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Reprinted from ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, Volume 78, No. 2, December 1958
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Received May 29, 1958

Introduction

On the basis of their investigations of the partial oxidation of cyclitols by *Acetobacter suboxydans* A.T.C.C. 621, Magasanik, Franzl, and Chargaff (1, 2) stated that the specificity requirements of this organism for cyclitol substrates can be defined by two rules based on the conformational analysis of cyclitols. [For a discussion of the theory involved, see the review by Klyne (3).] The rules were: 1. *Only axial*[2] *hydroxyls are oxidized*, and 2. *If an axial hydroxyl is to be oxidized, there must be an equatorial hydroxyl in the* meta *position, clockwise if the molecule be oriented so that the axial hydroxyl projects downward.* It was suggested (2) that an additional rule might also apply, *viz.*: 3. *There must be an equatorial hydroxyl in the* para *position.* A number of things still remained to be ascertained, such as: (*a*) whether the third rule does apply, (*b*) whether the rules accurately predict the oxidation of lower[3] cyclitols and cyclitols with functional groups other than hydroxyl, and (*c*)

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This investigation was supported in part by a research grant (G-3707) from the Division of Research Grants, U. S. Public Health Service.

[2] It was assumed that the inositols exist predominantly in that chair form which has the smaller number of axial hydroxyl groups. Subsequent evidence has supported this assumption (4).

[3] In this paper, the term "lower" is applied to cyclitols with four or fewer oxygen or analogous functions, and "higher" to cyclitols with five or six such functions.

whether the organism possesses one or several primary cyclitol dehydrogenases.

In the first paper of this series (5), it was reported that the organism failed to oxidize certain aminocyclitol derivatives which satisfy the specificity rules. It was also shown that the dehydrogenase for 1-*cis*-2-*cis*-3-cyclohexanetriol differed from the dehydrogenase(s) which attack the higher cyclitols. Since then, methods for the synthesis of several rarer inositols (6 OH's) and quercitols (5 OH's) have been developed in Sydney and a sizable number of inositol methyl ethers have become available in the course of chemical studies carried out in Madison and in Sydney. On the basis of limited tests with one of the new cyclitols (*neo*-inositol), Magasanik (6) concluded that the third rule does apply. The work reported in the present paper was designed to provide a more firmly based answer to this question, and an answer to the second question as it pertains to the methyl ethers. Tests of two cyclohex-5-ene-1,2,3,4-tetrols which happened to be available are included for the record.

MATERIALS[4]

Syntheses of the following cyclitols have been described in previous publications: *neo*-inositol (9); *allo*-inositol and (+)-1,2/3,4-cyclohexenetetrol (10); *cis*-inositol and *cis*-quercitol (11); DL-bornesitol (12); and 2-*O*-methyl-*myo*-inositol (13). (−)-1,2/3,4-Cyclohexenetetrol was prepared from D-inositol by the procedure described for the enantiomorph, and *myo*-inosose-5 ["*neo*-inosose," ref. (4)] was synthesized according to Allen (14). Natural D(+)-bornesitol[5] was kindly furnished by Professor F. E. King, Nottingham; the natural L(+)-ononitol[5] was a gift from M. Victor Plouvier, Paris. Dambonitol was isolated from the latex serum of the Malayan jelutong tree, *Dyera costulata* (13). D-1-*O*-Methyl-*allo*-inositol was derived from natural D(+)-pinitol by a procedure to be described later.[6] The sequoyitol was from the lot used in the previous investigation (5).

The naturally occurring inositol methyl ethers are invariably contaminated with small amounts of *myo*-inositol, even after numerous recrystallizations. Accordingly, the natural ethers used in the present work were purified by chromatography on cellulose columns. This procedure was also one of the steps in the

[4] Cyclitols are named and numbered according to Fletcher, Anderson, and Lardy (7), with the following exceptions: (*a*) The pentahydroxy compounds are *named* as quercitols, with trivial prefixes, as suggested by Angyal, Macdonald, and Matheson (8); and (*b*) the configurations of cyclohexenetetrols are designated by the conventional "fractional" system.

[5] Proof that the methyl groups in D- and L-bornesitol and L-ononitol occupy the positions here assigned (Fig. 1) will be provided in a forthcoming paper [L. Anderson and G. G. Post; see also Angyal, Gilham, and Macdonald (13)].

[6] S. J. Angyal and P. T. Gilham, unpublished.

preparation of each of the synthetic methyl ethers. The details of the method as it is used in the authors' respective laboratories have been described (13, 15).

L(−)-*Pinitol* (XIX) and *5,6-di-O-methyl-L-inositol* (XXIV) were prepared by hydrolysis of the corresponding diisopropylidene compounds (16)[7] following the method used by Anderson, MacDonald, and Fischer (17) for the enantiomorphs. L(−)-Pinitol had m.p. 185–186° and $[\alpha]_D^{24}$ −65.5° (c = 2 in H_2O); the dimethyl compound, m.p. 191–192° and $[\alpha]_D^{24}$ −73.2° (c = 2 in H_2O). Anderson and co-workers reported m.p. 185–187° and $[\alpha]_D^{25}$ +65.0°, and m.p. 191–193° and $[\alpha]_D^{27}$ +73.0°, respectively, for the enantiomorphs.

neo-*Quercitol* (IV).[8] *myo*-Inosose-5 (55 mg.) was dissolved in 5 ml. of 5% (v/v) H_2SO_4 in water, 15 mg. of platinum oxide was added, and the whole was shaken under hydrogen for 3 hr. Paper chromatography of the reaction mixture (acetone–water, 4:1, v/v) showed the presence of a quercitol (R_f 0.40) and *neo*-inositol (R_f 0.19). No spot was obtained with the ferricyanide-ferric sulfate reagent (2), showing the absence of inosose. The acid was neutralized with a hot solution of $Ba(OH)_2$, and the precipitated $BaSO_4$ was filtered off after standing overnight. The residue (38 mg.) obtained by evaporating the filtrate to dryness was taken up in acetone–water (4:1), and the components were separated by chromatography on a small cellulose column.

The quercitol fraction (27 mg.) was sublimed *in vacuo* to give colorless crystals, m.p. 238–239° (dec.).

Anal. Calcd. for $C_6H_{12}O_5$ (164.16): C, 43.90; H, 7.37. Found: C, 43.89; H, 7.35.

neo-*Quercitol Pentaacetate*.[8] A portion of the *neo*-quercitol was acetylated in an acetic anhydride–H_2SO_4 mixture. The product, after sublimation at reduced pressure, had m.p. 182°.

Anal. Calcd. for $C_{16}H_{22}O_{10}$ (374.34): C, 51.33; H, 5.92. Found: C, 51.42; H, 5.94.

The culture of *Acetobacter suboxydans* A.T.C.C. 621 employed was descended from the one previously used (5). It was maintained as before, on slants of Difco yeast extract, 0.5%; sorbitol, 0.5%; and agar, 1.5%; and subcultured on a liquid medium of Difco yeast extract, 1%; and sorbitol, 5%. The preparation of washed resting cells was as previously described, except that different incubation periods were used: 12–17 hr. in Madison, 48 hr. in Sydney. One milliliter of the preparation contained *ca.* 3 mg. bacterial N (Madison) or 90–100 mg. dry wt. (Sydney).

EXPERIMENTAL

Manometric Tests

The manometric tests for the susceptibility of a substrate to oxidation by washed resting cells were carried out in the Warburg apparatus in the usual way (1, 5). Each Warburg flask contained: suspension of resting cells, 0.5 ml.; 1/15 M phosphate buffer, pH 6.0, 2.0 or 2.2 ml.; substrate (in the side arm), 0.3 ml.; and 10% KOH (in the center well), 0.2 ml. Incubation was at 37° with air as the gas

[7] Designated as 3-*O*-methyl- and 3,4-di-*O*-methyl-1:2-5:6-di-*O*-isopropylidene-(−)-inositol, respectively, in the reference cited.

[8] The authors thank Miss Mary Pitman (Sydney) for carrying out this preparation.

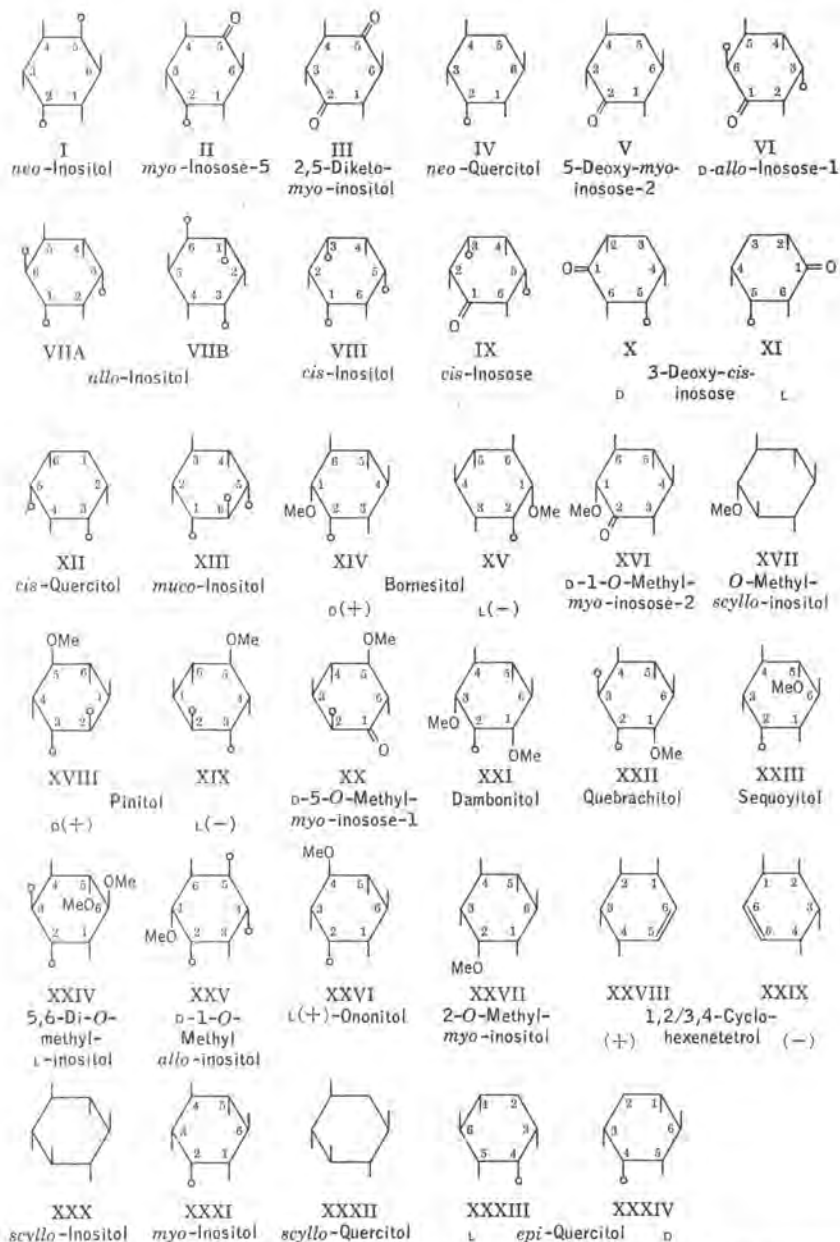

FIG. 1. Formulas of the cyclitols discussed. Vertical lines terminating in small circles represent axial hydroxyls; plain vertical lines represent equatorial hydroxyls [*cf.* Ref. (3)].

TABLE I
Oxygen Consumption Data

Substrate	Amount taken	Number of runs	Duration	Oxygen consumed	Rate data: Rate/Rate for *myo*[a]	Rate data: Remarks
	μmoles		*min.*	*g. atoms/mole substrate*[b]		
neo-Inositol (I)	7.4	1	270	0.39	0.04	Steady entire run
	7.4	1	1000[c]	1.45	0.03	Steady entire run
myo-Inosose-5 (II)	10.0	2	240	0.83 (±.03)	0.41	Slackening at end
neo-Quercitol (IV)	10.0	2	240	0.75 (±.04)	0.19	Steady entire run
allo-Inositol (VII)	12.0	3	210	1.23 (±.03)	0.87	1 g. atom in 70 min.
cis-Inositol (VIII)	10.0	2	230	0.66 (±.02)	0.07	Steady entire run
cis-Quercitol (XII)	10.0	2	230	0.40 (±.05)	0.04	Steady entire run
muco-Inositol (XIII)[d]	24–59	28	300	2.27 (±.28)	0.60[e]	Slackening at end
D(+)-Bornesitol (XIV)	13.0	3	250	0.81 (±.06)	0.23	Slackening at end
DL-Bornesitol (XIV + XV)	13.0	2	220	0.30 (±.01)	0.09	Zero at end
	26.0	1	320	0.28	0.18	Zero at end
L(−)-Pinitol (XIX)	10.0	2	240	1.05 (±.05)	0.13	Zero at end
Dambonitol (XXI)	13.8	2	150	0.05 (±0)	—	Zero at end
Sequoyitol (XXIII)	13.0	2	220	0.08 (±.03)	—	Zero at end
5,6-Di-*O*-methyl-L-inositol (XXIV)	10.0	1	240	0.04	—	Zero at end
D-1-*O*-Methyl-*allo*-inositol (XXV)	11.0	2	240	0.10 (±.01)	—	Zero at end
L(+)-Ononitol (XXVI)	10.0	2	240	0.05 (±.01)	—	Zero at end
2-*O*-Methyl-*myo*-inositol (XXVII)	10.0	2	230	0.04 (±.03)	—	Zero at end
(+)-Cyclohexenetetrol (XXVIII)	12.0	2	210	0.53 (±.01)	0.10	Steady entire run
(−)-Cyclohexenetetrol (XXIX)	12.0	3	210	0.08 (±.02)	—	Zero at end

[a] Ratios of the slopes of the flat portions of the oxygen uptake curves, determined for each individual run, then averaged.

[b] Figures in parentheses are the deviations of the individual values from their averages.

[c] For this run (16 hr.), the washed cells were prepared aseptically. Substrate, buffer, Warburg vessels, and plugs were all sterilized by autoclaving, and transfers were made with sterile pipets. Plugs and joints were greased with sterile lanolin. At the end of the run, the suspensions in the flasks were checked for contaminating gram-positive organisms, but none were found.

[d] Data taken from previous paper (5); 24 or 36 μmoles of *myo*- was used in the control flasks.

[e] Ratio for one individual run.

phase. In each run, two flasks were used for each substrate. In addition, two flasks without substrate and two flasks of *myo*-inositol (10 or 12 μmoles) were included. With the bacterial suspensions used, 15–45 min. was required for complete oxidation of the *myo*-inositol. The results are shown in Table I. Recorded oxygen consumption values are corrected for endogenous uptake.

Identification of the Oxidation Products[9]

Fermentation in shake flasks was used to obtain quantities of the oxidation products sufficient for their characterization. The media for the fermentations contained cyclitol, 3%; Difco yeast extract, 0.5%; and sorbitol, 0.1%. After inoculation with 5 vol.% of 3-day-old subculture (see *Materials*), the flasks were shaken continuously at 30°. During the fermentation period, the culture fluids were examined by paper chromatography. Inososes were specifically detected with the ferricyanide–ferric sulfate reagent of Magasanik, Franzl, and Chargaff (2). Oxidations were terminated by centrifuging the cells out (30 min., 15,000 × g), and the inososes were isolated directly from the supernatants.

All but two of the inososes were sirups. Characterization was accomplished, in general, by reducing the inosose with sodium amalgam to a mixture of the parent alcohol and its epimer(s), and identifying the latter by standard methods. The characterization work is presented in detail in the following paragraphs and summarized in Table II.

*2,5-Diketo-*myo*-inositol (III) from* neo*-Inositol and* myo*-Inosose-5.* One hundred milliliters of medium was used for 200 mg. *neo*-inositol (I), since the low solubility of this cyclitol prevented its inclusion at the usual level of 3%. A paper chromatographic test with phenol–water (4:1, *v/v*) on the fifth day showed that all of the starting material (R_f 0.24) had been replaced by a reducing substance with R_f 0.16. On the sixth day the bacteria were removed and the culture fluid was concentrated to 10 ml. under reduced pressure. Storage of the concentrate at 0° for 2 days yielded 100 mg. of colorless crystals. Analysis, after recrystallization from ethanol–water, indicated that the product was a diketoinositol (tetrahydroxycyclohexanedione), and this indication was confirmed when a bisphenylhydrazone was obtained (see below). In the Kofler apparatus, the compound did not melt but gradually decomposed, losing its crystalline appearance at 170–180°.

Anal. Calcd. for $C_6H_8O_6$(176.12): C, 40.91; H, 4.58. Found: C, 40.66; H, 4.78.

Forty milligrams of the product in 3 ml. water was shaken with 3% sodium amalgam (3 g., added in six batches); *N* H_2SO_4 was added as required to keep the solution slightly acidic. When reduction was complete (no reaction with alkaline copper), the solution was filtered and evaporated to dryness, and the residue was treated with acetic anhydride and anhydrous sodium acetate. The solid acetate obtained by dilution of the acetylation mixture was then fractionated by treatment with hot ethanol. The insoluble portion, on fractional sublimation under reduced pressure, yielded 6 mg. of colorless crystals of *scyllo*-inositol hexaacetate, m.p. 295°; mixed m.p. with an authentic sample, 294–295°. From the ethanol-soluble portion, on recrystallization (ethanol–water) and fractional

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[9] This section by S. J. Angyal and D. J. McHugh.

TABLE II

Positions Attacked by Acetobacter *in the Cyclitols Studied*

Substrate	Positions of axial hydroxyls[a]	Positions oxidizable according to		Observed oxidation	
		All three rules	Rules 1 and 2 only	Positions attacked	Oxidation product
neo-Inositol (I)	2, 5	None	2, 5	2, 5	2,5-Diketo-*myo*-inositol (III)
myo-Inosose-5 (II)	2	None	2	2	2,5-Diketo-*myo*-inositol (III)
neo-Quercitol (IV)	2	None	2	2	5-Deoxy-*myo*-inosose-2 (V)
allo-Inositol, form A (VIIA)	1, 3, 6[b]	1	1, 6	1 (and ?)	D-*allo*-Inosose-1 (VI)
allo-Inositol, form B (VIIB)	1, 3, 6	None	3, 6	?	+ Diketone
cis-Inositol (VIII)	1, 3, 5	None	None	1[c]	*cis*-Inosose (IX)
cis-Quercitol (XII)	3, 5	None	None	3 or 5[d]	3-Deoxy-*cis*-inosose (X, XI)[d]
muco-Inositol (XIII)	1, 5, 6	1, 6	1, 6	1 (?) (and ?)	Not identified
D(+)-Bornesitol (XIV)	2	2	2	2	D-1-*O*-Methyl-*myo*-inosose-2 (XVI)
L(−)-Pinitol (XIX)	2, 3	3	2, 3	3	D-5-*O*-Methyl-*myo*-inosose-1 (XX)
L(−)-Bornesitol (XV)[e]	2	2	2	None	—
Dambonitol (XXI)	2	2	2	None	—
Quebrachitol (XXII)[f]	2, 3	2	2	None	—
Sequoyitol (XXIII)[g]	2	None	2	None	—
D(+)-Pinitol (XVIII)[f]	2, 3	None	2	None	—
5,6-Di-*O*-methyl-L-inositol (XXIV)	2, 3	None	2, 3	None	—
D-1-*O*-Methyl-*allo*-inositol (XXV)	2, 4, 5	None	2	None	—
L(+)-Ononitol (XXVI)	2	None	None	None	—
2-*O*-Methyl-*myo*-inositol (XXVII)	None	None	None	None	—

[a] See text, footnote 2, for the assumptions involved.

[b] See *Discussion* regarding inositols with three axial hydroxyls (*allo*, *cis*, and *muco*). Hydroxyls which are axial in one of the possible chair forms are equatorial in the other. The two forms of *cis* and *muco* are identical, but those of *allo* are enantiomorphic. The enantiomorphs must be present in equal porportions, and are assumed to be freely interconvertible. In all three cases, the lowest numbers are here given to the axial hydroxyls, since it is desired to focus attention on them.

[c] See footnote *b*. The 6 OH's of *cis*-inositol are stereochemically equivalent in the classical sense. The one which is oxidized becomes No. 1 by definition.

[d] It is not known whether one or the other of these positions is attacked preferentially, since the product was not characterized as to enantiomorphic composition.

[e] Tried as the DL-compound.

[f] Magasanik and Chargaff (1) observed that these cyclitols were not oxidized. We have confirmed their observations.

[g] The previously reported (5) slow oxidation of this cyclitol could not be confirmed. That oxidation presumably depended on *myo*-inositol present as an impurity.

sublimation, there was obtained 10 mg. *myo*-inositol hexaacetate, m.p. 213°; mixed melting point with an authentic sample, 212–213°.

The conversion to *scyllo*-inositol (XXX) identifies the oxidation product of *neo*-inositol, for of the mono and diketones derivable from this inositol, only III, *2,5-diketo*-myo-*inositol*, could give *scyllo*-inositol on reduction. The formation of *myo*-inositol (XXXI) from III is, of course, also to be expected.

The configuration of *neo*-inositol (I) is such that *myo*-inosose-5 (II) must be an intermediate in its oxidation to the diketone III. And conversely, III must be the oxidation product of *myo*-inosose-5. Efforts to detect this inosose by paper chromatography at various stages in the oxidation of *neo*-inositol failed, but this is understandable in view of the fact that the inosose is oxidized much faster than *neo*-inositol (Table I). A solution of *myo*-inosose-5 which had been oxidized in the Warburg apparatus gave a reducing spot in the same position as 2,5-diketo-*myo*-inositol.

2,5-Diketo-myo-*inositol Bisphenylhydrazone*. The product from oxidation of *neo*-inositol was treated with phenylhydrazine according to Carter *et al.* (18). The phenylhydrazone, which decomposed without melting at 175–180°, gave the following analysis.

Anal. Calcd. for $C_{18}H_{20}O_4N_4$ (356.37): N, 15.72. Found: N, 15.54.

Oxidation Product of neo-*Quercitol*. A solution containing *neo*-quercitol (IV, 50 mg.) in water (15 ml.) was oxidized with a culture of *Acetobacter suboxydans* for 10 days; the culture fluid then reduced cold Fehling solution. The bacteria-free supernatant was shaken with 2% sodium amalgam, added in portions, until Fehling solution was no longer reduced; glacial acetic acid was added as required to keep the pH at 5–7. The filtered solution was concentrated and the residue run, in acetone–water 4:1, *v/v*, through a cellulose powder column. After some *neo*-quercitol (R_f 0.40), a fraction of R_f 0.31 was collected and evaporated, and the residue was crystallized from ethanol to give 9 mg. *scyllo*-quercitol (XXXII) melting at 233° alone and at 234–235° on admixture with an authentic sample. The conversion to *scyllo*-quercitol identifies the oxidation product of *neo*-quercitol as *5-deoxy*-myo-*inosose-2* (V).

D-allo-*Inosose-1 (VI) from* allo-*Inositol*.[8] A solution containing *allo*-inositol, VII (0.7 g.) in water (15 ml.) was oxidized with a culture of *Acetobacter suboxydans* for 7 days. At the end of this time, paper chromatography in acetone–water (4:1, *v/v*) showed the presence of *allo*-inositol (R_f 0.30) and two reducing compounds of R_f 0.37 and 0.25, respectively. The slower moving compound, which has not yet been fully characterized, appears to be a diketone.

The solution was filtered through a bed of kieselguhr, the filtrate concentrated to dryness *in vacuo*, and the oxidation product separated from *allo*-inositol by chromatography on a cellulose powder column in acetone–water (4:1). The monoketone fraction (R_f 0.37) was taken to dryness *in vacuo*, and the residue was crystallized from aqueous ethanol. The inosose (81 mg.) had m.p. 141–142° and $[\alpha]_D^{20}$ −24° (c = 1.55, H_2O).

Anal. Calcd. for $C_6H_{10}O_6$ (178.14): C, 40.45; H, 5.66. Found: C, 40.33; H, 5.61.

Seventy milligrams of the product in 5 ml. water was shaken with 3% sodium amalgam (3 g., added in batches); glacial acetic acid was added as required to keep the pH between 5 and 7. The filtered solution was then passed through a

Zeocarb 225 column to remove sodium ions and evaporated, and the residue was chromatographed on cellulose powder with acetone–water (4:1, *v/v*). The main product was *neo*-inositol, which was acetylated to give *neo*-inositol hexaacetate (103 mg.), m.p. 255° alone; mixed melting point with pure *neo*-inositol hexaacetate 254–256°. *allo*-Inositol and traces of *myo*- and D- or L-inositol were identified by paper chromatography in acetone–water and phenol–water.

The fact that the inosose from *allo*-inositol gives *neo*-inositol (I) on reduction identifies it as an allo-*inosose-1*. Its enantiomorphic configuration was not determined by chemical means but can be deduced from the optical rotation by Whiffen's method (19) which gives clear-cut results with cyclitols. The method has not been used with ketones; but on the assumption that the keto-group—like the methylene group—does not contribute to the rotation, the value of −50° is calculated for D-*allo*-inosose-1 (VI) and +50° for its enantiomorph. The actual value is smaller but sufficiently close—in view of our unproven assumption—to assign the D-configuration to the inosose. The same configuration is predicted by the specificity rules (see *Discussion*).

cis-*Inosose (IX) from* cis-*Inositol*. Six hundred milligrams of *cis*-inositol (VIII) was fermented for 7 days. This period was insufficient for complete transformation of the starting material, as determined by paper chromatography (acetone–water, 4:1, *v/v*), but previous experience had shown that longer oxidation resulted in considerable darkening of the solution. On concentration of the bacteria-free supernatant and dilution of the concentrate with ethanol, 210 mg. of crystalline material separated out. After recrystallization from ethanol–water, the compound melted at 179–180° (dec.), alone and in admixture with authentic *cis*-inosose (11). This inosose is the only monoketone derivable from *cis*-inositol.

Anal. Calcd. for $C_6H_{10}O_6$ (178.14): C, 40.45; H, 5.66. Found: C, 40.13; H, 5.65.

Oxidation Product of cis-*Quercitol*. Four hundred and fifty milligrams of *cis*-quercitol (XII) was fermented for 9 days, which sufficed for nearly complete transformation (paper chromatography). An attempt to prepare a phenylhydrazone was unsuccessful. Accordingly, the bacteria-free supernatant was shaken with 20 g. of 3% sodium amalgam, added in portions; glacial acetic acid was added as required to keep the pH at 5–7. The solution was decanted from the mercury and taken to dryness, and the residue was acetylated with acetic anhydride and sodium acetate. Chloroform extraction of the diluted reaction mixture yielded an oil which crystallized on the addition of ethanol.

The material so isolated was deacetylated with boiling 1% hydrochloric acid and separated by chromatography on cellulose powder (acetone–water, 4:1, *v/v*) into *cis*-quercitol and *epi*-quercitol (50 mg.), identified by paper chromatography. The *epi*-quercitol was acetylated; after recrystallization from ethanol, the acetate (10 mg.) melted at 131–141°. Fractional sublimation in vacuum gave a portion which melted at 138–140°, alone and in admixture with authentic DL-*epi*-quercitol (XXXIII + XXXIV) pentaacetate. D(−)-*epi*-Quercitol [XXXIV, Ref. (2)] was prepared[10] from the corresponding inosose and acetylated to provide a com-

[10] The authors thank Mrs. Veronica Bender (Sydney) for carrying out this preparation.

parison sample of optically active *epi*-quercitol pentaacetate. The melting point was 121°.

The fact that the oxidation product of *cis*-quercitol can be reduced to *epi*-quercitol identifies it as *3-deoxy*-cis-*inosose* (D-form, X; L-form, XI). The melting-point data obtained with the *epi*-quercitol pentaacetate indicate that the sample was probably a mixture of one of the optical isomers with a small amount of racemate. No optical rotation data were collected.

Oxidation Product of muco-*Inositol*.—The oxidation of *muco*-inositol (XIII), which produces a mono- and a diketone, is still under investigation. It is hoped to publish the results at a later date. The present evidence seems to indicate that the monoketone is one of the enantiomorphic forms of *muco*-inosose-1.

Oxidation Product of D(+)-*Bornesitol*. One and two-tenths grams of DL-bornesitol (XIV + XV) was fermented for 9 days, and the solution, after removal of the bacteria, was concentrated to 7 ml. Chromatography of the concentrate on cellulose powder (acetone–water, 4:1, *v/v*) yielded an oxidized and an unoxidized fraction.

The unoxidized fraction weighed 550 mg. After recrystallization from ethanol containing a little water, it melted at 204° and had $[\alpha]_D^{28}$ −32° (c = 3, water) and was therefore L(−)-bornesitol (XV). Plouvier (20) reports m.p. 203–204° and $[\alpha]_D$ −32°.

The oxidized fraction, after concentration to a small volume, was reduced with 15 g. of 3% sodium amalgam as described above for 3-deoxy-*cis*-inosose. The reduction product could not be satisfactorily purified as the acetate, so this was deacetylated. Chromatography on cellulose powder (acetone–water, 4:1, *v/v*) gave two fractions, one having R_f 0.35 on paper (acetone–water, 4:1), and one having R_f 0.29. The R_f 0.29 fraction was found to be bornesitol.

On recrystallization of the R_f 0.35 fraction from water–ethanol, 30 mg. of colorless crystals, m.p. 239–242° (dec.) was obtained. A portion of this material was demethylated by boiling with conc. hydriodic acid for 1 hr. Parts of the residue were examined by paper chromatography in acetone–water, 4:1; phenol–water, 4:1; and ethyl acetate–acetic acid–water, 3:1:1; and found to have R_f values identical with those of *scyllo*-inositol (XXX) in all three systems. The remainder was acetylated to yield 25 mg. of colorless crystals melting at 293–295° alone and 294–295° on admixture with authentic *scyllo*-inositol hexaacetate.

The R_f 0.35 compound was therefore O-*methyl*-scyllo-*inositol* (XVII), and the bacterial oxidation product of D(+)-bornesitol was D-*1*-O-*methyl*-myo-*inosose-2* (XVI).

O-*Methyl*-scyllo-*inositol pentaacetate* crystallized from ethanol as small, colorless needles, m.p. 192–194°. The compound appears to be dimorphous, changing to quite large needles at 170–175°.

Anal. Calcd. for $C_{17}H_{24}O_{11}$ (404.36): C, 50.49; H, 5.98. Found: C, 50.84; H, 5.98.

The Oxidation Product of L(−)-*Pinitol*. Five hundred and ten milligrams of the compound (XIX) was fermented for 9 days. After removal of the bacteria and concentration, the oxidized solution was chromatographed on a cellulose column with acetone–water, 4:1. The fractions which gave reducing spots (alkaline ferricyanide) on paper chromatograms were combined and taken to dryness to yield 190 mg. of brown gum. The gum was reduced with sodium amalgam (35 g.),

and the reduction product was acetylated as previously described. On standing 1 month, a solution of the oily acetate in ethanol–water deposited 25 mg. of brown cubic crystals which melted from 170 to 185°. These were further purified by fractional sublimation under reduced pressure; the final fraction melted at 198–200° alone and in admixture with authentic sequoyitol pentaacetate.

Reduction of the L-pinitol oxidation product thus gives sequoyitol (XXIII), and this identifies the oxidation product itself as D-*5-O-methyl*-myo-*inosose-1* (XX).

Discussion

The number of published papers dealing with the *Acetobacter* oxidation of cyclitols is now considerable. Posternak [(21, 22) and papers there cited] has tested most of the known, unsubstituted cyclitols with three or more contiguous hydroxyl groups, and a variety of miscellaneous cyclic alcohols, with resting cells of the Kluyver and de Leeuw strain of *A. suboxydans*. He also subjected many of these compounds to the action of growing cultures. With the completion of the present work, most of the known unsubstituted higher cyclitols,[3] a number of inosamines, and most of the known cyclitol methyl ethers have been tested with both resting cells and growing cultures of strain 621 from the American Type Culture Collection [see also Refs. (2, 5) and papers there cited]. The time would therefore seem ripe to attempt to put the accumulated results into perspective.

In most of the papers alluded to, the results presented are discussed in terms of whether they fulfill the predictions of Magasanik's specificity rules (see *Introduction*). Thus, in his latest paper (22), Posternak has reviewed his own data, and concluded that only rule 1 (that only axial hydroxyls are oxidized) has validity for the Kluyver and de Leeuw strain. Since it does not seem possible to attach any general biochemical significance to the specificity rules, there is little utility in detailed debate about the rigor with which they apply to a particular strain of the organism. The rules may be held to be useful if they (*a*) enable one to recall correctly the positions which are oxidized in a given cyclitol (e.g., when considering the availability of specific ketones for further synthetic work); (*b*) predict whether a given position in an untried cyclitol will be oxidized; and (*c*) as suggested by the authors who proposed them, indicate the points of attachment of substrate to enzyme.

The data of the present paper, obtained with the A.T.C.C. 621 strain, may now be discussed in the light of these considerations. First of all, it may be stated that there are apparently no exceptions to rule 1. The word "apparently" is used because, in inositols with three axial

hydroxyls (*allo*, *cis*, and *muco*), it is not possible to know the conformation of a given hydroxyl at the moment of oxidation. (This statement would also apply to cyclohexanetetrols with two axial hydroxyls.) The uncertainty arises because, in these cyclitols, the probabilities that a given hydroxyl has the axial or equatorial conformation are equal (see footnote *b*, Table II). There is no reason to suppose that these cyclitols are exceptions to rule 1, but they cannot be shown to conform to it.

The results obtained with the "*neo*-group" of cyclitols (*neo*-inositol, *myo*-inosose-5, and *neo*-quercitol) constitute a test of the proposed rule 3. These cyclitols have axial hydroxyls satisfying rule 2, and *para* to these an axial hydroxyl, a keto group, and no oxygen function, respectively. Since all of the *neo*-compounds were oxidized, rule 3 cannot hold in its original form. It will be noted that, at the concentrations employed in the manometric tests, the *neo*-cyclitols are oxidized much more slowly than *myo*-inositol (Table I) and other cyclitols which have equatorial hydroxyls in the *para* position (1). These rate differences do not have preparative significance, however, for a ketone was prepared from each of the *neo*-cyclitols by oxidation in growing culture.

The course of the oxidation of *allo*-inositol is an additional test of the proposed rule 3. If the conformation of the hydroxyl in the *para* position had no effect, both forms (see footnote *b*, Table II) of this inositol should be oxidized at appreciable rates to give a mixture of two diketones. On the other hand, if a *para* equatorial hydroxyl is important for oxidation, all of the *allo* should be oxidized as form A to D-*allo*-inosose-1, and this should only slowly be oxidized to diketone. In our hands, *allo*-inositol was only slowly oxidized after 1 gram atom oxygen/mole was taken up, and the principal product of the oxidation in culture was D-*allo*-inosose-1, which was accompanied by small amounts of a diketone.

On the basis of these results, we suggest that an amended rule 3 applies, viz.: *The oxidation of an axial hydroxyl in a higher cyclitol proceeds best when there is an equatorial hydroxyl in the* para *position, and when there is a choice, the axial hydroxyl satisfying this rule is attacked preferentially*.

The data obtained with the methyl ethers provide one case (L-pinitol) of additional support for the third rule. L-Pinitol has two axial hydroxyls satisfying rule 2, but only the one which also satisfies rule 3 is oxidized. However, three of the ethers having hydroxyls which satisfy all three rules are not substrates at all. It would therefore seem that the methyl

ether function must be listed, along with the amino function (5), as one which so alters the cyclitol molecule that satisfaction of the conformational rules is not sufficient to guarantee *Acetobacter* oxidation.

The fact that *cis*-inositol and *cis*-quercitol are oxidized is of interest, since none of the axial hydroxyls in these cyclitols satisfy Magasanik's rule 2. Both were converted to monoketones by a growing culture. They are thus the first clear-cut examples of oxidations contrary to rule 2 effected by the A.T.C.C. 621 strain. The rates (Table I) are considerably lower than that for *myo*-inositol.

An examination of Posternak's results (21, 22) shows that the behavior of the Kluyver and de Leeuw strain parallels in general that of strain A.T.C.C. 621; evidently, however, the former is less selective in oxidizing cyclitol hydroxyls. The experiments which have been done with the one strain have not been completely duplicated with the other; in particular, almost no work has been done on the cyclohexanetetrols with strain A.T.C.C. 621. Since Posternak observed with these compounds a number of oxidations contrary to the rules, the following conclusions relate specifically to the action of the A.T.C.C. 621 strain on higher cylitols.

In this frame of reference, we may say that: (*a*) Magasanik's rules 1 and 2, and the amended rule 3 presented here, are a generally reliable mnemonic guide to the positions oxidized; (*b*) predicted oxidations often do not take place in cyclitols with functions other than hydroxy or keto; and (*c*) it is still attractive to speculate that the hydroxyls mentioned in the rules are those through which the cyclitol substrates are bound to the dehydrogenase or dehydrogenases which act on them.

Extensive studies with the purified enzyme(s) will be required to test this latter speculation. It is hoped that such studies will eventually be undertaken. Since the rather rigid structure of the cyclitols makes it possible to determine their molecular geometry with considerable precision, the study of the topography of an enzyme surface which shows specificity toward them should yield an unusually accurate picture of an enzyme–substrate complex.

Summary

1. Several rare inositols and quercitols, most of the known inositol methyl ethers, and two cyclohexenetetrols have been tested as substrates for partial oxidation by *Acetobacter suboxydans* A.T.C.C. 621.

2. *neo*-Quercitol and several other cyclitols (methyl ethers, inososes, and derivatives) are described for the first time.

3. In all cases where oxidation occurred, the positions attacked have been identified by characterizing the reduction products of the inososes formed. The attack always took place on axial hydroxyl groups.

4. A modified version of the third specificity rule of Magasanik, Franzl, and Chargaff is proposed, and the present status of generalizations about specificity in the *Acetobacter* oxidation of cyclitols is reviewed.

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