Phytochemistry, 1968, Vol. 7, pp. 1 to 4. Pergamon Press. Printed in England

MICRO-BIOLOGICAL OXIDATION OF ALLITOL TO L-*RIBO*-HEXULOSE BY *ACETOMONAS OXYDANS*

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(*Received* 14 *June* 1967)

Abstract—Oxidation of allitol by *Acetomonas oxydans* yields L-allulose (L-*ribo*-hexulose) in accordance with the Bertrand–Hudson rule.

INTRODUCTION

ALLITOL has been found to be a major component of *Itea* plants,[1,2] the actual proportion of the hexitol in the leaves varying with the duration of photosynthesis.[3] The importance of allitol in the metabolism of *Itea* plants was shown by the rapid incorporation of ^{14}C into the hexitol when the leaves were allowed to photosynthesize in an atmosphere of $^{14}CO_2$.[4] Further biosynthetic studies,[5] involving the feeding of D-[1-^{14}C]- and D-[6-^{14}C]-glucose to the leaves, required a scheme for the degradation of allitol in order to determine the distribution of ^{14}C within the molecule. Since allitol (VII) is a completely symmetrical molecule, a purely chemical procedure would not differentiate between the two terminal carbon atoms. Consequently the micro-biological oxidation of allitol to a hexulose, namely, *ribo*-hexulose (VIII) (allulose), was investigated since subsequent degradation via the phenylosazone derivative[6] would lead to differentiation of the terminal carbon atoms.

RESULTS AND DISCUSSION

Many micro-organisms can use polyols as sources of carbon and energy but metabolism in acetic acid bacteria has been studied the most extensively.[7] Bertrand studied the oxidative action of *Acetobacter xylinum* and observed that the oxidation to a keto group of a hydroxyl group on the penultimate carbon of a polyol requires the presence, on three contiguous carbon atoms, of two secondary hydroxyl groups with either the D-*erythro* (I) or L-*erythro* (II) configuration, and a primary hydroxyl group.[8]

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[1] V. PLOUVIER, *Compt. Rend.* **249**, 2828 (1959).
[2] L. HOUGH and B. E. STACEY, *Phytochem.* **2**, 315 (1963).
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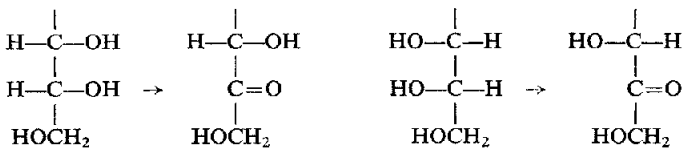
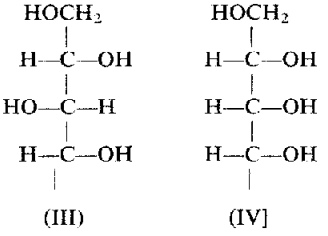


(I) (II)

Hann, Tilden and Hudson later showed that oxidation with another acetic acid bacterium named *Acetabocter suboxydans* (now called *Acetomonas oxydans*), was more specific in that only the D-*erythro* configuration of secondary hydroxyls (I) is oxidized.[9] Several ω-deoxyglycitols which do not possess configurations I or II are nevertheless oxidized by *Acetobacter suboxydans*. By considering the CH_3CHOH group as a substituted CH_2OH group, the Bertrand–Hudson rules were extended to include the action of the organism on ω-deoxyglycitols.[10]

The first report of a microbiological oxidation of allitol was by Steiger and Reichstein who used the bacterium *A. xylinum*;[11] the product was characterized as L-allulose, none of the D-isomer being detected. This result is contrary to the reported ability of the organism to oxidize both D- and L-*erythro* configurations of secondary hydroxyls [(I) and (II)][8] and shows a specificity for the D-*erythro* hydroxyls in allitol. The oxidation of allitol has also been effected with a rat-liver polyol dehydrogenase[12] in the presence of NAD^+. The product was considered to be D-allulose but insufficient material was available for measurement of its optical rotation. The identification was based on analogy with the products obtained from other polyols: thus, D-glucitol gave D-fructose whilst ribitol gave D-*erythro*-pentulose, the vulnerable configurations being considered to be (III) and IV).



(III) (IV]

Arcus and Edson[13] have presented evidence to support the existence of at least three polyol dehydrogenases in *A. suboxydans*. One of these enzymes, cytochrome-linked D-mannitol dehydrogenase, was shown to oxidize allitol to allulose but neither the optical rotation of the product nor that of its phenylosazone was measured. Since the substrate specificity of the preparation follows the Bertrand–Hudson rule, the product was presumably L-allulose. Kersters, Wood and De Ley[14] have recently isolated six different soluble polyol dehydrogenases from *Gluconobacter oxydans* (usually called *Acetomonas oxydans*) and have shown that one of these, an NAD-linked *erythro* dehydrogenase, will oxidize allitol to allulose. The latter was identified by paper chromatography and by its negative

[9] R. M. HANN, E. B. TILDEN and C. S. HUDSON, *J. Am. Chem. Soc.* **60**, 1201 (1938).
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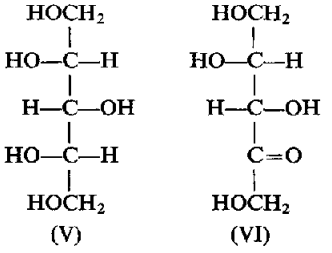
reactions with yeast hexokinase and the cysteine–sulphuric acid reaction thus distinguishing the product from D-fructose and sorbose, respectively. The configuration of the allulose was not determined but by analogy with the products of similar oxidations, for example, ribitol to D-*erythro*-pentulose, it would appear that allitol was in fact oxidized to D-allulose. The description of the enzyme as an "-*erythro* dehydrogenase" is, however, misleading, as it is also effective in oxidizing *threo*-hydroxyls as in the oxidation of xylitol (V) to D-*threo*-pentulose (VI).



(V) (VI)

Kersters *et al.* have also oxidized allitol with a particulate polyol dehydrogenase preparation obtained from the cytoplasmic membrane of *G. oxydans* (*Acetomonas oxydans*). Once again, lack of material prevented the determination of configuration of the allulose formed, but since other examples followed the Bertrand–Hudson rule (that is, only polyols with the configuration (I) were oxidized) it seems very probable that the product was L-allulose.

In the present work allitol was oxidized by cells of *Acetomonas oxydans* (strain CR49)[15] grown in a medium whose composition is described under experimental methods. Paper chromatography[16,17] of the product indicated almost complete conversion to allulose, only a trace of allitol remaining unoxidized (Table 1). No trace of any further product was revealed

TABLE 1. R_f VALUES IN SOLVENT (*c*)

Allulose (*ribo*-hexulose)	0·62
Fructose (*arabino*-hexulose)	0·56
Tagatose (*lxyo*-hexulose	0·51
Sorbose (*xylo*-hexulose)	0·45
2,5-*threo*-Hexodiulose	0·34
2,5-*erythro*-Hexodiulose	0·45

with *p*-anisidine hydrochloride,[18] and hence since the organism oxidized D-allulose to 2,5-D-*erythro*-hexodiulose (identified by partial reduction with sodium borohydride to allulose and tagatose), the product of allitol oxidation was considered to be L-allulose (unpublished results, by R.A.C. and G.C.W.). Characterization of the product as L-allulose was effected by conversion to the di-*O*-isopropylidene and phenylosazone derivatives. The former was prepared essentially by the method of Steiger and Reichstein[11] with the modification that purification of the product was effected by chromatography on a column of silica gel; this method has been found extremely useful for the separation of *O*-isopropylidene derivatives of other monosaccharides and has been used to obtain the pure D-allulose derivative from

[15] J. L. SHIMWELL and J. G. CARR, *Antonie van Leeuwenhoek, J. Microbiol. Serol.* **25**, 353 (1959).
[16] S. M. PARTRIDGE, *Nature* **158**, 270 (1946).
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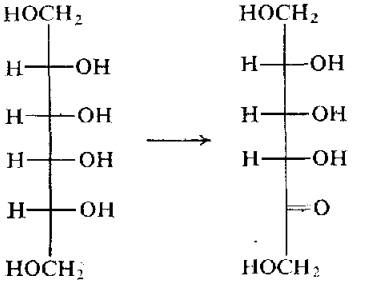
the mixture of products formed by the reaction of acetone with the mixture of carbohydrates contained in an alcoholic extract of *Itea* leaves.[5]

Hence, in common with other growing acetic acid bacteria, *Acetomonas oxydans* exhibits the specificity according to the Bertrand–Hudson rule. The oxidation of allitol to L-allulose in high yield through the agency of this bacterium has been subsequently used successfully as a first step in the degradation of ^{14}C-labelled allitol.[5]



(VII) (VIII)

EXPERIMENTAL

All evaporations were carried out at 40–45° on a rotary evaporator. Optical rotations were determined at 24°. Paper chromatography was carried out by the descending method on Whatman No. 1 filter paper using the following solvent systems: (*a*) butan-1-ol–pyridine–water (10:3:3 v/v); (*b*) ethyl acetate–acetic acid–water (9:2:2 v/v); (*c*) phenol–water (3:1 w/v) containing 1 per cent (v/v) of formic acid. The following spray reagents were used: (*a*) silver nitrate in ammonium hydroxide solution (4 per cent w/v),[16] 0·02 M sodium metaperiodate followed by ammoniacal silver nitrate;[17] (*b*) 5 per cent w/v solution of orcinol in butan-1-ol containing concentrated hydrochloric acid (2 per cent v/v);[18] (*c*) 3 per cent *p*-anisidine hydrochloride[18] in 90 per cent aqueous ethanol. Thin-layer chromatography (TLC) was carried out on silica gel (Merck), containing calcium sulphate hemihydrate (13 per cent) as binder, with chloroform/ether (3:1 v/v) as solvent. Substances were located by spraying with ethanolic sulphuric acid (5 per cent v/v), subsequent heating revealing non-volatile organic compounds as dark coloured spots.

Oxidation of Allitol with Acetomonas oxydans

Allitol (1·15 g) was made up as a broth containing the polyol (0·5 per cent w/v) and yeast extract (1 per cent w/v) and inoculated with a culture of *Acetomonas oxydans* (CR49).[15] After 3 weeks at 25°, the mixture was centrifuged, the supernatant liquid deionised by passage in turn through columns of ion-exchange resins, Amberlite IR 120 (H^+) and IRA 400 (CH_3COO^-) and concentrated (16 ml). Paper chromatography indicated the presence of a ketose detected by spray (*b*) together with a trace of allitol detected by spray (*a*).

Characterization of the Product as L-*Allulose*

(*a*) The phenylosazone derivative was prepared by treating a portion of the hexulose solution (1·25 ml) with 3 per cent acetic acid (3 ml), phenylhydrazine (0·2 ml) and a trace of sodium metabisulphite at 70–75° for 4 hr under nitrogen. Recrystallization of the crude product from aqueous ethanol (50 per cent v/v) yielded L-*ribo*-hexosephenylosazone (65 mg) having mp 161–164° and $[\alpha]_D+64°$ (*c*. 1·0 pyridine) [lit.[11,19] m.p. 173–174°, and 165°; $[\alpha]+78·6°\rightarrow 73·9°$ (pyridine)]. (*b*) A second portion of the hexulose solution was concentrated to a dry syrup (0·68 g) and shaken with anhydrous acetone (18 ml), anhydrous $CuSO_4$ (2 g) and conc H_2SO_4 (0·04 ml) for 48 hr. After neutralisation with anhydrous K_2CO_3, the supernatant was concentrated to a syrup. Examination by TLC revealed di-*O*-isopropylidene allulose (R_f 0·62) with traces of several other compounds, those with higher R_f values most probably being allitol derivatives.[5] The syrup was fractionated on a column of silica gel (28 g) with chloroform/ether (3:1 v/v) as solvent; fractions were collected by an automatic collector and monitored by TLC. The fraction corresponding to di-*O*-isopropylidene allulose yielded a syrup which crystallized (0·41 g) on trituration with light petroleum (b.p. 40–60°). Recrystallization from the same solvent yielded chromatographically pure di-*O*-isopropylidene L-allulose (0·33 g) m.p. 57–58°, $[\alpha]_D+96°$ (*c*. 1·0 acetone) with an i.r. spectrum identical to that of an authentic specimen (m.p. 56·5–57°, $[\alpha]_D^{20}+99°$).[11]

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