Immunoochemistry of the Cell Walls of *Listeria monocytogenes*

WILLIAM W. ULLMANN AND J. A. CAMERON

Laboratory Division, Connecticut State Department of Health, Hartford, Connecticut 06101, and Microbiology Department, University of Connecticut, Storrs, Connecticut

Received for publication 9 January 1969

The antigenic specificity of *Listeria monocytogenes* types I, II, III, IVa, and IVb was studied by immunochemical techniques. Immunologically active carbohydrates of the various types were extracted from cell walls and were chemically analyzed. Types I and II contained predominantly glucosamine and rhamnose; type III, galactose, rhamnose, and glucosamine; and types IVa and IVb, glucose and galactose. Quantitative precipitin inhibition tests with purified monosaccharides indicated that the major antigenic determinant of types I and II is rhamnose. Precipitin reactions could not be detected with type III carbohydrate and homologous or heterologous antisera. The major determinants of types IVa and IVb were found to be galactose and glucose, respectively. As much as 87% inhibition of the quantitative precipitin test for types I and II was obtained with rhamnose, 72% for type IVa with galactose, and 72% for type IVb with glucose. The immunochemical basis for the antigenic specificity of *L. monocytogenes* types I, II, IVa, and IVb was further confirmed by using agar gel diffusion. Cross-reactions among the various type-specific carbohydrates and heterologous antisera were also studied. Type II carbohydrate was found to contain galactose and react with type IVa antisera. This reaction could be blocked by galactose. Type I carbohydrate did not contain galactose nor did it react with antiserum prepared from type IVa cells. Therefore, the somatic antigens of type I and type II *L. monocytogenes*, previously thought to be identical, appeared to differ. The dominant immuno-specific group in the cross-reaction between type IVb carbohydrate and type IVa antiserum was found to be galactose. Type IVa absorbed antiserum did not produce a significant cross-reaction with type IVb carbohydrate. The results obtained from this investigation indicate a lesser degree of antigenic relationship between type IVa and type IVb *L. monocytogenes* than was previously believed to exist.

The immunological and serological behavior of *Listeria monocytogenes* is extremely complex. Cross-reactions, weak titers, and the presence of serum antibodies reactive with *L. monocytogenes* in a high percentage of normal vertebrates (10, 14, 17) indicate the need for clarification of the immunological behavior of the organism.

All available evidence indicates that the mucopolypeptide of the bacterial cell wall contains only amino sugars and amino acids (12). Other sugars may occur as oligo- or polysaccharides which are more readily solubilized than the mucopolypeptide. The purpose of this investigation was to determine and to compare the chemical nature of the cell walls of the five serological types of *L. monocytogenes* and to study the immunochemical properties of the cell wall carbohydrates. Special emphasis was placed on the delineation of the antigenic determinants involved in the reaction between the type-specific carbohydrates and homologous and heterologous antisera.

MATERIALS AND METHODS

**Growth and harvesting of cells.** Cultures of *L. monocytogenes* types I, II, III, IVa, and IVb were obtained from the late M. L. Gray of the Veterinary Research Laboratory, Montana State College, Bozeman, Mont. These were designated strains 5349, 144, 2469X, 5214, and 2470X, respectively. Stock cultures were maintained in the lyophilized state and on blood-agar slants. Large numbers of cells were obtained by inoculating 15 ml of 6-hr cultures into 10 liters of Trypticase Soy Broth. After stationary growth for 24 hr at 37 C, cells were heat-killed by steaming at 100 C for 10 min in an autoclave. Cells were harvested by centrifugation in a Sharpies Laboratory Model Super-Centrifuge and were washed twice with 0.05 M phosphate buffer (pH 7.4) in a Superspeed Refrig-
erated Centrifuge (Ivan Sorvall Inc., Norwalk, Conn.) at 2,000 X g.

For growth on Tryptic Soy Agar for 24 hr at 37 C, and Gram stains of the cultures, were examined with a binocular scanning microscope. Colonial morphology and Gram stains were used to verify purity of cultures.

**Cell disruption and preparation of wall fractions.**

Packed cells were diluted 1:4 (v/v) with phosphate buffer (pH 7.4) mixed with an equal weight of glass beads and disrupted in a Braun (model MSP) homogenizer (Bromwill Scientific, Rochester, N.Y.) cooled with liquid CO2.

After disruption, crude cell walls were isolated by differential centrifugation in 0.05 M phosphate buffer (pH 7.4). After resuspension in phosphate buffer, walls were treated with 1 mg of trypsin (Calbiochem) per ml of suspension and incubated at 37 C for 2 hr. A few drops of chloroform were added prior to each incubation to prevent bacterial growth. After incubation, the trypsinized walls were sedimented by centrifugation, resuspended in phosphate buffer, washed twice with buffer, and again resuspended; 1 mg of ribonuclease (Calbiochem) per ml of suspension was then added, and the suspension was again incubated for 2 hr at 37 C. The treated cell walls were again sedimented and resuspended in 0.05 M phosphate buffer. The walls were then washed by alternate centrifugation at 2,000 and 4,000 X g. The material sedimented at 2,000 X g was discarded and that sedimented at 4,000 X g was retained. The wash solution was analyzed for protein and nucleic acids by determining optical densities at wavelengths of 280 and 260 nm, respectively, in a Beckman DB spectrophotometer. Approximately seven 25-ml washes were required for each of the five cell types to reduce the optical density at both wavelengths to 0.10 or lower. When this value was reached, the walls were considered to be essentially devoid of nucleic acids and protein. The walls were then sedimented, washed twice with distilled water, and lyophilized in a VirTis Freeze-Dryer.

**Preparation of type-specific carbohydrate.** Whole cells, grown and harvested as described above, were suspended in distilled water and lyophilized. To 1.0 g of dried cells was added 40 ml of formamide, and the mixture was heated in an oil bath at 145 to 150 C for 20 min. After cooling, the solubilized cells were dialyzed overnight at 4 C against buffered saline (pH 7.4) to remove formamide. After dialysis, the solubilized cells were treated with 1.0 mg (per ml) of Pronase (Calbiochem) and incubated for 4 hr at 37 C to release protein. The treated cells were then dialyzed overnight at 4 C against distilled water. Purified cell wall carbohydrate was isolated by the method described by Fuller (1). The serological activity of the extract was checked against type-specific antisera by the capillary precipitin test.

Hydrolases of cell walls and mucopeptide residue, after formamide extraction of the type-specific carbohydrate, were prepared by treatment with 6 N HCl at 103 C for 18 hr. The extracted carbohydrates were hydrolyzed with 3 N HCl at 103 C for 1 hr. Hydrolysates were dried in a 37 C water bath under vacuum, made up to 1 ml with 10% isopropanol, and stored at 4 C prior to amino acid analysis. Those portions which formed white suspensions were dried over phosphorus pentoxide in a vacuum and made up to 1 ml with distilled water. Salts, amino acids, and amino sugars were removed by adding 100 mg of Dowex 50 (H+ form; Baker Chemical Co.). Dowex 1 (OH- form; Baker Chemical Co.) was then added until the pH of the mixture was approximately neutral. The mixture was filtered and the resins were washed on the filter with two 2-ml volumes of distilled water. The filtrate was dried under vacuum over phosphorus pentoxide and made up to 1 ml with 10% isopropanol. Amino acids and neutral sugars in hydrolysates were determined in a high-voltage paper electrophoresis apparatus (model D; Gilson Medical Electronics Co., Middleton, Wis.) by the method of Mabry and Todd (7) and Mabry, Gryboski, and Karam (6), respectively. All analytical results were verified by duplicate analysis.

Amino sugars could not be determined by electrophoresis because of trailing. Therefore, prior to analysis, amino sugars were converted to neutral sugars by ninhydrin oxidation (15). After ninhydrin treatment, the solution was treated batchwise with Dowex 1 and Dowex 50 as described for neutral sugars, to remove all charged compounds (including excess ninhydrin). The treated hydrolysates were then brought to dryness over phosphorus pentoxide in a vacuum and made up to 1 ml with 10% isopropanol, and sugars were determined as described above.

**Preparation of somatic antisera.** Type I and II antigens were prepared by scraping motile growth from the surface of 0.8% tryptose phosphate-containing semisolid agar which had been inoculated and incubated for 16 to 18 hr at 22 C. The organisms were suspended in 0.3% Formolized phosphate-buffered saline (pH 7.2) and diluted to yield a McFarland nephelometer reading of 4. Antigens of types III, IVa, and IVb were prepared by incoating 500 ml of Tryptose Soy Broth with 2 ml of broth culture, incubating overnight at 37 C, and heat-killing at 60 C for 30 min. Cells were harvested by centrifugation, suspended in 20 ml of saline, and boiled for 2 hr.

Nine consecutive intramuscular injections of antigen mixed with Freund's incomplete adjuvant were administered to rabbits as follows: 0.1 ml of vaccine and 0.1 ml of adjuvant the first day and increasing daily to 0.9 ml of vaccine and 0.9 ml of adjuvant on the ninth day. The rabbits were then rested for 5 days and test-bled from the ear. If titers were at least 1:32 by slide agglutination testing, the animals were bled out by cardiac puncture; otherwise, the animals were rested for 2 months and the schedule was repeated. Sera were obtained by using a refrigerated centrifuge, preserved with 1:5,000 Merthiolate, and stored in the frozen state.

**Preparation of absorbed antisera.** Absorbed antisera was prepared by adding purified type-specific homologous carbohydrate to the antisera (3). The concentration of carbohydrate added was equal to that found to produce the equivalence point for a given antisera. The mixtures were incubated at room temperature for 1 hr and held overnight at 4 C to
produce maximum precipitation. The antigen-antibody complex was then sedimented by centrifugation.

Precipitin tests. The method described by Swift, Wilson, and Lancefield (16) was used in performing the capillary precipitin test. The quantitative precipitin test used was that described by McCarty and Lancefield (8). All precipitin results, including inhibition studies, were verified by duplicate analysis.

Inhibition studies. Inhibition studies were conducted by adding 0.1 ml of a 10% solution of the sugar under study to 0.1 ml of antiserum, allowing the mixture to incubate for 1 hr at room temperature, and carrying out the quantitative precipitin test at the equivalence point as described above. Percentage inhibition was calculated by determining the ratio of the optical density obtained in the presence of the sugar compared to the optical density obtained in the absence of the inhibitor and multiplying by 100.

Agar diffusion precipitin test. The method used was an adaptation of the Ouchterlony (11) method as described by Muraschi and Tompkins (9); 0.1 ml of a 200 μg/ml solution of extracted carbohydrate in normal saline was added to the outer wells, and 0.1 ml of serum was added to the center well. Plates were held for at least 24 hr at room temperature before band formation could be detected. Plates were examined daily for 4 weeks. To study the effect of inhibitors, 10% solutions of the sugar being investigated were added to the liquefied media just prior to pouring the plate to yield a final sugar concentration of 1%.

RESULTS

Composition of cell walls. The results of high-voltage paper electrophoresis on cell wall hydrolysates (Table 1) revealed that types I, II, III, IVa, and IVb all contained muramic acid, glutamic acid, alanine, and diaminopimelic acid in essentially the same proportion. These are in agreement with the semiquantitative results obtained by Keeler and Gray (4). These authors also found low concentrations of aspartic acid and lysine, which could not be confirmed in this study. It is possible that the lysine and aspartic acid they reported were present in their preparations as membrane contaminants.

In light of the relatively high ratios of N-acetylglucosamine to muramic acid found in the cell walls of types I, II, and III, glucosamine must be present in excess of that found in the cell wall mucoprotein polymers of other organisms. This was borne out in later results obtained on hydrolysates of the formamide-extracted carbohydrate and the mucoprotein residue remaining after formamide extraction.

Considerable variations in amino sugar and neutral sugar concentrations (Table 1) were observed among the five types. Type I contained significant concentrations of glucosamine and the neutral sugars rhamnose and glucose. A small concentration of galactose was found in type II cell walls in addition to the rhamnose, glucose, and glucosamine present in those of type I. Type III consisted principally of galactose and glucosamine, with lower concentrations of galactosamine, rhamnose, and glucose. However, the galactose concentration of type III was considerably greater than in any of the other types except type IVa. Types IVa and IVb were similar in that each contained glucosamine, galactosamine, rhamnose, glucose, galactose, and mannose. Slight variations in concentrations, however, did exist, with a higher concentration of galactose present in the type IVa cell walls and a higher concentration of glucose in those of type IVb. Types IVa and IVb also contained considerably lower concentrations of glucosamine than did types I, II, and III.

Analysis of hydrolysates of the extracted carbohydrates of types I, II, III, IVa, and IVb are shown in Table 2. These results show that, with the exception of that portion of the glucosamine associated with the mucoprotein complex (Table 3), all sugars and amino sugars are included in this fraction. The extracted carbohydrate was also essentially devoid of amino acids, indicating that this material must have been almost completely free of mucoprotein residue. As in the results of analysis of cell walls (Table 1), the carbohydrates of types I and II were quite similar in that both contained rhamnose, glucose, and glucosamine. The type II carbohydrate, however, also contained galactose, which was not found in type I. Type III consisted primarily of galactose and glucosamine. Types IVa and IVb contained glucosamine, galactosamine, glucose, rhamnose, mannose, and galactose with only slight differences in concentrations. As with the

Table 1. Composition of cell walls of L. monocytogenes types I, II, III, IVa, and IVb

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Type I</th>
<th>Type II</th>
<th>Type III</th>
<th>Type IVa</th>
<th>Type IVb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muramic acid</td>
<td>4.3</td>
<td>4.3</td>
<td>4.1</td>
<td>4.1</td>
<td>4.1</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>14.3</td>
<td>14.3</td>
<td>12.5</td>
<td>6.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>1.0</td>
<td>3.8</td>
<td>1.0</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.9</td>
<td>5.8</td>
<td>5.4</td>
<td>5.3</td>
<td>5.5</td>
</tr>
<tr>
<td>Alanine</td>
<td>6.9</td>
<td>7.4</td>
<td>6.7</td>
<td>6.9</td>
<td>6.8</td>
</tr>
<tr>
<td>Diaminopimelic acid</td>
<td>6.9</td>
<td>7.6</td>
<td>8.4</td>
<td>7.4</td>
<td>6.8</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>14.1</td>
<td>12.2</td>
<td>7.0</td>
<td>1.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.0</td>
<td>1.8</td>
<td>2.2</td>
<td>6.6</td>
<td>10.7</td>
</tr>
<tr>
<td>Galactose</td>
<td>&lt;1.0</td>
<td>3.8</td>
<td>15.1</td>
<td>12.2</td>
<td>9.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>3.2</td>
<td>4.8</td>
</tr>
</tbody>
</table>

* Percentages based upon recovery from hydrolysates of 100 mg of purified cell walls. Minimum detectable concentration, 1%.
cell walls of types IVa and IVb, a higher concentration of galactose was present in the type IVa carbohydrate than in that of IVb, and IVb contained a higher concentration of glucose than that of IVa.

**Formamide residue.** Two products are obtained by hot formamide treatment of cell walls: the soluble carbohydrate and the insoluble mucoprotein residue precipitated by alcohol. Analysis of the mucoprotein residues of the five types (Table 3) indicated that for the most part the residues were identical. Each contained muramic acid, glutamic acid, alanine, diaminopimelic acid, and glucosamine. Since the glucosamine-muramic acid ratios for types III, IVa, and IVb exceed the 1:1 ratio of the mucoprotein polymer, some of the carbohydrate must be bound to the mucoprotein residue after extraction. However, detectable concentrations of neutral sugars were not found.

**Precipitin tests.** Quantitative precipitin reactions between extracted carbohydrate from serotypes I, II, III, IVa, and IVb and homologous and heterologous antisera are shown in Table 4. Types I and II exhibited strong cross-reactions, type IVa was specific, and type IVb carbohydrate showed a weak but consistently detectable cross-reaction with type IVa antiserum. Type III failed to demonstrate a detectable precipitin reaction. The precipitin test also showed a cross-reaction between type II carbohydrate and type IVa antiserum. This cross has not been demonstrated by agglutination reactions nor can it be explained by the assignment of the various somatic factors of Gray and Killinger (2).

**Inhibition of antigen-antibody precipitation by constituent sugars.** Quantitative precipitin tests with various inhibitors of types I, II, IVa, and IVb carbohydrates and their homologous antisera are shown in Table 5. These results show a high percentage of inhibition of the types I and II homologous reactions by rhamnose, with lesser degrees of inhibition by glucose, galactose, and glucosamine. Galactose and glucose strongly inhibited the types IVa and IVb homologous reactions, respectively. Other constituent sugars were much less effective as inhibitors. Inhibition of both reactions by the disacharride melibiose was high and greatly exceeded inhibition produced by other disacharrides tested.

The antigenic relationship between types I and II is illustrated in Fig. 1; quantitative precipitin analyses with both homologous and heterologous antisera illustrated the strong cross-reactions between the two types, both of which were almost completely inhibited by rhamnose. Types I and II antiserum absorbed with homologous antisera lost practically all reactivity for the heterologous carbohydrate.

The cross-reactivity between type II carbohy-
drate and type IVa antiserum was indicated by quantitative precipitin test results (Fig. 2). Cross-reactivity of type 1 carbohydrate with type IVa antiserum could not be demonstrated. The significant reduction in cross-reactivity after absorption of type IVa antiserum with homologous carbohydrate suggests that the same determinant group is involved as is in the type IVa homologous reaction. Quantitative precipitin tests, however, did not show significant inhibition of the type II carbohydrate-type IVa antiserum reaction by galactose, although the formation of the precipitate did appear to be retarded in the presence of the inhibitor. The results of a time study of precipitate formation, with galactose as the inhibitor (Fig. 3), verified this impression; at 5-min intervals, the increasing optical density resulting from precipitate formation was measured in a Beckman (model DB) spectrophotometer at a wavelength of 480 nm.

A significant cross-reaction occurred between type IVb carbohydrate and type IVa antiserum (Table 4). The results of a study to ascertain the determinant group responsible for this cross-reaction are illustrated in Fig. 4. Inhibition of both the IVa and IVb carbohydrate-IVa antiserum reactions by galactose was practically identical. Mellibiose also markedly inhibited these reactions. Practically complete removal of IVb reactivity was obtained when IVa antiserum, previously absorbed with purified IVa carbohydrate, was used.

Agar gel precipitin tests. Agar gel precipitin analysis of formamide-extracted carbohydrates

---

**Table 5. Inhibition of quantitative precipitin reactions between L. monocytogenes formamide-extracted carbohydrate (types I, II, IVa, IVb) and homologous type-specific antisera.**

<table>
<thead>
<tr>
<th>Inhibitor (1%)</th>
<th>Type I</th>
<th>Type II</th>
<th>Type IVa</th>
<th>Type IVb</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetylglucosamine</td>
<td>17</td>
<td>21</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>N-acetylgalactosamine</td>
<td>ND*</td>
<td>ND</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>87</td>
<td>80</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>Glucose</td>
<td>13</td>
<td>9</td>
<td>25</td>
<td>72</td>
</tr>
<tr>
<td>Galactose</td>
<td>17</td>
<td>20</td>
<td>72</td>
<td>38</td>
</tr>
<tr>
<td>Mannose</td>
<td>ND</td>
<td>ND</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Melibiose</td>
<td>ND</td>
<td>ND</td>
<td>80</td>
<td>70</td>
</tr>
<tr>
<td>Lactose</td>
<td>ND</td>
<td>ND</td>
<td>36</td>
<td>52</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>33</td>
</tr>
<tr>
<td>Maltose</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>6</td>
</tr>
<tr>
<td>Trehalose</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>23</td>
</tr>
</tbody>
</table>

*Not determined.

---

**Fig. 1. Inhibition of quantitative precipitin reactions.**

(A) Type I L. monocytogenes antisera and types I and II type-specific carbohydrates. (B) Type II L. monocytogenes antisera and type I and II type-specific carbohydrates. Type II CHO, no inhibitor (○); type I CHO, no inhibitor (□); type II CHO, 1% rhamnose (△); type I CHO, 1% rhamnose (●); type II CHO, type I absorbed antiserum (●); type I CHO, type II absorbed antiserum (△).

**Fig. 2. Inhibition of precipitin reaction between type IVa antiserum and type II-specific carbohydrate.** No inhibitor (○); 1% galactose (□); type IVa absorbed antiserum (△).
Fig. 3. Galactose retardation of precipitin formation between type IVa antisera and type II type-specific carbohydrate. No inhibitor (○); 1% galactose (□).

Fig. 4. Inhibition of quantitative precipitin reactions. (A) Type IVa L. monocytogenes antisera and type IVa type-specific carbohydrate; (B) type IVa L. monocytogenes antisera and type IVb type-specific carbohydrate. No inhibitor (○); 1% galactose (□); 1% melibiose (△); type IVa absorbed antisera (●).

is shown in Fig. 5. Reactions between types I and II carbohydrate with homologous and heterologous antisera in the absence of inhibitor show strong merging bands of identity, indicating that antigens with the same determinant groups are involved in both the homologous and heterologous reactions of types I and II. These reactions were completely inhibited by the inclusion of 1% rhamnose in the agar, providing further substantiation for the findings obtained with quantitative precipitin tests. The cross-reaction detected between type IVb carbohydrate and type I and II antisera was not detected in the quantitative precipitin procedure.

Agar gel precipitin testing also provided further confirmation of the results of quantitative precipitin testing of types IVa and IVb antisera with homologous and heterologous carbohydrates. The strong band of identity between IVa carbohydrate and its homologous antisera (Fig. 5) was completely inhibited by galactose. Galactose inhibition of the reaction between types II and IVb carbohydrates and type IVa antisera was also shown. For the reaction between type II carbohydrate and type IVa antisera, however, a second band was detected. This band could not be eliminated by the addition of any of the constituent sugars detected in the chemical analysis of the various type carbohydrates.
These results also show that only the homologous reaction occurred with type IVb antiserum and that this reaction was completely inhibited by glucose, confirming quantitative precipitin test results.

DISCUSSION

In our initial attempt to extract a serologically active carbohydrate from the cell walls of L. monocytogenes, the formamide extraction procedure described by Fuller (1) was used. The material recovered by this procedure consists of thermostable, acetone- and alcohol-precipitable, water-soluble substances which give negative protein reactions. Chemical analysis has revealed that this material consists principally of polysaccharides. These polysaccharides represent the soluble portion of the somatic antigen, to which they confer serological specificity (14) and offer an excellent means of studying the immunochromy of the bacterial cell wall by eliminating peptide-induced cross-reactions.

However, this procedure, although successfully applied to streptococci (5), failed to produce a material which was serologically active when applied to Listeria. The extracted material was found to be anthrone-negative (13) and therefore did not consist of carbohydrate. Anthrone determinations on the alcohol precipitates were positive, indicating that protein attached to the carbohydrate may result in its inclusion in this fraction. Therefore, the formamide was dialyzed out and the dialysate was treated with Pronase followed by ribonuclease (Calbiochem) to remove protein and nucleic acids from the carbohydrate prior to alcohol and acetone precipitation. This procedure produced approximately 20 mg of relatively pure carbohydrate from 1 g of dried cells and provided the serologically active material required to complete the study.

The fact that free rhamnose clearly inhibits the reactions between types I and II carbohydrates and their homologous antisera demonstrates that rhamnose residues are of primary importance in determining the specificity of the cell wall carbohydrates of these two types. The inhibitory effects of glucose, galactose, and glucosamine, while significant, are considerably less than that of rhamnose and cannot, therefore, be considered as being involved in the determination of primary specificity. The similarity of the inhibitory effect of rhamnose on the reactions of types I and II carbohydrates with their homologous antisera provides an explanation for the cross-reactions which occur between the two types of organisms during routine serological identifications.

All previous evidence has indicated that the somatic antigens of types I and II are identical. The results of precipitin tests, however, indicate a difference as reflected by a slight cross-reaction between the type II carbohydrate and type IVa antiserum, which was not duplicated by type I carbohydrate. The chemical composition of types I and II carbohydrate was found to differ in that type II contains galactose, which could not be detected in the carbohydrate of type I. Since the type II carbohydrate-type IVa antiserum reaction can be demonstrated by quantitative precipitin testing and agar gel diffusion, and since the latter is inhibited by galactose, the dissimilarity of the two becomes apparent. Failure to detect galactose inhibition in the quantitative precipitin test, as well as the apparent diminution of galactose inhibition with time, indicate that galactose may react reversibly with the antibody and be displaced by the type-specific carbohydrate in the precipitin test.

Inhibition studies of the quantitative precipitin reaction between types IVa and IVb antisera, respectively, and constituent sugars indicate that galactose is the primary determinant group for type IVa and that glucose is the primary determinant group for type IVb. Although other constituent monosaccharides did show a slight inhibitory effect, the inhibition was not of sufficient magnitude to be considered of primary significance. The high degree of inhibition by melibiose indicates that the galactose in the type IVa carbohydrate may be joined to glucose through a 1,6-β linkage. The lesser degree of inhibition by lactose than by melibiose rules out the possibility of a 1,4-β linkage. Melibiose inhibition of the reaction between type IVb carbohydrate and type IVb antiserum parallels the inhibition of the IVa reaction, indicating the possible existence of a 1,6-β linkage between glucose and galactose in the type IVb carbohydrate with glucose as the primary determinant.

A significant cross-reaction has been shown to occur between type IVb carbohydrate and type IVa antiserum. No detectable cross-reaction between type IVa carbohydrate and type IVb antiserum could be detected. More recent work, however, indicates that such a cross-reaction probably does occur. Galactose inhibition of the reaction of both IVa and IVb carbohydrate with IVa antisera is practically identical. Melibiose inhibition of the cross-reaction further illustrates the probability that galactose is joined to glucose through a 1,6-β linkage. Complete removal of IVb reactivity in IVa antiserum absorbed with purified IVa carbohydrate further substantiated the conclusion that the determinant group is the same for the type IVa homologous reaction as for the
type IVb carbohydrate-type IVa antiserum cross-
reaction. Although glucose, joined to galactose by
a 1, 6 linkage, is the major determinant for type
IVb carbohydrate, galactose serves as a minor
determinant and is responsible for the cross-
reaction with type IVa antiserum. Two possible
explanations for this phenomenon are that galac-
tose is present as a terminal group on a branch of
the main body of the carbohydrate, or that galac-
tose is exposed as a result of the extraction pro-
dure.

LITERATURE CITED
1. Fuller, A. T. 1938. The formamide method for the extraction
of polysaccharides from haemolytic streptococci. Brit. J.
Exp. Pathol. 19:130-139.
2. Gray, M. L., and A. H. Killinger. 1966. Listeria monocyt-
nochemistry. Charles C Thomas, Publisher, Springfield, IL.
biochemical properties of Listeria monocytogenes. I. Prepa-
arion and composition of cell wall material. J. Bacteriol. 84:
683-692.
structure of the streptococcal cell wall. I. The identifi-
cation of mucopentide in the cell walls of groups A and
identification and measurements of amino and oligo-
saccharides. An adaptation of high biological materials.
7. Mabry, C. C., and J. M. Todd. 1963. Quantitative measure-
ment of individual and total free amino acids in urine.
8. McCarty, M., and R. D. Lancefield. 1955. Variation in the
group-specific carbohydrate of group A streptococci. I.
Immunochimical studies on the carbohydrate of variant
9. Murachi, T. F., and V. N. Tompkins. 1963. Somatic precipi-
tinogens in the identification and typing of Listeria mono-
10. Osebold, J. W., and M. T. Sawyer. 1955. Agglutinating anti-
odies for Listeria monocytogenes in human serum. J.
Bacteriol. 78:350-351.
11. Ouchterlony, O. 1948. In vitro method for testing the toxin
peptides (mucopentide) and the sensitivity to enzymic
degradation. In The bacteriol cell wall. Elsevier Publish-
ing Co., Amsterdam.
52:373-379.
group A hemolytic streptococci by M precipitin reactions
17. Welshimer, H. J. 1962. Some serological reactions observed
with Listeria monocytogenes. 2nd Symp. Listeria Infections,