Pert, J. H., Zucker, M. B., Lundberg, A., Yankee, R., and Henderson, E. (1967), *Vox Sanguinis 13*, 119.
Sawardeker, J. S., Sloneker, J. H., and Jeans, A. (1965), *Anal. Chem. 37*, 1602.
Scheinthal, B. M., and Bettelheim, F. A. (1968), *Carbohydrate Res. 6*, 257.
Schulz, H. (1968), Electron Microscopy of Blood Platelets and Thrombosis, New York, N. Y., Springer-Verlag.
Shimada, A., and Nathenson, S. G. (1967), *Biochem. Biophys. Res. Commun. 29*, 828.
Shulman, N. R., Marder, V. J., Hiller, M. C., and Collier, E. M. (1964), *Progr. Hematol. 4*, 222.
Svennson, H. (1967), *Protides Biol. Fluids 15*, 515.
Warren, L. (1960), *Nature 186*, 237.
Wesemann, W., and Zilliken, F. (1968), *Z. Physiol. Chem. 349*, 823.
Winzler, R. J., Harris, E. D., Pekas, D. J., Johnson, C. A., and Weber, P. (1967), *Biochemistry 6*, 2195.

The *O*-Acetyl Derivatives of *N*-Acetylmuramic Acid*

Toshiaki Osawa,† Pierre Sinaÿ,‡ Margaret Halford, and R. W. Jeanloz§

ABSTRACT: The 4-*O*-acetyl, 6-*O*-acetyl, and 4,6-di-*O*-acetyl derivatives of *N*-acetylmuramic acid [2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose] were synthesized starting from benzyl 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside. The 4-*O*-acetyl derivative was obtained by preparation of the 4,6-*O*-benzylidene derivative of the benzyl ester, followed by removal of the benzylidene group, tritylation at O-6, acetylation at O-4, detritylation, and hydrogenolysis of both benzyl groups. The 6-*O*-acetyl derivative was obtained by formation of the internal ester at O-4 and acetylation at O-6, followed by hydrogenolysis of the benzyl aglycon group. The 4,6-di-O-acetyl derivative was obtained by acetylation at O-4 and O-6 of the benzyl ester of the starting material, followed by removal of both benzyl groups.

The various derivatives were characterized by infrared spectroscopy and by paper and gas–liquid partition chromatography. The 6-*O*-acetyl derivative was identical with a compound isolated from *Staphylococcus aureus* cell-wall.

The presence of *O*-acetyl groups has been reported in numerous polysaccharides of bacterial origin, and it has been suggested that the resistance of some bacterial cell walls toward egg-white lysozyme (Brumfitt *et al.*, 1958; Brumfitt, 1959) or to attack by bacteriophages (Brumfitt, 1960) was caused by *O*-acetyl groups linked to the cell wall structure.

After enzymic degradation of *Staphylococcus aureus* cell wall, Ghuysen and Strominger (1963) isolated a mono-*O*-acetyl derivative of *N*-acetylmuramic acid [2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose]. On the basis of the results of the periodate oxidation of this derivative and of the parent disaccharide, these authors proposed the structure of a 4-*O*-acetyl derivative. After it was shown that the *N*-acetylmuramic acid residue is linked in the bacterial cell wall at C-4 (Jeanloz *et al.*, 1963; Sharon *et al.*, 1966), the periodate oxidation of the monoacetyl derivative and of the parent disaccharide was reinvestigated, and the new results were interpreted to suggest a 6-*O*-acetyl derivative of muramic acid (Tipper *et al.*, 1965). Since the results of the periodate oxidation have in the past led to erroneous interpretations of structures containing muramic acid (Salton and Ghuysen, 1959, 1960; Ghuysen and Strominger, 1963), the synthesis of the various *O*-acetyl derivatives of *N*-acetylmuramic acid was accomplished in order to obtain standards for comparison with natural products.

* From the Laboratory for Carbohydrate Research, Department of Biological Chemistry and Medicine, Harvard Medical School, and Massachusetts General Hospital, Boston, Massachusetts 02114. *Received March 25, 1969.* This is Publication LIX of the series Amino Sugars and No. 479 of the Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital. This investigation has been supported by research grants from the National Institute of Allergy and Immunology, National Institutes of Health, U. S. Public Health Service (Grant AI-06692), and from the National Science Foundation (Grant GA-5031). A preliminary note has been published (Jeanloz and Osawa, 1964).

† Present address: Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

‡ Fellow from the Centre National de la Recherche Scientifique (France).

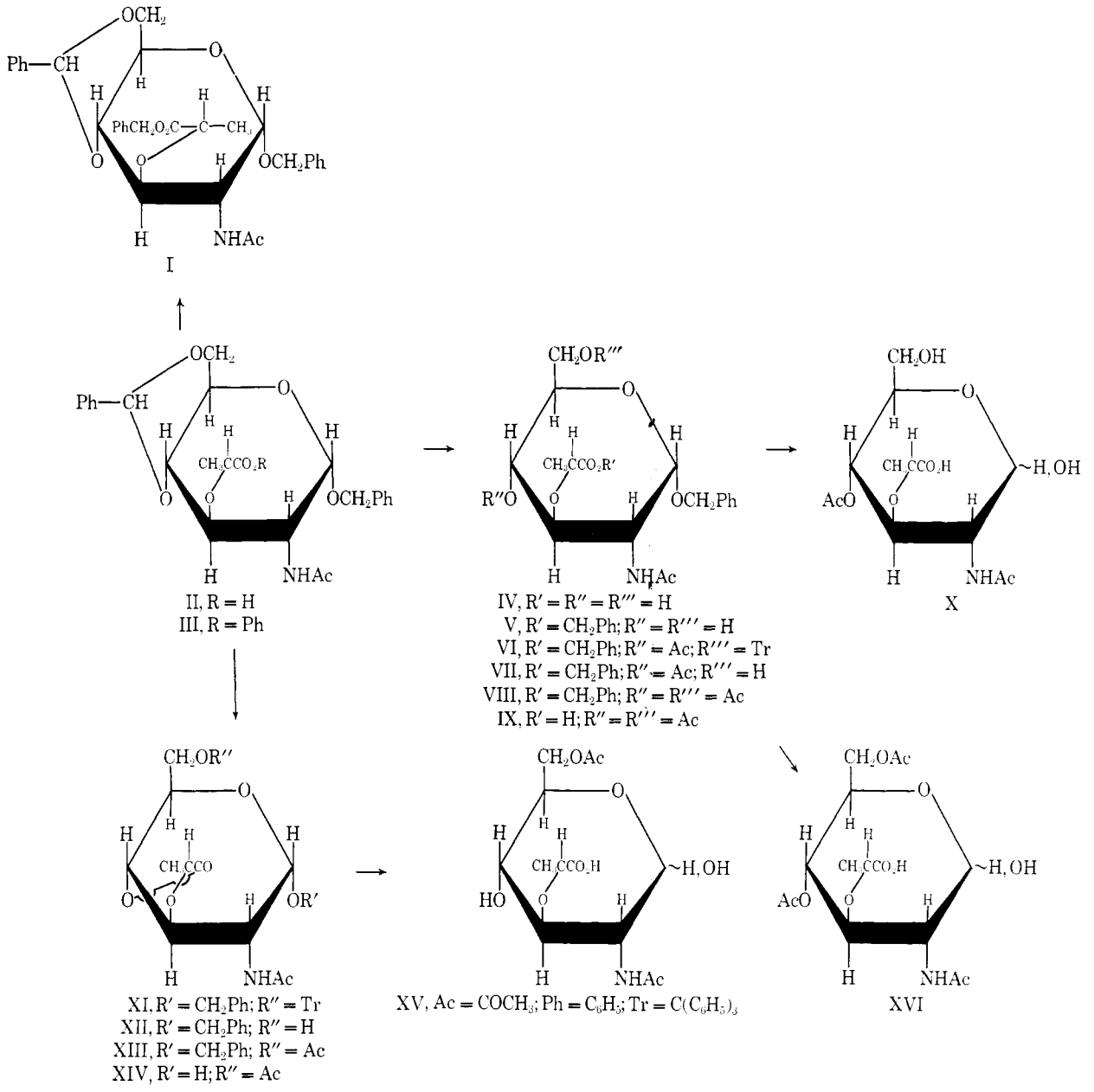
§ To whom inquiries should be sent.

Results and Discussion

Benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside (II) (Scheme I) (Flowers and Jeanloz, 1963; Osawa and Jeanloz, 1965; Jeanloz *et al.*, 1968) was selected as starting material for the synthesis of the three possible *O*-acetyl derivatives of *N*-acetylmuramic acid, 4-*O*-acetyl (X), 6-*O*-acetyl (XV), and 4,6-di-*O*-acetyl (XVI), because the mild conditions used for removal of the benzyl aglycon group by catalytic hydrogenation would not affect the *O*-acetyl groups.

For the preparation of the 4-*O*-acetyl derivative X, the carboxyl group of the lactyl side chain was protected by esterification with benzyl alcohol, in order to avoid the formation

SCHEME I



of the internal ester[1] with *O*-4, which occurs under the conditions of the acetylation. Like the benzyl glycoside group, the benzyl ester group may be split off under hydrogenolytic conditions that will not affect the *O*-acetyl groups. Since a preliminary experiment with pure compound II had shown that the alkaline conditions used for the preparation of the benzyl ester III from the acid II caused partial racemization of the lactyl side chain, impure compound II contaminated with a small proportion of L isomer was used as starting material. Separation of the benzyl ester of the L isomer I from the D isomer III was obtained easily by chromatography. The benzylidene group of compound III was removed by acid hydrolysis, and the resulting compound V was tritylated at C-6, and then acetylated at C-4 to give compound VI. During the removal of the benzylidene group with dilute acetic acid, a small proportion of the benzyl ester group was split off, despite the mildness of the conditions of acid hydrolysis; a similar observation has been made for various methyl ester derivatives of muramic acid and galactomuramic acid (Sinaÿ and Jeanloz, 1969). The hydrolysis of the benzyl ester group was established by the isolation of the internal ester[1] XI after tritylation and acetylation.

Removal of the trityl group of compound VI gave compound VII, which was catalytically hydrogenolyzed into the crystalline 4-*O*-acetyl derivative of *N*-acetylmuramic acid, 2-acetamido-4-*O*-acetyl-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (X).

[1] Erroneously referred as "lactone" in earlier literature.

TABLE I: Comparison of Various *O*-Acetyl Derivatives of *N*-Acetylmuramic Acid by Paper Chromatography and Morgan–Elson Test.

Compound	Paper Chromatography[a] Solvent A R_{GNAc}[d]	Paper Chromatography[a] Solvent B R_{GNAc}[d]	Gas–Liquid Partition Chromatography[b] t_R'	Morgan–Elson Test[c]
2-Acetamido-2-deoxy-D-glucose	1.00	1.00		1.00
N-Acetylmuramic acid	2.00	1.00	1.52	0.95
N-Acetylmuramic acid derivatives				
4-*O*-Acetyl (compound X)	2.20	1.20	1.63	
6-*O*-Acetyl (compound XV)	2.45	1.35	1.75	0.48
4,6-Di-*O*-acetyl (compound XVI)	2.70	1.45		0.28
6-*O*-Acetyl 1,4-internal ester (compound XIV)	3.30	1.80	1.81	
Compound isolated fron *S. aureus*[e]	2.45	1.35		0.49

[a] Paper chromatography on Whatman paper No. 1 (descending) in 1-butanol–acetic acid–water (6:1:1, v/v, solvent A) and in 1-butanol–pyridine–water (6:4:3, v/v, solvent B). [b] Gas–liquid partition chromatography of the tri(methylsilyl) glycosides–per-(trimethylsilyl) ethers on 3% OV-17 on Gas-Chrom A (60–80 mesh); the t_R' values were calculated according to Horváth (1967) relative to the per(trimethylsilyl) ether of *myo*-inositol. [c] Reissig *et al.* (1955) modification; the color was determined after 3 min at 544 nm, and the values obtained were compared with that given by an equimolar amount of 2-acetamido-2-deoxy-D-glucose under similar conditions. [d] GNAc, 2-acetamido-2-deoxy-D-glucose. [e] Kindly provided by Dr. J.-M. Ghuysen.

For the synthesis of the 6-*O*-acetyl derivative XV, benzyl 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-α-D-glucopyranoside (IV), obtained by acid hydrolysis of compound II, readily forms a cyclic internal ester[1] XII (Osawa and Jeanloz, 1965) under the conditions of acetylation, and thus gives directly the 6-*O*-acetyl derivative XIII. During catalytic hydrogenolysis of the benzyl aglycon, the internal ester ring was opened, by addition of water to the solution, to give the 6-*O*-acetyl derivative of *N*-acetylmuramic acid, 2-acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (XV). When the hydrogenolysis was performed in an anhydrous medium, the 6-*O*-acetyl derivative of the internal ester[1] (XIV) was obtained.

For the preparation of the di-*O*-acetyl derivative XVI, acetylation of the benzyl ester V gave the 4,6-di-*O*-acetyl derivative VIII, which was also obtained by acetylation of the 4-*O*-acetyl derivative VII. Hydrogenolysis of the benzyl aglycon and benzyl ester groups proceeded at very different rates. After 2 days, the benzyl ester group had been completely hydrogenolyzed, whereas most of the benzyl glycoside group was still present, and compound IX could be isolated in good yield. After 1 week, the 4,6-di-*O*-acetyl derivative of *N*-acetylmurmamic acid, 2-acetamido-4,6-di-*O*-acetyl-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (XVI), was isolated in amorphous state. Hydrogenolysis of the benzyl glycoside of muramic acid is a sluggish process (Flowers and Jeanloz, 1963; Osawa and Jeanloz, 1965; Jeanloz *et al.*, 1968), and introduction of acetyl groups into the muramic acid molecule further decreases the speed of this reaction. Consequently, the yields of compound XVI were erratic, and chromatography was necessary in order to obtain a pure product, sometimes in very low yield.

The two monoacetyl derivatives were well separated and could be identified with great ease from each other, as well as from *N*-acetylmuramic acid and from the diacetyl derivative, by paper chromatography in two solvent systems (Table I), gas–liquid partition chromatography on OV-17 (Table I), and by their infrared spectra (Figure 1). Although the reproducibility of the R_F values obtained with solvent B on paper chromatograms was not good (see also Sharon *et al.*, 1966), the relative speeds of migration of the various derivatives were found to be very reproducible (Table I). The infrared spectrum of the 6-*O*-acetyl derivative XV shows a typical broad adsorption at 1560–1660 cm^{-1}, which differentiates it clearly from the spectrum of the 4-*O*-acetyl derivative X. Extensive drying of the 6-*O*-acetyl derivative XV gave the internal ester XIV which showed an infrared spectrum different from that of both compounds X and XV (Figure 1).

Since acetyl groups may play a role in protecting the cell wall structure from lysozyme degradation (Brumfitt *et al.*, 1958; Brumfitt, 1959) and from attack by bacteriophages (Brumfitt, 1960), it is of interest to know the exact location of this group in the peptidoglycan structure. Use of the periodate oxidation to establish the location of substituent groups in a muramic acid residue having a free aldehyde group has resulted, in the past, in erroneous interpretations (Salton and Ghuysen, 1959, 1960; Ghuysen and Strominger, 1963; see discussion in Sharon *et al.*, 1966). Reduction of the aldehyde group with sodium borohydride before periodate oxidation (Tipper *et al.*, 1965) resulted in the concomitant loss of part of the *O*-acetyl groups, thereby complicating seriously the interpretation of the oxidation. The possible location of *O*-acetyl groups at position 4 of muramic acid residues is not excluded by the earlier demonstration that most of these residues are substituted at C-4 by 2-acetamido-2-deoxy-D-glucopyranosyl residues (Jeanloz *et al.*, 1963; Sharon *et al.*, 1966), since the degree of branching of the peptidoglycan structure

is unknown. Thus, each end group could possibly consist of a 4-*O*-acetyl-*N*-acetylmuramic acid residue. The availability of the synthetic acetyl esters of muramic acid will greatly facilitate their identification in the future.

The *O*-acetyl derivative isolated from *S. aureus* cell wall was compared with the synthetic samples reported in this study and found to be identical with the 6-*O*-acetyl derivative XV, on the basis of infrared spectra, paper chromatography in two solvent systems, and Morgan–Elson reaction. The large peak shown at 1560–1660 cm^{-1} in the infrared spectrum of the synthetic 6-*O*-acetyl derivative XV (Figure 1) was also observed by Ghuysen and Strominger (1963) for the infrared spectrum of the natural product. On paper chromatograms, as well as in the Reissig *et al.* (1955) modification of the Morgan–Elson test, both the natural product and the synthetic 6-*O*-acetyl derivative XV showed a similar behavior (Table I).

Experimental Section

General. Melting points were taken on a hot stage, equipped with a microscope, and correspond to "corrected melting point." Rotations were determined in semimicro or micro (for amounts smaller than 3 mg) tubes with lengths of 1 or 2 dm, using a polarimeter equipped with a Rudolph photoelectric attachment, Model 200, or with the Perkin-Elmer polarimeter no. 141; the chloroform used was analytical reagent grade and contained approximately 0.75% ethanol. Infrared spectra were determined on a Perkin-Elmer spectrophotometer, Model 237, on potassium bromide disks (Figure 1). Evaporations were performed *in vacuo*, with an outside bath temperature kept below 45°; amounts of volatile solvent smaller than 20 ml were evaporated under a stream of dry nitrogen. The microanalyses were performed by Dr. M. Manser, Zurich, Switzerland.

Chromatographies. Column chromatographies were made on "Silica Gel Davison," from the Davison Co., Baltimore 3, Md. (grade 950; 60–200 mesh) used without pretreatment. The sequence of eluents was hexane, benzene, or dichloroethane, ether, ethyl acetate, acetone, and methanol individually or in binary mixtures. The proportion of weight of substance to be adsorbed to weight of adsorbent was 1 to 50–100. The proportion of weight of substance in grams to volume of fraction of eluent in milliliters was 1 to 100. The ratio of diameter to length of the column was 1 to 20.

The homogeneity of the nonpolar substances was controlled by thin-layer chromatography (ascending) on plates covered by a mixture of silica gel G and silica gel GF (Merck) revealed by concentrated sulfuric acid. The polar substances were examined on thin-layer plates of microcrystalline cellulose (Avicel), and the spots were detected with the same reagents as on paper chromatograms.

The paper chromatographies were performed, descending, on Whatman paper no. 1, and the spots were detected with the silver nitrate reagent or with the alkaline reagent of Sharon and Seifter (1964). The results are reported in Table I.

The gas–liquid partition chromatographies were performed with a gas–liquid partition chromatograph, Perkin-Elmer, Model 900, equipped with a flame ionization detector, on a column of stainless steel (300 × 0.3 cm), packed with 3% OV-17 on Gas-Chrom A (60–80 mesh) (Applied Science Laboratories, State College, Pa.), after per(trimethylsilyl)ation with Tri-Sil (Pierce Chemical Co., Rockford, Ill.). The compounds were injected at 120°, and the temperature was raised at the rate of 5°/min. The times of elution were compared with that of the per(trimethylsilyl) ether of *myo*-inositol, and the t_R' values, calculated according to Horváth (1967), are reported in Table II.

Color Reaction. Compounds X, XI, and XVI (0.4–0.5 mg in 1 ml of water) were treated with the Morgan–Elson reagent, as modified by Reissig *et al.* (1955). The amount of color formed was determined after 3 min at 544 nm, and the result was compared with that given by an equimolar amount of 2-acetamido-2-deoxy-D-glucose (Table I).

Benzyl 2-Acetamido-3-O-[D-1-(benzyloxycarbonyl)ethyl]-4,6-O-benzylidene-2-deoxy-α-D-glucopyranoside (III) and the L Isomer (I). To a solution of benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1-carboxethyl)-2-deoxy-α-D-glucopyranoside (II) (2.35 g, dried over phosphorus pentoxide at 80–85° *in vacuo*, slightly contaminated with the L isomer) in dry, peroxide-free tetrahydrofuran (50 ml, freshly distilled over lithium aluminum hydride) was added sodium hydride (0.29 g of a 50% oil suspension; Metal Hydrides, Beverly, Mass.). The solution was heated at reflux under magnetic stirring. After 2 hr, a solution of benzyl bromide (1.71 g) in dry tetrahydrofuran (5 ml) was added, and the heating and stirring were continued for 24 hr. The reaction mixture was poured into ice–water (about 250 ml) and extracted once with pentane (100 ml). The aqueous layer and the precipitate were extracted with chloroform. The extracts were washed several times with water, dried with sodium sulfate, and concentrated to dryness. Investigation of the residue by thin-layer chromatography in chloroform–methanol (99.2:0.8, v/v) showed three compounds which were, in order of increasing R_F, the starting material II, benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-[L-(benzyloxycarbonyl)ethyl]-2-deoxy-α-D-glucopyranoside (I) and (III). The residue (2.46 g) was dissolved in ethylene dichloride, and the solution was chromatographed on silica gel (200 g). Elution with a mixture of ethylene dichloride and ether (6:1, v/v) gave crystalline fractions (1.09 g, 40%) which were recrystallized from methanol to give needles: mp 178–179°, $[\alpha]_D^{20}$ +109° (*c* 0.79, chloroform).

Anal. Calcd for $C_{32}H_{35}NO_8$: C, 68.44; H, 6.28; N, 2.49. Found: C, 68.40; H, 6.29; N, 2.74.

Further elution with the same solvent mixture gave a mixture of compounds I and III (1.05 g, 38%; compound III 30% and compound I 8%, according to thin-layer chromatography). Preparative thin-layer chromatography of this mixture (75 mg) with a mixture of chloroform–methanol (99.2:0.8, v/v) gave pure compound I (16.5 mg), which was recrystallized from methanol into needles (12.5 mg): double mp 229 and 237–239°, $[\alpha]_D^{20}$ +32° (*c* 0.54, chloroform).

Anal. Calcd for $C_{32}H_{35}NO_8$: C, 68.44; H, 6.28; N, 2.49. Found: C, 68.50; H, 6.36; N, 2.42.

Since pure compound II (free of L isomer) gave a small proportion of compound I, optical purity of the starting material II was not necessary.

Benzyl 2-Acetamido-3-O-[D-1-(benzyloxycarbonyl)ethyl]-2-deoxy-α-D-glucopyranoside (V). A mixture of compound III (1.1 g) and 60% acetic acid (5 ml) was heated for 1 hr on a boiling-water bath. Evaporation gave a glassy residue, which was freed from benzaldehyde and acetic acid by addition of water, followed by distillation, and then dried by addition of dry toluene and distillation. The residue was dissolved in

ethyl acetate and chromatographed on a silica gel column. Elution with the same solvent gave 0.69 g (74%) of crystalline material, which was recrystallized from a mixture of acetone, ether, and hexane to give needles: mp 121–122°, $[\alpha]_D^{20}$ +142° (*c* 0.81, ethanol).

Anal. Calcd for $C_{25}H_{31}NO_8$: C, 63.41; H, 6.60; N, 2.96. Found: C, 63.36; H, 6.59; N, 2.81.

Benzyl 2-Acetamido-4-O-acetyl-3-O-[D-*1-(benzyloxycarbonyl)ethyl]-2-deoxy-6-O-triphenylmethyl-α-*D-*glucopyranoside* (*VI*). To a solution of compound V (655 mg) in pyridine (10 ml) was added chlorotriphenylmethane (464 mg). The solution was heated for 1 hr on a boiling-water bath and then cooled to room temperature. Acetic anhydride (7 ml) was added, and the solution was kept overnight at room temperature. It was then poured into ice–water and extracted with chloroform. The chloroform layer was washed with a 10% potassium hydrogen sulfate solution and with water, dried, and evaporated. The residue was dissolved in benzene and chromatographed on silica gel. Elution with a mixture of benzene and ether (4:1, v/v) gave 760 mg (75%) of crystalline material, which was recrystallized from a mixture of acetone, ether, and pentane to give needles: mp 91–94°, $[\alpha]_D^{25}$ +91° (*c* 0.53, chloroform).

Anal. Calcd for $C_{46}H_{47}NO_9$: C, 72.90; H, 6.25; N, 1.85. Found: C, 72.89; H, 6.26; N, 1.92.

Benzyl 2-Acetamido-4-O-acetyl-3-O-[D-*1-(benzyloxycarbonyl)ethyl]-2-deoxy-α-*D-*glucopyranoside* (*VII*). A solution of compound VI (750 mg) in 60% acetic acid (20 ml) was heated for 30 min at 100°. The solution was evaporated, and the last traces of acetic acid were removed by addition of toluene, followed by distillation. The residue was dissolved in ethylene dichloride and chromatographed on silica gel. Ether–ethyl acetate (4:1, v/v) eluted 320 mg (63%) of crystalline fractions. They were recrystallized from a mixture of acetone, ether, and pentane to give needles: mp 130–132°, $[\alpha]_D^{25}$ +116° (*c* 1.03, chloroform).

Anal. Calcd for $C_{27}H_{23}NO_9$: C, 62.90; H, 6.45; N, 2.72. Found: C, 62.86; H, 6.47; N, 2.68.

Benzyl 2-Acetamido-3-O-[*(R)-1-carboxyethyl*]*-2-deoxy-6-O-triphenylmethyl-α-*D-*glucopyranoside 4-Internal Ester* (*XI*). Compound III (2.2 g) was hydrolyzed by 60% acetic acid as just described. The residue (1.6 g) was directly dissolved in pyridine (30 ml) and freshly prepared chlorotriphenylmethane (1.4 g) was added. The solution was heated for 1 hr on a boiling-water bath. After the solution cooled to room temperature, acetic anhydride (2.5 ml) was added. After 24 hr, the solution was poured into ice–water (400 ml) and extracted with chloroform. The extracts were washed with 10% potassium hydrogen sulfate, then with water, and dried with sodium sulfate. After evaporation, thin-layer chromatography of the residue in benzene–ether–methanol (50:50:3, v/v) showed the presence of several triphenylmethyl derivatives (yellow spots detected with sulfuric acid). The residue was dissolved in benzene, and the solution was chromatographed on a silica gel column. Benzene–ether (6:1, v/v) eluted triphenylcarbinol, benzene–ether (4:1, v/v) compound VI (300 mg, 10%), and benzene–ether (2:1, v/v) first a mixture of compounds VI and XI and then pure compound XI (750 mg, 31%). Recrystallization from acetone–pentane gave needles: mp 219–221°, $[\alpha]_D^{20}$ +103° (*c* 0.87, chloroform).

Anal. Calcd for $C_{37}H_{37}NO_7$: C, 73.13; H, 6.14; N, 2.30; O, 18.43. Found: C, 73.13; H, 6.70; N, 2.22; O, 18.51.

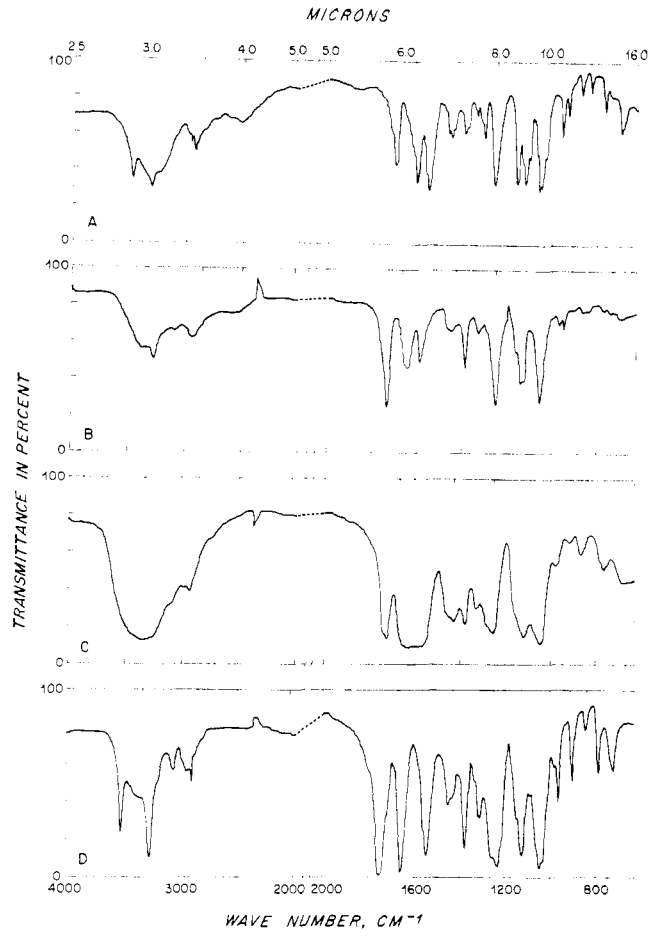


FIGURE 1: Infrared spectra determined on the Perkin-Elmer spectrophotometer, Model 237, on KBr disks. (A) 2-Acetamido-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (*N*-acetylmuramic acid), (B) 2-acetamido-4-*O*-acetyl-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (*N*-acetyl-4-*O*-acetylmuramic acid, X), (C) 2-acetamido-6-*O*-acetyl-3-*O*-(D-1-carboxyethyl)-2-deoxy-D-glucose (*N*-acetyl-6-*O*-acetylmuramic acid, XV), and (D) 2-acetamido-6-*O*-acetyl-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-D-glucose 4-internal ester (*N*-acetyl-6-*O*-acetylmuramic acid 4-internal ester, XIV).

2-Acetamido-4-O-acetyl-3-O-(D-*1-carboxyethyl)-2-deoxy-*D-*glucose* (*N-Acetyl-4-O-acetylmuramic Acid*) (*X*). A solution of compound VII (60 mg) in 80% ethanol (10 ml) was hydrogenolyzed for 24 hr at room temperature and pressure in the presence of 10% palladium on charcoal as catalyst (50 mg). After filtration, the solution was evaporated. The crystalline residue was recrystallized from ethyl acetate to give small needles (33 mg, 85%): mp 156–158°, $[\alpha]_D^{20}$ +82° (*c* 0.50, 90% ethanol). The product showed no mutarotation. The behavior of the compound on paper chromatograms and on gas–liquid partition chromatography of its per(trimethylsilyl) derivative is reported in Table I.

Anal. Calcd for $C_{13}H_{21}NO_9 \cdot 0.5H_2O$: C, 45.34; H, 6.44; N, 4.07. Found: C, 45.49; H, 6.19; N, 4.03.

Benzyl 2-Acetamido-6-O-acetyl-3-O-[*(R)-1-carboxyethyl*]*-2-deoxy-α-*D-*glucopyranoside 4-Internal Ester* (*XIII*). A solution of compound II (0.4 g) in 60% acetic acid (5 ml) was heated for 1 hr on a boiling-water bath. After being cooled to room temperature, the solution was evaporated to a syrupy residue and the last traces of acetic acid and benzaldehyde

were removed by addition of water followed by distillation. The residue was finally dried by addition of toluene, followed by distillation, and dissolved in pyridine (3 ml). Acetic anhydride (2 ml) was added and, after 24 hr, the excess of acetic anhydride was decomposed by addition of methanol under cooling. The solvents were evaporated, and the last traces of pyridine were removed by additions of toluene, followed by evaporation. The residual, crystalline mass was recrystallized from ethanol–ether to give needles (0.31 g, 90%): mp 170–172°, $[\alpha]_D^{20}$ +144° (*c* 1.28, chloroform).

Anal. Calcd for $C_{20}H_{25}NO_8$: C, 58.96; H, 6.19; N, 3.44. Found: C, 58.80; H, 6.00; N, 3.52.

The internal ester was hydrolyzed very slowly by water. After 4 days in ethanol–water (1:1, v/v), thin-layer chromatography showed the presence of some unreacted starting material.

2-Acetamido-6-O-acetyl-3-O-(D-*1-carboxyethyl*)*-2-deoxy-*D-*glucose* (*N-Acetyl-6-O-acetylmuramic Acid*) (*XV*). A solution of compound XIII (92 mg) in 80% ethanol (10 ml) was hydrogenated for 2 days at room temperature and pressure in the presence of 10% palladium on charcoal as catalyst (90 mg). After filtration, the solution was evaporated. The residual syrup (74 mg) was a mixture of compounds XV and XIV, as shown by thin-layer chromatography in butyl alcohol–acetone–water (4:5:1, v/v); the spots appeared after heating at 200° without spray. The residue was chromatographed on a silica gel column (6 g). Acetone eluted first a mixture of compounds XV and XIV (28 mg), then pure compound XV (13 mg). More compound XV was eluted with acetone–methanol (4:1, v/v), and finally with pure methanol. The fraction eluted with methanol was evaporated, dissolved in a small amount of ethanol, and precipitated by addition of ether to give an amorphous white powder: $[\alpha]_D^{20}$ +46° (*c* 1.10, 90% ethanol).

Anal. Calcd for $C_{13}H_{21}NO_9$: C, 46.57; H, 6.31. Found: C, 46.36; H, 6.74.

This amorphous material was recrystallized from moist methanol–acetone to give needles: mp 176–176.5°. The product showed no mutarotation in water: $[\alpha]_D^{20}$ +56° (*c* 0.63, water).

Anal. Calcd for $C_{13}H_{21}NO_9 \cdot 0.5H_2O$: C, 45.35; H, 6.44; N, 4.07; O, 44.14. Found: C, 44.89; H, 6.22; N, 4.36; O, 44.35.

2-Acetamido-6-O-acetyl-3-O-[(*R*)-*1-carboxyethyl*]*-2-deoxy-α-*D-*glucose 4-Internal Ester* (*N-Acetyl-6-O-acetylmuramic Acid 4-Internal Ester*) (*XIV*). A solution of compound XIII (203 mg) in absolute ethanol (25 ml) was hydrogenated for 4 days at room temperature and pressure in the presence of 10% palladium on charcoal (200 mg) as catalyst. After filtration, the solution was evaporated, and the residue (146 mg) was shown by thin-layer chromatography in chloroform–methanol (97:3, v/v) to be mainly compound XIV. It was dissolved in ethyl acetate–acetone (2:1, v/v) and chromatographed on a silica gel column (12 g). Elution with the same solvent mixture gave compound XV (97 mg, 61%): mp 197–199°, $[\alpha]_D^{20}$ +104 to +91° (after 14 hr, *c* 0.58, methanol). The substance was homogeneous, on descending paper chromatograms, in butyl alcohol–pyridine–water (6:4:3, v/v) and in butyl alcohol–acetic acid–water (6:1:1, v/v) (Table I).

Anal. Calcd for $C_{13}H_{19}NO_8$: C, 49.21; H, 6.04; N, 4.41. Found: C, 49.08; H, 6.08; N, 4.41.

Benzyl 2-Acetamido-4,6-di-O-acetyl-3-O-[D-*1-(benzyloxycarbonyl)ethyl*]*-2-deoxy-α-*D-*glucopyranoside* (*VIII*). To a solution of compound V (80 mg) in pyridine (3 ml) was added acetic anhydride (2 ml). The solution was kept overnight at room temperature. The excess of acetic anhydride was decomposed by addition of methanol under cooling, and the solution was evaporated. The remaining traces of pyridine were removed by addition of toluene, followed by evaporation. The crystalline residue was recrystallized from ether–pentane to give needles (80 mg, 85%): mp 86–88°, $[\alpha]_D^{20}$ +110° (*c* 0.79, chloroform).

Anal. Calcd for $C_{29}H_{35}NO_{10}$: C, 62.47; H, 6.33; N, 2.51. Found: C, 62.57; H, 6.38; N, 2.66.

An identical compound was obtained by acetylation of compound VII in pyridine with acetic anhydride.

Benzyl 2-Acetamido-4,6-di-O-acetyl-3-O-(D-*1-carboxyethyl*)*-2-deoxy-α-*D-*glucopyranoside* (*IX*). A solution of compound VIII (260 mg) in 90% ethanol (24 ml) was hydrogenated for 48 hr, at room temperature and normal pressure, in the presence of 10% palladium on charcoal (200 mg) as a catalyst. After filtration, the solution was evaporated, and the crystalline residue was recrystallized from dilute ethanol to give needles (130 mg, 60%): mp 135–136°, $[\alpha]_D^{20}$ +105° (*c* 1.19, 90% ethanol).

Anal. Calcd for $C_{22}H_{29}NO_{10}$: C, 56.53; H, 6.25. Found: C, 56.63; H, 5.86.

2-Acetamido-4,6-di-O-acetyl-3-O-(D-*1-carboxyethyl*)*-2-deoxy-*D-*glucose* (*N-Acetyl-4,6-di-O-acetylmuramic Acid*) (*XVI*). A solution of compound VIII (130 mg) in absolute ethanol (9 ml) was hydrogenated for 1 week at room temperature and normal pressure in the presence of 10% palladium on charcoal (50 mg). After filtration, the solution was evaporated to give a syrup which was dissolved in ethanol and precipitated by a large excess of ether (73 mg, 75%): $[\alpha]_D^{17}$ +66° (*c* 0.45, 90% ethanol). The substance was homogeneous on thin-layer chromatography in butyl alcohol–acetone–water (4:5:1, v/v) and on descending paper chromatograms in butyl alcohol–pyridine–water (6:4:3, v/v) and in butyl alcohol–acetic acid–water (6:1:1, v/v) (Table I). It retained tenaciously 0.5 mole of ether, after drying in high vacuum for 3 hr, at 60°.

Anal. Calcd for $C_{15}H_{23}NO_{10} \cdot 0.5(C_2H_5)_2O$: C, 49.15; H, 6.79; N, 3.37. Found: C, 48.96; H, 6.82; N, 3.23.

Acknowledgments

The authors thank Dr. V. Reinhold for the gas–liquid partition chromatographic determinations and Dr. J.-M. Ghuysen for a sample of 6-*O*-acetylmuramic acid isolated from *S. aureus* cell wall.

References

Brumfitt, W. (1959), *Brit. J. Exptl. Pathol. 40*, 441.

Brumfitt, W. (1960), *J. Pathol. Bacteriol. 79*, 1.

Brumfitt, W., Wardlaw, A. C., and Park, J. T. (1958), *Nature 181*, 1783.

Flowers, H. M., and Jeanloz, R. W. (1963), *J. Org. Chem. 28*, 2983.

Ghuysen, J.-M., and Strominger, J. L. (1963), *Biochemistry 2*, 1119.

Horváth, C. (1967), *in* The Practice of Gas Chromatography, Ettre, L., and Zlatkis, A., Ed., New York, N. Y., Wiley, p 129.

Jeanloz, R. W., and Osawa, T. (1964), *Intern. Symp. Fleming's Lysozyme 3*, 70 RT.

Jeanloz, R. W., Sharon, N., and Flowers, H. M. (1963), *Biochem. Biophys. Res. Commun. 13*, 20.
Jeanloz, R. W., Walker, E., and Sinaÿ, P. (1968), *Carbohydrate Res. 6*, 184.
Osawa, T., and Jeanloz, R. W. (1965), *J. Org. Chem. 30*, 448.
Reissig, J. L., Strominger, J. L., and Leloir, L. F. (1955), *J. Biol. Chem. 217*, 959.
Salton, M. R. J., and Ghuysen, J.-M. (1959), *Biochim. Biophys. Acta 36*, 552.
Salton, M. R. J., and Ghuysen, J.-M. (1960), *Biochim. Biophys. Acta 45*, 355.
Sharon, N., Osawa, T., Flowers, H. M., and Jeanloz, R. W. (1966), *J. Biol. Chem. 241*, 223.
Sharon, N., and Seifter, S. (1964), *J. Biol. Chem. 239*, PC 2398.
Sinaÿ, P., and Jeanloz, R. W. (1969), *Carbohydrate Res. 10*, 189.
Tipper, D. J., Ghuysen, J.-M., and Strominger, J. L. (1965), *Biochemistry 4*, 468.

Pentose Synthesis in *Escherichia coli**

Richard Caprioli† and David Rittenberg

ABSTRACT: *Escherichia coli* was grown on [1-^{18}O]-, [2-^{18}O]-, and [6-^{18}O]glucose and [2-^{18}O]fructose as the sole carbon sources. Growth was terminated in midexponential phase and the nucleic acids were isolated and degraded to the nucleoside level. The distribution of ^{18}O in the nucleosides was determined in a mass spectrometer by observing mass shifts of fragment ions in the mass spectrum of the nucleosides. A fragment ion containing ^{18}O exhibits a peak in the mass spectrum 2.0043 mass units higher than the normal ion containing ^{18}O. The relative intensities of the peaks can be related to the per cent ^{18}O in the fragment. When [1-^{18}O]- and [6-^{18}O]-glucose were used as carbon sources, 35 and 64%, respectively, of the original label of the hexose appeared in the 5′-oxygen atom of the nucleosides. No other oxygen atoms were labeled. When [2-^{18}O]glucose and [2-^{18}O]fructose were used as the substrates, the ribosides were similarly labeled with approximately 14% of the original label of the hexose in the 2′ position and 22% in the 4′ position (the deoxyribosides contained ^{18}O only in the 4′-oxygen atom). These results show that both the oxidative and nonoxidative pathways operate simultaneously to produce pentose phosphate. The major portion (about 70%) of the pentose in the nucleic acids was synthesized *via* the nonoxidative pathway and the remainder *via* the oxidative pathway. The above experiments also provide evidence which suggests that the enzyme aldolase in *E. coli*, in contrast to that of mammalian muscle, cleaves fructose 1,6-diphosphate without the obligatory loss of the C-2 oxygen atom.

D-Glucose is commonly utilized by living cells as a source of energy and intermediates necessary for the biosynthesis of vital cellular components. For the conversion of glucose into ribose, two major biosynthetic pathways coexist in most living cells; the oxidative pathway and the nonoxidative pathway.

When glucose is metabolized *via* the oxidative pathway to pentose, the reactions are accompanied by the formation of 2 moles of TPNH/mole of hexose utilized. The result is the conversion of 1 mole of hexose into 1 mole of pentose and 1 mole of CO_2.

When glucose is metabolized by the nonoxidative pathway, the reactions result in the conversion of 2.5 molecules of hexose phosphate into 3 molecules of pentose phosphate.

In addition to the oxidative and nonoxidative pathways, there are several other pathways by which pentose may be produced. The Entner–Doudoroff pathway produces glyceraldehyde 3-phosphate and pyruvic acid from hexose through the intermediate 2-keto-3-deoxy-6-phosphogluconate (Entner and Doudoroff, 1952). Pentose could be formed from this triose through the action of transketolase. However, it has recently been shown (Fraenkel and Levisohn, 1967; Zablotny and Fraenkel, 1967) that *Escherichia coli* mutants lacking gluconate 6-phosphate dehydrase and phosphoglucose isomerase do not utilize the Entner–Doudoroff pathway when grown on glucose. A second minor pathway for glucose metabolism, demonstrated in animal tissue, is the glucuronic acid pathway. In this pathway, glucose may be converted into ribose through the formation of 3-keto-L-gulonic acid, an intermediate in ascorbic acid biosynthesis (Hassan and Lehninger, 1956). However, Hiatt and Lareau (1958) have obtained evidence to show that it does not play a role in ribose biosynthesis.

The concurrent operation of the oxidative and nonoxidative

* From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, New York. *Received April 15, 1969.* This work was supported in part by grants from the National Institutes of Health (No. AM-08625) and the Atomic Energy Commission (No. AT(30-1)1803).

† Predoctoral fellow of the National Institutes of Health (No. Fl-GM-37691-10A1). This work has been submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.