Two sets of anti-glycosyl antibodies have been isolated by affinity chromatography methods from the antisera of rabbits immunized with a vaccine of nonviable cells of *Streptococcus faecalis*, strain N. Both types of antibodies are directed against a diheteroglycan of glucose and galactose present in the cell wall of this organism. The members of one set, anti-galactose antibodies, combine with the terminal galactose residues of the glycan and the members of the other set, anti-lactose antibodies, combine with terminal lactose residues of the same glycan. Each set of antibodies is composed of multiprotein components. The electrofocusing method had been used to isolate the individual antibody proteins in homogeneous states as shown by both electrophoresis and ultracentrifugation techniques. Since the components of each set combine with the same structural unit of the antigen, they have been designated as isoantibodies. The sedimentation constants, electrophoretic properties, carbohydrate constituents, and amino acid compositions of the two sets of antibodies are recorded.

Anti-glycosyl antibodies are defined as those antibodies which are induced in the host by a glycosyl antigen and which combine with a specific carbohydrate moiety of that antigen. In a preliminary note (1) we have described the isolation of two sets of such antibodies from antisera of rabbits immunized with a vaccine of nonviable cells of *Streptococcus faecalis*, strain N, which contains an antigenic diheteroglycan of glucose and galactose in its cell wall. The antibodies of one set are anti-galactose (anti-Gal) antibodies and these combine with the terminal galactose residues of the antigen while the antibodies of the other set are anti-lactose (anti-Lac) antibodies and these combine with terminal lactose residues of the same antigen. The isolation procedure involved adsorption of both types of antibodies on a lactosyl-Sepharose column and sequential elution with galactose and lactose solutions. Also in the earlier studies (1, 2) it was shown by gel electrophoretic and electrofocusing methods that the anti-Gal antibodies from a particular rabbit consisted of six distinct proteins and the anti-Lac antibodies consisted of 12 different proteins. Since the individual proteins of each set combine with the same type of structural moiety, i.e., terminal galactose or terminal lactose units of the antigen, the term isoantibody is suggested for the individual members of each set. This report describes details of the procedures for isolating the two sets of anti-glycosyl antibodies and records the sedimentation constants, electrophoretic properties, carbohydrate constituents and amino acid compositions of the two sets of anti-glycosyl antibodies. In addition results of electrofocusing experiments are presented showing that the isoantibodies can be resolved into individual components. By the latter technique antibodies directed against a natural antigen have been prepared in a homogeneous state (2). These antibodies should be especially useful for studies on antibody structure and homology (3-5), on the biosynthetic pathways (6) and regulatory mechanisms for antibody synthesis (7, 8).

**EXPERIMENTAL PROCEDURES**

*Preparation of Antigen, Vaccine, and Antiserum*—The antigenic glycan of glucose and galactose employed in this study was extracted from the cell wall of *Streptococcus faecalis*, strain N, with 10% trichloroacetic acid and purified to homogeneity by fractional precipitation with ethanol alcohol (9) and Bio-Gel filtration (10). The molecular structure of the antigen has been determined by use of a combined analytical scheme based on methylation, periodate oxidation, acetylation, and enzymic hydrolysis (10, 11). Hapten inhibition studies have shown that galactose and lactose are inhibitors of the precipitin reaction and, therefore, terminal galactose and terminal lactose units are immunodeterminant groups of this antigen (9).

A vaccine of nonviable cells of *S. faecalis*, strain N, was prepared from freshly grown cells collected from a 500-ml culture. Formaldehyde treatment was used to obtain a suspension of nonviable cells (10). After removal of the formaldehyde by washing the cells with 0.1 M phosphate buffer of pH 7.2 in 0.9% saline, the cells were suspended in 80 ml of sterile saline. The suspension gave an absorbance value of 1.5 at 600 nm, and this suspension has been used to immunize rabbits by intravenous injection. Details of the immunization procedure and regime have been described elsewhere (10). Antisera were prepared from blood samples drawn weekly from the rabbits by standard immunological methods. The antisera were analyzed for protein constituents by micronephelometry. To date unpoled antisera from six different rabbits have been prepared by the above methods and subjected to affinity chromatography as described in the next section.

*Isolation of Anti-Gal and Anti-Lac Antibodies*—Affinity chromatography (12) on lactosyl-Sepharose was employed for the fractionation of the anti-*S. faecalis* serum. The lactosyl-Sepharose (13) was prepared from 12 g of cyanogen bromide-activated Sepharose 4B
Biochemicals). The above quantities of materials were shaken in 20 ml of 0.1 M NaHCO₃, pH 9.0, for 24 h at 4°C. The reaction product was reacted groups on the Sepharose were blocked by treatment of the original value, the column was washed with 50 ml of 0.1 M galactose solution followed by 50 ml of 0.1 M lactose solution. The fractions corresponding to the 280-nm absorbing material which had been eluted by the galactose and those which were eluted by lactose were combined separately. An equal volume of saturated ammonium sulfate solution was added to the combined samples and precipitation was allowed to proceed for 24 h at 4°C. The precipitates which formed were collected by centrifugation and dissolved in 0.1 ml of saline/0.1 M phosphate buffer, pH 7.2. One milliliter of the antiserum was introduced onto the column and, after the serum protein had been washed through the column, 50 ml of 0.5 M lactose solution followed by 50 ml of 0.1 M phosphate buffer. The absorbance of the eluate was monitored at 280 nm with an ISCO UV analyzer. When the base-line had returned to the original value, the column was washed with 50 ml of 0.1 M galactose solution. The fractions corresponding to the 280-nm absorbing material which had been eluted by the galactose and those which were eluted by lactose were combined separately. The components which were separated were analysed individually in a Dupont 21-490 mass spectrometer, and the nature and abundance of the m/e fragments were determined.

Amino Acid Analysis - Samples of 0.5 ml of the anti-Gal and anti-Lac antibody preparations was taken to dryness by lyophilization. The dried sample and a standard sample containing 0.1 mg of each standard (fucose, mannose, galactose, glucosamine, galactosamine, and neuraminic acid) were subjected to electrophoresis on 5% polyacrylamide gels as described in a preceding section. Qualitative precipitin tests were performed with all fractions and the antigenic glycan.

Electrofocusing - Electrofocusing (22) of the antibody preparations was performed in an LKB instrument in a sucrose-stabilized pH gradient (Ampholine 6 to 8). Samples of approximately 10 mg of the antibody preparations were introduced into a 110-mL gradient and electrofocusing was performed at 800 V for 65 h. Fractions of 1 ml were collected and monitored for UV absorbance at 280 nm. Duplicate samples of 100 µl of the peak fractions of the polyacrylamide gel electrophoresis as described in a preceding section. Qualitative precipitin tests were performed with all fractions and the antigenic glycan.

RESULTS

Microzone electrophoretic patterns of anti-sera from rabbits immunized with nonviable cells of Strepococcus faecalis,
strain N. contained high levels of immunoglobulins in the blood samples collected after the 10th week of immunization. Agar diffusion tests revealed that these sera contained antibodies directed against a diheteroglycan of glucose and galactose isolated from the cell wall of the organism. Hapten inhibition data showed that both lactose and galactose were inhibitors of the reaction between the diheteroglycan and the antisera. This result was interpreted as evidence for the presence of two populations of antibodies, one which combined with the galactose moiety of the antigen. Fig. 1 shows affinity chromatography elution patterns for preimmune serum and serum collected from a single rabbit after the 13th week of immunization. It will be noted in the figure that the preimmune serum yielded only a UV-absorbing peak of serum proteins, but the immune serum yielded the serum protein peak and two other UV-absorbing peaks, one eluting with the galactose and the other eluting with lactose. The latter materials were recovered from the eluates as described under "Experimental Procedures." From 12.5 ml of antiserum, 40 mg of anti-Gal antibodies and 60 mg of anti-Lac antibodies were obtained. The antibody preparations were stored in saline/0.1 M phosphate buffer, pH 7.2, at a concentration of 0.5% protein in the frozen state. All analyses were performed on the antibodies isolated from the serum of a single rabbit. Examination of antisera from five other rabbits by the affinity chromatography technique revealed that all samples also contained two sets of antiglycosyl isoantibodies. However, the structural and immunological characterization of these samples has not yet been undertaken.

That both preparations react with the diheteroglycan to yield antigen-antibody precipitins is shown in the agar diffusion patterns in Fig. 2. Other agar diffusion tests showed that both types of antibodies reacted with goat anti-IgG serum, but not with goat anti-IgA or anti-IgM serum. Thus, both the anti-Gal and anti-Lac antibodies are of the IgG immunoglobulin type.

Data on the sedimentation patterns obtained by centrifugation on sucrose density gradients and in the analytical ultracentrifuge have been published (1, 2). Both antibody preparations yielded single symmetrical peaks on ultracentrifugation, characteristic of material of uniform molecular size. Utilizing these data and appropriate formulae (14, 15), sedimentation constants for both preparations were calculated to be 6.9 S and the molecular weights to be $1.5 \times 10^6$.

A variety of carbohydrates and derivatives were tested as potential inhibitors of the precipitin reaction. Some of these data are plotted in Fig. 3. It will be noted in the figure that only lactose and lactosyl derivatives functioned as inhibitors for the anti-Lac antibodies, but galactose, lactose, and glycosides of these compounds functioned as inhibitors for the anti-Gal antibodies. It should also be noted that a 40-fold difference in the concentration of inhibitors for 50% inhibition of the anti-Gal and the anti-Lac antibodies existed.

Gel electrophoretic patterns of the anti-Gal and the anti-Lac antibodies were published earlier (1), showing that the anti-Gal antibodies consisted of six distinct proteins and the anti-Lac antibodies consisted of 12 different proteins. Gel electrophoretic patterns of an equal mixture of the two types of antibodies yielded a composite pattern containing the individual bands of each set. Unstained gels of anti-Gal and anti-Lac antibodies were tested by the agar diffusion method to determine if the individual components were antibody proteins. In these tests it was found that all of the protein components in both antibody preparations yielded a precipitin band with the diheteroglycan. Other polyacrylamide gels of the anti-Gal and anti-Lac antibodies were stained with glycoprotein staining reagents, periodate-Schiff (16) and periodate-dansyl hydrazine (17). Positive tests were obtained with all components of the anti-Gal and the anti-Lac antibodies, showing that all of the components were glycoproteins.

On paper chromatographic analyses of dilute acid hydrolysates of the anti-Gal and the anti-Lac antibodies two principal monosaccharide constituents with $R_f$ values of 0.28 and 0.11 were obtained from both preparations. These compounds in the hydrolysate of the anti-Gal preparation were isolated by preparative paper chromatography. The compound with $R_f$ value of 0.28 yielded a positive test with galactose oxidase (23, 24) and with the hexosamine reagent (25) while the compound with $R_f$ value of 0.11 yielded a positive test with the thio-bar-
bituric acid reagent (26). These tests and the RF values establish the former to be galactosamine and the latter to be neuraminic acid. In the native antibodies the amino sugars are most likely in the N-acetylated form.

In the dilute acid hydrolysates, neutral sugars with RF values of fucose (0.50), mannose (0.41), and galactose (0.33) were also faintly visible. The presence of these monosaccharides in the antibody preparation was established by methylation analysis. The identities of the various components in the methylation mixture were established by retention times on gas chromatography on OV-225 at 170° and characteristic m/e fragments on mass spectrometry. Thus the carbohydrate derivatives identified from the anti-Lac preparation were: 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-fucitol, retention time of 0.55 and m/e fragments of 117 (100), 131 (40), 161 (20), and 175 (10); 1,3,4,5-tetra-O-acetyl-2-O-methyl-fucitol, retention time of 1.43 and m/e fragment of 117 (100); 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-mannitol, retention time of 0.97 and m/e fragments of 45 (70), 117 (100), 161 (50), and 205 (20); 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-galactitol, retention time of 1.17 and m/e fragments of 45 (80), 117 (100), 161 (60), and 205 (30); 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl-mannitol, retention time of 1.80; 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-mannitol, retention time of 2.02 and m/e fragments of 45 (40), 117 (100), and 233 (20) and 1,2,3,5-tetra-O-acetyl-4,6-di-O-methyl-mannitol, retention time of 2.72. The numbers in parentheses represent relative abundance of the fragments with the most abundant fragment assigned an arbitrary number of 100. These values are in agreement with published values (20) and values determined in this laboratory for standard compounds. Methyllated amino sugar derivatives were also detected on temperature program analysis of the methylation mixture but the exact nature of the derivatives has not yet been determined. Also the exact molecular structures of the oligosaccharide chains of the antibodies have not yet been determined. Work is in progress on this aspect of the structures of the anti-Gal and anti-Lac antibodies to determine whether these antibodies possess oligosaccharide chains with the basic structure of oligosaccharides from other types of immunoglobulins (27, 28).

Data on the amino acid composition of the antibodies have been obtained on the two sets of isoantibodies. The values recorded in Table I are average values of triplicate analyses on two different antibody preparations. Also correction factors of 1.13 and 1.25 have been employed in the calculations of the threonine and serine values (29). The number of residues of each amino acid per mole of antibody has been calculated for a molecular weight of 1.5 × 10⁶. Since cysteine, tryptophan, and methionine were not determined in our analyses, values for these amino acids were taken from Table I of Ref. 30 and assumed to be a reasonable estimate for these amino acids.

A partial separation of the anti-Lac isoantibodies was achieved by adsorption on lactosyl-Sepharose and elution with a lactose gradient of 0 to 0.5 M. The gel patterns obtained for several fractions from the column are reproduced in Fig. 4. The gradient elution technique resulted in a resolution of the components into several subsets. A resolution into individual components was achieved by electrofocusing. The electrofocusing pattern for the anti-Lac preparation and gel electrophoretic patterns for the individual components are shown in Fig. 5. The components are numbered with Roman numerals with

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Anti-Gal</th>
<th>Anti-Lac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>109</td>
<td>111</td>
</tr>
<tr>
<td>Threonine</td>
<td>165</td>
<td>170</td>
</tr>
<tr>
<td>Serine</td>
<td>160</td>
<td>159</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>133</td>
<td>132</td>
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<tr>
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<td>100</td>
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<tr>
<td>Glycine</td>
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<td>116</td>
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<tr>
<td>Alanine</td>
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<td>78</td>
</tr>
<tr>
<td>1/2 Cystine*</td>
<td>122</td>
<td>128</td>
</tr>
<tr>
<td>Methionine*</td>
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<td>47</td>
</tr>
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<td>Isoleucine</td>
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<td>84</td>
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</tr>
<tr>
<td>Arginine</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>Tryptophan*</td>
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</table>

* Not yet determined for the anti-Gal and anti-Lac antibodies and in the calculations for this table, the values for the myeloma immunoglobulin (Ref. 30) have been employed for these amino acids.

**Fig. 4.** Gel electrophoretic patterns for the anti-Lac antibody preparations and Fractions 1, 2, 3, and 4 obtained by elution of the antibodies from a lactosyl-Sepharose column with a lactose gradient.

**Fig. 5.** Electrophoresis and gel electrophoretic patterns for the anti-Lac isoantibodies and the individual components. Roman numerals, isoantibody components; M, original mixture of anti-Lac isoantibodies; R, reconstituted sample from the individual anti-Lac isoantibodies.
I being the component with the slowest electrophoretic mobility. Similar results for the anti-Gal preparation have been published (2).

**DISCUSSION**

Two sets of antibodies directed at two different immunodeterminant groups of the same glycan have been isolated from antisera of a rabbit immunized with a vaccine of non-viable cells of *Streptococcus faecalis*, strain N, containing an antigenic glycan of glucose and galactose in its cell wall. The isolation was achieved by affinity chromatography on lactosyl-Sepharose and sequential elution with galactose and lactose solutions. One set, anti-Gal antibodies, is directed against terminal galactose units of the antigen and the other, anti-Lac antibodies, is directed against terminal lactose units of the same antigen. Gel electrophoresis and electrofocusing experiments revealed that both sets of antibodies consisted of several protein components with each component exhibiting antibody activity. Since the members of each set combine with the same structural unit of the antigen, i.e. terminal galactose or terminal lactose units, such antibodies are appropriately termed isoantibodies.

The isoantibodies of the anti-Gal set were separated into individual components by electrofocusing, and six distinct protein species were obtained. The isoantibodies of the anti-Lac set were also separated into individual components and 12 isoantibodies were obtained from this set. The analytical data reported in this paper have been obtained on the antibodies from a single rabbit. However, the antisera from five additional rabbits have been analyzed and in every case two sets of isoantibodies were obtained. Isoantibodies are produced not only against streptococcal glycans, but recent reports show that other antigens such as pneumococcal glycans (31, 32), staphylococcal ribonuclease (33) and synthetic carbohydrate-protein conjugates (34) elicit immune responses, resulting in the synthesis of sets of isoantibodies directed at different structural units of the same antigen. This phenomenon is apparently a general property of the immune response.

To ascertain the chemical nature and the physical properties of the two types of antibodies, experiments have been performed on the mixture of isoantibodies of each set which have been isolated from the serum of a single rabbit. Thus ultracentrifugation by the density gradient method and in the analytical ultracentrifuge yielded sharp symmetrical sedimentation patterns for both sets of antibodies, indicating that the antibodies of each set are of identical molecular size. Further, a mixture of the two types of antibodies yielded a symmetrical pattern showing the molecular weights of the two sets of antibodies were also identical. The molecular weight calculated for both types of antibodies from these data was $1.5 \times 10^6$.

Agar diffusion tests (Fig. 2) showed that the anti-Gal and anti-Lac preparations reacted with the diheteroglycan. Hapten inhibition data (Fig. 3) verify that the anti-Gal antibodies combine with terminal galactose units and the anti-Lac antibodies combine with terminal lactose units of the same antigen. Agar diffusion tests were also used to show that the individual components of each set possessed antibody activity and all are of the IgG class of immunoglobulins. Equine anti-galactose antibodies against the same diheteroglycan have been prepared in another laboratory (35), but these antibodies were shown to be of the IgM class. The immunization regime and the species difference may be responsible for the difference in immunoglobulin types. Also, anti-galactose antibodies directed against terminal galactose units of other antigens (36, 37) have been observed and such antibodies would be useful for comparative studies.

Some differences in the ability of the anti-Lac isoantibodies to bind lactosyl-Sepharose do exist. On elution of the antibodies from lactosyl-Sepharose with a lactose gradient, the anti-Lac antibodies eluted in subsets of two to five components. Apparently the binding constants for the lactosyl moiety and the individual isoantibodies vary sufficiently to allow for separation of the components. With the availability of the individual pure components, this property of the isoantibodies can be more fully explored.

The carbohydrate content and composition of the two sets of antibodies is very similar. In dilute acid hydrolysates of both preparations galactosamine and neuraminic acid, which in the native antibodies are likely acetylated, were present in highest concentration. The neutral sugars, fucose, mannose and galactose were detected as their alditol acetate derivatives on methylation analysis. The structure of the oligosaccharide side chains in the antibodies has not yet been determined. On the basis of the derivatives thus far identified, it appears that the oligosaccharides in the antibody proteins differ somewhat in structure from the oligosaccharides in myeloma immunoglobulins (27, 28). The above finding is not surprising in view of a recent report (38) on the variations in carbohydrate composition of IgG antibodies isolated from rabbits and chickens immunized with fluoresceinyl-globulin conjugates. The amino acid composition of the two sets of isoantibodies is very similar (Table I). The structural differences in the two sets of antibodies and among the individual members of each set most probably reside in the hypervariable regions of the antibody molecules and the electrophoretic differences may be due to different numbers of amide groups in the individual antibodies.

Our findings show that two different structural units of an antigenic diheteroglycan elicit immune responses leading to the synthesis of different types of proteins. Whether the phenomenon is related to the genetic constitution of the animals (39-41), to the manner of attachment of the antigen at the receptor sites or to some yet unknown factor needs to be ascertained. Some evidence for a role for the receptor sites has been presented in recent studies on the nature of the receptor sites of membrane glycoproteins for various types of lectins (42, 43). The overall result with the *S. faecalis* diheteroglycan is the initiation of two series of biochemical reactions, one leading to synthesis of anti-galactose isoantibodies and the other to the synthesis of anti-lactose isoantibodies. Since it has been possible to separate the isoantibodies into individual components, these materials should be useful for studies on questions relating to antibody homology, antibody diversity, biosynthetic pathways and regulatory mechanisms for the synthesis of antibodies.

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