Galactomuramic Acid

CHEMICAL SYNTHESIS, PROPERTIES, ASSAY, AND SURVEY IN SEVERAL BACTERIAL SPECIES*

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SUMMARY

Synthetic D-galacto- and D-glucuronic acids were prepared from the condensation of L-α-chloropropionic acid with benzyl-2-acetamido-4,6-O-benzylidene-2-deoxy-α-D-galacto- or glucopyranoside. The configuration of the D( ) lactate moiety (i.e. 3-O-(o-1-carboxyethyl) D( ) —) obtained by alkaline β elimination, was proven by use of specific D( )- and L( )-lactic acid dehydrogenases. Standard methods commonly used for the detection of muramic acid were found to be unsuitable for distinction between the two products. Thus, although the Barker and Summerson, Morgan-Elson, Park-Johnson, and Rondle-Morgan tests were shown to detect both gluco and galacto epimers of muramic acid (color yields differed slightly), such colorimetric assays could not differentiate between the analogues. Ionophoresis did not distinguish the forms of muramic acid, and thin layer chromatography was found to be suitable for distinction only under carefully controlled conditions.

Cation exchange chromatography, according to Gardell, was used successfully in the separation of the glucuronic and galactomuramic acids, but it was found that glucosamine co-elutes with galactomuramic acid. The most rapid and precise detection method was that in which a commercial amino acid analyzer was used. Detection of nanomole quantities of material was possible. The muramic acid content of 16 bacterial species was determined. Only the glucuronic acid analogue was found.

Muramic acid has been detected by alkaline silver staining or ninhydrin reactivity on chromatograms of hydrolyzates of the

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1 R. W. Wheat, unpublished observations.
paper chromatography. Naturally occurring muramic acid (glucuronic acid) was isolated from Micrococcus lysodeikticus (8). Glucuronic acid was also chemically synthesized by use of both the α-benzyl and α-methyl glucoside derivatives by using established methods and routes (10, 13-17). A sample of synthetic glucuronic acid was furnished by Dr. J. T. Park, Department of Microbiology, Tufts University School of Medicine. \( \nu (-) \)-Lactic dehydrogenase from Lactobacillus casei and a \( \nu (-) \)-lactic acid standard were the kind gifts of Drs. Rebecca C. Garland and N. O. Kaplan, Brandeis University. The \( \nu (+) \)-lactic acid standard was purchased from Sigma. 3-Acetylpyrindine DPN+ was obtained from P-L Biochemicals, and \( \nu (+) \)-lactic dehydrogenase was purchased from Worthington.

Acetone powdered cells of Pseudomonas sacchariphila were obtained from Dr. A. Ita, University of Kentucky, Lexington; Rhizobium japonicum cells were donated by Dr. Gerald Elkan, North Carolina State University, Raleigh; a R. sphaeroides cell wall preparation was generously given by Dr. David Platt, University of Pittsburgh. Veillonella parvula cells were received from Dr. Stephen Mengenhagen, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland; Micrococcus lysodeikticus and Clostridium acetobutylicum cells were purchased from Worthington. The other organisms were grown and harvested and acetone powders and cell walls were prepared as described previously (8, 11, 18-21).

**Partition Chromatography**—Ascending thin layer cellulose chromatography and descending paper chromatography were carried out with the following solvents: (a) 1-butanol-pyridine-water (0.4:4:3); (b) 1-butanol-acetic acid-water (5:1:2); (c) ethylacetate-pyridine-acetic acid-water (5:5:1:3); (cZ) phenol-water (70:30). Spots were visualized with ninhydrin and alkaline silver nitrate.

**Ion Exchange Chromatography**—Amino sugars were separated according to Gardell on Dowex 50-H columns, 1 x 60 cm, eluted with 0.30 x n HCl (22). Separation of galactomuramic acid by use of a Beckman/Spinco 120C amino acid analyzer was achieved on a column, 55 x 0.9 cm, of Dowex 50-H form Aminex A-4 resin (Bio-Rad Laboratories, Richmond, California), eluted at 68 ml per hour at 50°, first with citrate buffer, pH 3.15, 0.2 N NaCl, then and with a citrate buffer, pH 4.25, 0.20 N NaCl. The second buffer contained 3% n-propanol and 2.3% benzyl alcohol.

The buffer change made at 75 min was detected on recorder tracings just after alanine. The amino acid analyzer was modified to split the column effluent and analyze for amino groups by ninhydrin reactivity and reducing groups by a coupled ferri-cyanide-arsenomolybdate assay, details of which will be published separately.

**Electrophoresis**—Ionophoresis was carried out on Whatman No. 1 paper in 0.05 M pyridine acetic acid buffer, pH 6.4, at 8 volts per cm with a Saffant flatbed electrophoresis apparatus.

**Analyses**—Reducing sugars were determined by the ferricyanide assay of Park and Johnson (23). The Roodle-Morgan (24) modification of the Elson-Morgan assay was used for the determination of 2-amino sugars. Amino sugars and 2- acetamido sugars were also determined by a modification of the Morgan-Elson procedure (25). Lactic acid was assayed chemically by the method of Darker and Summerson (26), with the use of lithium lactate as standard. Spectra were recorded on a Cary 14 spectrophotometer. Following \( \beta \) elimination by alkaline treatment of muramic derivatives according to the procedure described by Tipper (27), the enzymatic assay of \( \nu (-) \) and \( \nu (+) \)-lactic acid was utilized as described by Dennis (28) and modified by Drs. N. O. Kaplan and Rebecca Garland as follows to establish the configuration of the 1-carboxethyl (lactate) moiety. \( \nu (-) \)-Lactic acid assays were run with 1.0 M Tris buffer at pH 9.0, while glycine buffer, 1.0 M at pH 10.0, was utilized for \( \nu (+) \)-lactic acid assays. Enzyme assays were followed at 365 mp with a Gilford model 222 photometer with recorder and 210 D cuvette positioner. Melting points were obtained with a Thomas Hoover capillary melting point apparatus and are not corrected. Optical rotations were obtained with a Rudolph and Sons, Inc., model 80 polarimeter. Microanalyses were done by Galbraith Laboratories, Inc., Knoxville, Tennessee; the analytical samples were dried over \( \text{P}_2\text{O}_5 \) in vacuo for 2 hours at 60°.

**EXPERIMENTAL RESULTS**

**Synthesis of \( \nu \)-Galactomuramic Acid (2-Amino-3-O-(D-1-carboxethyl)-2-deoxy-L-galactopyranose)**

The galacto analogue of muramic acid was synthesized via the route described by Strange and Kent (10), as modified by Matsushika and Park (14) and Flowers and Jeanloz (16), for the synthesis of glucuronic acid as follows. Benzyl-2-acetamido-4,6-0-benzylidene-2-deoxy-\( \alpha \)-D-galactopyranoside (I), (17) was condensed with L-\( \alpha \)-chloropropionic acid (13-15) in the presence of sodium hydride to give benzyl-2-acetamido-3-O-(D-1’-carboxethyl)-4,6-0-benzylidene-2-deoxy-\( \alpha \)-D-galactopyranoside (II). Configuration was assigned to the carboxyethyl group in analogy to the fact that \( \nu \)-glucuronic acid results by the use of L-\( \alpha \)-chloropropionic acid under similar conditions (13, 14, cf. Reference 29). Hydrolysis of II with 3 N HCl gave the galacto analogue of muramic acid, a product homogeneous chromatographically and electrophoretically. Removal of the benzylidene group from II by 60% aqueous acetic acid gave benzyl-2-acetamido-3-O-(D-1’-carboxethyl)2-deoxy-\( \alpha \)-D-galactopyranoside (III). Hydrogenolysis of III yielded a compound (N-acetylgalacturonic acid) which, on hydrolysis and treatment as described in the case of the hydrolysis of II, yielded free galactomuramic acid.

Benzyl-2-acetamido-3-O-(1-carboxethyl)-4,6-0-benzylidene-2-deoxy-\( \alpha \)-D-galactopyranoside (II)

Benzyl-2-acetamido-4,6-0-benzylidene-2-deoxy-\( \alpha \)-D-galactopyranoside (1.10 g, 0.00275 mole) was dissolved in 80 ml of dry dioxane at 60°. To this solution 0.9 g of sodium hydride suspended in 2.2 ml of mineral oil were added, the temperature was raised to 95°, and the mixture was stirred for 1 hour. After cooling to 65°, a solution of 1.3 ml (0.018 mole) of L-\( \alpha \)-chloropropionic acid in 20 ml of dry dioxane was added dropwise and the reaction mixture was stirred for an hour. Then 1.32 g of sodium hydride suspended in 2.2 ml of mineral oil were added, and the stirring was continued overnight at 65°. After cooling to room temperature, ice-cold water was added slowly until a clear solution was obtained. The dioxane was removed by distillation under reduced pressure at 50°, water (50 ml) was added to the residue, and the aqueous solution was extracted with chloroform to remove mineral oil. The aqueous solution was then cooled to 0°, and upon neutralization by addition of cold
6 N HCl, a white solid separated which was extracted immediately with 50 ml of chloroform. The chloroform layer was washed with water and dried over anhydrous sodium sulfate, and the solvent was removed by distillation in a vacuum. The solid residue was recrystallized from aqueous alcohol. The yield was 0.7 g; the mother liquor yielded an additional 0.1 g (61% total). After two recrystallizations, the melting point was 208-210°, $[\alpha]_D^{25} +208^\circ$ (c, 0.86, in methanol).

**C₆H₁₆O₇N (471.49)**

Calculated: C 63.69, H 6.20, N 2.97
Found: C 63.65, H 6.33, N 2.93

**Benzy-2-acetamido-3-O-(1'-carboxyethyl)-2-deoxy-α-D-galactopyranoside (III)**

A solution of II (0.472 g, 0.001 mole) in 20 ml of 60% aqueous acetic acid was refluxed for 1 hour. The solution was removed by distillation at 40° in a vacuum, water was added, and the mixture was again distilled in a vacuum to dryness. The residue was dissolved in 25 ml of water and 2 ml of N NaOH were added. After 2 hours the solution was titrated with Dowex 50-H⁺ to remove Na⁺, filtered, and taken to dryness in a vacuum at 40°. The crystalline residue was recrystallized from methanol-ethyl acetate. The yield was 0.29 g (75%), m.p. 178° (shrinkage, 158°), $[\alpha]_D^{25} +175^\circ$ (c, 1, in methanol).

**C₆H₁₆O₇N (383.39)**

Calculated: C 56.38, H 6.57, N 3.65
Found: C 56.38, H 6.64, N 3.78

**2-Amino-3-0-(1'-carboxyethyl)-2-deoxy-α-D-galactose (Galactosamine Analogue of Muramic Acid, or Galactomuramic Acid (IV))**

In a sealed tube, 0.2 g of II was heated with 10 ml of 3 N hydrochloric acid at 100° for 4 hours. The solution was evaporated to dryness under reduced pressure at 50°, and the residue was extracted with water (5 ml) and shaken with ether (10 ml). Lactones were hydrolyzed as follows by a modification of the procedure described by Matsushima and Park (14) and Matsushima, Park and Montague (15). The aqueous layer was titrated with Dowex 1 carbonate form to pH 5.5 and then filtered. The filtrate was carefully titrated with Dowex 50 H⁺ to pH 5.5 and filtered. The filtrate was lyophilized, yielding a white powder. It was crystallized from methanol-ethyl acetate (1:2) and recrystallized from the same mixture three times to give 21 mg (20% yield) of a hygroscopic compound (m.p. 130-131°, with decomposition). The product was homogeneous chromatographically and electrophoretically. The $R_f$ was 0.18 on Whatman No. 1 paper, solvent system B, with the use of silver nitrate and ninhydrin as staining agents, and $[\alpha]_D^{25} +138^\circ$ at 3 min to +128° was constant after 6 min (c, 1, in water).

**C₅H₁₀O₆N (251.24)**

Calculated: C 43.05, H 6.97, N 5.58
Found: C 43.19, H 6.98, N 5.23

**Assay and Configuration of Lactic Acid (3-O-(1-carboxyethyl)-) Substituent**

Measurement of the lactic acid (3-O-(1-carboxyethyl)-) moieties of muramic acid by the Barker and Summermson procedure

**TABLE I**

**Determination of $\beta$(-) and $\alpha$-(+)-lactic acid (i.e. 1-carboxyethyl) content of α-benzyl-N-acetylgalactomuramic and -glucosamuramic acids**

<table>
<thead>
<tr>
<th>Treatment and assay</th>
<th>Galactomuramic acid</th>
<th>Glucosamuramic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmoles/ml</td>
<td>%</td>
<td>nmoles/ml</td>
</tr>
<tr>
<td>1. Prior to treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. By weight</td>
<td>532 100</td>
<td>600 100</td>
</tr>
<tr>
<td>b. By amino acid analyzer assay as in Fig. 6</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>c. By 3'-lactic dehydrogenase</td>
<td>428 80.5</td>
<td>380 63.3</td>
</tr>
<tr>
<td>2. After acid hydrolysis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. By amino acid analyzer</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>b. By 3'-lactic dehydrogenase</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>3. After alkaline hydrolysis of acid hydrolyzate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. By amino acid analyzer</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>b. $\beta$(-)-lactic dehydrogenase</td>
<td>0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>c. $\alpha$-(+)-lactic dehydrogenase</td>
<td>436 82.0</td>
<td>420 70.0</td>
</tr>
</tbody>
</table>

(20), as suggested by Kent and Strange (30), indicated that the two muramic acid analogues gave approximately the same color yield. Standard curves showed that glucosamuramic acid gave 95% and galactomuramic acid 105% the color values, per mole, of the lithium lactate standard.

The configuration of the 1-carboxyethyl groups was determined by the $\beta$ elimination procedure described by Tipper (27). α-Benzyl-N-acetyl derivatives of glucosamuramic acid and galactomuramic acid were hydrolyzed with 3 N HCl at 75 min at 123°. The hydrolysates were dried in vacuo at 28° and re-evaporated several times after addition of water to remove HCl. Alkaline treatment at pH 12.5 for 2 hours followed, according to Tipper (27). The results of enzymatic lactic acid dehydrogenase assays of the alkaline-treated samples are shown in Table I. Somewhat greater quantities of product were detected enzymatically (i.e. D-lactate) than by means of the amino acid analyzer (i.e. free
muramic acids). This can be attributed to the enzymatic detection of lactate produced from free muramic acid, acetamidomuramic acid and lactam forms of muramic acid. The last two forms of muramic acid are not detected by ion exchange chromatography. The carboxyethyl moiety of the synthetic glucomuramic and galactomuramic acids is thus shown to be in the D configuration.

Properties of Galactomuramic Acid (IV)

The properties of natural and synthetic glucomuramic acid and of the analogue, galactomuramic acid, were examined in the following ways. (a) Reactivities in several colorimetric assays were compared; (b) mobilities were compared with those of glucosamine-HCl on thin layer cellulose and paper chromatograms and on ionophosphogamy; and (c) elution positions relative to glucosamine were compared on various cation exchange elution systems.

Colorimetry: Amino Sugar and Reducing Sugar Assays

Rondle-Morgan Reaction—The expected orange color seen with glucomuramic acid was also observed with galactomuramic acid when assayed by the Rondle-Morgan modification of the Elson-Morgan assay. In Fig. 1, the absorption spectra of glucomuramic and galactomuramic acids ($\lambda_{\text{max}}$ of both occurs at 505 nm) are compared with that of glucosamine ($\lambda_{\text{max}}$ at 530 nm to 540 nm).

Morgan-Elson Reaction—Galactomuramic acid yielded about half the color value of glucomuramic acid under conditions optimal for glucosamine in a modified Morgan-Elson reaction (25).

Reducing Sugar Assay—Both muramic acids reacted in the fericyanide reducing sugar assay of Park and Johnson (23). However, compared with a glucosamine standard (100%), galactomuramic acid (55%) was much less sensitive than glucomuramic acid (85%) in this assay.

Partition Chromatography

As shown in Table II and Fig. 2, galactomuramic acid and glucomuramic acid can be separated in several solvent systems on paper or thin layer cellulose plates. However, as indicated in Fig. 2, mobilities are close enough in some solvents so that variability in application of the compound to the chromatogram or unfortunate choice of solvents could cause confusion between the two compounds.

Ionophoretic Mobility

Fig. 3 shows the similarity of mobility of the two muramic acid analogues compared with glucosamine upon ionophoresis at pH 6.4.

Cation Exchange Chromatography: Gardell Column Chromatography

The elution pattern on a Gardell column of galactomuramic acid, glucosamine, and glucomuramic acid is shown in Fig. 4. Each compound was eluted separately under identical conditions from a Dowex 50-H (X8, 200 to 400 mesh) column, 1 X 60 cm, with 0.30 N HCl collecting 2.5 ml fractions. Aliquots removed

![Fig. 1. Absorption spectra of amino sugars in the Rondle-Morgan reaction. Glucosamine-HCl (A), galactomuramic acid (B), and glucomuramic acid (C).](http://www.jbc.org/)

![Fig. 2. Thin layer cellulose chromatogram comparing mobilities of glucosamine-HCl (1) with glucomuramic acid (2) and galactomuramic acid (3); solvent system B; ninhydrin stain.](http://www.jbc.org/)
were neutralized with an equal volume of 0.3 N NaOH and assayed for reducing sugar by the ferricyanide assay. The same elution pattern, as seen in Fig. 4, of two peaks designated Peak I and Peak II, was observed when the three compounds were mixed together and then eluted. The two peaks were pooled separately, dried in a vacuum at 50°, and redissolved in 1 ml of water and then assayed by the Rondle-Morgan procedure. The presence of galactomuramic acid in the usual glucosamine peak could be detected by a shift toward 505 mp in the wave length of maximum absorbance which was dependent on the ratio of glucosamine and galactomuramic acid. When these two peaks were chromatographed on thin layer cellulose, the presence of galactomuramic acid and glucosamine in Peak I was confirmed, as was the presence in Peak II of glucomuramic acid (Fig. 5). However, spots due to formation of lactones or lactams (14, 15, 29) were also observed in peaks containing either of the muramic acids which were evaporated in vacuo to remove acid. These could be removed by careful de-ionization or both alkaline hydrolysis and de-ionization as outlined in the above section on synthesis, followed by lyophilization. The bulk of the glucosamine HCl could be removed from the mixed sample in Peak I by an initial treatment near pH 5.5 to 6.0 with Dowex 50-H+, leaving galactomuramic acid, because the latter compound exists as a dipolar ion at this pH, whereas glucosamine exhibits a net positive charge in acid solutions. As shown in Fig. 5, Spots 3 and 7, the same respective lactones could be produced from the chromatographically homogeneous glucomuramic and galactomuramic acid samples by merely evaporating them to dryness at 40-50° in 0.3 N HCl.

Separation and Identification by Use of Commercial Amino Acid Analyzer

As shown in Fig. 6, the separation of galactomuramic acid and glucomuramic acid in the presence of expected or usual bacterial cell wall hydrolyzate components, i.e. glucosamine, alanine, glutamic acid, and diaminopimelic acid, as well as other protein component amino acids, can be achieved with a buffered elution system with commercially available amino acid analyzers. This separation is much faster than our previously reported system (32, 33). Galactosamine uronic acid, like galactomuramic acid, is eluted by 0.30 N HCl in the same peak with glucosamine from Dowex 50-H+ columns by the procedure of Garidel (22). However, under the conditions used for the separation shown in Fig. 6, galactosamine uronic acid is eluted with methionine. Galactosamine uronic acid therefore does not interfere with identification of either glucosamine or the muramic acids in this system.

Assay for Glucomuramic and Galactomuramic Acids in Bacteria

Cell wall preparations of varied purity and whole acetone powdered bacteria from species of several taxonomic groups were scanned for the presence of glucomuramic acid and galactomuramic acid by the procedure shown in Fig. 6. Hydrolyzates of Citrobacter freundii 8090 and C. ballerup phenol-insoluble residues were used to compare recoveries from 18-hour 6 N HCl at 100° hydrolyzates with 2-hour 3 N HCl at 125°. Approximately equivalent amounts of glucomuramic acids were observed in both

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**Fig. 3.** Electrophoretic mobilities of glucosamine-HCl (A), glucomuramic acid (Micrococcus lysodeikticus) (B), synthetic glucomuramic acid (C and E), and galactomuramic acid (D). The conditions are as described in the text. Spots are detected with silver nitrate.

**Fig. 4.** Cation exchange chromatography of amino sugars according to Garidel (22). Glucosamine-HCl (A), galactomuramic acid (B), and glucomuramic acid (C). The conditions are as described in the text.
Fig. 5. Paper chromatogram of peaks from Gardell column chromatography of amino sugars shown in Fig. 4. Glucosamine-HCl standard (1), Peak I (3), galactomuramic acid heated in 0.30 M HCl (5), galactomuramic acid standard (4), glucomuramic acid standard (6), Peak II (6), glucomuramic acid heated in 0.3 M HCl (7); solvent system B; ninhydrin stain.

Fig. 6. Beckman/Spinco model 120C amino acid analyzer trac- ing. The numbers below the peaks correspond to 0.1 µmole each of hydroxyproline (1), aspartic acid (2), threonine (3), serine (4), galactomuramic acid (5), glucomuramic acid (6), glutamic acid (7), proline (8), glycine (9), alanine (10), cystine (11), valine (12), α,ε-diaminopimelic acid (13), methionine or galactosamine uronic acid (14), isoleucine (15), leucine (16), norleucine (17), tyrosine (18), phenylalanine (19), glucosamine (20), and galactosamine (21). The conditions are as described in the text under “Ion Exchange Chromatography” in “Methods.” Solid lines, 570 µm; dotted lines, 440 µm.

hydrolyzates, although amino acid recoveries were slightly greater in the 18-hour 6 N HCl procedure. With the shorter 2-hour 3 N HCl procedure, only glucomuramic acid was observed in hydrolyzates of the various bacterial preparations examined, as listed in Table III. Values listed are results of integration of areas under the curves, with the use of Technicon integrator calculator (model AAG). Serine and glutamic acid values are included for comparison with those found for glucomuramic acid.
with galactomuramic acid. The most rapid and precise detection of galactomuramic acid, but it was found that glucosamine co-elutes with muramic acid. Thus, although the Barker and Summerson, Morgan-Morgan (4, 27, 34) was proven by use of the specific D(-)- and L(+)lactic acid dehydrogenases. This had not been previously shown for muramic acid prepared by this route of chemical synthesis. For muramic acid analogues other than the gluco form has been shown feasible, from various species (4, 35-37). The recognition of muramic acid analogues other than the glucose form has been shown feasible, and alert observations by investigators in the future may reveal a muramic acid of a configuration other than that of D-glucouramuramic acid. It may further be expected that the glucosamine moiety of murain might also be substituted with galactosamine or some other sugar or amino sugar. Detection of nanomole quantities of material was possible. The survey of muramic acid content of 16 bacterial species by means of an amino acid analyzer system revealed the presence of only the glucouramuramic analogue under our conditions of assay. It is not unreasonable to believe that the galactouramuramic acid analogue may yet be found in the peptidoglycan cell wall structure of unexamined species in analogy to the occurrence of genetic drift exhibited by the variety of substitutions in both the muramyl peptides and bridging peptides recently recognized in murein from various species (4, 35-37). The recognition of muramic acid analogues other than the glucose form has been shown feasible, and alert observations by investigators in the future may reveal a muramic acid of a configuration other than that of D-glucouramuramic acid. It may further be expected that the glucosamine moiety of murain might also be substituted with galactosamine or some other sugar or amino sugar. Detection of nanomole quantities of material was possible. The survey of muramic acid content of 16 bacterial species by means of an amino acid analyzer system revealed the presence of only the glucouramuramic analogue under our conditions of assay. It is not unreasonable to believe that the galactouramuramic acid analogue may yet be found in the peptidoglycan cell wall structure of unexamined species in analogy to the occurrence of genetic drift exhibited by the variety of substitutions in both the muramyl peptides and bridging peptides recently recognized in murein from various species (4, 35-37). The recognition of muramic acid analogues other than the glucose form has been shown feasible, and alert observations by investigators in the future may reveal a muramic acid of a configuration other than that of D-glucouramuramic acid. It may further be expected that the glucosamine moiety of murain might also be substituted with galactosamine or some other sugar or amino sugar.

**DISCUSSION**

Synthetic D-galacto- and D-glucouramuramic acids were prepared from the condensation of L-α-chloropropionic acid with benzyl-2-acetamido-4,6-O-benzylidene 2 deoxy α D galacto or glucopyranoside. The configuration of the D(-)-lactate moiety (i.e. 3-O-(1-carboxyethyl)) obtained by alkaline β elimination (4, 27, 34) was proven by use of the specific D(-)- and L(+)-lactic acid dehydrogenases. This had not been previously shown for muramic acid prepared by this route of chemical synthesis (10, 14, 16) and confirms expectations based on knowledge of chemical mechanisms (13). Standard methods commonly used for the detection of muramic acid were found to be unsuitable for distinction between the two configurational isomers of muramic acid. Thus, although the Barker and Summerson, Morgan-Ellson, Park-Johnson, and Rondle-Morgan tests were shown to detect both forms of the synthetic muramic acid (color yields differing slightly), such colorimetric assays could not differentiate between the analogues. Ionophoresis did not distinguish the forms of muramic acid, and thin layer chromatography was found to be suitable for distinction only under carefully controlled conditions.

Cation exchange chromatography, according to Gardell, was used successfully in the separation of the glucouramuramic acid and galactouramuramic acid, but it was found that glucosamine co-elutes with galactouramuramic acid. The most rapid and precise detection method was that in which a commercial autoanalyzer was used.
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