Although the substrate specificity does not conform to a simple rule, the enzyme oxidizes mannitol vigorously and is named DPN-linked D-mannitol dehydrogenase. Similar, but not identical activity occurs in extracts of *C. utilis*.

3. The problem of multiplicity of DPN-linked polyol dehydrogenases is discussed briefly.

We are much indebted to Dr N. K. Richtmyer, National Institutes of Health, Bethesda, U.S.A., and to Dr D. R. D. Shaw for generous gifts of rare polyols; and to the Virus Research Laboratory, Medical School, for the use of a Spinco Model L centrifuge.

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Polyol Dehydrogenases

3. GALACTITOL DEHYDROGENASE AND D-IDITOL DEHYDROGENASE

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(Received 22 February 1956)

Although dulcitol occurs in many plants and is used as a substrate in fermentations which help to distinguish members of the family Enterobacteriaceae, very little is known about its metabolism. It appears that dulcitol is not metabolized by mammals (Embden & Griesbach, 1914; Carr & Krantz, 1934; Edson, 1936; Smith, Finkelstein & Smith, 1940; Todd, 1954). On the other hand, most species of *Salmonella* can ferment dulcitol but the initial rate is slow in certain strains, suggesting that enzymic induction may precede dissimilation (Teague & Morishimu, 1920; Kristensen, 1943). Apart from reports of 'acid' and 'gas' formation there seems to be no description of dulcitol catabolism in the Enterobacteriaceae.

Hermann & Neuschul (1931) have reported that *Acetobacter gluconicum* converts dulcitol into a substance that reduces Fehling's solution but gives a negative Seliwanoff reaction. Without further evidence they considered the substance to be galactose. Some strains of staphylococci 'dehydrogenate' dulcitol as well as other polyols (Ömer, 1940), and the yeast *Hansenula anomala* takes up oxygen in the presence of dulcitol and other polyhydric alcohols (Usami, 1942). In the acetone-butanol fermentation of *Clostridium acetobutylicum* dulcitol gives a higher ratio of butyric acid to acetic acid than glucose (Simon, 1947). Shockley & Randles (1954) found that non-proliferating cell suspensions of *Lactobacillus* sp. form acid from dulcitol when the

cells have been grown in the presence of dulcitol or sorbitol, and suggested that tagatose was an intermediate.

Although the primary product of dulcitol catabolism was not identified by these authors, the observations indicate that a dehydrogenase mechanism analogous to the known polyol dehydrogenases may convert dulcitol into tagatose. Since the polyol dehydrogenases of mammalian liver and *Acetobacter suboxydans* do not attack dulcitol, it is likely that the catabolism of dulcitol will depend on a galactitol dehydrogenase specific for configuration (I) or (II), and that the enzyme will be found in plants and micro-organisms (Edson, 1953).

After an unsuccessful attempt to extract a stable enzyme from a dulcitol-fermenting strain of *Escherichia coli*, a satisfactory preparation of galactitol dehydrogenase was eventually made from an airborne organism isolated in the laboratory.

MATERIALS AND METHODS

Organism. The micro-organism used in this study was isolated by elective culture. The medium (25 ml.) contained 0.2% of dulcitol, 0.25% of KH_2PO_4 , 0.01% of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01% of anhydrous CaCl_2 and 0.1% of $(\text{NH}_4)_2\text{SO}_4$, and the pH was adjusted to 7.0 with NaOH. It was exposed to the air of the laboratory for 24 hr. in a wide-necked flask, which was then plugged with cotton wool and incubated at 30° for 48 hr. The primary culture was plated out on the above medium, to which 0.01% of Difco yeast extract and 2% of agar had been added. Single colonies were picked off and subcultured four times. The colonies appeared to be homogeneous when specimens were stained by Gram's method and examined microscopically.

One organism of the three strains isolated in pure culture was selected for further study. It grew in the synthetic medium described above, and utilized dulcitol as the sole carbon source. The following characteristics were noted: Gram-negative rods occurring singly or in chains, no spores apparent, old cultures cocco-bacillary; motile in nutrient broth. On the synthetic medium small white colonies appeared slowly, but colonies on nutrient agar grew rapidly, were cream coloured and released into the medium a pigment with a strong green fluorescence. Growth did not occur at 38° but took place readily at 30°. Neither acid nor gas was formed during growth in 1% peptone-0.5% NaCl medium supplemented by 0.5% of glucose, sucrose, lactose, maltose, xylose, sorbitol, mannitol or dulcitol. In beef-infusion broth (pH 7.4) or in peptone water, growth occurred mainly at the surface. Gelatine-stab cultures showed no liquefaction. Phenazine pigment was not detected in chloroform extracts of alkaline suspensions of the bacteria

(Tobie, 1945). The organism was tentatively classified as a fluorescent species of *Pseudomonas*.

Washed suspensions of the bacterial cells. Cultures of the organism were maintained on nutrient-agar slopes and transferred to nutrient broth for 16 hr. before use as an inoculum. For experimental purposes the bacteria were grown for 24 hr. at 30° on nutrient agar which was supplemented with 1% (w/v) of carbohydrate where indicated in the text. The cells were harvested by washing from the agar with 0.9% (w/v) NaCl solution followed by centrifuging and further washing (three times) with the same solution. The yield of cells was usually 1.5 g. wet wt./70 ml. of medium contained in a Roux bottle.

Cell-free extracts. These were prepared in a Raytheon sonic oscillator from 50% (w/v) bacterial suspensions in 0.02M potassium phosphate buffer, pH 7.4. The cup assembly was set up in a cold room at 2–3° and the cup cooled by circulation of ice water at 0–1°. The suspensions were subjected to sonic treatment for 45 min., clarified by centrifuging (20000 g) in a refrigerated centrifuge at –3° and dialysed against distilled water at 2–3°.

Acyclic polyols. *L-Rhamnitol* (m.p. 122–123°) was prepared by the catalytic reduction of *L-rhamnose* by the method of Gätzi & Reichstein (1938). *L-Iditol* (m.p. 73–5°) was prepared by hydrolysis (Hann & Hudson, 1945) of the hexaacetate which was prepared from *L-sorbose* by the method of Meyer & Reichstein (1946). *D-Iditol* (m.p. 73–74°) was prepared by sodium amalgam reduction (Glatthaar & Reichstein, 1938) of *D-idonolactone* followed by catalytic reduction of the *D-idose*. *D-Idonolactone* was prepared by cyanhydrin synthesis from *D-xylose* (Hudson, Hartley & Purves, 1934) and separated from the crystalline epimeric *D-gulonolactone*. *L-Talitol* (syrup) was obtained by acid hydrolysis (Lobry de Bruyn & Alberda van Ekenstein, 1899) of tribenzylidene-*L-talitol* (m.p. 204–206°; $[\alpha]_D^{25} = +40.8^\circ$, c, 1.25, CHCl_3). The benzylidene derivative was prepared from the catalytic reduction product of *L-talose*. *L-Talose* (not isolated) was prepared by the method of Glatthaar & Reichstein (1938) from *L-galactonic acid*, which in turn was prepared by catalytic reduction of *D-galacturonic acid* (Pizzarello & Freudenberg, 1939).

Sugars. *D-Tagatose* (m.p. 130°; $[\alpha]_D^{25} = -2.9^\circ$, c, 5, water) was prepared by the method of Reichstein & Bosshard (1934) except that the epimeric aldoses were removed by bromine oxidation, followed by removal of ions with Amberlite IR 4-B and IR 120. *L-Rhamnose hydrate* and *D-galacturonic acid* were products of the Eastman Kodak Co., Rochester, U.S.A. The sources of the other polyols and sugars used in this work are given in Part I (McCorkindale & Edson, 1954).

Diphosphopyridine nucleotide (DPN). This compound and also triphosphopyridine nucleotide (TPN), and reduced diphosphopyridine nucleotide (DPNH) were the chromatographically purified products of the Sigma Chemical Co., St Louis, U.S.A.

Analytical methods

Oxygen consumption was measured by the conventional Warburg technique. Carbon dioxide was absorbed by 0.2 ml. of 10% (w/v) KOH contained in the inseal.

Reduction and reoxidation of DPN were followed by measuring optical densities at 340 mμ. in a Uvispek spectrophotometer. Polyol-dehydrogenase activity was determined by measuring the initial rate of reduction of DPN by

polyols in the presence of buffered enzyme solutions. Optical density (340 $m\mu$) readings were taken at intervals of 15 sec. in the case of rapid reactions or at longer intervals for slow reactions. It was found (see Fig. 6) that the rate of reduction was linear until about one-third of the DPN was reduced.

Protein. This was estimated turbidimetrically by the method of Stadtman, Novelli & Lipmann (1951).

Identification of polyol oxidation products. The reaction mixtures were deproteinized with $\text{Ba}(\text{OH})_2$ and ZnSO_4 (Somogyi, 1945) or with 0.1 vol. of 7% metaphosphoric acid, and residual dye was removed by addition of Merck's activated charcoal. Reducing sugar and ketose in the protein-free decolorized filtrates were detected by methods described earlier (McCorkindale & Edson, 1954). Sugars were identified by paper chromatography and by conversion into phenylsazones (Edson, 1940).

Chromatography. Three solvents were employed for descending paper chromatography of sugars on Whatman no. 1 paper. Water-saturated phenol, containing 0.04% of 8-hydroxyquinoline (Block, 1950) was used at 23–27° and gave satisfactory separations of ketohexoses and ketopentoses (R_F values in brackets): L-sorbose (0.41), D-allulose (0.44), D-tagatose (0.46), D-fructose (0.52), D-xylulose (0.59) and L-ribulose (0.64). Butanol-pyridine-water (6:4:3, by vol.) (Kenner & Richards, 1954) and ethyl acetate-pyridine-water (2:1:2, by vol.) (Isherwood & Jermyn, 1951) were used at 15–20°.

Ketoses were detected by an orcinol reagent (Klevstrand & Nordall, 1950). Reducing sugars were detected by a silver nitrate reagent [1% of AgNO_3 (w/v) in 5N- NH_3 soln.] which does not detect polyols under the conditions used (cf. Hough, 1950). After spraying, the papers were heated for 5–10 min. at 95°.

RESULTS

Oxidation of polyols by cell suspensions of the organism isolated

The initial rate of oxygen uptake by non-proliferating cell suspensions in the presence of polyols depends on the conditions under which the organism was grown. Dulcitol is oxidized without a preliminary period of induction by cells which were cultivated on a medium containing dulcitol or

sorbitol (Figs. 2, 3). The latent period before oxidation begins in cells grown on an unsupplemented medium (Fig. 1), or on a medium containing glucose or mannitol, is 1–2 hr. This induction period is interpreted as the time required for the production of an enzyme oxidizing dulcitol.

Fig. 2. Oxidation of polyols by cells grown on nutrient agar supplemented by 1% of sorbitol. Conditions are the same as in Fig. 1.

Fig. 3. Oxidation of polyols by cells grown on nutrient agar supplemented by 1% of dulcitol. Conditions are the same as in Fig. 1.

Fig. 1. Oxidation of polyols by cells grown on unsupplemented nutrient agar. Warburg flasks contained 50 mg. (wet wt.) of washed cells, 50 μ moles of potassium phosphate buffer, pH 7.4, and 50 μ moles of substrate (added from side bulb after equilibration). Volume, 1.4 ml. Gas, air. Temp. 30°. ●, No substrate; □, xylitol; ▽, mannitol; ○, sorbitol; Δ, dulcitol.

Fig. 4. Oxidation of polyols by cells grown on nutrient agar supplemented by 1% of mannitol. Conditions are the same as in Fig. 1.

Fig. 3 shows that cells adapted to dulcitol during growth are also adapted to sorbitol but not to mannitol, whereas cells adapted to sorbitol during cultivation can oxidize sorbitol, mannitol and dulcitol without further induction (Fig. 2). Cells grown in the presence of mannitol (Fig. 4) oxidize mannitol and sorbitol, but not dulcitol, without subsequent induction. It will be seen that sorbitol is oxidized at a slow rate by cells grown on the unsupplemented medium (Fig. 1).

Cells grown in the presence of glucose showed subsequently the same adaptive pattern as those cultivated in the unsupplemented medium, but the endogenous respiration and the rates of polyol oxidation were greater. Xylitol is oxidized at a slow constant rate by cells grown under all conditions (Figs. 1-4).

In each case the polyol used to supplement the medium is oxidized at a faster rate than the other substrates. Since the excess oxygen uptake due to the addition of hexitols (50 μ moles) is greater than the theoretical amount (25 μ moles) required for conversion of polyol into ketose, the sugar is probably catabolized further.

Oxidation of polyols in cell-free extracts of the organism

Dialysed extracts (40 hr.) of cells grown on sorbitol oxidized sorbitol, mannitol and dulcitol under anaerobic conditions with methylene blue as hydrogen acceptor (Table 1); but the ability to oxidize mannitol disappeared almost completely on further dialysis or on storage at 0-2° for 24 hr. The same phenomenon occurred in extracts of cells grown on dulcitol.

The oxidations of mannitol and dulcitol are presumably due to two different enzymes. The lability of the mannitol activity was a fortunate coincidence, since it became possible to study the properties of the enzyme responsible for dulcitol oxidation by using extracts which had been dialysed for 48-60 hr. These extracts did not take up oxygen in the presence of mannitol.

The differences in the enzyme-inducing properties of dulcitol and sorbitol which were shown by whole cells (Fig. 1-4) are reflected in the properties of the extracts. Extracts from cells grown on sorbitol oxidized sorbitol more rapidly than dulcitol, and the reverse was true of extracts from cells grown on dulcitol. Table 2 shows that extracts from cells grown on sorbitol do not exhibit appreciable oxygen uptake with xylitol, whereas extracts from cells grown on dulcitol oxidize xylitol as rapidly as sorbitol. Cells harvested from all the media used oxidize xylitol at a slow rate which does not change in 4 hr. Although the vigorous oxidation of xylitol by extracts from cells grown on dulcitol was unexpected, it is possible that the bacterial cell

membranes may show selective permeability towards polyols.

Coenzyme requirements. In the absence of added DPN methylene blue is not reduced anaerobically and there is no oxygen uptake in manometric experiments. TPN cannot be substituted for DPN. Spectrophotometric observations confirmed the specificity for DPN.

pH optimum. The optimum pH for oxygen consumption is about 10 in the presence of sorbitol and dulcitol (Fig. 5). Although curves were not determined in other cases it was found that D-gulitol, D-talitol, 6-deoxy-L-gulitol and xylitol are oxidized about twice as rapidly at pH 10 (glycine buffer) as at pH 8 (phosphate buffer).

Cytochromes. Examination of thick bacterial cell suspensions with a direct-vision spectroscope showed the presence of two cytochrome-like absorption bands: an intense band centred at 555 m μ . but extending from 550 to 562 m μ ., and a diffuse band at 525 m μ . On dilution of the suspension the broad band centred at 555 m μ . was resolved into a strong band at 553 m μ . and a very weak band at 560 m μ . Cell-free extracts were reddish brown in colour and the same absorption

Table 1. *Oxidation of hexitols by extracts from cells grown on sorbitol*

Evacuated Thunberg tubes contained: 0.25 ml. of extract from cells grown on sorbitol, 20 μ moles of potassium phosphate buffer, pH 7.8, 20 μ moles of substrate, 10⁻¹ μ mole of DPN and 2.8 $\times$ 10⁻¹ μ mole of methylene blue. Volume, 1.25 ml. Temp., 30°.

Substrate	Reduction time (min.)	
	Extract dialysed for 40 hr.	Extract dialysed for 64 hr.
Nil	24	69
Mannitol	1.5	54
Sorbitol	0.5	2
Dulcitol	1.5	4

Table 2. *Oxidation of xylitol by cell-free extracts*

Each Warburg flask contained: 1.0 ml. of bacterial extract, 20 μ moles of potassium phosphate buffer, pH 7.8, 4 $\times$ 10⁻¹ μ mole of DPN and 80 μ moles of substrate (added from side arm after equilibration). Volume, 1.6 ml. Gas, air. Temp., 25°. Time, 30 min.

Extract from ...	O ₂ uptake (μ moles)	
	Sorbitol-grown cells	Dulcitol-grown cells
Substrate		
Nil	0.27	1.29
Sorbitol	7.73	3.94
Dulcitol	5.85	5.45
Xylitol	0.31	4.02

bands appeared on the addition of a trace of $\text{Na}_2\text{S}_2\text{O}_4$, or of dulcitol or sorbitol together with DPN. The bands disappeared when oxygen was bubbled through the solution of reduced pigment. A difference spectrum, obtained in a manner similar to that described by Smith (1955), showed absorption maxima at 423, 524 and 553 $\text{m}\mu$. The pigment closely resembles that observed in extracts of *Pseudomonas fluorescens* and identified as cytochromes *b* and *c* by Stanier, Gunsalus & Gunsalus (1953).

Presence of natural hydrogen carrier. Extracts took up oxygen at pH 8 in the presence of substrate and DPN without the addition of an accessory hydrogen carrier (methylene blue). The presence of cytochrome pigment suggested that a cytochrome oxidase or an autoxidizable cytochrome might have mediated the oxygen uptake. Table 3 shows that the

natural hydrogen carrier was inhibited at pH 10, since there was little oxygen uptake at this pH in the absence of methylene blue. Although it was not necessary to add methylene blue to promote oxygen consumption at pH 8, the rate of consumption was stimulated by the dye.

Spectrophotometric observations showed that DPNH was oxidized in the presence of extract buffered at pH 8. The oxidation of DPNH was inhibited by cyanide ($2 \times 10^{-3}\text{M}$) or by raising the pH to 10. The reduction of the coenzyme by dulcitol at pH 10 was so much more rapid (about 300 times) than its re-oxidation that spectrophotometric measurements of dehydrogenase activity could be made at pH 10 with negligible interference by the hydrogen carrier.

Substrate specificity of extracts from cells grown on sorbitol

The dehydrogenase activity was separated from the natural hydrogen carrier in order to obtain a preparation free from DPNH oxidase.

The extract was dialysed free from endogenous substrate and showed no oxygen uptake in the presence of mannitol. A sample (30 ml.) was cooled to 0° and brought to 0.3 saturation by addition of solid ammonium sulphate. The quantity of ammonium sulphate required to give a desired saturation was calculated from a nomogram (Dixon, 1953). The mixture was stirred for 15 min. and centrifuged to give a brown precipitate containing cytochrome (fraction *O*) and a pale-yellow supernatant (fraction *P*, 26 ml.). Fraction *O* was suspended in water (12 ml.) and an equal volume of 0.6 saturated ammonium sulphate solution was added. The mixture was centrifuged to give a red-brown precipitate (fraction *Q*) and a pale-yellow supernatant (fraction *R*, 23 ml.). Fractions *P* and *R* were combined and brought to 0.5 saturation by the addition of solid ammonium sulphate. The resulting precipitate (fraction *S*) was dissolved in water (10 ml.) and dialysed against water for 3 days at $0-2^\circ$. This fraction contained 80% of the original activity towards dulcitol and 30% of the protein. A protocol of the fractionation is shown in Table 4. The supernatant from 0.5 saturation with ammonium sulphate was discarded (fraction *T*), as was fraction *Q*. The fine particles containing cytochrome pigment were aggregated at 0.3 saturation. Precipitation of these particles may cause co-precipitation of the dehydrogenase, but this can be recovered by extraction with 0.3 saturated ammonium sulphate solution.

While cell-free extracts took up oxygen in the presence of substrate and DPN at pH 8 (Table 3), fraction *S* was free from hydrogen carrier, since oxygen was not consumed in the presence of dulcitol and DPN at pH 8 unless methylene blue was added.

Fig. 5. Optimum pH for oxidation of sorbitol and dulcitol by extracts of cells grown on dulcitol-nutrient agar. Warburg flasks contained 0.5 ml. of bacterial extract, 40 μmoles of substrate, $2 \times 10^{-1}\mu\text{mole}$ of DPN, 1.0 μmole of methylene blue and 30 μmoles of buffer. Volume, 1.0 ml. Gas, air. Temp. 25° . Buffers: $\circ$, potassium phosphate; $\bullet$, glycine.

Table 3. *Effect of methylene blue on oxygen uptake*

Each Warburg flask contained: 0.5 ml. of extract from cells grown on dulcitol, 30 μmoles of buffer, 40 μmoles of dulcitol (added from side arm after equilibration), $2 \times 10^{-1}\mu\text{mole}$ of DPN, and 1.0 μmole of methylene blue where indicated. Volume, 1.0 ml. Gas, air. Temp., 25° . Time, 30 min. There was no oxygen consumption in the absence of dulcitol.

Buffer	Methylene blue	O ₂ uptake (μmoles)
Potassium phosphate, pH 8.0	Absent	2.50
	Present	3.26
Glycine, pH 10.0	Absent	0.22
	Present	7.23

Table 4. *Fractionation of extracts from cells grown on sorbitol*

Cuvettes initially contained in a volume of 2.0 ml.: 0.05–0.2 ml. of enzyme solution, 40 μ moles of glycine buffer, pH 10.0, 10 μ moles of dulcitol and 2×10^{-3} μ mole of DPN. Controls contained no substrate. Temp., 18°. Fraction Q was suspended in water (10 ml.) before testing. The initial rate of reduction of DPN was measured. The unit of enzymic activity is the amount of enzyme which will bring about an increase of 1.000 in optical density (340 m μ)/min.

	Protein (mg.)	Enzyme (units)
Original extract ...	335	23.60
Fraction		
O	189	9.00
P	156	13.26
Q	134	0.73
R	46	7.50
S	101	18.80
T	89	0.92

Fraction S was tested for substrate specificity with all available polyols. The activity was very stable at low temperature. After storage for 6 months in a frozen state at -20° a sample of this preparation lost less than 5 % of its original activity towards sorbitol and dulcitol.

Fig. 6 shows the relative rates of oxidation of certain polyols (5×10^{-3} M). Of the hexitols sorbitol is attacked most rapidly, dulcitol and L-iditol being next in sequence; D-iditol is oxidized very slowly and D-mannitol, D-gulitol and D-talitol are not attacked. In a subsequent experiment L-talitol was oxidized very slowly (3 % of the rate of sorbitol, 5×10^{-3} M). L-Arabitol and xylitol are oxidized slowly, the latter at 1 % of the rate of sorbitol; ribitol and D-arabitol are not oxidized. L-gulo-D-talo-Heptitol (β -sedoheptitol) and L-gulo-D-gala-heptitol are oxidized faster than the pentitols but slower than L-iditol; D-manno-D-gala-heptitol (perseitol) is oxidized very slowly (increase of optical density 0.001/min.) and is not shown in the figure. Of the deoxypolyols tested L-rhamnitol is not attacked but 6-deoxy-L-gulitol is oxidized rapidly.

Identification of the products of reaction. The enzyme solution (2–10 ml. of fraction S) containing polyol (0.1 M), glycine–NaCl buffer, pH 9.8 (2×10^{-2} M), DPN (2×10^{-4} M) and methylene blue (10^{-3} M) was shaken in air for 4 hr. at 25° . After deproteinization and removal of dye the oxidation products were identified as follows.

Sorbitol was oxidized to a product which reduced Fehling's solution and gave a red resorcinol reaction. Chromatography (phenol–water) gave a single ketose spot which did not separate from authentic fructose but separated from sorbose and tagatose. The product was D-fructose.

Dulcitol formed a reducing substance which gave a red resorcinol test. The phenylosazone crystallized from pyridine–water in large yellow blades, m.p. (recryst.) $199\text{--}200^\circ$ (decomp.), $[\alpha]_D^{25} = +81^\circ$ in pyridine–absolute ethanol (2:3, v/v), c, 0.25 (cf. Levene & La Forge, 1915). The osazone was identified as D-galactosazone. Chromatography of the sugar (phenol–water) gave a single spot which did not separate from authentic tagatose. The sugar was D-tagatose.

L-Iditol formed a product which reduced Fehling's solution and gave a red resorcinol reaction. Chromatography (phenol–water) showed a single spot which did not separate from authentic sorbose. The product was L-sorbose.

L-Arabitol was oxidized to a reducing substance which gave a green resorcinol reaction. Chromatography with ethyl acetate–pyridine–water as well as with phenol–water gave a single spot which separated from authentic D-xylulose. With the latter solvent the R_F value of the sugar was 0.64 and the R_F value of D-xylulose was 0.59. Futterman & Roe (1955) give R_F values of 0.63 and 0.58 for ribulose and xylulose, respectively. On these grounds the product is identified as L-ribulose.

Xylitol formed a reducing substance which gave a green resorcinol test. Chromatography with the above two solvents showed that the sugar formed a single spot which travelled at the same rate as authentic xylulose. The product is therefore xylulose but the rotation was not determined.

Effect of DPN concentration on the rate of sorbitol oxidation. This was determined over the range $1\text{--}100 \times 10^{-5}$ M. The concentration found to give half the maximum velocity was 7.3×10^{-5} M. The concentration of DPN selected as optimum was 2×10^{-4} M.

Effect of substrate concentration. Values of K_m were determined spectrophotometrically for sorbitol, dulcitol, L-iditol, L-arabitol and xylitol in order to obtain some indication of the enzyme–substrate affinities. The K_m values for sorbitol and dulcitol are much lower than those for L-iditol, L-arabitol and xylitol (Table 5), and tend to follow an order which is the reverse of that giving the relative rates of oxidation of the substrates at 5×10^{-3} M concentration (see Fig. 6).

Observation of the relative rates of oxidation of these substrates (Table 5) was repeated at a higher substrate concentration (1×10^{-1} M). The oxidation rate for dulcitol is not changed relative to that for sorbitol but the relative rates for the other three polyols are increased; L-iditol is attacked more rapidly than dulcitol at the higher concentration.

Reversibility of polyol oxidation. Fig. 7 shows that D-fructose and D-tagatose oxidized DPNH rapidly in the presence of the enzyme but L-sorbose reacted very slowly. The slow reduction of L-sorbose

was unexpected since L-iditol is oxidized more rapidly than dulcitol under similar conditions (cf. Table 5).

Substrate specificity of extracts from cells grown on dulcitol

The ability of extracts from cells grown on dulcitol to oxidize polyols was tested with unfractionated material. Table 6 shows that the rate of oxygen uptake with D-gulitol, 6-deoxy-L-gulitol, D-talitol, dulcitol, xylitol and sorbitol exceeded 1 μ mole/30 min. at pH 8. The reaction flasks were shaken for 4 hr. After deproteinization and removal of dye dilute solutions of the reaction product from each of

these polyols reduced Fehling's solution and gave a positive resorcinol test. The same tests were positive in concentrated solutions of the reaction products from all other flasks, except those which originally contained no substrate, erythritol, allitol, D-mannitol or volemitol.

Table 5. *Relative oxidation rates of substrates in fraction S of the extract from cells grown on sorbitol*

Cuvettes contained in a volume of 2.0 ml.: 40 μ moles of glycine-NaCl buffer, pH 9.8, 4×10^{-1} μ mole of DPN, 200 μ moles of substrate and 0.1 ml. of enzyme (threefold dilution of fraction S). Temp., 18°. The blank cuvette contained water in place of substrate solution.

Substrate	Increase in optical density (at 340 m μ .)($\times 10^3/15$ sec.)	Relative rates referred to sorbitol as 100	K_m values
Sorbitol	56.0	100	$6.22 \times 10^{-3} M$
Dulcitol	22.8	41	$3.92 \times 10^{-3} M$
L-Iditol	25.1	45	$6.89 \times 10^{-3} M$
L-Arabitol	9.2	16	$8.66 \times 10^{-3} M$
Xylitol	3.5	6	$1.41 \times 10^{-1} M$

Fig. 6. Rates of reduction of DPN in the presence of polyols by an enzyme preparation (fraction S) from cells grown on sorbitol-nutrient agar. Cuvettes contained 0.2 ml. of enzyme solution, 40 μ moles of glycine buffer, pH 10.0, 2×10^{-1} μ mole of DPN and 10 μ moles of substrate. Temp. 18°. The blank cuvette contained the same mixture without substrate.

Fig. 7. Reversibility of polyol oxidation. The reaction mixture (2.0 ml.) contained initially 0.1 ml. of enzyme solution (threefold dilution of fraction S), 40 μ moles of glycine-NaCl buffer, pH 9.8, 2.7×10^{-1} μ mole of DPNH and 200 μ moles of ketose. Temp. 20°.

Table 6. *Substrate specificity of extracts from cells grown on dulcitol*

Each Warburg flask contained: 0.5 ml. of extract from cells grown on dulcitol, 20 μ moles of potassium phosphate buffer, pH 8.0, or glycine buffer, pH 10.0, 20 μ moles of substrate (added from the side arm after equilibration), 2×10^{-1} μ moles of DPN and 1.0 μ mole of methylene blue. Volume, 0.85 ml. Gas, air. Temp., 25°. Time, 30 min. Experiments at pH 10 were performed with a different extract.

Substrate	O ₂ uptake (μ moles)	
	pH 8.0	pH 10.0
Nil	0.14	0.09
Erythritol	0.18	—
D-Arabitol	0.41	1.87
L-Arabitol	0.27	0.41
Ribitol	0.31	—
Xylitol	1.52	—
Allitol	0.18	—
D-Mannitol	0.00	—
L-Gulitol (sorbitol)	1.29	—
D-Gulitol	3.35	1.34
D-Talitol	3.04	1.12
Galactitol (dulcitol)	2.19	5.13
6-Deoxy-L-gulitol	5.13	—
D-manno-D-talo-Heptitol (volemitol)	0.18	—
D-manno-D-gala-Heptitol (perseitol)	0.54	0.36
L-gulo-D-gala-Heptitol	0.36	2.32
L-gulo-D-talo-Heptitol (β -sedoheptitol)	0.31	1.07

Some polyols which were oxidized slowly at pH 8 were tested at pH 10, which may provide optimum conditions for measuring oxygen consumption. Table 6 shows the larger oxygen consumption at pH 10 of certain pentitols and heptitols in comparison with that of dulcitol, D-gulitol and D-talitol.

Identification of the products of reaction. Dulcitol gave a product which reduced Fehling's solution and gave a red resorcinol reaction. The phenylosazone crystallized from pyridine-water, m.p. (recryst.) 200–201° (decomp.), $[\alpha]_D^{18} = +79^\circ$ in pyridine-absolute ethanol (2:3, v/v), c , 0.25. When mixed with authentic D-galactosazone the osazone isolated gave m.p. 199–200°. On chromatographic analysis with phenol-water and with ethyl acetate-pyridine-water the sugar formed a single spot which did not separate from authentic tagatose but separated from fructose and sorbose. The product was D-tagatose.

Sorbitol formed a reducing sugar which gave a red resorcinol reaction. The phenylosazone crystallized from 30% ethanol (v/v) in sheaves of needles typical of phenylglucosazone, m.p. 208–209°, $[\alpha]_D^{18} = -60^\circ$ in pyridine-absolute ethanol (2:3, v/v), c , 0.25. Chromatography (phenol-water) gave a single spot which did not separate from authentic fructose but separated from sorbose. The sugar was completely fermented by baker's yeast. The product was D-fructose.

Xylitol was oxidized to a reducing sugar which gave a green resorcinol reaction. The phenylosazone crystallized from aqueous ethanol in long needles, m.p. (recryst.) 161–162° (decomp.), $[\alpha]_D^{18} = +10^\circ$ (initial) and $+42^\circ$ (after 25 hr.) in pyridine-absolute ethanol (2:3, v/v), c , 1.0. Levene & La Forge (1915) give m.p. 164°, $[\alpha]_D^{20} = -10^\circ$ (initial) and -43° (after 24 hr.) for D-xylose phenylosazone. The product from xylitol was L-xylulose.

D-Gulitol (4 mg.) gave rise to a product which reduced Fehling's solution and gave a red resorcinol reaction. The phenylosazone crystallized from 30% aqueous ethanol (v/v) in sheaves of needles characteristic of glucosazone, m.p. 200–203° (decomp.). Chromatographic analysis of the product gave a single spot which did not separate from authentic fructose but separated from sorbose. The sugar was not fermented by baker's yeast. The product was L-fructose.

D-Talitol (4 mg.) formed a reducing sugar which gave a red resorcinol reaction. The phenylosazone, unlike galactosazone, recrystallized well from 30% aqueous ethanol (v/v) and poorly from aqueous pyridine to give small matted needles, m.p. (recryst.) 180–182° (decomp.). Levene & Jacob (1910) reported the melting point of D-allosazone as 183–185° (decomp.). The quantity of osazone was insufficient for the measurement of optical rotation.

Since D-talitol could give rise either to D-tagatose or to D-allulose the ability of the product to separate from authentic tagatose was tested on a paper chromatogram. A definite but incomplete separation of the spots was achieved in three different solvents (phenol-water, ethyl acetate-pyridine-water and butanol-pyridine-water). The product of D-talitol oxidation was therefore D-allulose.

6-Deoxy-L-gulitol gave a product which readily reduced Fehling's solution. It gave a pale orange-yellow resorcinol reaction which was similar to that given by 6-deoxy-L-sorbose (personal communication from Mr R. G. Kulka), but the behaviour of the product differed from that of 6-deoxy-sorbose in an attempted preparation of a phenylosazone; the reaction mixture became pale yellow in colour and deposited only a small quantity of yellow crystalline material. The product was not investigated further.

Evidence for the presence of more than one polyol dehydrogenase in extracts from cells grown on dulcitol. The oxidation (Table 6) of the enantiomorphs, sorbitol (L-gulitol) and D-gulitol, to D- and L-fructose respectively, indicates that extracts from cells grown on dulcitol contain two polyol dehydrogenases.

One enzyme appears to be specific for the L-threo configuration (I) and the other for the D-threo configuration (II) (see page 395). The enzyme with specificity I could account for the oxidation of dulcitol to D-tagatose and sorbitol to D-fructose; whereas the enzyme with specificity II could account for the oxidation of D-iditol to D-sorbose (see Table 8), D-gulitol to L-fructose, D-talitol to D-allulose, xylitol to L-xylulose and, possibly, dulcitol to L-tagatose.

Undialysed extracts from cells grown in the presence of dulcitol were fractionated with ammonium sulphate to separate the polyol dehydrogenase activity from the natural hydrogen carrier. The procedure was essentially the same as that described for the fractionation of extracts from cells grown on sorbitol except that the polyol dehydrogenase activity was precipitated between 0.3 and 0.7 saturation with ammonium sulphate, and after solution and dialysis, the enzyme was stored at -20° in the frozen state (fraction D).

Fraction D was refractionated with ammonium sulphate, a partial separation of the xylitol and sorbitol activities being achieved. Table 7 shows that a fraction which precipitated at 0.3 saturation with ammonium sulphate contained a higher ratio of xylitol to sorbitol activity than the original material, but the total xylitol activity remaining in the various fractions was only 41% of the original activity, and the recovered sorbitol activity was 70%. Other methods for protein separation were tested but the destruction of the xylitol-oxidizing component was so rapid that none was found satisfactory for purification.

Table 7. *Fractionation of extracts from cells grown on dulcitol*

A freshly thawed sample (18 ml.) of fraction *D* (see text) made from an extract of cells grown on dulcitol was brought to 0.3 saturation by the addition of solid ammonium sulphate. The precipitate (fraction *E*) was removed by centrifuging and dissolved in water (5 ml.). The supernatant (17.5 ml., fraction *F*) was brought to 0.45 saturation by the addition of solid ammonium sulphate and the precipitate (fraction *G*) was separated as before from the supernatant (fraction *H*) and dissolved in water (10 ml.). The activities of the various fractions towards xylitol and sorbitol were assayed under the conditions described in Table 8. The unit of enzyme activity is the amount of enzyme which will bring about an increase of 1.000 in optical density (340 m μ ./min).

Original sample Fraction	Enzymic activity		Ratio (Xylitol:sorbitol)	Percentage initial activity	
	Sorbitol units	Xylitol units		Sorbitol (100)	Xylitol (100)
	15.2	39.6	2.6		
<i>E</i>	0.9	6.4	7.1	6	16
<i>F</i>	10.5	13.0	1.2	70	32
<i>G</i>	8.1	7.5	0.9	54	19
<i>H</i>	1.5	2.6	1.7	10	6

Table 8. *Oxidation of polyols by fraction D from cells grown on dulcitol*

A sample of fraction *D* (see text) was kept for 4 days at 2–3°. The activity to various polyols was determined initially and after storage. Cuvettes contained in a volume of 2.0 ml.: 0.05 ml. of enzyme, 40 μ moles of glycine–NaCl buffer, pH 9.8, 4×10^{-1} μ mole of DPN and 10 μ moles of substrate. Temp., 18°.

Substrate	Increase of optical density (340 m μ ./min.)		Activity remaining after storage (%)
	Initial	After storage	
Xylitol	0.266	0.026	10
D-Gulitol	0.182	0.010	6
D-Iditol	0.344	0.024	7
D-Talitol	0.074	0.005	7
D-Arabitol	0.016	0.008	50
L-Rhamnitol	0.009	0.007	78
Dulcitol	0.195	0.169	87
Sorbitol	0.105	0.094	90
6-Deoxy-L-gulitol	0.466	0.366	79
L-Iditol	0.009	0.008	89
L-Arabitol	0.012	0.010	83
Ribitol	0.015	*	0
D-Mannitol	*	*	—
Nil	*	*	—

* No change of optical density.

Differential destruction of activities. Table 8 shows the effect of storage at 2–3° on the polyol dehydrogenase activities of fraction *D*. The original ability of fraction *D* to oxidize D-iditol, D-talitol, D-gulitol and xylitol diminished by 90 % or more. This is consistent with the view that a single enzyme (II) may be responsible for the oxidation of these polyols. By contrast the ability to oxidize dulcitol and sorbitol, which possess the structures required for oxidation by enzyme I, decreased by no more than 10–20 %. After 16 days' storage, when the xylitol activity had fallen to 2 % of its original rate,

the oxidation of dulcitol and sorbitol proceeded at 72 % of their original rates.

Properties of the stable activities. To study the stable activities of the extract (fraction *D*) from cells grown on dulcitol and to compare them with the activities of the extract (fraction *S*) made from cells grown on sorbitol, the experiments shown in Table 9 were performed. The K_m values are greater than the values for the corresponding activities in cells grown on sorbitol (Table 5). It will be seen that the relative rates of polyol oxidation in fraction *D* (Table 9) follow a different order from that observed in fraction *S* (Table 5). Dulcitol is oxidized faster than sorbitol by the enzyme from cells grown on dulcitol and L-arabitol more rapidly than L-iditol.

Lability of activities towards mannitol and ribitol. The rapid loss of the capacity to oxidize mannitol has already been stressed. Ribitol activity was originally low and quickly lost (Table 8). The high order of the K_m values for the stable activities suggested that the oxidation of mannitol and ribitol might be detected after storage if higher substrate concentrations were used. Table 10 shows that the effect of increased substrate concentration on reaction velocity is much greater with mannitol and ribitol than with sorbitol or xylitol.

DISCUSSION

Enzymes oxidizing polyols are formed adaptively by a micro-organism which has many characteristics of a species of *Pseudomonas*. The enzyme is induced by growing the organism on nutrient agar supplemented by a polyol or by incubating washed suspensions in the presence of a polyol. The induction phenomenon has some well-defined features. Dulcitol is oxidized by cells grown in the presence of dulcitol or sorbitol; mannitol by cells grown on

mannitol or sorbitol; and sorbitol by cells grown in the presence of any one of these hexitols. Hence the ability to oxidize dulcitol can be induced with either sorbitol or dulcitol.

The enzymes are DPN-linked dehydrogenases. A natural hydrogen carrier oxidizing DPNH was present in crude extracts but interference from this source was obviated by conducting spectrophotometric experiments at pH 9–10. In manometric experiments methylene blue can be substituted for the natural carrier.

Mannitol oxidation. The adaptive pattern in culture and in washed-cell suspensions (Figs. 2–4) suggests that the organisms form a mannitol dehydrogenase. A DPN-linked dehydrogenase of this type was detected in extracts of cells grown on sorbitol or dulcitol, but the activity was too labile to be studied in detail (Tables 1 and 8). The observations of Sebek & Randles (1952) suggest that a mannitol dehydrogenase is formed adaptively by *Ps. fluorescens* grown in the presence of mannitol or sorbitol.

Galactitol dehydrogenase. Extracts from cells grown on sorbitol oxidize dulcitol to D-tagatose and sorbitol to D-fructose. Specificity tests with the available pentitols and hexitols showed that those fully hydroxylated polyols containing an L-threo configuration (I) adjacent to a primary alcohol group were attacked (Fig. 6). In cases where a satisfactory identification was made, the products conformed to the hypothesis (e.g. Fig. 8). It is probable that a single enzyme is responsible for the oxidation of these polyols, although the relative

Table 9. *Stable activities in extracts of cells grown on dulcitol*

Cuvettes contained in a volume of 2.0 ml.: 40 μ moles of glycine-NaCl buffer, pH 9.8, 4×10^{-1} μ mole of DPN, 200 μ moles of substrate, 0.2 ml. of enzyme solution (four-fold dilution of fraction D stored for 16 days at 2–3°). Temp., 18°. The blank cuvette contained water in place of substrate solution.

Substrate	Increase in optical density (at 340 m μ .)($\times 10^3/15$ sec.)	Relative rates of oxidation referred to sorbitol as 100	K_m values
Sorbitol	29.3	100	9.47×10^{-3} M
Dulcitol	56.7	193	1.00×10^{-2} M
L-Iditol	5.7	19	1.89×10^{-1} M
L-Arabitol	13.4	46	1.09×10^{-1} M
Xylitol	6.0	20	—*

* Lineweaver-Burk plot non-linear.

Table 10. *Effect of substrate concentration on reaction velocity with mannitol and ribitol*

Conditions were the same as in Table 8 except for substrate concentrations. Enzyme (fraction D) was stored 4 days at 2–3°.

Substrate	Increase in optical density (340 m μ .) $\times 10^3/15$ sec. at substrate concentration of		Ratio of reaction rates at 5×10^{-3} M/ 5×10^{-2} M substrate
	5×10^{-3} M	5×10^{-2} M	
Mannitol	0.1	1.2	12.0
Ribitol	1.8	12.4	6.9
Xylitol	22.6	69.7	3.1
Sorbitol	16.6	40.3	2.4

Fig. 8

oxidation rates differ considerably (Fig. 6, Table 5). It is proposed to call the enzyme *galactitol dehydrogenase*.

The relative oxidation rates of the substrates of galactitol dehydrogenase from cells grown on sorbitol (Table 5) are different from those observed in the fractionated extracts of cells grown on dulcitol (Table 9). If a single enzyme is responsible for the oxidation of dulcitol, sorbitol, L-arabitol and L-iditol in extracts from cells grown on dulcitol it cannot be identical with the galactitol dehydrogenase induced by sorbitol, although it has the same type of specificity.

D-Iditol dehydrogenase. Freshly prepared extracts from cells grown on dulcitol (Tables 6, 8) showed vigorous activity not only towards dulcitol and sorbitol but also towards some polyols which are not known to occur in nature (D-iditol, D-gulitol, D-talitol and xylitol). The identified products of oxidation are shown in Fig. 9. The oxidation of sorbitol to D-fructose and of dulcitol to D-tagatose suggested strongly that galactitol dehydrogenase was present in the extracts but the oxidation of D-gulitol to L-fructose showed that the extracts also contained an enzyme with enantiomorphic specificity. The lability of the activities towards D-iditol, D-gulitol, D-talitol and xylitol supports the hypothesis that a single enzyme is concerned with the oxidation of these substrates. The lability foiled attempts to effect a satisfactory chemical separation although a partial separation was achieved (Table 7).

A simple hypothesis to explain the existence of the labile group of activities is that dulcitol, which has a D-threo (II) configuration at one end of the molecule, induces the formation of a dehydrogenase specific for the oxidation of fully hydroxylated polyols containing a D-threo configuration adjacent to a primary alcohol group. Since D-iditol is an unambiguous substrate which must give rise to D-sorbose whichever end of the molecule is attacked,

the name proposed for the enzyme is *D-iditol dehydrogenase*. This enzyme could account for the products which have been found, and it would be expected to oxidize D-arabitol to D-ribulose and dulcitol to L-tagatose. The experiments on differential inactivation of the enzymes provide no evidence suggesting that dulcitol can be oxidized to L-tagatose. If this reaction occurs it must be very slow. Although dulcitol induces the formation of D-iditol dehydrogenase, the failure of an inducer to serve as a substrate for an adaptive enzyme is not without precedent (Monod & Cohn, 1952). At the present juncture no definite conclusions can be reached about the nature and number of polyol dehydrogenases present in extracts of cells grown on dulcitol. The substrate-specificity patterns are complicated by several features. The labile mannitol dehydrogenase activity may account for the oxidation of ribitol but the stable activity towards D-arabitol is difficult to explain on the same grounds. The oxidation of heptitols possessing an L-threo configuration at one end of the molecule could be ascribed to galactitol dehydrogenase but the site of oxidation is uncertain since the products have not been identified.

6-Deoxy-L-gulitol, which is oxidized by a stable dehydrogenase more rapidly than any other polyol, presents a special problem. The unidentified product is a reducing sugar which does not behave like 6-deoxy-L-sorbose. It is conceivable that it is 1-deoxy-D-fructose, or a 3-keto sugar (cf. Richtmyer, Stewart & Hudson, 1950), and that 6-deoxy-L-gulitol is attacked by an enzyme which is not a galactitol dehydrogenase.

The occurrence of certain polyhydric alcohols in plants, alone (e.g. dulcitol and sorbitol in *Bostrychia scorpioides*) or in association with ketoses (e.g. perseitol and mannoheptulose in the avocado), may well be due to the reduction of ketoses by polyol dehydrogenases similar to those described in this paper.

Fig. 9

SUMMARY

1. A micro-organism identified as a species of *Pseudomonas* forms adaptive enzymes which oxidize a large number of polyols. The properties and specificities of the enzymes have been studied in cell-free extracts. They are diphosphopyridine nucleotide-linked dehydrogenases converting polyols into ketoses.

2. Sorbitol and dulcitol induce the formation of galactitol dehydrogenase, which oxidizes dulcitol to D-tagatose and accounts for the oxidation of other fully hydroxylated polyols containing an L-threo configuration adjacent to a primary alcohol group.

3. Dulcitol also induces the formation of a less stable enzyme (D-iditol dehydrogenase) which accounts for the oxidation of certain fully hydroxylated polyols containing a D-threo configuration adjacent to a primary alcohol group.

4. A labile mannitol dehydrogenase occurs in the extracts of cells grown in media supplemented with sorbitol.

I am greatly indebted to Professor N. L. Edson for suggesting this problem and for his invaluable advice and criticism throughout the course of this work.

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A New Method for the Isolation of Ribonucleic Acids from Mammalian Tissues

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(Received 12 April 1956)

The isolation of ribonucleic acids (RNA's) from mammalian tissues has been the subject of several procedures (cf. Magasanik, 1955) and none has been entirely satisfactory. The method described by Kay & Dounce (1953) involved separation of the nuclei, and an adjustment of the pH to 4.5 before

two treatments with sodium dodecyl sulphate. Grinnan & Mosher (1951) also found it essential to remove cell nuclei. They used guanidine hydrochloride to denature the protein, but it was necessary to heat the preparation at 90–100° and finally to treat with chloroform and butanol to remove the protein. Volkin & Carter (1951) also used guanidine hydrochloride and kept the temperature at 40°, but

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