Alkali-Catalyzed Elimination of D-Lactic Acid from Muramic Acid and Its Derivatives and the Determination of Muramic Acid*

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ABSTRACT: Treatment of muramic acid and of derivatives of muramic acid whose reducing group is free with alkali at 37° results in the elimination of the D-lactate moiety. A chromogen which reacts directly with *p*-dimethylaminobenzaldehyde to give a typical Morgan–Elson absorption spectrum is formed from reducing derivatives of *N*-acetylmuramic acid. The lactic acid derived from muramic acid in hydrolysates of several cell walls has been demonstrated to have the D configuration. Determination with D-lactate dehydrogenase of the D-lactate liberated by this procedure can be used to quantitate muramic acid in hydrolysates of cell wall materials. Elimination is more rapid when the lactic acid moiety is peptide substituted, and the chromogen isolated from such a material after incubation at 37° and pH 10.5 has been tentatively identified as Δ-2,3-*N*-acetylglucosamine (Δ-2,3-2-acetamido-2-deoxy-D-glucoseen), the predicted result of nucleophilic attack on the C_2 proton and consequent elimination of the C_3 substituent.

The repeating unit in the cell wall peptidoglycan of *Staphylococcus aureus* strain Copenhagen consists of a disaccharide (4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid) linked by the carboxyl group of its D-lactate residue to a tetrapeptide (L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine) which is in turn substituted by a pentaglycyl unit on the ε-amino group of its lysine residue. The disaccharides are polymerized by β-1,4 linkages to form the glycan, and the peptide units are cross-linked by D-alanyl-glycine linkages. This peptidoglycan, together with the teichoic acid which is covalently linked to it, makes up at least 95% of the cell wall weight in this organism. The *Chalaropsis* B enzyme solubilizes these cell walls by hydrolyzing all of their *N*-acetylmuramyl linkages giving a glycopeptide consisting of oligomers of the disaccharide–peptide unit still interlinked by their intact D-alanyl-glycine cross-bridges. Some of this glycopeptide remains linked to teichoic acid (Tipper *et al.*, 1964).

The purpose of this paper is to describe how mild alkali treatment of this glycopeptide causes quantitative elimination of D-lactyl peptide with concomitant formation of an unsaturated disaccharide which has been tentatively identified as the 4-*O*-β-*N*-acetylglucosaminyl derivative of Δ-2,3-2-acetamido-2-deoxy-D-glucoseen, the predicted result of β elimination. The unsaturated sugar, when unsubstituted, is a Morgan–Elson chromogen with a very high molar extinction coefficient. While the work presented here was in progress, it was learned that similar conclusions had been independently reached following work in the laboratory of Dr. J.-M. Ghuysen on the structure of the cell walls of *Streptococcus faecalis* (Ghuysen *et al.*, 1967). It is now also demonstrated that alkali treatment of free muramic acid results in quantitative elimination of D-lactate whose specific determination with D-lactate dehydrogenase can be used to quantitate muramic acid in hydrolysates of complex mixtures. The presence of lactic acid in the acid hydrolysis products of a peptide fragment obtained by alkali treatment of an *N*-acetylmuramyl peptide derived from *Corynebacterium poinsettiae* was briefly mentioned by Perkins (1967).

Materials and Methods

S. aureus glycopeptide was prepared from cell walls of *S. aureus* strain Copenhagen by solubilization with *Chalaropsis* B enzyme as previously described (Tipper *et al.*, 1964, 1965). After reduction of the products with $NaBH_4$, none of the muramic acid remained, demonstrating that hydrolysis of *N*-acetylmuramyl linkages had been complete. The teichoic acid free *S. aureus* glycopeptide (about 75% of the total) was separated from the teichoic acid–glycopeptide complex by chromatography on ECTEOLA-cellulose and the glycopeptide was fractionated into oligomers by G-50 and G-25 Sephadex chromatography as previously described (Tipper, 1966; D. J. Tipper and J. L. Strominger, submitted for publication). The disaccharide decapeptide fraction is called glycopeptide monomer, glycopeptide oligomers being peptide-linked multiples of this unit. UDP[1]-*N*-acetylmuramyl pentapeptide from penicillin-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DMAB, *p*-dimethylaminobenzaldehyde; GlcNAc-MurNAc disaccharide, 4-*O*-β-*N*-acetylglucosaminyl-

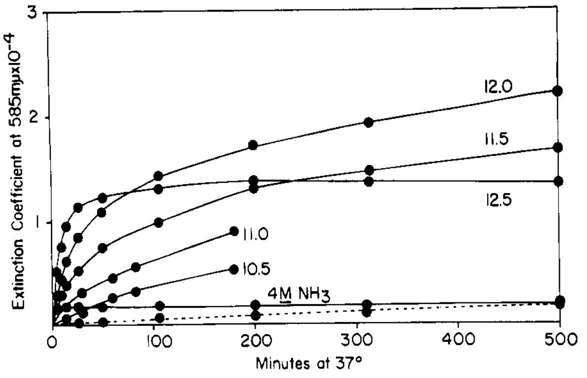

FIGURE 1: Release of chromogen from *N*-acetylmuramic acid at various pH values. *N*-Acetylmuramic acid (64 mμmoles) or *N*-acetylglucosamine (150 mμmoles) was incubated in 130 μl of buffers at the indicated pH values at 37°. Aliquots (10 μl) were removed at intervals, acidified with 0.2 M acetic acid (5 μl), and Morgan–Elson chromogen was subsequently quantitated by the direct procedure (*cf.* Methods). The dashed line is the release of chromogen from *N*-acetylglucosamine at pH 12.5. Buffers used were sodium carbonate–bicarbonate (10, 10.5, and 11.0), ionic strength 0.1, and 0.04 M sodium phosphate (11.5, 12.0, and 12.5).

treated cells of *S. aureus* was a gift of Dr. J. L. Strominger. GlcNAc-MurNAc disaccharide was prepared from *S. aureus* glycopeptide by digestion with the *Streptomyces albus* *N*-acetylmuramyl-L-alanine amidase as previously described (Ghuysen and Strominger, 1963). Muramic acid and *N*-acetylglucosamine were commercial preparations from Cyclo Chemical Corp. and Sigma Chemical Corp., respectively, and *N*-acetylmuramic acid was a gift from Lederle Laboratories. The muramic acid had a specific optical rotation of +115°, indicating that all of its lactic acid moiety had the D configuration. D-Lactate dehydrogenase (1.7 mg/ml) isolated from *Leuconostoc mesenteroides* was a gift of Dr. N. O. Kaplan. L-Lactate dehydrogenase was a commercial preparation from Sigma Chemical Corp. These enzymes are specific for the stereoisomers of lactic acid indicated. β-*N*-Acetylglucosaminidase was a preparation from pig epididimis as previously described (Sanderson *et al.*, 1962). Treatment with this enzyme was performed in 0.01 M sodium citrate buffer (pH 4.2) containing 0.01% (w/v) bovine serum albumin and 0.2 M NaCl, at 37°. This enzyme preparation (10 μl) in 250 μl of this buffer was able to hydrolyze 1 mole of GlcNAc-MurNAc disaccharide in 2 hr at 37°. D-Lactate (K^+ salt) and L-lactate (Li^+ salt) were commercial preparations from Calbiochem. APDPN was a commercial preparation from Pabst Laboratories.

Gel filtration on columns of Sephadex G-50 and G-25 (Pharmacia Fine Chemicals, Inc.) and Bio-Gel P2 (Bio-Rad Laboratories) was performed at room temperature using deionized water as eluent. Reducing power, total hexosamines, total phosphates, and N- and C-terminal amino acids were determined as previously described (Ghuysen *et al.*, 1966). Determination of periodate uptake and formaldehyde production during periodate oxidations were performed as previously described (Ghuysen *et al.*, 1966). Total amino acids were determined on a Beckman–Spinco amino acid analyzer after hydrolysis for 16 hr at 105° in 6 N HCl. Total glucosamine and muramic acid were similarly determined following hydrolysis for 6 hr at 100° in 3 N HCl using a hydrolyzed sample of GlcNAc-MurNAc disaccharide as standard. Hydrolysates were not dried but were applied directly to the column after dilution with appropriate buffer.

Determination of N-Acetylhexosamines and Derivatives by Morgan–Elson Procedures. 1. HEATING PROCEDURE (7 MIN). Samples in 15 μl of 1% (w/v) $K_2B_4O_7\cdot5H_2O$ were heated for 7 min in a boiling-water bath, cooled, mixed with 90 μl of the DMAB reagent previously described (Ghuysen *et al.*, 1966), and incubated for 20 min at 37°. Absorbance was measured at 585 mμ.

2. HEATING PROCEDURE (30 MIN). Samples in 40 μl of 0.5% (w/v) $K_2B_4O_7\cdot5H_2O$ were heated for 30 min in a boiling-water bath, cooled, lyophilized, redissolved in water (15 μl), and then treated as in the 7-min procedure.

3. DIRECT PROCEDURE. Samples (15 μl) were mixed with 90 μl of DMAB reagent and treated as in the 7-min procedure.

Determination of D- *and* L-*Lactates.* Samples were hydrolyzed in 3 N HCl for 8 hr at 100° in sealed tubes and neutralized with 10 N NaOH. The subsequent procedure followed closely that described by Dennis (1962) but using smaller volumes. Aliquots were diluted with water to 75 μl, 1.0 M Tris-HCl buffer (pH 9.0) (10 μl), and 10 mg/ml of APDPN solution (10 μl) were added. The reaction was started by addition of D-lactate dehydrogenase (4 μl of a 1.7-mg/ml solution) or L-lactate dehydrogenase (1 μl of a 5-mg/ml suspension) and the reduction of APDPN followed by the increase in absorbancy at 363 mμ at room temperature. Under these conditions the reactions were half complete by 2 min but further rapid reduction of APDPN on addition of L- or D-lactate to the appropriate incubation mixture showed the enzymes to be stable for at least 1 hr. Multiple D-lactate samples were quantitated by measuring the change in absorbance at 363 mμ on incubation for 30 min at 37°. Standard curves were approximately linear up to 16 mμmoles of D-lactate with an extinction coefficient of 5800, corresponding to 74% oxidation of lactate, since APDPNH has an extinction coefficient of 7800 (Kaplan and Ciotti, 1956). Racemization of D- and L-lactates under these conditions of hydrolysis (or on incubation at pH 12.5 for 2 hr at 37°, see below) was less than 3% and liberation of D-lactate from free *N*-acetylmuramic acid or from *S. aureus* glycopeptide was less than 5%. Destruction of D- and L-lactates in this procedure was less than 7%.

Results

Elimination of D-*Lactyl Peptide from S. aureus Glycopeptide. S. aureus* glycopeptide (7.8 μmoles of total glutamic acid, chain length greater than two disaccha-

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N-acetylmuramic acid; APDPN, the 3-acetylpyridine analog of DPN, diphosphopyridine nucleotide; APDPNH, the reduced form of APDPN; Δ-2,3-GlcNAc, Δ-2,3-2-acetamido-2-deoxy-D-glucoseen; GlcNAc-Δ-2,3-GlcNAc disaccharide, 4-*O*-β-*N*-acetylglucosaminyl-Δ-2,3-2-acetamido-2-deoxy-D-glucoseen.

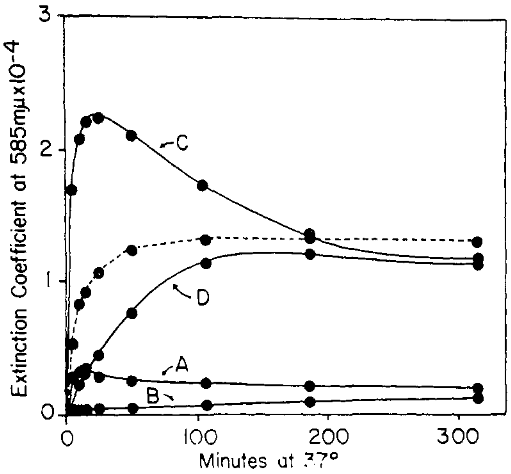

FIGURE 2: Release of chromogen at 37° from 4-*O*-β-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharide and *S. aureus* glycopeptide at pH 12.5. *N*-Acetylmuramic acid (128 mμmoles), GlcNAc-MurNAc disaccharide (150 mμmoles), and *S. aureus* glycopeptide (150 mμmoles) were separately incubated at 37° in 260 μl of 0.04 M phosphate buffer (pH 12.5). Duplicate aliquots were removed at intervals, acidified with acetic acid, and frozen. One set of each was then hydrolyzed for 3 hr at 37° at pH 4.5 in 20 μl of 0.01 M citrate buffer with 0.4 μl of the β-*N*-acetylglucosaminidase solution. Morgan–Elson chromogen was then determined in both aliquots by the direct procedure. The *N*-acetylmuramic acid aliquots gave identical curves with and without preincubation with β-*N*-acetylglucosaminidase (dashed line). The lower two lines are the release of chromogen from (A) glycopeptide and from (B) disaccharide. The upper two lines are the release of chromogen from (C) glycopeptide and (D) disaccharide when the alkali-treated samples were subsequently hydrolyzed with β-*N*-acetylglucosaminidase.

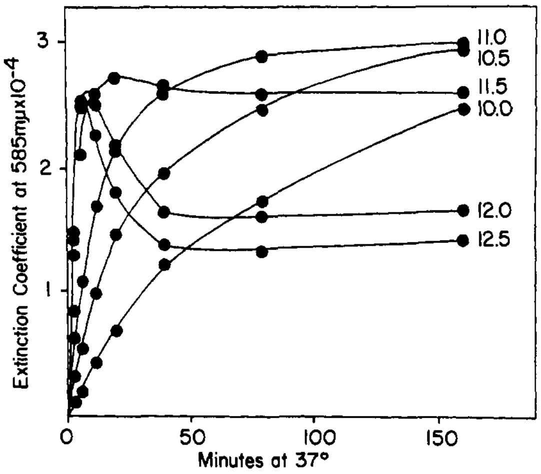

FIGURE 3: Release of chromogen from *N*-acetylmuramy peptide oligomer at various pH values. Samples of *N*-acetylmuramyl peptide oligomer (35 mμmoles) were incubated in 150 μl of buffers at the indicated pH values at 37°. The release of Morgan–Elson chromogen was quantitated in aliquots (14 μl) at intervals by the direct procedure. UDP-MurNAc pentapeptide (0.3 μmole) was hydrolyzed for 5 min at 100° in dilute HCl (pH 1.5–2.0, 140 μl) followed by neutralization with NaOH to give a 1.7 mM solution of MurNAc pentapeptide. Aliquots (20 μl) of this solution were treated as above, and gave virtually identical curves for Morgan–Elson chromogen production.

ride–peptide units) was incubated in 200 μl of 4 M NH_3 for 2 hr at 37° and lyophilized. The product was fractionated on a column (100 × 2.5 cm) of Sephadex G-25. A single symmetrical peak with K_D[2] = 0.9 contained all the reducing power and total hexosamines and was pooled. Analysis of hydrolysates of this material on the Beckman–Spinco amino acid analyzer showed it to contain 7.9 μmoles of glucosamine and no other components (it was subsequently found that the unsaturated GlcNAc derivative which should also have been present did not give a peak on the analyzer after hydrolysis; see below). Peptide was detected in aliquots of fractions by measurement of free amino groups after hydrolysis for 18 hr in 6 N HCl at 105°. Two peaks with K_D values near zero contained virtually all the peptide and were pooled. This fraction contained glutamic acid, alanine, glycine, and lysine in the molar proportions of 1:2:1:5, respectively, but no amino sugars or N-terminal alanine. This material also contained 1.02 moles of D-lactate and 0.03 mole of L-lactate per mole of glutamic acid. Thus treatment with ammonia resulted in quantitative elimination of D-lactyl peptide with loss of the disaccharide moieties and disappearance of all of the muramic acid.

Production of Morgan–Elson Chromogen from N-Acetylmuramic Acid Derivatives at 37°. Alkali-catalyzed β elimination of C-3 substituents from *N*-acetylmuramic acid derivatives should yield Δ-2,3-GlcNAc as the initial carbohydrate product. This is one of the structures proposed for Morgan–Elson chromogen I by Kuhn and Kruger (1956).

The facile production of chromogen from free *N*-acetylmuramic acid was confirmed by incubation at pH values from 10.5 to 12.5 at 37° as shown in Figure 1. The rate of chromogen production increased with pH, but the maximal color yield was reduced at pH 12.5. No similar production of chromogen occurred at 37° in the presence of 4 M NH_3, although as shown above, elimination probably occurred under these conditions. There was negligible production of chromogen at 37° from *N*-acetylglucosamine at pH 12.5. Production of chromogen at 37° from GlcNAc-MurNAc disaccharide and *S. aureus* glycopeptide at pH 12.5 could not be directly demonstrated (Figure 2). However, if the products were hydrolyzed with *N*-acetylglucosaminidase, it became apparent that chromogen production had occurred cryptically (Figure 2). After hydrolysis of *S. aureus* glycopeptide with *N*-acetylglucosaminidase to give *N*-acetylmuramyl peptide oligomer, chromogen production in alkali could be directly demonstrated (Figure 3).

A fraction of *S. aureus* glycopeptide with a chain length of five disaccharide peptide units (1.4 μmoles) was incubated at 37° in 80 μl of citrate buffer (pH 4.2) containing 10 μl of the β-*N*-acetylglucosaminidase preparation. The liberation of free *N*-acetylglucosamine

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[2] $K_D = (V_e - V_0)/V_i$, where V_e is the elution volume, V_0 the void volume (V_e for Blue Dextran), and V_i the internal volume of the gel. $V_0 + V_i = V_e$ for NaCl.

was followed by determination of 7-min Morgan–Elson color at intervals and was maximal at 6 hr. A further 10 μl of enzyme was added and incubation was continued for 6 hr. The mixture was then fractionated at room temperature on a column (120 × 2 cm) of Sephadex G-50. The column was eluted with water at 0.3 ml/min and aliquots (30 μl) of the fractions (4 ml) were analyzed for reducing power. A peak of reducing power was found with K_D = *ca.* 0.27, as expected for material with the molecular weight of glycopeptide pentamer. The only other peak had a K_D of 1 and consisted of free *N*-acetylglucosamine, as demonstrated by paper chromatography. The earlier peak was pooled and is called *N*-acetylmuramyl peptide oligomer. An aliquot of this material was hydrolyzed for determination of total amino acids and hexosamines. It contained glutamic acid, lysine, alanine, glycine, and muramic acid in the molar proportions of 1.0:0.97:2.31:4.83:0.92, but it contained no detectable amounts of glucosamine.

The rate of Morgan–Elson chromogen production from this material at pH 10 (Figure 3) was approximately the same as at pH 12 with unsubstituted *N*-acetylmuramic acid (see Figure 1). The approximate relative initial rates of chromogen production at pH 12.5 from GlcNAc-MurNAc disaccharide, *N*-acetylmuramic acid, *S. aureus* glycopeptide, and *N*-acetylmuramyl peptide oligomer were 0.25, 1.0, 5.5, and 15, respectively.

Preparation of Δ-2,3-GlcNAc from Alkali-Treated S. aureus Glycopeptide and Its Identification. A. ISOLATION. *S. aureus* glycopeptide–teichoic acid complex[3] (520-μmoles total phosphate and 50 μmoles of disaccharide peptide units) was dissolved in carbonate–bicarbonate buffer (pH 10.5) (ionic strength–0.1, 12 ml) and incubated at 37°. Aliquots (2 μl) were removed at intervals, neutralized with 0.1 M acetic acid (10 μl), and frozen for subsequent determination of Morgan–Elson chromogen by the direct procedure after hydrolysis with β-*N*-acetylglucosaminidase. The pH was constantly adjusted by the addition of 2 N NaOH to maintain a value of 10.5. Chromogen release was maximal after 3 hr. After 4.5 hr the pH was adjusted to 6.0 with HCl and the mixture was concentrated to 4 ml and desalted on a column (3 × 45 cm) of Sephadex G-10. The column was eluted with water and fractions of 2 ml were collected each at 12 min. Analysis of the fractions showed good separation of total phosphate and reducing power from salt (detected with silver nitrate). All fractions (excluding the salt peak) were recombined, concentrated to 5 ml, and applied to a column (4 × 100 cm) of Sephadex G-50 (V_0 = 185 ml; V_i = 210 ml). The column was eluted with water and fractions of 2.8 ml were collected each at 8 min. The fractions were analyzed for total phosphate, reducing power, and total amino groups after hydrolysis (6 N HCl, 16 hr, 105°).

An initial peak containing organic phosphate (520 μmoles, teichoic acid) at the excluded volume of the column also contained free amino groups after hydrolysis (450 μmoles, polypeptide) and reducing power (24 μmoles, corresponding to about 1 mole of disaccharide or unsaturated disaccharide per mole of teichoic acid of chain length 30 ribitol phosphate units; *cf.* Ghuysen *et al.*, 1965). A peak of reducing power (50 μmoles), devoid of phosphate and peptide, was eluted with a mean K_D of 0.75 (disaccharide). Total eluted reducing power (74 μmoles) is equivalent to 50 μmoles of disaccharide (see Table I). The disaccharide peak was pooled and concentrated to 5 ml. An aliquot (0.9 ml) was incubated with β-*N*-acetylglucosaminidase solution (100 μl) in 2.5 ml of citrate buffer (pH 4.2). The liberation of chromogen was determined by the direct Morgan–Elson reaction. A maximum of 7 μmoles (extinction coefficient 49,500, see below) was released after 3 hr, and after 9 hr the mixture was applied to a column (1.7 × 26 cm) of Bio-Gel P-2 and eluted with water at 0.3 ml/min. The fractions (3 ml) were analyzed for reducing power and for Morgan–Elson chromogen. Retardation of the unsaturated sugar by the column allowed its separation from *N*-acetylglucosamine. A peak of reducing power without chromogen at K_D = 0.6 (free *N*-acetylglucosamine), partially overlapped a peak with both reducing power and chromogen at K_D = 0.72 (Δ-2,3-*N*-acetylglucosamine). The fractions containing chromogen were pooled, concentrated, and fractionated on a second Bio-Gel P-2 column (1.7 × 77 cm) as before, resulting in almost complete resolution of the two compounds. Those fractions with a constant ratio of chromogen to reducing power were pooled (Δ-2,3-GlcNAc).

B. PREPARATION OF THE 7-MIN MORGAN–ELSON CHROMOGEN. *N*-Acetylglucosamine (7.0 mg) was dissolved in 5% $K_2B_4O_7 \cdot 5H_2O$ (200 μl) and heated for 7 min in a boiling-water bath. After neutralization with glacial acetic acid (5 μl), the solution was lyophilized and then dried three times from solution in dry methanol, to volatilize methyl borate. The residue was dissolved in water to give a solution 10 mM with respect to the original quantity of *N*-acetylglucosamine. As expected, the extinction coefficient of this material in the direct Morgan–Elson reaction was equal to that of *N*-acetylglucosamine itself in the 7-min procedure (Table I).

C. EXTINCTION COEFFICIENTS OF Δ-2,3-GLCNAC, GLCNACMURNAC DISACCHARIDE AND GLCNAC-Δ-2,3-GLCNAC DISACCHARIDE IN THE MORGAN–ELSON REACTIONS. After hydrolysis for 3 hr at 100° in 3 N HCl, Δ-2,3-GlcNAc gave no recognizable peak on the Beckman–Spinco amino acid analyzer. It was therefore possible to quantitate the GlcNAc-Δ-2,3-GlcNAc disaccharide by measurement with the amino acid analyzer of the glucosamine released on its hydrolysis. On this basis, the unsaturated disaccharide had the same extinction coefficient in the 30-min Morgan–Elson reaction as did GlcNAc-MurNAc disaccharide (Table I). This result was predictable since presumably the reactions occurring at 37° at pH 10.5 occur very rapidly in borate at 100° and are the first steps in production of the 7- and 30-min Morgan–Elson chromogens. Δ-2,3-GlcNAc is thus also expected to have the same extinction coefficient in the 7-min Morgan–Elson procedure

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[3] Glycopeptide–teichoic acid complex was used as the starting material for this preparation since it was available in large amounts. It was the gift of Dr. Mary Jo Carter.

TABLE I: Molar Extinction Coefficients in Morgan–Elson and Reducing Power Procedures and Results of Periodate Oxidations of GlcNAc, Δ-2,3-GlcNAc, and Derivatives.[a]

	Extinction Coefficients $\times 10^{-3}$				Periodate Oxidation			
	Morgan–Elson Reactions (585 mμ)			Reducing Power (690 mμ)	Formaldehyde Produced (0.001 M IO_4^-)	IO_4^- Uptake (0.01 M)		
Substance	Direct	7 min	30 min			0.1 hr	5 hr	50 hr
GlcNAc	0	20.0	15.8	100	<0.05	<0.1		
MurNAc	0	20.0	15.6	78	<0.05	<0.1		
GlcNAc-MurNAc disaccharide	0	2.0	9.4	150	<0.05	<0.1		
GlcNAc-Δ-2,3-GlcNAc disaccharide	0.8	5.0	9.4	159	0.15			
Δ-2,3-GlcNAc	49.5	20.0	15.0	77	1.01	0.8	1.35	3.0
7-min Morgan–Elson chromogen	19.6	18.4	15.4	88				
Reduced GlcNAc	0	0		0	1.0	3.0	3.15	3.9
Reduced MurNAc	0	0		0	1.0			
Reduced GlcNAc-MurNAc disaccharide	0	0		2	1.1	1.1	2.25	2.8
Reduced GlcNAc-Δ-2,3-GlcNAc disaccharide	0	0		56	1.2	1.2	2.0	4.0
Reduced Δ-2,3-GlcNAc	0.2	0		4	1.2	1.7	2.0	3.6

[a] Details of procedures are given in the text. Periodate oxidation data are expressed as moles of formaldehyde produced or periodate consumed per mole of substance. Blank spaces indicate not determined.

as GlcNAc, and this was confirmed as follows. First, hydrolysis of GlcNAc-Δ-2,3-GlcNAc disaccharide with β-*N*-acetylglucosaminidase resulted in the maximal liberation of the expected 2.0 moles of 7-min Morgan–Elson color relative to a standard of *N*-acetylglucosamine. The maximum yield of direct Morgan–Elson color was 2.4 moles/mole, relative to a standard of 7-min Morgan–Elson chromogen. Second, *N*-acetylmuramyl peptide oligomer gave a maximal extinction coefficient of 30,000 in the direct Morgan–Elson procedure at pH 10.5–11.5 at 37° (Figure 3). However, if the products formed after any time of incubation at these pH values were submitted to the 7-min procedure, extinction coefficients of 20,000 were uniformly found. Third, the Δ-2,3-*N*-acetylglucosamine, whose isolation has been described above, quantitated on the basis of an extinction coefficient of 20,000 in the 7-min Morgan–Elson reaction, gave the predicted yield of 1 mole of formaldehyde on periodate oxidation (see below). On this basis, this material had an extinction coefficient of 49,500 in the direct Morgan–Elson reaction (Table I).

D. Effect on color produced in the direct Morgan–Elson reaction from Δ-2,3-GlcNAc of further incubation at alkaline pH. Samples of Δ-2,3-GlcNAc were incubated at 37° in buffers at pH values 10.5–12.5 as before. Determinations of residual chromogenicity in the direct Morgan–Elson reaction were made at intervals (Figure 4). A control sample incubated at pH 6.5 lost no chromogenicity, but at higher pH values the loss was progressive.

E. Chromatography of *N*-acetylglucosamine, Δ-2,3-*N*-acetylglucosamine, and their derivatives. Samples of GlcNAc, MurNAc, GlcNAc-MurNAc disaccharide, GlcNAc-Δ-2,3-GlcNAc disaccharide, 7-min Morgan–Elson chromogen, and Δ-2,3-GlcNAc were chromatographed on paper and on thin layers of silica gel G (prepared in 0.1 M boric acid) in the solvents listed in Table II. All compounds were detectable on paper by fluorescence after heating in alkali as described by Sharon (1964). The monosaccharides were detectable on paper with silver nitrate and NaOH, and both procedures demonstrated the presence of several components in the preparation of 7-min Morgan–Elson chromogen. Δ-2,3-GlcNAc gave a single spot in all solvents, and by all detection methods. This spot was detected most efficiently, both on paper and thin layers, by the DMAB reagent described by Salton (1959). Only the fastest moving component of the 7-min Morgan–Elson chromogen was detectable with this reagent, and this spot was chromatographically indistinguishable from Δ-2,3-GlcNAc.

F. Reduction and periodate oxidation of Δ-2,3-*N*-acetylglucosamine and unsaturated disaccharide. Samples (0.4 μmole) of GlcNAc, MurNAc,

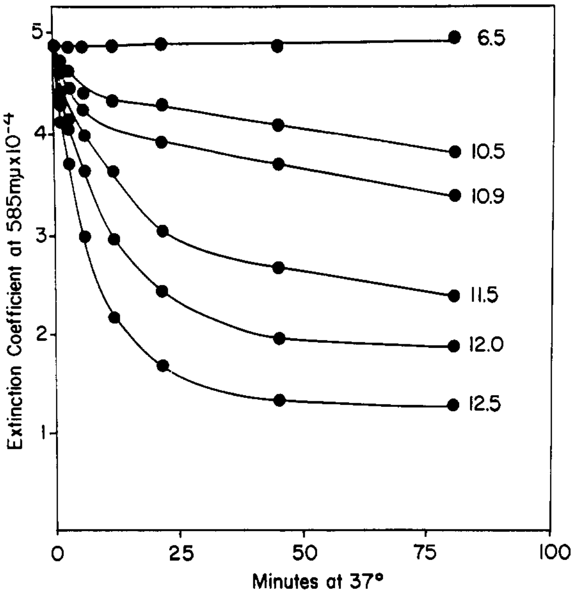


FIGURE 4: Effect of incubation at various pH values on color produced in the direct Morgan–Elson reaction from Δ-2,3-*N*-acetylglucosamine. Δ-2,3-GlcNAc (21 mμmoles) was incubated in buffers (110 μl) at the indicated pH values at 37°. Aliquots (12 μl) were removed at intervals, acidified with 0.2 N acetic acid (6 μl), and frozen. Chromogen was subsequently determined by the direct Morgan–Elson reaction.

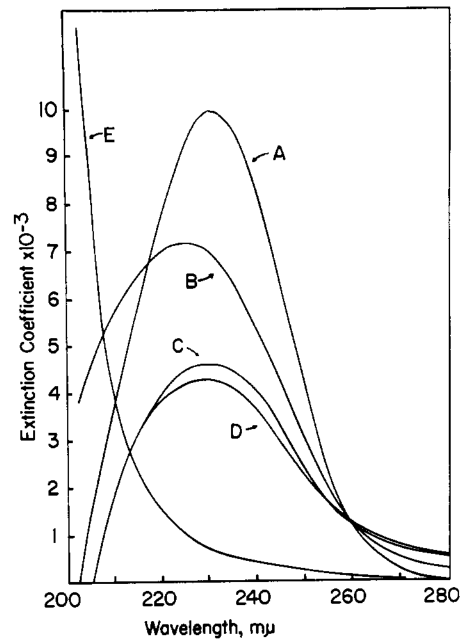


FIGURE 5: Absorption spectra of Δ-2,3-*N*-acetylglucosamine and derivatives. (A) Δ-2,3-GlcNAc (blank, *N*-acetylglucosamine), (B) reduced Δ-2,3-GlcNAc (blank, *N*-acetylglucosaminitol), (C) GlcNAc-Δ-2,3-GlcNAc disaccharide (blank, GlcNAc-MurNAc disaccharide), and (D) reduced GlcNAc-Δ-2,3-GlcNAc disaccharide (blank, reduced GlcNAc-MurNAc disaccharide). The absorption spectrum of only one of the blanks, GlcNAc-MurNAc disaccharide, is given (curve E, blank water). The other blanks were all similar in shape, but lower in intensity at all wavelengths.

GlcNAc-MurNAc disaccharide, GlcNAc-Δ-2,3-GlcNAc disaccharide, and Δ-2,3-GlcNAc were reduced for 9 hr at room temperature in 0.2 M $NaBH_4$ (50 μl). The pH of the solutions was adjusted to about 4.5 by the addition of 4 M acetic acid (5 μl), and the resulting solutions were analyzed for reducing power and in the Morgan–Elson procedures (Table I). Low residual reducing power indicated that reduction of all had been at least 95% complete with the exception of GlcNAc-Δ-2,3-GlcNAc disaccharide. Conversely, digestion of the reduced GlcNAc-Δ-2,3-GlcNAc disaccharide with β-*N*-acetylglucosaminidase resulted in the liberation of only 0.85 mole of 7-min Morgan–Elson color, and no direct Morgan–Elson color, indicating that complete reduction had occurred (see Discussion).

TABLE II: R_F Values for Paper (A–C) and Thin-Layer (D and E) Chromatography of GlcNAc, Δ-2,3-GlcNAc, and Their Derivatives.[a]

	A	B	C	D	E
GlcNAc	0.24	0.52	0.47		
MurNAc	0.52	0.52	0.21		
GlnNAc-MurNAc disaccharide	0.25	0.38	0.11		
GlcNAc-Δ-2,3-GlcNAc disaccharide	0.35	0.55	0.38		
Δ-2,3-GlcNAc	0.55–0.8	0.63	0.65	0.61	0.41
7-min Morgan–Elson chromogen	0.55–0.8	0.63	0.65	0.61	0.41

[a] The figures given for the 7-min Morgan–Elson chromogen are for the spots detected with DMAB reagent. The solvents used were: (A) butanol–acetic acid–water (3:1:1), (B) isobutyric acid–1 N ammonia (5:3), (C) butanol–pyridine–water (6:4:3), (D) methyl ethyl ketone–acetic acid–water (28:10:12), and (E) butanol–ethanol–water (65:15:20).

Aliquots (10 μl) of the solutions of reduced materials were oxidized in 110 μl of 0.001 M $NaIO_4$ at room temperature in the dark. Aliquots (80 mμmoles) of unreduced GlcNAc-Δ-2,3-GlcNAc disaccharide and Δ-2,3-GlcNAc were similarly oxidized. Determinations of released formaldehyde were made at intervals, and reached constant levels which are given in Table I. All compounds yielded 1 mole of formaldehyde after reduction but only Δ-2,3-GlcNAc yielded a mole of formaldehyde prior to reduction.

Some of these samples were also oxidized in 0.01 M $NaIO_4$ (32 μl) under the same conditions, and uptake of IO_4^- was determined at intervals by the decrease in absorbancy at 224 mμ. Corrections were applied for the absorbancies of the oxidized materials using blanks determined by addition of excess ethylene glycol after 50-hr oxidation. The data obtained at 0.1, 5, and 50 hr are given in Table I.

G. ULTRAVIOLET AND INFRARED SPECTRA. Absorb-

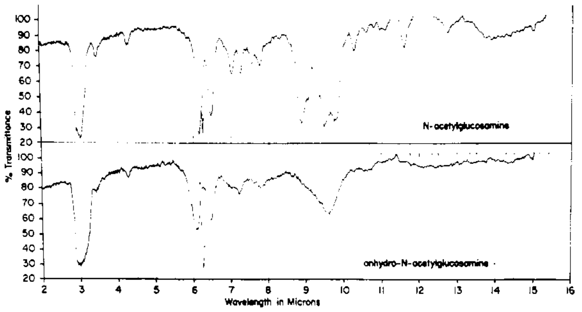


FIGURE 6: Infrared adsorption spectra of *N*-acetylglucosamine (upper tracing) and Δ-2,3-*N*-acetylglucosamine (lower tracing) in KBr pellets. The wavelength of each run was calibrated from the 6.24-μ peak of polystyrene.

ancies of solutions (0.1 mM) of reduced and unreduced GlcNAc, GlcNAc-MurNAc disaccharide, GlcNAc-Δ-2,3-GlcNAc disaccharide, and Δ-2,3-GlcNAc were determined over the range of 200–290 mμ using a Zeiss PMQ II spectrophotometer (Figure 5). Samples of reduced and unreduced Δ-2,3-GlcNAc were also oxidized for 1 hr at 37° in 0.005 M IO_4^-, after which excess IO_4^- was reduced with ethylene glycol, and spectra were determined as before, using a solution containing no carbohydrate as blank. The products, still absorbed at about 230 mμ, but with lower extinction coefficients (3000 and 6700, respectively).

Samples (0.4 μmole) of GlcNAc and Δ-2,3-GlcNAc were dissolved in water (200 μl) containing KBr (15 mg) and lyophilized. Samples (*ca.* 5 mg) of the residues were compressed into micropellets and analyzed in a Beckman Model IR5 spectrophotometer. The spectra of similar amounts of the two compounds are given in Figure 6.

Determination of Muramic Acid in Hydrolysates of Cell Wall Materials. D-Lactate was quantitatively eliminated from muramic acid and its *N*-acetyl derivative at pH 12.5, 37°, at very similar rates (Figure 7). Measurement with D-lactate dehydrogenase of the D-lactate released on treatment of acid hydrolysis products with alkali was therefore used for the determination of the muramic acid content of hydrolysates of cell wall materials. In order to test the effects of various cell wall constituents (teichoic acid, neutral polysaccharides, and proteins) on determination of D-lactate with D-lactate dehydrogenase, samples of muramic acid (50 mμmoles) were mixed with the following compounds and hydrolyzed for 18 hr at 100° in 4 N HCl. (a) Teichoic acid extracted with trichloracetic acid from cell walls of *S. aureus* strain Copenhagen (150 mμmoles total phosphate); (b) galactosamine-containing type-specific polysaccharide isolated by mild acid treatment of cell walls of *Lactobacillus casei* strain R094 (50-mμmoles total hexosamines); (c) protein solubilized by autolysis from washed and trypsin-digested cell walls of *Bacillus sphaericus* strain 9602 (160 μg); and (d) *N*-acetylglucosamine (150 mμmoles).

Similar amounts of these materials were hydrolyzed in duplicate without added muramic acid, and muramic acid (50 mμmoles) was added to one of each pair of duplicates subsequent to hydrolysis. The hydrolysates were neutralized and incubated in 0.04 M phosphate buffer (pH 12.5) (60 μl) for 1 hr at 37°. D-Lactate was then determined on aliquots (20 μl).

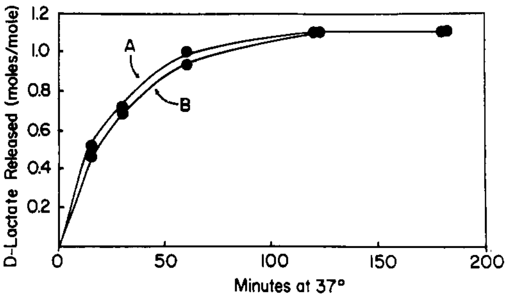


FIGURE 7: Release of D-lactate from free muramic acid and *N*-acetylmuramic acid at pH 12.5. Samples of muramic acid and *N*-acetylmuramic acid (50 mμmoles) were incubated in 0.04 M Na_3PO_4 buffer (pH 12.5) (50 μl), and the D-lactate content of aliquots (10 μl) was determined at intervals. (A) Muramic acid and (B) *N*-acetylmuramic acid.

In the absence of added muramic acid none of these compounds released D-lactate on hydrolysis. The yield of D-lactate from added muramic acid varied from 95 to 102%, whether the muramic acid was added before or after the hydrolysis.

The muramic acid content of several cell walls was quantitated by the Beckman–Spinco amino acid analyzer and in each case was virtually identical with the glutamic acid content of the cell wall peptidoglycan. Samples of cell walls, containing about 50 mμmoles of total muramic acid, were hydrolyzed under the same conditions as above, neutralized, and incubated at pH 12.5 as above (final volume 60 μl).

Determination of the D-lactate content of aliquots (10 μl) gave figures for the muramic acid contents of the original cell walls almost identical with those determined by the amino acid analyzer (Table III). Similar results were obtained with larger aliquots (20 μl) except in the case of *L. casei*, where brown color, probably resulting from interaction of neutral sugars and amino acids, caused interference. None of these materials were found to contain appreciable amounts of L-lactate.

TABLE III: Analyses of Muramic Acid in Bacterial Cell Walls.[a]

Bacterial Strain	Detd by Amino Acid Analyzer	Detd by D-Lactate Liberated
S. aureus strain copenhagen	0.42	0.40
B. subtilis strain 168	0.32	0.30
B. sphaericus strain 9602	0.25	0.24
L. casei strain R094	0.2	0.21

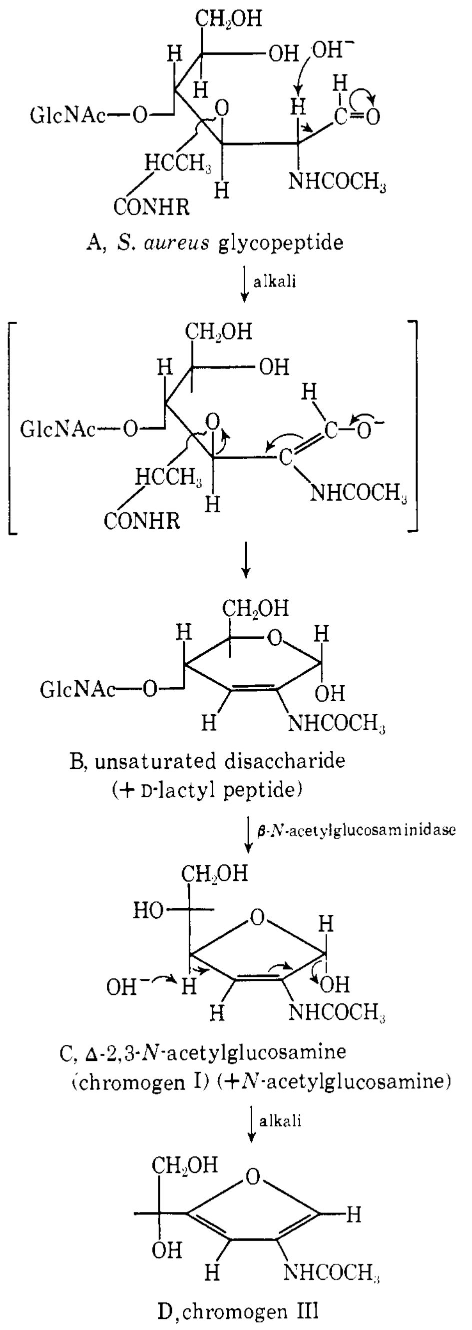
[a] Data are given as micromoles per milligram dry weight.

Discussion

All of the *N*-acetylmuramic acid residues in *S. aureus* glycopeptide had their reducing group free, and treatment with alkali resulted in quantitative β elimination of D-lactyl peptide from these residues. Similarly, alkali treatment of the products of acid hydrolysis of four cell walls resulted in the production of D-lactate in amounts equal to their muramic acid content. Thus the configuration of the lactyl moieties in the muramic acid residues of these organisms has been directly confirmed, since configuration of lactate should be conserved during β elimination (see Figure 8). This procedure is potentially useful for the determination of muramic acid in complex materials. Configurations of lactyl residues in muramic acid samples have previously depended on comparison of their specific rotations with that of stereospecifically synthesized muramic acid (*cf.* Matsushima and Park, 1962).

The primary carbohydrate product of alkali-catalyzed β elimination of substituents on position 3 of *N*-acetylmuramic acid derivatives should be Δ-2,3-GlcNAc (see Figure 8). Kuhn and Kruger (1956) proposed the furanose form of this compound and its Δ-3,4 analog as two alternatives for the structure of Morgan–Elson chromogen I, which is converted into D-5-dihydroxy-3-acetamidofuran (chromogen III) on further alkali treatment (Kuhn and Kruger, 1957). Mild alkali treatment of MurNAc derivatives was indeed found to produce Morgan–Elson chromogen, and a β-elimination mechanism for its production is consistent with the effect of C-3 substituents on the rate of chromogen production from these derivatives, on the basis of the relative efficiencies of such substituents as electron-accepting leaving groups. Thus OH^- (in GlcNAc) is less efficient than $CH_3CHO^-COO^-$ (in MurNAc) which is also predictably less efficient than CH_3CHO^-CONHR (in MurNAc peptide oligomer). Kuhn and Kruger demonstrated that C-4 substituents prevent the formation of chromogen from GlcNAc, and hypothesized that dehydration to an unsaturated derivative occurred only in the furanose form. The present studies demonstrate that while the presence of a 4-*O*-β-*N*-acetylglucosaminyl substituent in *S. aureus* glycopeptide and GlcNAc-MurNAc disaccharide retards the formation of unsaturated sugar (and the elimination of lactyl peptide or lactate) from MurNAc by a factor of 3–4, it does not prevent it. However, it masks the chromogenicity of the product which is only revealed on subsequent hydrolysis with β-*N*-acetylglucosaminidase. Chromogenicity may therefore depend on rearrangement to a furanose form.

Chromogen is formed most rapidly from peptide derivatives of MurNAc, and so the *S. aureus* glycopeptide was used for the preparation of Δ-2,3-GlcNAc under the mildest possible conditions. The reactions involved are depicted in Figure 8. The product predictably had the same molar extinction coefficients in the 30- and 7-min Morgan–Elson determinations and in the reducing power determination as MurNAc (Table I) while it reacted directly with DMAB to give an absorption spectrum superimposable upon that given by the 7-min Morgan–Elson Chromogen (peaks at 547 and 487 mμ and a shoulder at 510 mμ) but with



A, *S. aureus* glycopeptide

B, unsaturated disaccharide (+ D-lactyl peptide)

C, Δ-2,3-*N*-acetylglucosamine (chromogen I) (+*N*-acetylglucosamine)

D, chromogen III

FIGURE 8: Action of alkali on *S. aureus* glycopeptide, preparation of Δ-2,3-GlcNAc, and its conversion into chromogen III. *S. aureus* glycopeptide (structure A, NHR indicating the peptide) is converted into a mixture of D-lactyl peptide and unsaturated disaccharide (structure B), presumably by a β-elimination mechanism involving nucleophilic attack on the C_2 proton as illustrated. The Δ-2,3-GlcNAc residue in the unsaturated disaccharide exists in solution predominantly as a pyranose ring form, but on liberation by the action of β-*N*-acetylglucosaminidase, it appears to assume preferentially a furanose ring form (structure C) in which it can interact with DMAB to give a Morgan–Elson color. Further treatment with alkali converts this material into chromogen III (structure D) probably by a second nucleophilic attack, this time on the C_4 proton.

a much higher extinction coefficient. Chromogenicity decayed on further incubation in alkali (Figure 4), the two stages in this process being distinguishable in the production of chromogen from MurNAc peptide oligomer (Figure 3) but not from MurNAc itself, where the initial reaction is too slow (Figure 1). This decay of chromogenicity probably involves a second nucleophilic attack on the furanose form of Δ-2,3-GlcNAc, whose relative planarity would favor the introduction of a second double bond (Figure 8). This process is initially rapid even at pH 10.5 (Figure 4), and is probably inhibited during production of the unsaturated disaccharide, in which the Δ-2,3-GlcNAc residue cannot assume the furanose configuration. However, the presence of the double bond in the unsaturated disaccharide should labilize its glycoside linkage to alkali. Hence after vigorous alkali treatment, as in the 30-min Morgan–Elson and reducing power determinations, GlcNAc-MurNAc disaccharide is hydrolyzed and has a strong Morgan–Elson reaction and a high reducing power. In contrast, MurNAc-GlcNAc disaccharide, with no easily eliminated β substituent, has a low Morgan–Elson reaction and reducing power (*cf.* Ghuysen *et al.*, 1966). The unsaturated disaccharide, in contrast to GlcNAc-MurNAc disaccharide, also retained considerable reducing power after complete reduction (Table I).

The purity of the Δ-2,3-GlcNAc preparation is indicated by its chromatographic behavior and the equality of its reducing power to that of MurNAc, since chromogen III is nonreducing. It also contained none of the nonchromogenic components in the 7-min Morgan–Elson chromogen preparation, although it was not distinguished chromatographically from the chromogenic component of this preparation. The periodate oxidation data confirm its purity and are consistent with its existence (predominantly) in a closed pyranose ring form in the unsaturated disaccharide and in a furanose ring form when free. Unreduced GlcNAc, MurNAc, GlcNAc-MurNAc disaccharide, and unsaturated disaccharide all gave rise to very little formaldehyde on oxidation in 0.001 M periodate, but all rapidly gave rise to approximately 1 mole after reduction (Table I). In contrast, Δ-2,3-GlcNAc rapidly gave rise to 1 mole of formaldehyde under these conditions both before and after reduction (Table I), and in 0.01 M periodate, rapidly consumed 1 mole of periodate before reduction and about 2 moles after reduction (Table I). At 50 hr, consumption by Δ-2,3-GlcNAc was 3 moles, as reported for chromogen I by Kuhn and Kruger (1956).

The ultraviolet spectrum of Δ-2,3-GlcNAc is consistent with conjugation of its double bond with a carbonyl grouping. It has a peak at 230 mμ (Figure 5) as observed for chromogen I (Kuhn and Kruger, 1956) and as described earlier by Aminoff *et al.* (1952) for Morgan–Elson chromogens. A similar absorption peak is given by 3-*O*-(β-D-Δ-4,5-glucoseenpyranosyluronic acid)-*N*-acetylglucosamine, the product of bacterial hyaluranidase digestion of hyaluronic acid (Linker *et al.*, 1956). The unsaturated disaccharide had a similar absorption peak at 230 mμ but with a lower extinction coefficient (Figure 5), so that conjugation is less efficient when a 4-substituent fixes the pyranose ring form of Δ-2,3-GlcNAc. Reduction with $NaBH_4$ reduced the 230-mμ absorption of Δ-2,3-GlcNAc, but had little effect on that of the unsaturated disaccharide, which is probably due to interaction with the acetamido group. The infrared absorption spectrum of Δ-2,3-GlcNAc (Figure 6) resembled that published by Kuhn and Kruger (1956) for chromogen I, and had typical acetamido absorption peaks at 6.05 and 6.5 μ (Figure 6). Its spectrum resembled that of *N*-acetylglucosamine also in having minor absorption peaks at 7.2 and 7.8 μ, and a major peak (COH stretching) at 9.6 μ. However, like chromogen I, the 8.9-μ peak (ether CO stretching) found in *N*-acetylglucosamine, and presumably due to the pyranose ring oxygen, is not apparent.

Rigorous identification of the structure of Δ-2,3-GlcNAc will depend on the isolation of larger amounts for more extensive analyses. This isolation is in progress.

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