acetylated on position 4 would

be expected to yield two moles of formaldehyde for each mole of starting material. If the acetyl group were located at position 2 or 6, however, one mole of acetylated disaccharide would yield only one mole of formaldehyde. The formaldehyde yields from four experiments agreed with the latter prediction (Table XIV). Therefore, the acetyl group must occupy either position 2 or 6 of the galactosyl residue, on the assumption that no migration has taken place.

The distinction between substitution on position 2 or 6 was made on the basis of periodate oxidation of the unreduced acetylated disaccharide. The analysis of the products was simplified by the availability of the disaccharide containing uniformly ^{14}C-labeled galactose. The periodate oxidation was performed in 0.1 M sodium metaperiodate buffered with 4 mM sodium acetate, pH 4.5, in a final volume of 0.125 ml. After one hour, the excess periodate was destroyed by the addition of ethylene glycol (18 μmoles). The oxidation products were reduced with sodium borohydride, subjected to complete acid hydrolysis and then analyzed by paper chromatography in solvents 1 and 2. Glycerol was the only radioactive product recovered from a methyl galactoside control. The product from the disaccharide formed a single radioactive spot, which was chromato-

TABLE XIV

Formaldehyde Formed by Periodate Oxidation of Acetylated and Deacetylated L-Rhamnosyl-(1→3)-D-Galactose

Expt. No.	Substrate	μmole formaldehyde			Per cent yield	
		Theoretical		Experimental		
		C_4 or unacetylated	C_2 or C_6		C_4 or unacetylated	C_2 or C_6
1	Rha-Gal	0.040	-	0.042	105	-
	O-Acetyl Rha-Gal	0.040	0.020	0.021	52	105
2	Rha-Gal	0.040	-	0.031	77	-
	O-Acetyl Rha-Gal	0.040	0.020	0.015	37	75
3	Rha-Gal	0.040	-	0.021	52	-
	O-Acetyl Rha-Gal	0.040	0.020	0.010	25	50
4	Rha-Gal	0.040	-	0.026	65	-
	O-Acetyl Rha-Gal	0.040	0.020	0.012	30	60

The procedures used are described in the text.

graphically identical to lyxitol. The galactose in the un-acetylated disaccharide should have been split between positions 1 and 2, if it had been oxidized in the pyranoside form, resulting in the formation of 5-O-formyl-D-lyxose. If it had been oxidized in the open-chain form, cleavage also would have occurred between positions 4 and 5, and 5 and 6, resulting in the appearance of glycerol after the reduction and hydrolysis. Since no glycerol was found, the oxidation must have occurred predominantly on the ring form. The compound acetylated at position 2 would be expected to be resistant to oxidation. It was found that oxidation of the acetylated disaccharide did occur and lyxitol was recovered after borohydride reduction. Thus, the acetyl group must have been on either position 4 or 6 of galactose. The elimination of position 2 in this experiment and the elimination of position 4 in the previous experiment leaves only position 6 as the site of the acetyl group.

C. Effect of Phage Conversion on the Synthesis of O-10 Transacetylase

The original objective of the work presented in this thesis was to elucidate the mechanism by which the phage ε^{15} causes the loss of O-acetyl groups from the LPS of the host bacterium. The enzyme responsible for the acetylation of LPS in the uninfected

cells has been described. Some experiments designed to elucidate the mechanism that controls enzyme activity will now be described.

The mutants of ϵ^{15} isolated by Uetake and Uchida have been shown to cause structural changes in the O-antigen (48). These changes, however, differ in some respects from those caused by ϵ^{15}. The presence of acetyl groups in the LPS of strains lysogenic for ϵ^{15a} and ϵ^{y} is of particular interest since it indicates that the phage can lose the ability to affect the acetylation reaction. The levels of O-10 transacetylase in strains lysogenic for the various phages were measured to determine whether these structural variations were correlated with the presence or absence of O-10 transacetylase (Table XV). The strains lysogenic for either ϵ^{15a} or ϵ^{y} contain O-10 transacetylase. The variations between levels of the enzyme are probably caused by the differences in the amount of endogenous acceptor present, which competes with the exogenous assay substrate. As expected, strains lysogenic for ϵ^{15} or ϵ^{15b} contained no O-10 transacetylase. The loss of enzyme activity is thus caused by a phage determined function that can be lost through mutation.

The changes in the level of O-10 transacetylase during infection were examined. Growing cultures of S. anatum A_1 were

TABLE XV

Levels of 0-10 Transacetylase, LPS and LPS-Bound O-Acetyl Found in S. anatum A_1 and Lysogenic Derivatives of S. anatum A_1

Strain	0-10 Transacetylase Specific Activity	LPS[c] (moles rha/ mole heptose)	O-Acetyl[c] (mole/ mole rha)
A_1	1.01[a]	5.7	0.54
$A_1(\varepsilon^{15})$	<0.05[a]	6.4	<0.10
$A_1(\varepsilon^{34})$	1.27[b]		
$A_1(\varepsilon^{15}\varepsilon^{34})$	<0.05[b]		
$A_1(\varepsilon^{15a})$	0.71[a]	6.0	0.71
$A_1(\varepsilon^{15b})$	<0.05[a]	1.2	<0.10
$A_1(\varepsilon^{y})$	1.60[a]	0.90	0.40

[a] Each reaction mixture contained about 60 µg of particles; 13.2 mµmoles of β-hexasaccharide flavazole; 52 mµmoles of ^{14}C-acetyl-CoA and 2.5 µmoles of phosphate buffer, pH 7.4, in a total volume of 0.1 ml, and was incubated at 30°. At intervals of 4.5 min, a 25 µl aliquot was added to 0.1 ml of neutral 2 M hydroxylamine. After 5 min, 0.1 ml of 2 M acetic acid was added. The flavazole was adsorbed to 250 µg of acid washed Norite A, which was collected on filters and analyzed as described in the standard charcoal assay procedure.

[b] The assay procedure was identical to that described in Figure 9.

[c] From reference 48.

infected with various phages at a multiplicity of about 6, which would be expected to cause infection of essentially all the bacteria in the culture. Samples of the cultures were poured over ice at 45 and 170 minutes after infection. Particles were prepared in the normal fashion and assayed for total enzyme activity with both the endogenous and exogenous acceptors (Table XVI). The amount of enzyme present 45 minutes after infection with either ϵ^{15} or ϵ^{15b} was considerably lower than in cells infected with ϵ^{15a} or the uninfected control. Since the assays were performed in the presence of equal amounts of acetyl-CoA, the difference in enzyme levels cannot be ascribed to differences in the <u>in vivo</u> levels of acetyl-CoA. The fact that the total amount of enzyme present in the cell does not change between 45 and 170 minutes after infection with ϵ^{15} argues against the appearance of an inhibitor of 0-10 transacetylase. But, it does not exclude an inhibitor that is synthesized and bound into the membrane with newly formed enzyme. A more plausible model would be that the phage represses the synthesis of 0-10 transacetylase by a mechanism similar to that postulated by Jacob and Monod for repression of the lactose operon in <u>E. coli</u> (81). The phage ϵ^{15a} would thus be a mutant that has lost the ability to repress enzyme

TABLE XVI

Levels of 0-10 Transacetylase During Early Stages of Phage Infection

Infecting phage	Endogenous acetyl incorporation[a]		Total enzyme units (charcoal assay[b])	
	45 min	170 min	45 min	170 min
Uninfected control	0.28	0.43	56	580
ϵ^{15}	0.26	0.06	26	23
ϵ^{15a}	0.35	0.33	53	400
ϵ^{15} vir	0.22	<0.02	23	<2

[a] A modified particle assay was used to determine these values. The standard incubation mixture was increased four-fold and 0.1 ml samples were added to 0.1 ml of neutral 2 M hydroxylamine at 5, 10 and 20 min after the reaction was started. All values presented above are expressed as mμmoles of acetyl transferred per min per mg of protein.

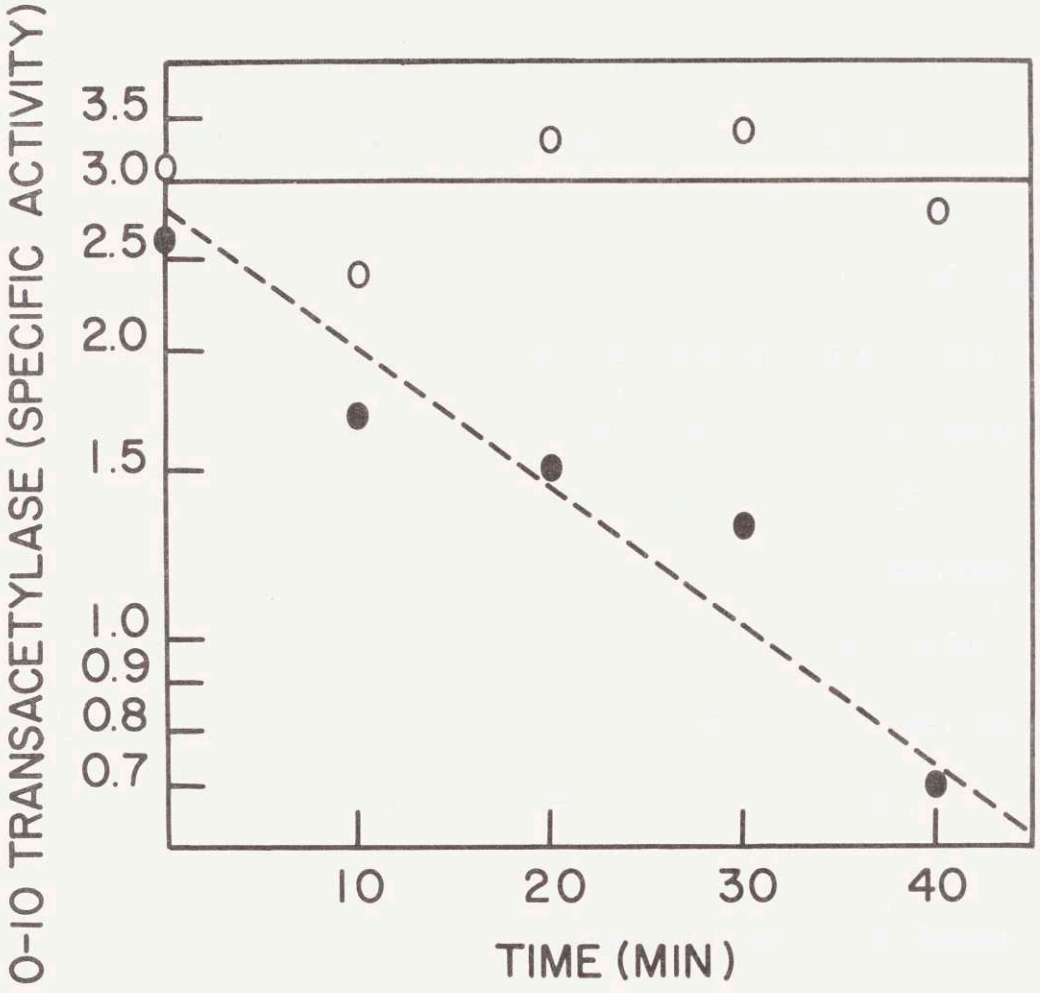
[b] The standard charcoal assay procedure was used except that reactions were stopped with neutral 2 M hydroxylamine as described above.

synthesis. The results of kinetic analysis of 0-10 transacetylase prepared from A_1 and $A_1(\epsilon^{15a})$ cells support the idea that the enzymes in the two strains are identical (Table XII).

An attempt was made to determine how fast the proposed repression of enzyme synthesis occurs. A growing culture of A_1 was infected with ϵ^{15} at a multiplicity of about 10. Particles were prepared from samples removed from the culture at 10-minute intervals (Figure 18). The results were expressed in terms of specific activities rather than total units because of material losses during enzyme preparations. The specific activity in the uninfected control remained essentially constant. The specific activity in the infected culture, however, dropped off exponentially from almost the time of infection. The effect of the phage on the synthesis of 0-10 transacetylase was thus quite rapid and probably occurred within 5 to 10 minutes after infection.

Figure 18. Effect of ϵ^{15} Infection on the Specific Activity of 0-10 Transacetylase

At the indicated time after infection 100 ml samples were removed from a 550 ml culture and poured over ice. Particles were prepared in the standard fashion and assayed as described in Figure 9.


O-10 TRANSACETYLASE (SPECIFIC ACTIVITY)
3.5
3.0
2.5
2.0
1.5
1.0
0.9
0.8
0.7
10
20
30
40
TIME (MIN)

DISCUSSION

The infection of Salmonella group E_1 strains by the phage ϵ^{15} is known to cause the disappearance of O-antigen 10. Chemical and immunological methods had previously been used to demonstrate that this antigen was partially determined by O-acetyl groups located in the LPS of the bacterial cell wall. Specifically, evidence was obtained suggesting that the O-acetyl group was attached to the 6 position of the D-galactosyl residue in the trisaccharide α-D-galactosyl-(1→6)-α-D-mannosyl-(1→4)-L-rhamnose. Since the smooth O-antigen was believed to contain more than two repeating units and since at least half of these trisaccharide units were acetylated, one could deduce that non-terminal acetylated galactosyl residues are present.

This thesis describes O-10 transacetylase, the enzyme responsible for the presence of the O-acetyl groups that form a major part of O-antigen 10. This enzyme appears to be localized in particulate material isolated from disrupted S. anatum A_1 cells. The presence of O-10 transacetylase in the cell wall-membrane particulate material is not surprising, since

the O-antigen is an extracellular macromolecule. Many enzymes involved in the biosynthesis of extracellular macromolecules have been found recently in the cell wall-membrane fraction of disrupted cells (43). Whether or not soluble O-10 transacetylase exists is an unresolved question. The failure to find this enzymatic activity in the cell cytoplasmic fraction could be the result of a rapid breakdown of the acetyl donor in the presence of soluble cell extracts.

The particles that contain O-10 transacetylase also contain acetyl acceptor. This endogenous acetyl acceptor was extracted from the particles with hot phenol and identified as LPS by the criteria of sedimentation during high-speed centrifugation and precipitation with anti-3, 10 serum. Partial elucidation of the LPS biosynthetic pathway has made it possible to test various intermediates in this pathway as acetyl acceptors. The intermediate L-rhamnosyl-(1→3)-D-galactose phospholipid was demonstrated to be an essentially inactive acetyl acceptor. Since the enzyme preparation catalyzed both the synthesis of the disaccharide-phospholipid and the transfer of acetyl groups, intermediates that are formed prior to disaccharide-phospholipid were also eliminated as acetyl acceptors. The presence of an endogenous acetyl acceptor in the rough mutant L-14 suggests that the membrane-bound form

of soluble antigen, which is believed to be present in L-14, is an active acetyl acceptor. Whether soluble antigen is an intermediate in LPS biosynthesis or a side product has not been established. Nevertheless, the results indicate that O-10 transacetylase catalyzes the transfer of acetyl groups to the polymerized trisaccharide repeating units either before or after the polysaccharide is transferred to the LPS core. The high charge carried by the acetyl donor, acetyl-CoA, makes it likely that the transfer occurs before movement into or across the membrane. The possibility that the acetyl group is first transferred to some type of lipophilic donor within the membrane, however, has not been excluded and is considered in more detail elsewhere in this discussion.

The results of substrate specificity studies demonstrate that the O-acetyl group is esterified to the D-galactosyl units in the smooth O-antigen. The enzyme will catalyze the acetylation of D-galactose, but not of D-mannose or L-rhamnose. In addition, results obtained with other acetyl acceptors have demonstrated that the specificity of O-10 transacetylase is strongly determined by the presence of glycosyl units covalently bonded to D-galactose. This conclusion is derived from the

results of experiments in which initial rates of acetylation were measured in the presence of varying amounts of acetyl acceptors. Comparisons of enzymatic activities, as measured by initial rates, were made at a substrate concentration that was within the range where these activities are linearly proportional to substrate concentration. Within this range, enzymatic activities with L-rhamnosyl-(1→3)-D-galactose, D-galactose and β-D-galactosyl-(1→6)-D-mannose are in the ratio of 10:1:0.65. The presence of the 1→3 linked L-rhamnosyl residue causes a large increase in the rate of acetylation. In contrast, the difference in rates observed with the substrates D-galactose and β-D-galactosyl-(1→6)-D-mannose is minor.

This difference, even though of minor significance, is more pronounced in a comparison of enzymatic activities obtained with the substrates melibiose [α-D-galactosyl-(1→6)-D-glucose], D-galactose and β-D-galactosyl-(1→6)-D-mannose. When measured under the conditions described in the previous paragraph, the ratio of enzymatic activities is 1.7:1.0:0.65, respectively. Since melibiose is acetylated 2.6 times faster than β-D-galactosyl-(1→6)-D-mannose, the anomeric configuration of the D-galactosyl bond may be important in determining

substrate specificity. The role of the structural differences between the D-glucose and D-mannose, present as terminal reducing residues in these two disaccharides, has not been determined. The linkage position of the bond may also be important since melibiose is a substrate but lactose [β-D-galactosyl-(1→4)-D-glucose] is not. This conclusion, however, is tentative because the relative contributions of anomeric configuration, linkage position and monosaccharide structure have not been determined.

The hexasaccharide flavazole substrates contain both the L-rhamnosyl residue and the α- or β-D-galactosyl-(1→6)-D-mannose linkage. The Michaelis constant of the alpha derivative is 4 to 5 times higher than that of the beta isomer. If the *in vivo* transacetylation were limited by the concentration of acetyl acceptor, one might expect that a smooth O-antigen containing α-D-galactosyl linkages would be acetylated less efficiently and thus contain fewer O-acetyl groups. However, the O-antigens from A_1 and $A_1(\epsilon^{15a})$ cells contain about the same number of O-acetyl groups. Thus, the acetyl acceptor is probably not the limiting factor in the physiologically important reaction. The maximum velocity of acetylation of the alpha hexasaccharide flavazole is at least 2.2 times higher

than that of the beta isomer. Therefore, in the presence of saturating amounts of acetyl acceptor, the higher maximum velocity with the substrate containing α-D-galactosyl linkages apparently makes up for the less efficient reaction at limiting concentrations of acetyl acceptor. This interpretation must be considered as tentative since the effect of the bulky flavazole nucleus on the acetylation reaction is not known. In addition, the measurement of the O-acetyl groups in LPS is not very reproducible (see Materials and Methods). Thus, the possibility that essentially all of the galactosyl residues in the LPS from both strains are acetylated is not excluded. If this were true, the argument presented above might not be relevant to the in vivo situation.

As has been discussed, the acetyl group is esterified to the D-galactosyl residues within the smooth O-antigen. Furthermore, the acetylation reaction is considerably enhanced by the presence of an L-rhamnosyl residue linked through a glycosidic bond to carbon 3 of D-galactose. These facts strongly support the conclusion that antigen 10 is determined by internal acetylated galactosyl residues as deduced from the original chemical studies of the E_1 O-antigen (9). The methylation studies of the LPS had demonstrated that less than 20 per cent

of the D-galactosyl residues were at non-reducing ends (see Introduction). The analysis of O-acetyl content indicated that there was at least one O-acetyl for every two D-galactosyl residues. Thus, some non-terminal D-galactosyl residues carry O-acetyl groups. The recent demonstration that the smooth O-antigen is synthesized by a polymerization of the trisaccharide units α-D-mannosyl-(1→4)-L-rhamnosyl-(1→3)-D-galactose leads to the prediction that no non-reducing terminal D-galactosyl residues are present. Those that were found may have been derived from galactose present in the core.

The acetyl group that partially determines O-antigen 10 had originally been assigned to the primary hydroxyl group on carbon 6 of the D-galactosyl residues (9). This assignment was based on the results of complement fixation assays with chemically acetylated trisaccharides derived from group E LPS. Solutions containing the trisaccharides α- or β-D-galactosyl-(1→6)-α-D-mannosyl-(1→4)-L-rhamnose were treated with acetic anhydride at room temperature until 0.5 to 2.62 moles of O-acetyl were present per mole of trisaccharide. The unacetylated alpha trisaccharide, at a concentration of 1.3 mM, did not inhibit anti-10 serum as determined by a complement fixation assay. The alpha trisaccharide containing 2.18 to 2.62 moles

of O-acetyl produced 50 per cent inhibition at concentrations of about 0.013 mM. When only 0.5 mole of O-acetyl was attached per mole of trisaccharide, 50 per cent inhibition was observed at a concentration of 0.066 mM. The acetylated beta trisaccharide had considerably less activity. The α-D-galactosyl linkage therefore also partly determines O-antigen 10. The position of the O-acetyl residue in the active oligosaccharide was assigned on the basis of the reactivity of the hydroxyl groups in the acetylation reaction. Thus, the hemiacetal hydroxyl group of the L-rhamnose and the primary hydroxyl group on position 6 of the D-galactosyl residue should have been acetylated. The chemically acetylated trisaccharides were not purified and, therefore, the possibility that a minor component in the reaction mixture is the active derivative cannot be excluded.

A general problem that must be considered in any attempt to assign the position of an acyl group in a polyhydroxylated compound is the facile migration of such acyl groups (82). The phenomenon of acyl migration was first reported by Fischer, who suggested that it probably occurred via an orthoester intermediate (83). It subsequently was demonstrated that 1, 2, 3, 4-tetra-O-acetyl-β-D-glucose was converted into the 1, 2, 3, 6-tetra-O-acetyl derivative by treatment with either 0.001 N

sodium hydroxide or the alkali present in soft glass (84, 85). Since then, numerous instances of acid- as well as alkali-catalyzed acyl migrations have been reported, which can all be explained in terms of the orthoester mechanism (86, 87).

The kinetics of base catalyzed acyl migration have been studied in several different systems. The 2' to 3' isomerization of 2'-O-(N-acetylvalyl)adenosine was found to occur so rapidly that rates in aqueous solutions above pH 7.0 could not be measured (88). However, the steric restraints caused by the furanose ring structure and adjacent cis hydroxyl groups make migration a very favorable reaction. The rate of beta to alpha isomerization in β-O-acetyl-glycerol was found to be 0.1 to 0.2 times that of the adenosine derivatives (89). The difference in rates probably resulted from the free rotation of the carbon-carbon bonds in the glycerol derivative. The kinetics of the conversion of 3-O-acetyl-1,2-isopropylidene-D-glucofuranose to the 6-O-acetyl derivative have also been studied (90). This rearrangement occurs at about one-half the rate observed for β-O-acetyl-glycerol. The role of the glucofuranose ring structure in this migration from carbon 3 to 6 is not known.

The kinetics of acyl migration in partially acylated glycopyranose derivatives have not been studied in detail. As mentioned above, weakly alkaline conditions are known to cause migration in this type of structure. Nevertheless, the identification of the acetyl position in some O-acetyl glycopyranose derivatives has been reported. Whether the product characterized is identical to that formed by enzymatic action has not been established. Some group B Salmonellae carry O-antigen 5, which is determined by an O-acetyl group. This determinant is believed to be specified by 2-O-acetyl-D-galactosyl residues since N-acetyl-D-galactosamine, but not D-galactose or N-acetyl-D-glucosamine, inhibits a serological reaction specific for O-antigen 5 (91). However, the position suggested by the inhibition studies will have to be demonstrated by structural analysis. The enzyme thiogalactoside transacetylase is believed to catalyze the transfer of acetyl groups to position 6 of β-galactosides (92). This conclusion was based on the production of one mole of formic acid per mole of isopropyl O-acetyl-β-D-thiogalactoside by oxidation with periodate at pH 4.5. It should be noted, however, that the acetylated compound analyzed was isolated from whole cells

grown in the presence of the acetyl acceptor isopropyl β-D-thiogalactoside. Since the effect of the intracellular pH on acyl migration is unknown, the identity of the compound analyzed with the enzymatic product is questionable.

The acetyl group that partially determines O-antigen 10 was shown to be esterified to the primary hydroxyl group on carbon 6 of D-galactose. The unavailability of the 2-O-acetyl- and 4-O-acetyl-D-galactose derivatives prevented a study to determine if migration could occur during the procedures used. Nevertheless, it is possible to indicate those procedures during which migration may have occurred. The periodate oxidation study, which was used to eliminate position 2 as the site of acetylation, was carried out under mildly acid conditions (pH 4.5). Alkali-catalyzed migration should thus be at a minimum. However, the oxidized compound had been enzymatically prepared at pH 7.2. If migration occurs at this pH, the isolated material may not be the direct product of O-10 transacetylase.

The method used to eliminate the 4 position of D-galactose involved a borohydride reduction in methanol of enzymatically acetylated L-rhamnosyl-(1→3)-D-galactose. In aqueous solutions, sodium borohydride rapidly decomposes until enough borate is

formed to raise the pH to the alkaline range in which borohydride is stable. Gaylord reports that sodium borohydride in methanol breaks down at a constant rate because the neutral product does not appreciably change the pH of the solution (93). If this were true, alkali-catalyzed migration may not have occurred during the reduction described. The use of model compounds will be necessary to demonstrate the validity of this conclusion.

The loss of O-acetyl groups from LPS in the E_1 strains lysogenic for ϵ^{15} has been shown to be correlated with the loss of O-10 transacetylase activity. The mechanism by which enzymatic activity is lost, however, is not clear. An explanation based on the loss of the acetyl donor acetyl-CoA was experimentally excluded. As stated earlier, however, a membrane-bound acetyl donor should be considered. Membrane-bound intermediates in the biosynthesis of several extracellular macromolecules have been found (94, 53, 95). These membrane-bound intermediates consist of a highly lipophilic residue linked to sugar or sugar derivatives by either a phosphate or pyrophosphate unit (43, 44). Recently, Robbins and coworkers have suggested that these lipid compounds may represent a new class of activated sugars, which would be in many ways analogous to

the nucleoside diphosphate sugars (43). The importance of this class of activated sugars is that they would be present either at or near the lipophilic membrane, which is the biosynthetic site of many extracellular polysaccharides. The possible existence of a lipophilic acetyl donor, perhaps similar to the lipophilic sugar donors, has not been studied. The acetyl groups would presumably be transferred to this donor from acetyl-CoA, since acetyl-CoA does function as a donor. If a lipophilic donor did exist, the effect of phage conversion could result from the loss of either this lipophilic acetyl donor or one of the acetyl-transferring enzymes.

A loss of acceptor is not responsible for the loss of enzymatic activity since the enzyme will catalyze the acetylation of either α- or β-D-galactosyl residues. This raises the possibility of the existence of two transacetylases, one specific for alpha linkages and one for beta linkages. There is no experimental evidence to refute this possibility. There are two pieces of information, however, which bear on this problem. The enzymatic activities are always both present or absent. Infection with ϵ^{15} results in the disappearance of both enzymatic activities. On the other hand, cells lysogenic for ϵ^{15a} retain both activities. Secondly, since the smooth O-antigen of group E_1 strains contains no β-D-galactosyl linkages,

there is no apparent reason for the non-lysogenic cells to contain the beta galactosyl acetylating activity.

At least two other explanations for the loss of 0-10 transacetylase activity should be mentioned. The first of these is an O-antigen deacetylase. This possibility was partially excluded by the fact that the acetylated hexasaccharide flavazole was not affected when incubated together with particulate or soluble material from $A_1(\epsilon^{15})$ cells. But, the existence of a deacetylase that had specificity requirements not found in the flavazole derivatives was not excluded. The other possible explanation for the loss of transacetylating activity is an enzyme inhibitor. Since acetyl-CoA is rapidly destroyed in the presence of cytoplasmic fractions from either A_1 or $A_1(\epsilon^{15})$ cells, a search for a soluble enzyme inhibitor was not undertaken. Although the enzymatic reaction was not affected by the presence of particulate material isolated from $A_1(\epsilon^{15})$, inhibitors that are bound to the membrane with newly synthesized enzyme would not have been detected. Definitive testing of these possibilities will probably depend upon the isolation of soluble enzyme.

Infection of A_1 cells by ϵ^{15} was shown to cause a halt in the production of 0-10 transacetylase. Samples taken at various

times after infection of growing cultures contained about the same amount of enzyme as that present at the time of infection. This result suggests that the phage causes a rapid repression of the synthesis of O-10 transacetylase. Presumably, the enzyme present at the time of infection is diluted as it is distributed among the progeny cells. Since the repression of O-10 transacetylase occurs after infection by ϵ^{15} or ϵ^{15} vir, the expression of the phage gene(s) responsible for the repression is independent of whether the infection is followed by lysogenization or production of mature phage particles. Knowledge concerning the biochemical events that accompany ϵ^{15} infection may supply information pertinent to the understanding of the mechanism of O-antigen 10 conversion.

The phage conversion of O-antigen 10 is not unique. The work of Uetake (96) and Le Minor (97) have demonstrated the existence of numerous converting systems among Salmonella strains (Table XVII). The structures of the smooth O-antigens that are involved in many of these conversions are as yet unknown. Nevertheless, in the light of present knowledge concerning the biosynthesis of LPS, conversions appear to belong to one or both of two general classes (Table XVIII). The first

TABLE XVII

Lysogenic Conversions of Salmonella O-Antigens[a]

O-antigen(s)	Phage	K-W group[b]	Antigen conversion
1	P 22	B	4, 12 → 1, 4, 12
		B	4, 5, 12 → 1, 4, 5, 12
		D	9, 12 → 1, 9, 12
	phage 1 (40)	R	40_1, 40_2 → 1, 40_1, 40_2
	phage 1 (42)	T	42 → 1, 42
	iota	A	2, 12 → 1, 2, 12
		B	4, 12 → 1, 4, 12
		D	9, 12 → 1, 9, 12
1, 37	phage 37	G_1	13, 22, 36 → 1, 13, 22, 36, 37
		G_2	13, 23, 36 → 1, 13, 23, 36, 37
6, 14	phage 6, 14 (18)	K	18 → 6, 14, 18
14	phage 14 (6, 7)	C_1	6, 7 → (6), (7), 14
15	ϵ^{15}	E_1	3, 10 → 3, 15
		E	(1), 3, 10, (19) → 3, 15
		E_4	1, 3, 19 → 1, 3, 15, 19
		D_2	3, 9, 46 → 3, 9, 15, 46

TABLE XVII (cont'd)

O-antigen(s)	Phage	K-W group[b]	Antigen conversion
20	phage 98	C_2	6, 8 → 6, 8, 20
27	phage 27	A	2, 12 → 2, 12, 27, 27_A
		A	1, 2, 12 → 1, 2, 12, 27, 27_A
		B	4, 12 → 4, 12, 27, 27_B
		B	4, 5, 12 → 4, 5, 12, 27, 27_B
		B	1, 4, 5, 12 → 1, 4, 5, 12, 27, 27_B
		D	9, 12 → 9, 12, 27, 27_D
34	ϵ^{34}	E_2	3, 15 → (3), (15), 34
34, 12_2	ϵ^{34}	$D_2(\epsilon^{15})$	3, 9, 15, 46 → (3), 9, 12_2, (15), 34, 46

[a] This table is composed of data from references 96, 97, and 10.

[b] Kauffmann-White group

TABLE XVIII

Two Classes of Lysogenic Conversions of Salmonella O-Antigens

	Phage	Structural Change[a]	Ref.
Class I	ϵ^{15}	...-D-(6-OAc)gal-...→...-D-gal-...	9
	ϵ^{34}	...-D-gal-...→ ...-D-[α-D-glc-(1→4)]gal-...	8
Class II	ϵ^{15}	...-α-D-(6-OAc)gal-...→ ...-β-D-gal-...	8,9

[a] ..., represents the continuation of the smooth O-antigen.

class includes those conversions that result in the gain or the gain and loss of enzymes that modify the preformed smooth O-antigen. Examples of this class are the group E_2 to E_3 conversion caused by ϵ^{34} (Dr. A. Wright, unpublished observations; 98; 99) and the group E_1 to E_2 conversion caused by ϵ^{15}. The second general class includes those conversions that involve linkage changes in the polysaccharide backbone of the smooth O-antigen. The α- to β-D-galactosyl change caused by ϵ^{15} is an example of this class. The important feature of this linkage is that it connects the trisaccharide repeating units in the smooth O-antigen. If this classification is validated, the biosynthesis of the repeating unit would not be affected in any of the conversions. Knowledge of the location on the bacterial chromosome of the various prophages and genes involved in these conversions may add to our understanding of the relationship between phage conversion and transduction.

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