GAS–LIQUID CHROMATOGRAPHY OF CARBOHYDRATE DERIVATIVES: 
THE SEPARATION AND QUANTITATIVE DETERMINATION OF 
SYNTHETIC AND NATURALLY OCCURRING GLYOSYL 
DERIVATIVES OF AMINO ACIDS*

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ABSTRACT

A gas–liquid chromatographic separation of the trimethylsilyl derivatives of 
several synthetic and naturally occurring glycosyl derivatives of amino acids is 
described. This technique may be used for the qualitative and quantitative determination of several compounds of biological interest including 3-<i>O</i>-<i>β</i>-<i>D</i>-xylopyranosyl-
<i>L</i>-serine, 3-<i>O</i>-<i>α</i>-<i>L</i>-arabinopyranosyl-<i>L</i>-serine, 5-<i>O</i>-<i>β</i>-<i>D</i>-galactopyranosyloxy-<i>D</i>,<i>L</i>-lysine, 
and 2-acetamido-<i>N</i>-(<i>L</i>-aspart-4-oyl)-2-deoxy-<i>β</i>-<i>D</i>-glucopyranosylamine. In addi-
tion, the mobilities of these glycosyl derivatives of amino acids in paper chromato-
graphic and paper electrophoretic systems are reported.

INTRODUCTION

Substituted and unsubstituted glycosyl derivatives of amino acids are found in a number of biological fluids and tissues. In tissues, they comprise the branch point between protein and covalently bound carbohydrate residues in acid mucopolysaccharides, glycoproteins, and collagen. For purposes of classification, protein–carbo-
ydrate compounds may be divided into two groups. In one group, the bond or branch point involves the amide nitrogen of <i>L</i>-asparagine, whereas in the second, the carbohydrate portion is linked through a glycosidic bond to the hydroxyl group of an amino acid contained in the polypeptide chain. Unsubstituted glycosyl derivatives of amino acids occur in urine. These are believed to originate from the catabolism of tissue protein–carbohydrate complexes.

During the course of studies concerned with the metabolism of vertebrate glycoproteins, a method was required that would allow separation and estimation of glycosyl derivatives of amino acids in microgram quantities. The efficacy of gas–
liquid chromatography for the analysis of biological compounds, including carbo-
ydrates and amino acids prompted development of the method described in

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this paper. We have selected the per(trimethylsilyl)ation procedure introduced by Sweeley et al. and the internal standard technique of Ray.

**RESULTS AND DISCUSSION**

The resolution of a mixture of standard glycosyl derivatives of amino acids at programmed temperature and isothermal modes is shown in Fig. 1. Of the seven compounds tested, only 3-\(\alpha\)-D-glucopyranosyl- and 3-\(\beta\)-D-galactopyranosyl-L-serine exhibited a significant peak overlap at an isothermal temperature (270°); when a temperature program was utilized, complete resolution was obtained. Some variation in the resolution of 3-\(\alpha\)-D-glucopyranosyl- and 3-\(\beta\)-D-galactopyranosyl-L-serine was observed with column packings prepared at different times and with commercially prepared packing. These differences in relative retention time (<5%) are not attributable to variability in the manner in which the columns were packed, since those prepared from the same batches of packing material gave essentially identical results. In no instance was the overlap of 3-\(\alpha\)-D-glucopyranosyl- and 3-\(\beta\)-D-galactopyranosyl-L-serine too extensive for identification or quantitation when the gas-liquid chromatograph was temperature programmed. In general, as

found with the trimethylsilyl derivatives of free sugars, there is an increase in retention time as the number of carbon atoms is increased. Each test compound could be reliably determined under the conditions described. This was indicated by (a) the appearance of a single peak with each test compound; (b) a linear detection response (Fig. 2); and (c) a variation of ±1.2% in relative retention time on duplicate derivatizations and injections. All trimethylsilyl derivatives except that of 2-acetamido-\(N\)-(L-aspart-4-oyl)-2-deoxy-\(\beta\)-D-glucopyranosylamine were stable over a 12-h period when maintained under anhydrous conditions. The latter derivative exhibited a second peak (relative retention time 1.69) after being kept 4–5 h at room temperature.

Although a linear detector response was obtained for each trimethylsilyl derivative, the slope of the detector response for the derivative of 2-acetamido-\(N\)-(L-aspart-4-oyl)-2-deoxy-\(\beta\)-D-glucopyranosylamine was less than that noted with the other glycosyl derivatives of amino acids tested.

In the initial phases of this work, each of the chemically synthesized glycosyl derivatives of amino acids (including 3-\(O\)-\(\alpha\)-L-arabinopyranosyl-, 3-\(O\)-\(\beta\)-D-xylopyranosyl-, 3-\(O\)-\(\beta\)-D-glucopyranosyl-, and 3-\(O\)-\(\beta\)-D-galactopyranosyl-L-serine) exhibited double peaks (Table I). In all cases, the second minor peak disappeared after recrystal-
TABLE I
GAS CHROMATOGRAPHY OF TRIMETHYLSILYL DERIVATIVES OF MONOSACCHARIDES AND GLYOSYL DERIVATIVES OF AMINO ACIDS

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Temperature Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Isothermal (270°)</td>
</tr>
<tr>
<td>3-O-α-L-Arabinopyranosyl-L-serine</td>
<td>0.29</td>
</tr>
<tr>
<td>3-O-β-D-Xylopyranosyl-L-serine</td>
<td>0.41</td>
</tr>
<tr>
<td>3-O-β-D-Galactopyranosyl-L-serine</td>
<td>0.55</td>
</tr>
<tr>
<td>3-O-β-D-Glucopyranosyl-L-serine</td>
<td>0.62</td>
</tr>
<tr>
<td>Sucrose</td>
<td>1.00</td>
</tr>
<tr>
<td>5-O-β-D-Galactopyranosylxy-D,L-lysine</td>
<td>1.52</td>
</tr>
<tr>
<td>2-Acetamido-N-(aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine</td>
<td>2.48</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td></td>
</tr>
<tr>
<td>D-Xylose</td>
<td></td>
</tr>
<tr>
<td>D-Galactose</td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td></td>
</tr>
</tbody>
</table>

The values given in this Table are retention times relative to per(trimethylsilylated) sucrose at an isothermal and at a programmed temperature mode. Each value represents an average of 4 runs; a maximum deviation in retention time of ±0.02 was found. The retention time for the internal standard, per(trimethylsilylated) sucrose, was 8.9 min in the isothermal mode and 28.3 min in the programmed temperature mode. Trimethylsilyl derivatives of L-hydroxylysine, L-aspartic acid, and L-serine had retention times of less than 0.25 when columns were programmed at 200–299°C. Retention time for a minor component detected in each of the crude preparations of chemically synthesized glycosyl derivatives of amino acids.

Resolution of the anomers would be expected in view of the results of Sweeley et al. In addition, Sweeley et al. found that the trimethylsilyl derivatives of di- and tri-saccharides could be separated on silicone columns at elevated temperatures. In the present study, an attempt was made to establish the retention time for per(trimethylsilylated) O-α-D-glucopyranosyl-(1→2)-β-D-galactopyranosylxy-(1→5)-D,L-lysine. No peak was obtained under the isothermal or programmed gas-chromatographic conditions described in this paper or under a variety of other experimental conditions including (a) incubation in the presence of Regisil for prolonged-time periods, (b) heating with Regisil at 75°C for periods up to 2 h, and (c) maintaining an isothermal mode of 300°C for periods up to 4 h. These results suggest that the upper limit for detection of per(trimethylsilylated) glycosyl derivatives of amino acids in this system approximates 27 carbon atoms.

The present study of urine specimens fractionated on Dowex 2 ion-exchange resin suggests that several pentoses and hexoses occur in urine covalently bound to...
compounds containing negatively charged groups. The trimethylsilyl derivative of 3-0-β-D-xylopyranosyl-L-serine isolated from urine or from chondromucoprotein exhibited the same relative retention time as the trimethylsilyl derivative of the chemically synthesized compound. Estimation by this procedure of 3-0-β-D-xylopyranosyl-L-serine in four 24-h urine specimens from a normal individual gave an average of 14.7 μmoles of 3-0-β-D-xylopyranosyl-L-serine/m²/24 h. Subsequent

studies with a less specific, but somewhat simpler, procedure (Fig. 3) have shown a range of 18.0 to 42.7 (mean value 24.0) μmoles of 3-0-β-D-xylopyranosyl-L-serine/m²/24 h for urine from normal individuals and concentrations as high as 146 μmoles of 3-0-β-D-xylopyranosyl-L-serine/m²/24 h in patients suffering from connective tissue diseases.

The presence of covalently bound arabinose in human urine and brain tissue, and a report that L-arabinose occurs as a linkage point between peptide and hyaluronic acid, perhaps in a manner analogous to that found for D-xylose in chondroitin 4-sulfate, prompted the chemical synthesis of 3-0-α-L-arabinopyranosyl-L-serine. Free 3-0-α-L-arabinopyranosyl-L-serine has not been demonstrated in human urine, but the synthetic compound serves as an acceptor for D-glucose residues when incubated in the presence of uridine-5-(α-D-glucopyranosyl pyrophosphate) and a novel glucosyltransferase obtained from embryonic-chicken brain. These results suggest a possible biological role for this compound.

*Excretion relative to body surface.
**In a recent report, Katzman concluded that arabinose is not a component of bovine-cerebral hyaluronic acid nor a component of crude mucopolysaccharide from bovine brain.
EXPERIMENTAL

Methods. — Descending paper chromatography was conducted on Whatman No. 40 and Schleicher and Schuell No. 589 Green papers with the following solvent systems (v/v): ethyl acetate–pyridine–water (50:2:7, upper layer, solvent A); 2-methyl-2-propyl alcohol–30% formic acid–water (5:1:1, solvent B); 2-butanone–propionic acid–water (3:1:12, solvent C); butyl alcohol–acetic acid–water (4:1:5, upper layer, solvent D); and ethyl acetate–pyridine–water (8:2:1, solvent E). Paper electrophoresis was conducted in a Gilson High Voltage Electrophorator (Model D) with Whatman 3MM paper and Isopar H (Humble Oil Co., Houston, Texas) as the coolant. The following conditions were used: 0.15M formic acid–1.6M acetic acid, pH 2.1, for 45 min at 58v/cm (buffer A); 50mM pyridine acetate, pH 6.5, for 30 min at 47 v/cm (buffer B). The chromatograms and electrophoretograms were air dried, sprayed with 0.2% ninhydrin in 95% acetone containing 2% pyridine, and heated at 100° to locate the compounds.

Gas–liquid chromatography. — Two Packard gas chromatographs were used in this study, a Series 7508 and a Model 7401. Each instrument was equipped with a temperature programmer and a dual-flame ionization detector. Coiled glass columns (4 mm i.d. and 244 cm in length) were packed with 3% OV-17 on 80/100 mesh Gas Chrom Q (Applied Sciences Laboratories, State College, Pa., Lot 767/60). The packed columns were conditioned with nitrogen at 270° for one week prior to use. This treatment reduced bleeding of the stationary phase to a level at which an electronic differential operation to compensate for baseline drift was not required. A number of column septa were employed. Of these, silicone rubber septa (Applied Science Laboratories, Type W-10) were found to withstand the high detector-cell temperature (370°) with minimal contribution to baseline response. The use of stainless steel in place of polypropylene tubing for detector leads, or stainless steel columns in place of glass columns resulted in degradation of the trimethylsilyl derivatives and the formation of extraneous peaks. Unless otherwise specified, the g.l.c. conditions were: column temperature 210–299°, at a program rate of 2.5°/min; detector cell temperature, 370°; injection temperature, 330°; hydrogen flow to detector, 30 ml/min; air to detector burner, 300 ml/min; carrier nitrogen, 45 ml/min; and chart speed 76 cm/h. Satisfactory resolution was obtained at program rates as high as 5°/min.

Column calibration and calculations. — The peak area was determined by multiplying the height of the peak by the width at half height. A detection-response curve was obtained for each glycosyl derivative of an amino acid by chromatographing varying amounts of the corresponding trimethylsilyl derivative in the presence of a constant amount of per(trimethylsilyl)ated sucrose. The ratio of the total area of the derivative peak to that of the standard was plotted against the ratio of the weight of the amino acid derivative to that of sucrose, the internal standard.

Preparation of trimethylsilyl derivatives. — Aliquots containing 5 nmoles of sucrose as internal standard and suitable amounts of amino acid derivatives (1–25 nmoles) were pipetted in Kontes Microflex tubes, 0.3-ml capacity (Kontes Glass Carbohydr. Res., 22 (1972) 369–379
Co., Vineland, New Jersey) and evaporated to dryness on a rotary desiccator in vacuo. N,N-Dimethylformamide (5 μl), and Regisil [N,N-bis(trimethylsilyl)trifluoroacetamide and 1% trimethylchlorosilane (Regisil Chemical Co., Chicago, Illinois)] (25 μl) were added under anhydrous conditions. The tubes were sealed with teflon caps, vigorously shaken, and kept for 90 min at room temperature. Suitable aliquots (less than 10 μl of the reaction mixture) were injected into the gas–liquid chromatograph.

The quantity of N,N-dimethylformamide used was not critical and could be varied for quantitative studies. The time required for maximal yields of the trimethylsilyl derivatives was studied using 3-O-β-D-xylopyranosyl-L-serine as a model compound. A series of tubes containing 3-O-β-D-xylopyranosyl-L-serine (5 nmoles), N,N-dimethylformamide (5 μl), and Regisil (25 μl) were incubated at room temperature. During the first hour, tubes containing reaction mixtures were removed at 15-min intervals, and thereafter, at 30-min intervals for a period of 5 h. At each time period, each tube was immediately opened, per(trimethylsilylated sucrose (5 nmoles) added, the solution mixed, and a suitable aliquot injected into the gas–liquid chromatograph; the maximum yields were obtained in 30 min. Subsequent studies with 2-acetamido-N-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine gave variable results at this time interval, but consistent qualitative per(trimethylsilylation) was obtained at 90 min for each of the test compounds, and all subsequent studies were conducted at this longer reaction time.

Materials. — D-Glucose, D-galactose, D-xylose, L-arabinose, and sucrose were obtained from Pfanzstieh Laboratories, Inc. (Waukegan, Illinois); and 2-acetamido-N-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine, L-aspartic acid, and L-serine from Cyclo Chemical Co. (Los Angeles, California). 3-O-β-D-Xylopyranosyl-L-serine-t (104 mCi/ml) was prepared by the New England Nuclear Corporation (Boston, Massachusetts). The method of Kum and Roseman' was used to synthesize 3-O-β-D-xylopyranosyl-L-serine, 3-O-β-D-glucopyranosyl-L-serine, and 3-O-β-D-galactopyranosyl-L-serine. 5-O-β-D-Galactopyranosyloxy-D,L-lysine and O-α-D-glucopyranosyl-(1 → 2)-β-D-galactopyranosyl-(1 → 5)-D,L-lysine were generous gifts of Dr. R. G. Spiro and were obtained by alkaline degradation of glomerular-basement membranes, prepared from bovine-renal cortex. In addition, 5-O-β-D-galactopyranosyloxy-L-lysine was isolated from human urine by the method of Cunningham et al. The isolated compound gave a single ninhydrin-positive spot when cochromatographed on paper with authentic 5-O-β-D-galactopyranosyloxy-D,L-lysine (Table II). After acid hydrolysis, the neutral fraction was shown to contain galactose by g.l.c.6 Paper chromatography (solvent D) of the hydrolyzate gave a material that migrated at the same speed as that of authentic 5-hydroxy-L-lysine.

3-O-α-L-Arabinopyranosyl-L-serine. — 2,3,4-Tri-O-acetyl-L-arabinopyranosyl bromide, [α]D +282° (c 2, chloroform) (1.65 g), was condensed with N-carbobenzyloxy-L-serine benzyl ester as described by Brendel and Davidson for the corresponding D-xylose derivative. After catalytic hydrogenation and separation by column chromatography on microcrystalline cellulose, the white crystalline 3-O-(2,3,4-tri-O-acetyl-L-arabinopyranosyl)-L-serine was recrystallized from ethanol, 1.1 g (62%),
### Table II

**Paper Chromatographic and Electrophoretic Behavior of Glycosyl Derivatives of Amino Acids**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Solvents $^{a}$</th>
<th>Electrophoretic mobility (cm)$^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RF $^{c}$</td>
<td>$A$</td>
</tr>
<tr>
<td>$3-O-\beta-D$-Xylopyranosyl-L-serine</td>
<td>0.47</td>
<td>0.59</td>
</tr>
<tr>
<td>$3-O-\alpha-L$-Arabinopyranosyl-L-serine</td>
<td>0.42</td>
<td>0.53</td>
</tr>
<tr>
<td>$3-O-\beta-D$-Glucopyranosyl-L-serine</td>
<td>0.41</td>
<td>0.46</td>
</tr>
<tr>
<td>$3-O-\beta-D$-Galactopyranosyl-L-serine</td>
<td>0.34</td>
<td>0.44</td>
</tr>
<tr>
<td>2-Acetamido-$N$-(aspart-4-oyl)-2-deoxy-$\beta-D$-glucopyranosylamine</td>
<td>0.27</td>
<td>0.44</td>
</tr>
<tr>
<td>$5-O-\beta-D$-Galactopyranosylxyoxy-$D,L$-lysine</td>
<td>0.30</td>
<td>0.39</td>
</tr>
<tr>
<td>$\alpha-D$-Glucopyranosyl-(1$\rightarrow$2)-$\beta-D$-galactopyranosylxyoxy(1$\rightarrow$5)-$D,L$-lysine</td>
<td>0.02</td>
<td>0.21</td>
</tr>
<tr>
<td>$L$-Serine</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

$^{a}$Solvent A, ethyl acetate–pyridine–water (50:2:7, upper layer); solvent B, 2-methyl-2-propyl alcohol–30 per cent formic acid–water (5:1:1); solvent C, 2-butanone–propionic acid–water (3:1:12); solvent D, butyl alcohol–acetic acid–water (4:1:5, upper layer). Whatman No. 40 paper was used for descending paper chromatography except for solvent A, where Schleicher and Schuell No. 589 Green paper was used. The glycosyl derivatives of amino acids were detected by spraying with 0.2% ninhydrin in 95% acetone containing 2% pyridine. $^{b}$Paper electrophoresis was conducted in a Gilson High Voltage Electrophorator (Model D) with Whatman 3MM paper and Isopar H as the coolant. The following conditions were used: Buffer A, 0.15 M formic acid–1.6 M acetic acid, pH 2.1 (45 min at 58 v/cm); buffer B, 50 M pyridine acetate, pH 6.5 (30 min at 47 v/cm). The compounds were detected as described above.

m.p. 212–215° (dec), $[\alpha]_{D}^{25} +18.1°$ (c 1, water). This compound (1 g) was deacetylated with ammonia in methanol. The solvent was removed in vacuo, and on being kept overnight the syrupy residue crystallized. The crystals were dissolved in water (15 ml) and methanol (30 ml), and acetone was added until the mixture became turbid. On being kept at 5°, crystals (750 mg) were formed. After two additional recrystallizations, the product showed a constant $[\alpha]_{D}^{25} +0.3°$ (c 4.4, water) and m.p. 141–144° (dec). Analysis of this product by g.l.c. showed a single homogeneous peak.

Anal. Calc. for C$_{8}$H$_{15}$NO$_{7}$: C, 40.50; H, 6.37; N, 5.91. Found C 40.65; H, 6.47; N, 5.90.

The product was homogeneous on paper chromatograms and electrophoreograms (Table II), and gave only serine and arabinose after acid hydrolysis. The molar rotation ([M]$_{D}$ +76°) corresponded more closely to that of methyl $\alpha-L$-arabinopyranoside$^{19}$ ([M]$_{D}$ +2,840°) than to that of the $\beta-D$ anomer ([M]$_{D}$ +40,300°), suggesting that the compound has an $\alpha-L$ configuration.

Isolation of 3-O-$\beta-D$-xylopyranosyl-$L$-serine from human urine. — A 24-h specimen of normal human urine (100 ml), stored at 4° under toluene, was deproteinized with picric acid$^{20}$. Excess picric acid was removed by three extractions with 3 vol of water-saturated ethyl acetate. The deproteinized sample was transferred to a column of Dowex 2 (X-8, OH$^{-}$, 200–400 mesh, 4 × 30 cm). The column was washed...
Fig. 4. Paper electrophoresis of chemically prepared glycosyl derivatives of amino acids with an anionic fraction obtained from normal human urine. To compensate for the effect of salt on the migration of the compounds, a mixture containing 0.25 μmole of each of the amino acid glycosides was bandstreaked to overlap the urine fraction. The conditions for paper electrophoresis and urine fractionation are described in the text. The amino acids and glycosyl derivatives of amino acids were detected with 0.2% ninhydrin in 95% acetone containing 2% pyridine. Ser, L-serine; Xyl-Ser, 3-O-β-D-xylopyranosyl-L-serine; Ara-Ser, 3-O-α-L-arabinopyranosyl-L-serine; GlcNAc-Asn, 2-acetamido-N-(aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine; Glc-Ser, 3-O-β-D-glucopyranosyl-L-serine; Gal-Ser, 3-O-β-D-galactopyranosyl-L-serine.

with 10 vol of distilled water, and the amino acid fraction was eluted with 3 acetic acid. In separate studies where 3-O-β-D-xylopyranosyl-L-serine-t was added to urine, 93 ±3% of the tritium was recovered in the acetic acid eluate. Acetic acid was removed in vacuo, and the residue was purified by preparative paper electrophoresis on Whatman 3MM in buffer A (35 min at 58 v/cm). The ninhydrin-positive band corresponding to 3-O-β-D-xylopyranosyl-L-serine (Fig. 4) was eluted and chromatographed on Whatman No. 40 with solvent C. The material that migrated at the same speed
as 3-\(\beta\)-d-xylopyranosyl-L-serine was extracted with water and readorsorbed on column of Dowex 2 (X-8, OH\(^{-}\), 1 x 18 cm). The column was washed with water and the xylosyl derivative of L-serine eluted in the manner just described. Use of latter column of ion-exchange resin was necessary to remove the neutral mater that were solubilized from the paper matrix and found to interfere with the silylation reaction.

The yield of product was 0.35 mg. It migrated at the same rate as chemic synthesized 3-\(\beta\)-d-xylopyranosyl-L-serine in solvents A, B, C, and D and in buffer A and B (Table II). The sample was hydrolyzed in 0.4 M sulfuric acid for 1 h at 100°; products of hydrolysis were found to migrate, on paper chromatograms in solvents and E, at the same speed as those of L-serine and D-xylose, respectively. Xylose also identified by g.l.c.\(^{8}\). The pentose (orcinol)\(^{22}\) to serine (ninhydrin)\(^{23}\) ratio found to be 0.94:1.00.

Preparation of 3-\(\beta\)-d-xylopyranosyl-L-serine from chondromucoprotein
Chondromucoprotein and chondromucoprotein glycopeptides were prepared from bovine-nasal septa in the manner described by Lindahl and Rodén\(^{24}\). The pure glycopeptide fraction (50 mg) was dissolved in 30\(\text{mm}\) hydrochloric acid (25 ml) pH was adjusted to 1.5, and the solution heated for 4 h at 100° in a sealed-glass tube. The hydrolyzate was neutralized with 4M ammonium hydrogen carbonate, evaporated to dryness; water was added and evaporated repeatedly, and the residue dried until all excess of ammonium hydrogen carbonate was removed. The residue dissolved in water (25 ml) and fractionated on Dowex 2 in the same manner described for the deproteinized urine specimens. The resulting compound mig at the same speed as that of 3-\(\beta\)-d-xylopyranosyl-L-serine on paper chromatograms (Table II). After acid hydrolysis, colorimetric analysis showed a xylose to serine of 0.91:1.0.

ACKNOWLEDGEMENTS

We thank Dr. Donald Kay for providing the results of the g.l.c. of 3-\(\beta\) xylopyranosyl-L-serine found in urine for normal individuals and patients connective tissue disease. In addition, the valuable technical assistance of Mrs. Czuma and Mrs. Wanda Torzewski is acknowledged.

REFERENCES

