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THE SYNTHESIS OF ACETAMIDO-DEOXY KETOSES BY ACETOBACTER SUBOXYDANS

PART I[1]

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ABSTRACT

The microbiological oxidation of 2-acetamido-2-deoxy-D-glucitol by *Acetobacter suboxydans* yielded a crystalline ketose which is shown to be 5-acetamido-5-deoxy-L-xylohexulose.

INTRODUCTION

The oxidation of unsubstituted polyhydric alcohols by proliferating cells of *Acetobacter suboxydans* has been studied intensively (1, 2, 3, 4, 5, 6), and has given rise to the Bertrand–Hudson rule for oxidations occurring in the pH range 5 to 6.5 (7, 8). Thus, a polyhydric alcohol possessing the D-erythro configuration (I) is oxidized mainly at the secondary alcohol group adjacent to the terminal primary alcohol to give a ketose (II). Bollenback and Underkofler (9), Richtmyer, Stewart, and Hudson (10), Hough, Jones, and Mitchell (11), and Jones and Mitchell (12) investigated the microbiological oxidation of terminal-substituted polyhydric alcohols. The latter authors reported the oxidation of several alcohols, giving chromatographic evidence for the presence of oxidation products, and characterizing completely the ketoses produced from 1-deoxy-1-*S*-ethyl-D-glucitol (11) and 1-deoxy-1-*S*-ethyl-D-arabitol (12).

We now wish to report the isolation and proof of structure of the ketose 5-acetamido-5-deoxy-L-xylohexulose (IV), produced by microbiological oxidation at pH 5 to 6.5 of 2-acetamido-2-deoxy-D-glucitol (III). Oxidation was slow when the culture medium contained only 2-acetamido-2-deoxy-D-glucitol, and was more rapid when glycerol (0.3%) was included. However, oxidation was not complete after 21 days.

The product (IV) was obtained as a white crystalline solid. Analysis indicated an acetamido-deoxy hexulose and the compound gave absorptions in the infrared corresponding to OH, NH, and the amide linkage. On periodate oxidation of (IV) in unbuffered solution it was found to consume 2.85 moles of periodate with the liberation of 0.85 mole formic acid and 0.8 mole formaldehyde per mole of sugar, which indicated that the ketose (IV) possessed a pyranose ring form. The ketose (IV) gave a crystalline phenylosazone (5-acetamido-5-deoxy-L-xylohexose phenylosazone) (V, acyclic form) which possessed an acetamido-deoxy group and gave absorptions in the infrared corresponding to OH, NH, the aromatic ring, and the amide linkage. The phenylosazone (V), when oxidized with periodate by the method of Hough, Powell, and Woods (13), consumed 1 mole of periodate immediately and released no formic acid or formaldehyde. Periodate oxidation gave an immediate precipitate of the 1,2-bisphenylhydrazone of mesoxalaldehyde (VI) (14),

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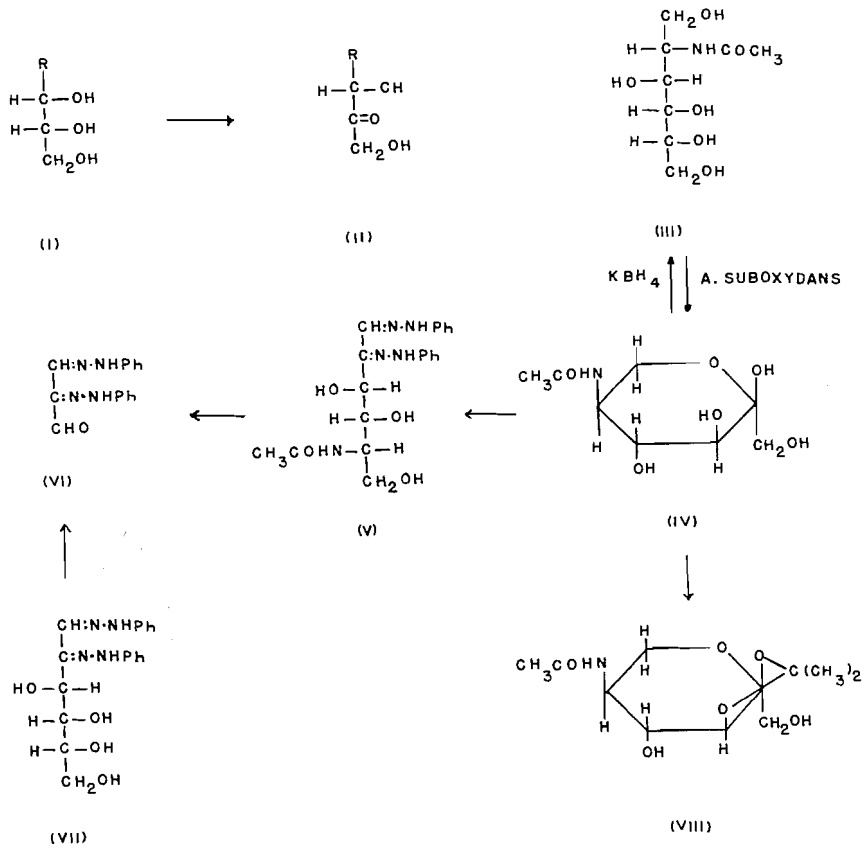
which was identified by mixed melting point and comparison of its infrared spectrum with that of an authentic sample obtained from the periodate oxidation of D-glucose phenylosazone (VII, acyclic form). These results indicated that the osazone (V) possessed hydroxyl groups at carbons 3 and 4 and that the acetamido-deoxy group was located at carbon 5. Considered with the preceding evidence, these results indicate that the site of biological oxidation in 2-acetamido-2-deoxy-D-glucitol (III) was at carbon 5, in accordance with the well-known enzyme specificity for oxidations at pH 5 to 6.5.

The ketose (IV) was shown to be a derivative of L-sorbose by its reduction with sodium borohydride to 2-acetamido-2-deoxy-D-glucitol, which had melting point, optical rotation, and infrared spectrum identical with those of an authentic specimen. The ketose (IV) gave, with acidified acetone, a non-reducing *O*-isopropylidene derivative (VIII) which was not attacked by periodate under unbuffered conditions, indicating that the mono-*O*-isopropylidene group spanned carbons 2 and 3 as in the case of 2,3-mono-*O*-isopropylidene-L-sorbose.



The microbiological oxidations of 1-acetamido-1-deoxy-*N*-methyl-D-glucamine and 1,2-dideoxy-2-acetamide-D-glucitol have been carried out and structural studies are in progress on the ketoses isolated.

EXPERIMENTAL

Solutions were concentrated under reduced pressure (ca. 15 mm) below 40° C. Melting points are uncorrected and optical rotations were determined in water at 23±3° C unless

otherwise stated. Paper chromatography was carried out on Whatman No. 1 paper using the descending method (15). The following solvent systems were used (v/v):

(*a*) ethyl acetate – acetic acid – formic acid – water (18:3:1:4);
(*b*) butan-1-ol – pyridine – water (5:3:2);
(*c*) butan-1-ol – ethanol – water (9:3:3).

Reducing compounds were detected with the *p*-anisidine hydrochloride spray reagent (16) and ketoses with the orcinol/trichloroacetic acid spray reagent (17). Non-reducing compounds were detected with the alkaline silver nitrate spray reagent (18). Rates of movement of the sugars on paper chromatograms are given relative to that of rhamnose (R_{rh}). Infrared spectra were measured as a 6% solution in chloroform or as a 0.8% dispersion in a potassium bromide disk, using a Perkin–Elmer Model 21 spectrophotometer.

Acetobacter suboxydans (A.T.C.C. No. 621) was obtained from the American type culture collection and maintained at 5° C on slopes of agar (3% w:v) containing sorbitol (5% w:v), yeast extract powder (0.5% w:v), potassium dihydrogen phosphate (0.05% w:v), and D-glucose (0.05% w:v). At approximately 2-month intervals a broth containing sorbitol (7% w:v), yeast extract powder (0.5% w:v), and potassium dihydrogen phosphate (0.05% w:v) in tap water was inoculated from the slopes. After 72 hours, when growth of the organism was apparent, fresh slopes were inoculated from the broth and left 2–3 days at room temperature when cream-colored colonies became visible. The slopes were then stored at 5° C.

*5-Acetamido-5-deoxy-*L*-xylohexulose*

A broth consisting of 2-acetamido-2-deoxy-D-glucitol (8.5 g), prepared by sodium borohydride reduction of 2-acetamido-2-deoxy-D-glucose, glycerol (0.5 g), yeast extract powder (0.8 g), and potassium dihydrogen phosphate (0.16 g) in tap water (160 ml) was distributed among eight 250-ml conical flasks giving a surface:volume ratio of ca. 2.5:1. The broths were then autoclaved at 15 p.s.i. for 30 minutes, allowed to cool, and each inoculated with one drop of a 48-hour culture of *Acetobacter suboxydans* grown in glycerol (6%). The broths were stored at room temperature in the dark, without agitation, for 21 days. At intervals small samples (2 ml) were withdrawn using sterile pipettes and the broth examined on paper chromatograms. The gradual disappearance of glycerol and the appearance of dihydroxyacetone (1,3-dihydroxypropanone) and a spot which had R_{rh} 0.59 (solvent (*c*)), 0.73 (solvent (*a*)), 0.65 (solvent (*b*)), and gave a yellow color with orcinol/trichloroacetic acid spray were noted. A quantitative measure of the rate of oxidation was obtained by estimation of the copper-reducing power of the broth using the Somogyi method (19). The results are recorded in Table I. After 21 days the broths

TABLE I

Time (days)	% yield of ketose (corrected for the presence of dihydroxyacetone)
3	4.5
11	11.25
17	14.55

were combined and poured into 2 volumes of ethanol. The cell debris was removed by filtration and the filtrate was evaporated to about 20 ml and then deionized by

passage through columns of Amberlite IR 120 (H) and Duolite A4 (OH) resins. The eluate was concentrated to a syrup which crystallized. The crystalline material was washed with ethanol and dried, m.p. 154–155° C, mixed m.p. with 2-acetamido-2-deoxy-D-glucitol, 154–155° C. The mother liquors from the crystallization were concentrated to a syrup (2.4 g) which was deposited on cellulose powder and packed as a level band on top of a cellulose column (54 cm × 4.5 cm). The column was irrigated with butan-1-ol half saturated with water, and 5-ml fractions of the eluate were collected using an automatic fraction collector (20). The fractions were examined on paper chromatograms run in solvent (*c*) and sprayed with alkaline silver nitrate spray reagent. A good separation of the sugars was obtained and the appropriate fractions were evaporated to dryness yielding crystalline 5-acetamido-5-deoxy-L-xylohexulose. One recrystallization from ethanol/ether gave crystals m.p. 174–176° C, $[\alpha]_D$ −62. Anal. Calc. for $C_8H_{15}O_6N$: C, 43.4; H, 6.8; N, 6.3. Found: C, 43.3; H, 6.9; N, 6.35. Absorptions in the infrared due to OH and NH (broad fused peak at 3300 cm^{-1}) and the amide linkage (1625 cm^{-1} and 1570 cm^{-1}) were observed. For infrared spectrum see Appendix (A).

Periodate Oxidation of 5-Acetamido-5-deoxy-L-xylohexulose

The ketose (50.62 mg) was dissolved in about 40 ml of water, 0.3 *M* sodium metaperiodate (5.0 ml) was added, and the volume was made up to 50 ml with water. A blank was prepared with 0.3 *M* sodium metaperiodate (5.0 ml), which was made up to 50 ml with water. The solutions were stored in the dark at room temperature and 5-ml aliquots were removed at intervals from each of the two solutions for the determination of periodate uptake (21) and formic acid production (22). Formaldehyde production was measured by the chromotropic acid method (23). The results are recorded in Tables II and III.

TABLE II

Oxidation of 5-acetamido-5-deoxy-L-xylohexulose

Time (hr)	Periodate uptake (moles/mole)	Formic acid production (moles/mole)
0.12	1.15	0.31
1.83	1.79	0.59
8.5	2.68	0.82
24	2.85	0.85

TABLE III

Time (hr)	Formaldehyde production (moles/mole)
0.2	0.36
1	0.53
25	0.62
45	0.80

5-Acetamido-5-deoxy-L-xylohexose Phenylosazone

5-Acetamido-5-deoxy-L-xylohexulose (45 mg) was dissolved in water (2.4 ml), and acetic acid (0.24 ml) and freshly distilled phenylhydrazine (0.24 ml) were added. The mixture was heated in a boiling-water bath for 15 minutes, after which time the phenylosazone had crystallized. The solution was cooled and the phenylosazone was collected by filtration, washed with ice-cold dilute acetic acid, ice water, and cold (−60° C)

methanol, and then dried. One recrystallization from ethanol gave a yellow powder (small needles) m.p. 202–203° C (decomposes). (An optical rotation could not be measured due to the intense color of the osazone.) Anal. Calc. for $C_{20}H_{25}O_4N_5$: C, 60.15; H, 6.3; N, 17.5. Found: C, 59.7; H, 6.35; N, 17.3.

Absorptions in the infrared due to OH and NH (broad fused peak at 3300 cm^{-1}), the aromatic ring (3030 cm^{-1}, 1605 cm^{-1}, 1500 cm^{-1}, 745 cm^{-1}, 690 cm^{-1}), and the amide linkage (1647 cm^{-1}, 1583 cm^{-1}) were observed. The infrared spectrum is given in the Appendix (B).

*Periodate Oxidation of 5-Acetamido-5-deoxy-*L*-xylohexose Phenylosazone*

The method of Hough, Powell, and Woods (13) was used. The results are shown in Table IV. (Formaldehyde was estimated by the chromotropic acid method (23).)

TABLE IV

Time (hr)	Periodate uptake (moles/mole)	Formic acid (moles/mole)	Formaldehyde (moles/mole)
0.25	1.1	0.00	0.00
3.15	1.14	0.00	0.00
18.65	1.22	0.00	0.00
40.65	1.18	0.00	

On oxidation a bright yellow precipitate of the 1,2-bisphenylhydrazone of mesoxalaldehyde was obtained. The material was collected by filtration, washed with water, recrystallized from aqueous ethanol, and obtained as orange needles, m.p. 188–189.5° C.

1,2-Bisphenylhydrazone of Mesoxalaldehyde (14)

D-Glucose phenylosazone was prepared (300 mg) and dissolved in 50% aqueous ethanol (500 ml). Sodium metaperiodate (20 ml; 0.3 *M*) was added with vigorous stirring and after about 15 seconds a curdy mass of fine orange-yellow needles was precipitated. The mixture was allowed to stand for 15 minutes, then the crystals were collected by filtration, and washed with cold 50% aqueous ethanol and dried. One recrystallization from aqueous ethanol gave orange needles, m.p. 188.5–190.5° C.

The two specimens of the 1,2-bisphenylhydrazone of mesoxalaldehyde had mixed m.p. 188–189.5° C and had identical R_{rh} values on paper chromatograms run in solvents (*a*), (*b*), and (*c*) (R_{rh} 2.03, 1.58, and 2.9 respectively). The infrared spectra were identical and showed absorptions due to NH (3310 cm^{-1}), the aromatic ring (3050 cm^{-1}, 1605 cm^{-1}, 1500 cm^{-1}, 745 cm^{-1}, 687 cm^{-1}), and the unsaturated aldehyde group (1675 cm^{-1}). Further details of the infrared spectrum are given in the Appendix (C).

*Potassium Borohydride Reduction of 5-Acetamido-5-deoxy-*L*-xylohexulose to 2-Acetamido-2-deoxy-*D*-glucitol*

5-Acetamido-5-deoxy-L-xylohexulose (100 mg) was dissolved in water (10 ml) and the solution was cooled in ice. A solution of potassium borohydride (100 mg) in water (10 ml) was added dropwise, with stirring, over a period of 30 minutes and the solution was then stirred at 0° C for 3 hours, after which time it was non-reducing towards Fehling's solution. Amberlite IR-120 (H) resin was added to decompose excess potassium borohydride and the solution was then passed through a column of Amberlite IR-120 (H). The eluate and washings from the column were concentrated to a white amorphous residue which was repeatedly evaporated to dryness with methanol to remove boric acid as

methyl borate. The residue was dissolved in hot methanol and ether added to incipient turbidity. On cooling, white needles (57 mg) of 2-acetamido-2-deoxy-D-glucitol were deposited. A further crop of crystals (30 mg) was obtained on concentration of the mother liquors. The material had m.p. 154–156° C, mixed m.p. with an authentic specimen of 2-acetamido-2-deoxy-D-glucitol, 153–156° C, and $[\alpha]_D$ −10.8°. The crystalline material gave one spot when run on paper chromatograms in solvents (*a*), (*b*), and (*c*), detected with the alkaline silver nitrate reagent. The spot had R_{rh} values identical with those of the authentic specimen (solvent (*a*) 0.79; solvent (*b*) 0.62; solvent (*c*) 0.50). The infrared spectra of the derived and authentic specimens were identical and showed absorptions due to OH and NH (very broad peak at 3300 cm^{-1}) and the amide linkage (1645 cm^{-1}, 1587 cm^{-1}). Further details are given in the Appendix (D).

2,3-Mono-O-isopropylidene-5-acetamido-5-deoxy-L-xylohexulose

5-Acetamido-5-deoxy-L-xylohexulose (100 mg) was shaken with dry acetone (50 ml) containing concentrated sulphuric acid (2 drops) for 24 hours. At the end of this time the clear solution was neutralized with barium carbonate, filtered, and the filtrate was concentrated to a clear colorless syrup (120 mg) which was examined on paper chromatograms run in solvents (*a*), (*b*), and (*c*). The *p*-anisidine hydrochloride spray reagent showed only a very faint spot due to 5-acetamido-5-deoxy-L-xylohexulose. However, the orcinol/trichloroacetic acid spray reagent revealed also a strong spot due to the *O*-isopropylidene compound with the following R_{rh} values (Table V). An ethanol solution of

TABLE V

	R_{rh} (f = faint, s = strong)	
Solvent	*p*-Anisidine hydrochloride	Orcinol/trichloroacetic acid
(*a*)	0.73 (f)	0.73 (f)+1.86 (s)
(*b*)	0.65 (f)	0.65 (f)+1.73 (s)
(*c*)	0.59 (f)	0.59 (f)+1.95 (s)

the syrup was applied to Whatmann 3 mm paper and developed in solvent (*a*) overnight. The section containing the *O*-isopropylidene ketose was cut out and eluted with 50% aqueous ethanol. The eluate was passed through Amberlite IR-120 (H) and Duolite A-4 (OH) resins and then concentrated to a clear colorless syrup (108 mg) which was chromatographically pure. The syrup had $[\alpha]_D$ −51° (*c*, 2.16 in ethanol), and was non-reducing towards Fehling's solution. The infrared spectrum showed absorptions due to OH and NH (broad peak at 3360 cm^{-1}), the amide linkage (1665 cm^{-1}, 1560 cm^{-1}), and the C-methyl group (1460 cm^{-1}, 1380 cm^{-1}). A considerable reduction of the OH peak as compared to that of 5-acetamido-5-deoxy-L-xylohexulose was observed. Further details are given in the Appendix (E).

Periodate Oxidation of 2,3-Mono-O-isopropylidene-5-acetamido-5-deoxy-L-xylohexulose

The *O*-isopropylidene ketose (75 mg) was dissolved in 50% aqueous ethanol (ca. 40 ml). A 0.3 *M* quantity of sodium metaperiodate (2 ml) was added and the volume made up to 50 ml with 50% aqueous ethanol. A blank solution was prepared containing 0.3 *M* sodium metaperiodate (2 ml), which was made up to 50 ml with 50% aqueous ethanol. The solutions were stored in the dark at room temperature and 5-ml aliquots removed at intervals for estimation of periodate uptake (22). No periodate was taken up by the *O*-isopropylidene ketose.

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APPENDIX

Infrared Spectra

The following abbreviations are used: s = strong, m = medium, w = weak; wave numbers expressed in cm^{-1}.

*(A) 5-Acetamido-5-deoxy-*L*-xylohexulose (0.8% in KBr)*

3300 (s), 2950 (m), 2880 (m), 1625 (s), 1570 (s), 1510 (m), 1475 (m), 1440 (s), 1400 (m), 1380 (m), 1360 (m), 1330 (s), 1310 (m), 1280 (m), 1255 (m), 1225 (w), 1185 (m), 1160 (s), 1120 (s), 1100 (s), 1065 (s), 1030 (s), 955 (m), 905 (m), 825 (s), 785 (m), 685 (m), 670 (m).

*(B) 5-Acetamido-5-deoxy-*L*-xylohexose Phenylosazone (0.8% in KBr)*

3300 (s), 3030 (m), 2960 (m), 2910 (m), 1647 (s), 1605 (s), 1583 (s), 1540 (s), 1500 (s), 1450 (m), 1420 (m), 1385 (m), 1305 (m), 1257 (s), 1170 (m), 1150 (m), 1105 (m), 1055 (m), 1010 (s), 960 (w), 890 (w), 880 (w), 785 (w), 745 (m), 690 (m).

(C) 1,2-bis Phenylhydrazone of Mesoxalaldehyde (Authentic and Derived Specimens) (0.8% in KBr)

3310 (s), 3050 (w), 2840 (w), 1675 (s), 1605 (s), 1550 (s), 1535 (s), 1500 (s), 1355 (m), 1280 (s), 1245 (s), 1200 (m), 1170 (m), 1165 (m), 1155 (m), 745 (s), 705 (m), 687 (m).

*(D) 2-Acetamido-2-deoxy-*D*-glucitol (Authentic and Derived Specimens) (0.8% in KBr)*

3300 (s), 2940 (s), 1645 (s), 1587 (s), 1447 (s), 1385 (s), 1315 (s), 1277 (s), 1255 (m), 1215 (w), 1145 (w), 1130 (w), 1090 (s), 1065 (s), 1010 (s), 970 (m), 940 (w), 895 (w), 880 (m), 765 (m), 690 (s).

*(E) 2,3-Mono-O-isopropylidene-5-acetamido-5-deoxy-*L*-xylohexulose (6% in Chloroform)*
3360 (s), 3100 (w), 3030 (m), 2970 (w), 2910 (w), 1725 (w), 1665 (s), 1560 (s), 1460 (m), 1435 (m), 1390 (s), 1380 (s), 1315 (m), 1240 (s), 1190 (s), 1115 (s), 1095 (s), 1075 (s), 1035 (m), 975 (m), 950 (w), 900 (s), 885 (s), 835 (w), 815 (w), 685 (w), 657 (w).