The Catabolism of Acyclic Polyols by Yeasts

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SUMMARY

Eight polyols were employed in turn as sole carbon source for aerobic growth tests with sixteen yeasts. The yeasts studied varied from those using none to others using all the polyols. Mean generation times in aerated, liquid, shaken medium for five yeasts and four polyols, were from 2 to 7.5 hr.

With seven yeasts, oxygen uptake was measured for different polyols as sole carbon source, of washed, starved yeast cells, harvested in the exponential phase of growth. No yeast respired a substrate on which it did not grow, or vice versa. Except when glucose, erythritol or galactitol (dulcitol) were employed, respiration rates were not greatly affected by the carbon source for growth.

Coenzyme-linked polyol dehydrogenase activity was measured with crude cell-free extracts of four yeasts and several polyols or sugars. The enzymes had a low affinity for their substrates. In certain cases the polyol oxidation products were examined chromatographically.

The polyol dehydrogenases of four yeasts were separated from crude cell extracts by gel electrophoresis, and detected on the gels by their activity with different polyols, and with NAD⁺ or NADP⁺. One strain of *Torulopsis candida* appears to synthesize at least eight polyol dehydrogenases which differ in their specificity for polyols, coenzyme or inducer. Similarly, four polyol dehydrogenases were found in a strain of *Candida utilis*. Most of these enzymes were inducible.

There was no evidence that phosphorylation was the first step in polyol catabolism.

From experiments with ¹⁴C-labelled polyols, it seems that *Candida utilis* and *Pichia membranaefaciens* do not utilize D-glucitol (sorbitol) or D-mannitol because these substrates do not enter the yeast cells.

INTRODUCTION

Differences between yeasts in their ability to use polyols as sole carbon sources for aerobic growth have been employed for classifying and identifying yeasts (e.g. Wickerham, 1951; Kudriavzev, 1954; Kreger-van Rij, 1964; Poncet & Arpin, 1965).

Work on the metabolism of the acyclic polyols has been reviewed by Edson (1953), Touster & Shaw (1962), Hollmann & Touster (1964), Lewis & Smith (1967*a*) and Barnett (1968). The following three kinds of enzymatic reaction are capable of initiating a metabolic attack on polyols.

- (a) Polyol + NAD⁺ (or NADP⁺) → ketose + NADH (or NADPH) + H⁺.
- (b) Polyol + NADP⁺ → aldose + NADPH + H⁺.
- (c) Polyol + ATP → polyol phosphate + ADP.

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The polyol phosphate, formed in reaction (c), may be oxidized to give a sugar phosphate by reactions analogous to (a) and (b). The sugars or sugar phosphates formed from the polyols are probably metabolized by the Embden–Meyerhof pathway or the pentose cycle.

All-or-none differences in the ability of yeasts to utilize polyols presumably reflect the presence or absence of (i) enzymes necessary to metabolize the polyols, or (ii) mechanisms for the entry of the polyols into the yeast cells. The present paper reports investigations of biochemical systems which may underlie differences between yeasts in their utilization of polyols.

The aim of this work was to study a number of yeasts and to relate differences in the behaviour of whole organisms to differences between their internal mechanisms. There are, however, special difficulties in showing why one yeast uses a particular substrate for growth and respiration, and another does not. For example, *Pichia delftensis* can grow on ribitol, D-glucitol or D-mannitol, whilst *P. membranaefaciens* cannot grow on any of the polyols (Table 1). Beech (1965) used this difference to distinguish these two species. Now, although succinate-grown *P. delftensis* may be compared with succinate-grown *P. membranaefaciens*, one or more enzymes essential for the catabolism of, say, D-glucitol may be inducible so that it is necessary to examine D-glucitol-grown *P. delftensis* in order to find out how the yeast metabolizes D-glucitol. For such reasons, a single strain of *Torulopsis candida* (NCYC 576), chosen for its polyol-using versatility (Table 1), was studied extensively.

Candida utilis (LTS 23) was also studied for two reasons. First, *C. utilis* contrasted with *Torulopsis candida* in using no polyols (Table 1). Secondly, Horecker and his colleagues (Chakravorty *et al.* 1962) had already examined the polyol metabolism of this strain using enzyme purification methods, and it was considered that their results would be useful for comparison with those obtained here with unfractionated extracts.

A possible reason for a yeast's inability to use a polyol is the absence of enzymes capable of catabolizing the product of the oxidation of the polyol. For example, Horecker (1962*a*) drew attention to the absence of kinase activity with either ribose or erythro-pentulose in both *Candida utilis* and *C. albicans*. Neither species can utilize ribose for growth (Dr B. L. Brady, 1965: personal communication), yet extracts of both yeasts could interconvert D-ribose and D-erythro-pentulose via ribitol (Horecker, 1962*a*):

Provided that ribitol enters these yeasts, lack of kinases may be said to block the utilization of ribitol.

METHODS

Yeasts

The following organisms have been used in this work: *Candida utilis* NCYC 359 and LTS 23 (received from B. L. Horecker); *Debaryomyces phaffii* (CBS 4346); *D. vanriji* NCYC 577 (received from V. I. Kudriavzev as *D. konokotinae*); *Hansenula anomala* CBS 1981; *Kluyveromyces (Saccharomyces) drosophilae* CBS 2896 (received from V. I. Kudriavzev as *Zygothrauxospora krasnikovi*); *Pichia delftensis* LTS 24 (received from F. W. Beech); *P. membranaefaciens* NCYC 54; *Saccharomyces acidifaciens* CBS 749; *S. cerevisiae* NCYC 231; *S. rouxii* CBS 732; *S. rouxii* var. *polymorphus* CBS 713; *Torulopsis candida* NCYC 576 (or *T. famata*, received from V. I. Kudriavzev as

Table 1. *Auxanographic growth tests on sixteen yeasts*

The yeast was washed off a slope into 10 ml. of water. For each auxanogram, 0.5 ml. of this suspension was added to a tube containing 12 ml. of melted agar medium without carbon source, at 47°. After mixing, the medium was poured into a Petri dish, cooled, and the surface dried in an incubator. Test carbon sources were placed on the agar surface of each plate, which was incubated and examined daily for 4 days. The results are given in terms of + (growth) and - (no growth); n, means not tested.

Yeast	Substrate															
	Erythritol	Ribitol	D-Arabinitol	L-Arabinitol	Xylitol	D-Glucitol	Galactitol	D-Mannitol	L-Arabinose	D-Arabinose	D-Xylose	D-Ribose	L-Sorbose*	D-Tagatose	Succinate	Acetate
<i>Saccharomyces cerevisiae</i> (NCYC 231)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
<i>S. rouxii</i> v. <i>polymorphus</i> (CBS 713)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	n
<i>Candida utilis</i> (NCYC 359)	n	-	-	-	-	-	-	-	-	-	+	-	-	-	+	+
<i>C. utilis</i> (LTS 23)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
<i>Pichia membranaefaciens</i> (NCYC 54)	-	-	-	-	n	+	-	+	-	n	-	-	n	-	+	+
<i>S. rouxii</i> (CBS 732)	-	-	-	-	-	+	-	+	-	-	-	-	-	-	+	n
<i>Kluyveromyces drosophilaram</i> (CBS 2896)	-	-	-	-	+	-	-	+	-	-	-	-	-	-	?	n
<i>Trigonopsis variabilis</i> (NCYC 378)	-	-	-	-	-	+	-	+	-	-	-	-	-	-	+	n
<i>S. acidifaciens</i> (CBS 749)	-	+	+	+	-	+	-	+	-	-	-	-	-	-	+	n
<i>Hansenula anomala</i> (CBS 1981)	+	-	+	+	-	+	-	+	-	-	-	-	-	-	+	+
<i>P. deliensis</i> (LTS 24)	-	+	+	+	-	+	-	+	-	-	-	-	n	-	+	n
<i>Torulopsis capsuligenus</i> (CBS 4736)	-	+	+	+	-	+	+	+	-	n	+	n	+	-	+	n
<i>T. pseudoveria</i> (LTS 19)	-	+	+	+	+	+	+	+	+	-	+	+	+	-	+	n
<i>T. candida</i> (NCYC 576)	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	n
<i>Debaryomyces vanriji</i> (NCYC 577)	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	n
<i>D. phaffii</i> (CBS 4346)	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	n

* L-xyl/o-Hexulose.

Debaryomyces mucosus); *Torulopsis capsuligenus* CBS 4736; *T. pseudaria* LTS 19 (received from J. Zsolt); *Trigonopsis variabilis* NCYC 378.

Each strain of yeast was taken from a single colony isolated after streaking out twice on Y-M agar in a Petri dish (*Topley & Wilson's Principles*, 1964). After incubation, stock slopes of each strain were kept at $+1^{\circ}$ and subcultured not less often than every 6 months (cf. Kirsop, 1954). The identity of each strain was checked at least by (i) microscopic examination, and (ii) auxanographic tests of its ability to use certain carbon compounds (e.g. Lodder & Kreger-van Rij, 1952).

Media

The following media were used to cultivate the yeasts: (i) Difco Bacto Y-M agar (pH 6.2); (ii) A2 (pH 4.8) a chemically defined, carbon-free medium (Wickerham & Burton, 1948; modified by Barnett & Ingram, 1955), plus a source of carbon usually at 50 mM. Succinate was added as the sodium salt in solution at pH 4 and acetate as ammonium acetate. Vitamins and glucose were filter-sterilized and added aseptically to the autoclaved medium (120° for 15 min.). Autoclaved carbon compounds were sterilized separately. 2 % (w/v) Difco Special Agar-Noble was added to certain media.

Growth rate

For determination of growth rates, T-tubes (Kay & Fildes, 1950; Barnett & Ingram, 1955) of 40 ml. total capacity, containing 10 ml. A2 medium with 0.5 mmole test substrate, were inoculated with 0.1 ml. yeast suspension (about 10 μ g. dry weight of yeast). The suspension was prepared by suspending the growth from an A2 agar slope to a suitable density. The tubes were rocked at 1 cycle/sec. at 25° . Growth was followed with either an E.E.L. nephelometer or automatically with a recording nephelometer (Dr H. Laser, private communication) and the readings converted to mg. dry weight by means of a standard curve.

'Induction or selection' experiments

For each strain, a culture was grown for about 18 hr in 10 ml. medium A2 with D-glucose as carbon source, in a T-tube as described above. The yeast, treated aseptically, was then washed twice in 10 ml. 0.1 M- KH_2PO_4 and resuspended in 100 ml. 0.1 M- KH_2PO_4 . Plates containing 15 ml. A2 agar, with 750 μ moles of a polyol or glucose, were inoculated from serial dilutions of this suspension in 0.1 M- KH_2PO_4 , and colonies were counted after 2 days at 25° .

Auxanograms

The auxanographic methods used were similar to those described by Barnett & Kornberg (1960).

Manometry

The methods used for these experiments correspond closely to those used by Barnett & Kornberg (1960). Yeast from a 1-day-old slope was washed into 100 ml. medium A2 (+ appropriate carbon-source) in a 1 l. Pyrex Fernbach culture flask. The flask was rocked at 1 cycle/sec. through an angle of 12° (6° either side of horizontal) for 20 hr. During this time air, passed through 5 % (v/v) H_2SO_4 and sterilized by cotton-wool filters, was passed through the upper part of the flask at 5 l./hr.

Aseptically, the contents of the Fernbach flask were centrifuged and the yeast

washed and re-suspended in 100 ml. 0.1 M-potassium phosphate buffer (pH 5.8). To reduce the endogenous rate of oxygen uptake the suspension was replaced in a Fernbach flask and rocked and aerated for 4 hr. The yeast cells were then centrifuged and re-suspended in 0.1 M-potassium phosphate buffer (pH 5.8) at a final concentration of about 2 mg. dry wt/ml.

Oxygen uptake on addition of substrates was measured with single side arm Warburg manometers containing 2 ml. yeast suspension in the main compartment, 20 μ moles substrate in 0.2 ml. water in the side arm, and a strip (3.5 $\times$ 1 cm.) of Whatman no. 40 filter paper and 0.2 ml. 2 N-KOH in the centre well. The gas phase in the manometers was air and the flasks were equilibrated at 25° for 10–20 min. before the contents of the side arms were mixed with those of the main compartments. After each experiment the contents of every flask were examined microscopically to ascertain that there were no bacterial contaminants.

Substrate penetration experiments

For experiments on the penetration of a substance into a yeast which did not use it, the yeast was grown on a suitable carbon source in the presence of that substance (0.025 M). This procedure allowed for cases where the mechanism for translocation might be inducible, as for maltose with *Saccharomyces cerevisiae* (Harris & Thompson, 1961). Otherwise growth, harvesting and starving the yeast suspension were performed exactly as described for the manometric experiments.

Incubation. Nine ml. of the yeast suspension (about 3 mg. dry weight of yeast/ml.) in 0.1 M-potassium phosphate buffer (pH 5.8) were placed in each of two 100 ml. Erlenmeyer flasks (A and B) containing a glass-covered rod magnet. Flask A was held in a water bath at 30° and flask B in a vessel packed with ground ice. The second flask allowed for radioactive material adsorbed by the cell walls and outside the cell membranes (Cirillo, 1961). Both flasks were over magnetic stirrers to keep the suspensions mixed and aerated. After 10 min. equilibration, to each flask were added at zero time either: (i) 0.1 ml. M-D-glucitol and 1.0 ml. [U-¹⁴C]D-glucitol (10 μ C, 1.2 μ moles/ml.), or (ii) 0.1 ml. M-erythritol and 0.5 ml. [U-¹⁴C]erythritol (10 μ C, 0.5 μ moles/ml.). Samples (1.0 ml.) were taken from each flask as near as practicable to zero time (within 20 sec.) and thereafter at 15 min. intervals for 1 hr. Each sample was sucked quickly through a membrane filter (MF 50, Membranfilter GmbH, Göttingen, diam. 30 mm.). The retained yeast was quickly washed twice with 1 ml. water at 1° (cf. Cirillo & Wilkins, 1964; Kotyk & Kleinzeller, 1963) and the membrane, with the yeast *in situ*, was removed immediately and transferred to a counting vessel containing 15 ml. toluene with 0.3 % (w/v) PPO (2,5-diphenyloxazole) at 0°. The counting vessels were glass bottles (27 mm. diam. 48 mm. height) with plastic screw caps (Wheaton Vials, Ekco Electronics Ltd.). Samples were stored at 0° until counted with a Packard Tri-carb coincidence liquid scintillation spectrometer (55–66 % maximum efficiency) and were counted at least twice for 30 min. at approx 2°. No corrections were made for quenching or for coincident disintegrations.

Cell-free extracts

For preparation of cell-free extracts 1 litre A2 medium, containing a suitable source of carbon, was inoculated with 100 ml. of a culture in a similar medium and

incubated at 25° in a rotating (100 to 110 rev./min.) 10 l. flask (Mitchell, 1949). After 20 hr, the yeast was harvested by centrifugation at about 10,000 *g* for 30 min. at -3° and stored at -20°.

Extracts were prepared with a Nossal disintegrator (Nossal, 1953). A closed stainless steel capsule of 20 ml. capacity contained 2-4 g. wet weight of frozen yeast, 10 g. washed ballotini no. 12 (Jencons Ltd.), 100 μ moles tris/g. of yeast and 7.5 ml. water. For some experiments 1 mmole sodium phosphate (pH 7.0 at 18°) was used in place of tris. The capsule was shaken reciprocally at room temperature for 30 sec. at about 6000 cycles/min., 15 mm. travel. During shaking, the temperature of the capsule's contents rose from 0° to between 5° and 10°. The capsule was carried to and from the disintegrator in ice. The suspension of disintegrated material was at about pH 7.

The suspension was centrifuged at about 10,000 *g* for 15 min. at -3° and the supernatant fluid centrifuged again in a refrigerated preparative ultracentrifuge (Spinco) at about 100,000 *g* for 2 hr. Without such severe centrifugation, electrophoresis was unsatisfactory; material, perhaps nucleoprotein, accumulated at the origin slot and interfered with the free-running of the protein.

The supernatant was dialysed for 4 hr. at 1° against 1000 ml. of 2 mM-tris HCl (pH 7.8), to which 1 mole of sucrose was added (final concentration 0.82 M) when the extract was prepared for electrophoresis. For certain experiments, 5 mM-sodium phosphate (pH 7.0) was used instead of tris buffer.

Estimation of protein

For routine purposes the concentration of protein in the cell-free extract was estimated by the method of Warburg & Christian (Layne, 1957), though in certain cases by that of Lowry, Rosebrough, Farr & Randall (1951).

Measurement of dry weight

For yeast suspensions in phosphate buffer, samples of from 2 to 10 ml. (as large as practicable) of the suspension were pipetted into weighed tubes or beakers and dried at 103-105° to constant weight. For yeast suspensions in growth medium with carbon source, a similar procedure was followed except that the samples were pipetted into weighed centrifuge tubes in which they were finally dried. Before drying, the samples were centrifuged, washed twice and re-suspended in buffer.

Measurements of enzyme activity

Coenzyme reduction and oxidation by crude extracts in the presence of polyols, ketoses or aldoses, were followed spectrophotometrically by measuring changes in extinction at 340 m μ with a Beckman automatic recording spectrometer (DK 2). For most experiments, the assay mixture contained 200 μ moles buffer [glycine pH 9.0 for NAD(P) reduction or phosphate pH 6.8 for NAD(P)H oxidation], 1.0 mmole substrate (polyol, ketose or aldose), 0.1 ml. enzyme-containing extract (about 0.4 mg. protein), 4.5 μ moles NAD(P)⁺ or 0.45 μ moles NAD(P)H, and water to 3.0 ml. The reference cuvette contained water only. Reactions were started by addition of co-enzymes. Recordings were begun within 10 sec. of the addition, and were usually continued for 3 min. In every experiment, control recordings were made without added substrate, without added coenzyme and without added extract. Assays were

carried out at 30° using a constant temperature cell and $E_{340m\mu}$ values were converted to NAD(P)H concentrations using $\epsilon_{mM} = 6.22$.

Products of polyol oxidation

The reactions were carried out in T-tubes containing 750 μ moles tris buffer (pH 8.0), 0.5 μ moles polyol, 2.0 ml. crude extract (approx. 10 mg. protein), 5.0 μ moles NAD⁺ or NADP⁺, 200 μ moles sodium pyruvate, about 400 units crystalline lactate dehydrogenase (capable of oxidizing about 0.2 μ moles NADH/min. at 25° and pH 7.5) and water to 10 ml. Extract or coenzyme was omitted from control tubes. Pyruvate and lactate dehydrogenase were included to reoxidize the coenzyme (cf. Fossitt, Mortlock, Anderson & Wood 1964). The tubes were covered and shaken gently for 3 hr. at 30°.

At the end of the incubation the mixture was checked for absence of microbial contamination, acidified to pH 5.5 or 6.0 with about 10 μ l. acetic acid, heated for 2 min. at about 100°, cooled, and centrifuged at about 30,000 *g* for 30 min. The clear supernatant fluid was put through about 2 g. cation exchange resin (200–400 mesh AG 50 W-X 8 Bio Rad H⁺ form) and the effluent dried under reduced pressure in a rotary evaporator at 40–50°.

Chromatography. The residue was taken up in about 1 ml. water and applied in 1 μ l. quantities to a silica-gel layer, made up in 0.1 M-boric acid and spread on glass as a layer 250 μ thick. Authentic sugar samples were also applied in different positions. The chromatograms were run vertically for a distance of 150 mm. with one of the following solvents: ethyl acetate + pyridine + water (10 + 4 + 3, by vol.), *n*-butanol + acetone + water (4 + 5 + 1, by vol.), *n*-butanol + ethanol + water (4 + 1 + 5, by vol.), or water-saturated phenol. The chromatograms were dried at laboratory temperature in a strong stream of air and were usually run again in the same solvent exactly as before. They were then treated to locate carbohydrates with the periodate-benzidine reagent of Gordon, Thornburg & Werum (1956) or with slightly modified naphthoresorcinol or aniline phthalate reagents of Hollmann & Touster (1964) in which ethanol and water-saturated *n*-butanol were replaced with an equal volume of methanol or methanol + water (80 + 20, v/v) respectively. Boric acid, when present in the silica-gel layer, interfered with the action of these reagents (cf. Lewis & Smith, 1967*b*) and methanol prevents this by removing boric acid as its volatile methyl ester (Wolfrom & Thompson, 1963). At least two (usually three) solvents were used for each identification. In two experiments (oxidation of D-glucitol by extracts of succinate- and D-glucitol-grown *Torulopsis candida*), chromatographic identification of fructose and xyllo-hexulose was confirmed by means of the cysteine-carbazole reaction of Dische & Borenfreund (1951) and the cysteine-sulphuric acid reaction of Dische & Devi (1960).

Gel electrophoresis

The best separations of the polyol dehydrogenases were obtained by vertical starch gel electrophoresis (Smithies, 1959), using a modification of the discontinuous buffer system of Poulik (1957) at pH 7.8. The use of M-urea to stabilize the starch gels at low temperatures and also operation at pH 8.6 each led to inactivation of the polyol dehydrogenases. The buffer discontinuity was achieved by using two different gel-buffers. The two end gel blocks were of borate buffer (Scopes, 1963), between which

was poured an inner gel containing tris-DETPA (diethylenetriamine penta-acetic acid) buffer (Scopes, 1964). This inner gel included the sample slots.

Inner gel buffer (pH 7·8 at laboratory temperature, i.e. about 18°) consisted of 2 mM-DETPA + 6·7 mM-tris. The solution of DETPA was prepared by the method of Scopes (1964).

Outer gel buffer (pH 7·8) consisted of 0·2 M-boric acid + 0·053 M-tris + 2 mM-HCl.

The top (cathode) tray buffer (pH 8·6) contained 0·2 M-boric acid + 0·05 M-NaOH + 0·03 M-NaCl.

The bottom (anode) tray buffer (pH 8·6) was 0·1 M-tris + 0·025 M-H₂SO₄.

Most of the extracts were also run in an alternative continuous buffer system, composed of *gel buffer* (pH 7·0): 3 mM-Na₂HPO₄ + 0·4 mM-citric acid (cf. Fine & Costello, 1963) and *tray buffer* (pH 7·0) of 30 mM-Na₂HPO₄ + 4 mM-citric acid.

The starch gels were prepared as follows. *Outer gel*: 25 g. hydrolysed potato starch were mixed with 250 ml. outer gel buffer in a 1000 ml. conical flask. The buffer and starch were heated over a Bunsen burner with continuous agitation. The gel first thickened and then liquefied. Heating was continued until the gel was almost boiling, and the dissolved air was removed by boiling under reduced pressure for about 30 sec. The two end-blocks of the gel tray were then filled with the gel, the latter was prevented from filling the centre part by pieces of perspex. After 30 min., the pieces were removed and the inner gel was poured. *Inner gel*: 21·5 g. starch were mixed with 180 ml. inner gel buffer, heated as described for the outer gel, and poured into the tray between the two outer gels. The slot-former was immediately put in place towards one end of the inner gel. Usually 16 slots were used of 3 mm. width (capacity 10 μ l.), 4 mm. apart; but 10 slots of 7 mm. width (capacity 25 μ l.), 4 mm. apart were used for some experiments. After 30 min., the whole gel was covered with a sheet of polythene to prevent drying; it was kept overnight at 10°, and put in a room at 1° for 30 min. before the extracts were inserted.

Dialysed, concentrated extract (about 15 mg. protein/ml.) was inserted into each slot as a free solution and the slots sealed with liquid petroleum jelly, mixed with CCl₄ to lower the melting point. Bovine serum albumin, stained with excess naphthalene black and then dialysed against distilled water at 1° to remove excess stain, was added to the concentrated extracts at a final concentration of 0·05 mg./ml. extract. The stained albumin acted as a visible marker throughout electrophoresis and served as a guide when cutting the gel into strips and as a reference substance for the identification of enzyme bands by their relative positions.

To prevent evaporation during electrophoresis, the gel was covered with a thin sheet of polythene held in position by petroleum jelly. Electrophoresis was carried out vertically for 3·5–5 hr in a constant temperature room at 1° at about 10 V/cm. Initially the current was about 24 mA, falling to about 18 mA during electrophoresis. The temperature of the gel did not rise above 10°.

Polyacrylamide gels were prepared as described by Raymond (1964) and Lund (1965) and were used with the starch-gel buffers at half the above concentrations.

Buffers

The recipes used for buffers were based on those given by Gomori (1955) and by Datta & Gryzbowski (1961). The buffers are listed below; the concentrations in which they were used are given with the specific experimental methods:

pH range	Buffer system
1.5-2.0	Hydrochloric acid + potassium chloride
2.1-3.5	Glycine + hydrochloric acid
3.6-4.5	Formic acid + sodium formate
5.0-5.6	Potassium hydrogen phthalate + sodium hydroxide
5.0-6.0	Citric acid + <i>di</i> -potassium hydrogen orthophosphate
5.8-7.5	Potassium dihydrogen orthophosphate + <i>di</i> -potassium hydrogen orthophosphate
7.6-8.6	Tris (hydroxymethyl) aminomethane + hydrochloric acid
8.8-10.5	Glycine + sodium hydroxide

Chemicals, enzymes and coenzymes

Most of the chemicals were obtained from British Drug Houses; and, where possible, they were of Analar grade. D-mannitol was recrystallized twice from water. D-glucitol was recrystallized twice from the pyridine-glucitol complex (Strain, 1934, 1937), and then recrystallized twice from methanol. Galactitol was recrystallized twice from water. L-iditol was a gift from Dr F. A. Isherwood. Ribitol and erythritol were recrystallized twice from ethanol, then twice from water by the addition of acetone. Xylitol was prepared by reduction of D-xylose with sodium borohydride and was recrystallized twice from methanol (Hayward & Wright, 1963). D- and L-Arabinitol (obtained from L. Light and Co. Ltd.) were recrystallized twice from methanol. The melting-point and optical rotation were checked on each preparation.

Uniformly labelled polyols [$U-^{14}C$]D-glucitol and [$U-^{14}C$]erythritol, were obtained from the Radiochemical Centre, Amersham. D-mannitol 1-phosphate and D-glucitol 6-phosphate were prepared by the sodium borohydride reduction of D-mannose 6-phosphate and D-glucose 6-phosphate, respectively (Wolff & Kaplan, 1956).

D-Erythro-pentulose was prepared by reacting its *o*-nitrophenylhydrazone (obtained from Calbiochem Ltd.), with benzaldehyde (Schmidt & Treiber, 1933). D-threo-Pentulose, and L-glycero-tetrol were gifts from Dr R. J. Ferrier, and D-erythrose was obtained from Sigma Chemical Co.

Sodium pyruvate was prepared by (i) redistilling pyruvic acid three times, and (ii) preparing the sodium salt according to the method of Price & Levintow (1952).

Starch for gel-electrophoresis was obtained from the Connaught Medical Research Laboratories, Toronto. Silica Gel G for thin layer chromatography came from E. Merck, A.G., Darmstadt.

Alcohol dehydrogenase, lactate dehydrogenase, NAD^+ , $NADP^+$, NADH and NADPH were obtained from the Sigma Chemical Co.

RESULTS

Polyol utilization by growing cells

The results of the auxanographic growth tests (Table 1) agreed generally with other published work on the growth of yeasts on polyols (e.g. Wickerham, 1951; Kudriavzev, 1954; Kreger-van Rij, 1964). The number of polyols employed as test substrates by

those authors was extended in the present work to include xylitol and D- and L-arabinitol. The following points are of particular interest: (i) The yeasts varied from those like *Saccharomyces cerevisiae* that used no polyols as sole carbon-source to others such as *Torulopsis candida* which used all that were offered. (ii) All yeasts that used any polyol used D-mannitol. (iii) No yeast used D-*lyxo*-hexulose (D-tagatose), an expected oxidation product of galactitol (Shaw, 1956, 1962), not even those able to grow on galactitol. (iv) Most of the yeasts could use succinate, and some were found able to use acetate. Hence these compounds could be employed as carbon substrates for growing yeasts in order to study their constitutive polyol dehydrogenases. (v) The identity of the strain LTS 23 was confirmed as that of *Candida utilis* by its responses to the growth tests. These were identical to the reactions given by *C. utilis* strain NCYC 359. Some strains of this species have been reported to use certain polyols; unpublished results of van Uden (B. L. Brady, personal communication, 1965) show that of 13 strains tested one used erythritol, four used ribitol and five used D-mannitol. L-*xylo*-Hexulose, D- and L-arabinose, D-ribose, galactitol and D-glucitol were not used by any strain, whereas D-xylose was used by all 13. Hence *C. utilis* appears to use D-xylose characteristically but not L-arabinose, although *C. utilis* has been reported as able to use L-arabinose for growth (Chakravorty, Veiga, Bacila & Horecker, 1962; Horecker, 1962*a*, pp. 21, 30). (vi) Growth tests with *Pichia delftensis* confirmed the observations of Beech (1965) that it can use ribitol, D-mannitol and D-glucitol, but not erythritol. The strain tested also used D-arabinitol, though not xylitol, L-arabinitol or galactitol (Table 1).

Table 2. *Rates of growth on polyols and glucose as substrates by five yeasts*

Cultures in T-tubes were rocked and incubated at 25°. Growth was measured nephelometrically every 30 min. and recorded automatically. n means not measured. In each case, mean generation (doubling) time was estimated from the exponential phase of growth (cf. Fig. 1).

	D-Glucose	D-Glucitol	D-Mannitol	Galactitol	Erythritol
<i>Torulopsis candida</i> (NCYC 576)	121	134	141	147	163
<i>Debaryomyces vanriji</i> (NCYC 577)	132	404	451	153	234
<i>D. phaffi</i> (CBS 4346)	115	128	n	139	180
<i>Hansenula anomala</i> (CBS 1981)	163	152	257	n	168
<i>Kluyveromyces drosophilorum</i> (CBS 2896)	102	210	160	n	n

Rates of growth during the exponential phase, in terms of mean generation (doubling) time, are given in Table 2 for five yeasts. These rates varied from about 2 to 7.5 hr. The growth of *Torulopsis candida* on four polyols or D-glucose was measured in terms of dry weight (Fig. 1). The inoculum, the same for each substrate, was grown in liquid culture on succinate, and harvested and washed before the end of the exponential phase of growth. The lag phases were particularly long for erythritol, galactitol and D-mannitol. The length of the lag phase could be reduced in each case by growing the inoculum on the same substrate as that on which the yeast's growth was to be measured. Generally, it was possible to harvest the yeasts just before the end of the exponential phase (about 20 hr after inoculation) with a yield of from 3 to 5 mg./ml.

Table 3. *Rates of respiration on polyols and sugars as substrates by intact cells of seven yeasts*

Manometer flasks contained approx. 5 mg. dry wt of washed, starved yeast in 2 ml. of 0.1 M-potassium phosphate buffer (pH 5.9) in the main compartments; 20 μ moles of substrate (as indicated) in 0.2 ml. of water in the side arms; 0.2 ml of 2 N-KOH with filter paper in each centre well. Incubation was in air at 25°, and substrates were added at zero time. 20 hr cultures were used. The figures for Q_{O_2} have not been corrected for endogenous respiration. n means not tested.

Carbon source for growth	Respiratory substrate										
	Glycerol	Erythritol	Ribitol	D-arabinitol	L-arabinitol	D-glucitol	D-mannitol	Galactitol	L-sorbose*	D-glucose	Endogenous
	Q_{O_2} (μ l. O_2 consumed/mg. dry wt yeast/hr)										
<i>Torulopsis candida</i>											
D-Glucose	29	8	11	10	8	12	10	8	36	64	8
Succinate	58	4	16	12	4	42	28	4	45	70	3
Glycerol	79	7	9	36	6	57	35	6	67	83	4
Erythritol	60	65	15	40	4	50	24	4	68	67	4
Ribitol	60	20	14	31	10	40	17	9	56	65	3
D-Glucitol	59	5	11	49	9	60	27	11	64	80	5
D-Mannitol	54	5	10	31	8	46	45	4	59	80	4
Galactitol	56	9	33	36	40	39	28	76	59	60	5
<i>Debaryomyces vanriji</i>											
Erythritol	17	24	9	12	12	17	15	7	18	32	4
Ribitol	14	6	9	12	8	16	16	10	10	32	6
D-Glucitol	16	8	10	14	9	16	17	7	16	27	5
D-Mannitol	10	6	10	10	6	16	13	6	10	35	5
Galactitol	13	6	12	15	n	18	17	42	17	55	6
<i>Hansenula anomala</i>											
D-Glucose	n	6	6	4	5	9	7	3	6	30	4
Succinate	n	5	3	5	4	21	21	4	5	65	3
Glycerol	60	6	4	3	2	17	16	3	3	45	2
Erythritol	84	73	11	3	3	27	17	5	5	66	3
D-Mannitol	47	7	14	6	4	25	16	6	8	39	5
<i>Saccharomyces acidifaciens</i>											
D-Glucose	20	4	3	2	2	6	3	2	4	74	2
Acetate	22	4	n	6	3	76	15	2	3	38	2
Ribitol	32	4	25	28	10	53	28	4	7	48	5
D-Glucitol	32	3	n	7	3	66	25	3	5	61	2
<i>D. phaffii</i>											
Erythritol	25	40	30	28	10	40	33	16	45	60	5
D-Glucitol	16	10	39	41	11	63	44	19	60	68	6
Galactitol	9	5	14	21	n	25	16	84	n	70	5
<i>Pichia delftensis</i>											
Succinate	n	5	6	6	5	17	4	4	n	47	5
D-Glucitol	n	17	48	65	16	69	60	17	n	102	16
D-Mannitol	n	4	23	36	2	60	59	3	n	55	2
<i>Kluyveromyces drosophilaram</i>											
Succinate	n	5	5	5	5	5	6	5	5	100	5
D-Glucitol	n	9	13	40	10	57	80	9	62	163	9
D-Mannitol	n	4	4	21	3	39	34	4	52	126	3

* L-xylo-Hexulose.

Nature of polyol utilization by intact yeasts: induction or selection?

Is the ability to utilize polyols a function of a majority of yeast cells or, is it due to the growth of yeast cells of a minority genotype (cf. Mortlock & Wood, 1964)? Surface colony counts were made with inocula from serial dilutions, for *Torulopsis candida* (NCYC 576) and *Pichia delftensis* (LTS 24) growing on D-glucose or a polyol. There was no appreciable difference between the numbers of yeast cells that grew on the different substrates. Hence polyol utilization in these two yeasts is probably not attributable to the selection of a minority genotype.

Oxidation of polyols by suspensions of washed yeast cells

The rates of oxidation of various polyols, L-xylo-hexulose and D-glucose by seven strains of yeast are shown in Table 3. Starvation generally lowered the endogenous rate to a Q_{O_2} of about 5 μ l. O_2 /mg. dry wt. of yeast/hr; so that rates of at least $Q_{O_2} = 15$ in the presence of a polyol clearly indicated that it was utilized.

No yeast respired a substrate upon which it could not grow, or vice versa. With certain exceptions, the rates of respiration of the polyols were little affected by the nature of the growth substrate. Notable exceptions were provided by glucose, erythritol and galactitol.

The respiration of polyols by yeasts grown on D-glucose was usually much slower than that by yeasts grown on other substrates, even on succinate which, presumably, does not induce the formation of polyol dehydrogenases. For example (Table 3) rates (Q_{O_2}) for D-glucitol by *Torulopsis candida* (NCYC 576) were: (i) D-glucose-grown 12 (endogenous 8), (ii) succinate-grown 42 (endogenous 3) and (iii) D-glucitol-grown 60 (endogenous 5). This effect, also observed with *Hansenula anomala* and with *Saccharomyces acidifaciens*, is not surprising, since glucose is known to inhibit the formation of a number of enzymes in yeasts (MacQuillan & Halvorson, 1962) and in bacteria (e.g. Magasanik, 1957). However the polyol dehydrogenase activity of glucose-grown yeast was investigated only with *Candida utilis*. For this yeast, the level of inducible polyol dehydrogenase activity was about as low in glucose- as in acetate-grown cells (Table 6). The activity of the NADP-specific polyol dehydrogenase of the same strain is also low in glucose-grown cells (Scher & Horecker, 1966a).

Only yeasts grown on erythritol could respire erythritol. Similarly, only yeasts grown on galactitol respired galactitol. But the ability to respire other polyols did not depend on prior growth on a specific polyol (Table 3). *Torulopsis candida* respired L-arabinitol much faster when grown on galactitol than on the other substrates (Table 3). The rate for D-arabinitol was higher for that yeast when polyol-grown than when grown on succinate or D-glucose. On the other hand, the rates at which *T. candida* respired D-glucitol or D-mannitol varied much less with the growth substrate (succinate or polyol) than the rates at which other polyols were respired. These rates for D-glucitol and for D-mannitol were, however, fastest when the yeast was grown on the respiratory substrate itself.

Respiration rates were generally constant for the first 2 hr with no pronounced initial lag, though D-mannitol oxidation by *Torulopsis candida* grown on D-mannitol provided a striking exception (Fig. 2). The rate of respiration of this polyol gradually increased to a maximum at about 1.5–2 hr. after zero time.

Polyol dehydrogenase activities of cell-free extracts

Crude cell-free extracts oxidized several polyols at the expense of NAD^+ or of NADP^+ . Similarly, the extracts reduced certain ketoses and aldoses in the presence of reduced coenzyme. Extracts of *Torulopsis candida*, however, did not oxidize either D-glucitol 6-phosphate or D-mannitol 1-phosphate in the presence of NAD^+ or NADP^+ .

Fig. 1

Fig. 2

Fig. 1. Growth of *Torulopsis candida* (NCYC 576) on polyols or glucose. T-tubes contained initially 10 ml. of medium A 2, 0.5 mmole of substrate (as indicated), and about 10 μg . dry wt of succinate-grown, washed yeast. Each point on the curves corresponds to an analysis of a single tube. The T-tubes were rocked and incubated at 25° and sterile air was passed through the upper part of the tube at 5 l./hr.

Fig. 2. Oxidation of polyols by intact cells of *Torulopsis candida* (NCYC 576) grown on D-mannitol as the sole source of carbon. Warburg manometer flasks contained: 7.4 mg. dry wt of washed, starved yeast and 20 μmoles of substrate as indicated. Other contents were as in Methods. Incubated in air at 25° ; substrate added at zero time.

Effect of pH value. The effect of pH on polyol oxidoreductase activity of crude extracts of yeasts was studied for: (i) *Torulopsis candida*, D-glucitol $\rightleftharpoons$ D-fructose (NAD) (see Fig. 3), D-glucitol $\rightleftharpoons$ L-sorbose (NADP); (ii) *Pichia delftensis*, D-glucitol $\rightleftharpoons$ D-fructose (NAD); (iii) *Candida utilis*, D-glucitol $\rightarrow$ D-fructose (NAD^+), D-fructose $\rightarrow$ D-glucitol + D-mannitol (NADH) (cf. Chavravorty *et al.* 1962). In each case, maximum rates for the polyol oxidation were found at or above pH 9, and between pH 5 and 7 for the ketose reduction. Buffers at pH values near these optima were used for further experiments. Different pH optima for oxidation and reduction are characteristic for the pyridine coenzyme-linked dehydrogenases, e.g. for L-idoitol dehydrogenase (Smith, 1962). By contrast, the cytochrome-linked mannitol dehydrogenase of *Acetobacter suboxydans* catalyses the oxidation of polyols with an optimum at about pH 5 (Arcus & Edson, 1956).

Effect of substrate concentration. Lineweaver–Burk plots of $1/v$ against $1/s$ (Dixon & Webb, 1964) were linear, though high concentrations of L-xylo-hexulose or D-fructose caused marked inhibition. With concentrations above 1.2 M-D-fructose, for example, there was a sharp fall in the rate of oxidation of NADH. The affinities for various substrates (Table 4) were low, as is not unusual with polyol dehydrogenases (e.g. Horecker, 1962*a*; Scher & Horecker, 1966*a*). Hence it was impracticable to compare activities at optimum substrate concentrations.

Table 4. *Michaelis constants for polyol dehydrogenase activity of crude extracts of yeasts*

Standard assay conditions as described in Methods		
Crude extract from	Reaction studied	K_m
Succinate-grown <i>Torulopsis candida</i> (NCYC 576)	$\text{D-Glucitol} \xrightleftharpoons[\text{NADH}]{\text{NAD}^+} \text{D-fructose}$	$\left\{ \begin{array}{l} 0.26 \text{ M-D-glucitol} \\ 0.86 \text{ M-D-fructose} \\ 0.16 \text{ mM-NAD}^+ \end{array} \right.$
	$\text{Ribitol} \xrightarrow{\text{NAD}^+} \text{ribulose}$	0.060 M-ribitol
	$\text{L-xylo-Hexulose} \xrightarrow{\text{NADH}} \text{L-iditol}$	1.3 M-L-xylo-hexulose
D-glucitol-grown <i>Pichia delftensis</i> (LTS 24)	$\text{D-Glucitol} \xrightarrow{\text{NAD}^+} \text{D-fructose}$	0.33 M-D-glucitol
	L-xylo-hexulose	

Substrate and coenzyme specificities of crude cell-free extracts. The rates of NAD- and NADP-linked oxidations of polyols and reductions of sugars by crude extracts were nearly linear for at least 2 min. after the addition of coenzyme. The activities varied according to the substrate on which the yeast was grown (Tables 5, 6). With *Torulopsis candida* erythritol was oxidized only by erythritol-grown material; and the only NAD⁺-linked oxidation of D-mannitol occurred with D-glucitol-grown yeast. For *Candida utilis*, the polyol dehydrogenase activity of extracts of D-xylose-grown cells was about ten times that of cells grown on acetate or glucose (Table 6).

The present observations on the polyol dehydrogenase activity of *Candida utilis* are compared in Table 6 with those made by Horecker and his colleagues (Chakravorty *et al.* 1962; Chakravorty & Horecker, 1966; Horecker, 1961; 1962*a, b*) with a partly purified enzyme ($\times 35$) obtained from the same strain of yeast (presumably D-xylose-grown, see Horecker, 1962*b*) by fractionation with ammonium sulphate and acetone (Chakravorty *et al.* 1962; Chakravorty & Horecker, 1966). The last two columns show that the relative rates of NAD⁺-linked polyol oxidation were similar for the crude extracts of D-xylose-grown yeast used in the present experiments and for the fractionated preparation of Chakravorty *et al.* (1962). This similarity is particularly remarkable in view of differences between the two sets of experiments (*a*) in concentrations of polyol and of NAD⁺, and (*b*) in pH.

Even larger differences were found between the activities of extracts of succinate-grown *Pichia delftensis* (LTS 24) and of the same yeast grown on D-glucitol (Table 5). There was much greater NADP-linked polyol dehydrogenase activity in extracts of *Torulopsis candida* than in those of the *Pichia* species.

No direct evidence was found of coenzyme-linked oxidation of galactitol or of L-arabinitol by extracts of *Torulopsis candida* (Table 5), even when the yeast had been grown on galactitol. Extracts of D-glucitol-grown *T. candida*, however, reduced both D-galactose and L-arabinose in the presence of NADPH (Table 5). From their configura-

Table 5. *Polyol dehydrogenase activity by crude extracts of four yeasts*

Activity was measured by following change of $E_{340m\mu}$ as described in Methods. Control cuvettes without alcohol or sugar gave negligible rates. Rates were calculated from the ΔE_{340} between 15 and 45 sec. after addition of coenzyme. n means not measured. o means < 0.005.

Enzyme substrate	Specific activity of extracts (μ moles of co-enzyme reduced or oxidized/mg. of protein/min.)											
	<i>Torulopsis candida</i> (NCYC 576)				<i>Pichia delfiensis</i> (LTS 24)				<i>P. membranefaciens</i> (NCYC 54)		<i>Saccharomyces acidifaciens</i> (CBS 749)	
	Succinate-grown	D-Mannitol-grown	D-Glucitol-grown	Erythritol-grown	D-Glucitol-grown	Succinate-grown	D-Glucitol-grown	Succinate-grown	Succinate-grown	D-Glucitol-grown	D-Glucitol-grown	D-Glucitol-grown
	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺	NAD ⁺ NADP ⁺
D-Glucitol	0.11	0.17	0.09	0.08	0.43	0.41	0.72	0.21	1.8	0	0.020	0.31
D-Mannitol	0	0.22	0	0.10	0.09	0.71	0	0.33	3.1	0.006	0	0.53
L-Iditol	0.04	0	0.04	0	0.07	n	0.51	0.01	0.81	n	n	0.70
Ribitol	0.08	0	0.04	0	0.23	0.01	0.43	0.01	0.92	0	0	0.78
Xylitol	0.20	0.04	0.06	0.01	0.70	0.10	0.57	0.04	1.4	n	0.012	0.08
D-Arabinitol	0	0.07	0	0.02	0.33	0.16	0.01	0.07	1.6	n	0.10	0.06
L-Arabinitol	0	0	0	0	0	0	0	0	0	0	0	0
Erythritol	0	0	0	0	0	0	0.06	0	1.3	n	0.019	0
Ethanol	0	0	0	0	0	0	0	0	0.51	n	0.74	0
Galactitol*	0	0	0	0	0	0	0	0	0	n	n	n
	NADH	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADPH	NADH	NADH
D-Fructose	0.27	0.43	0.25	0.16	0.28	0.81	0.35	0.40	3.4	0.11	0.51	2.3
L-Xylo-Hexulose	0.14	0.40	0.10	0.12	0.13	0.71	0.18	0.34	0.84	0.031	0.044	0.99
D-Glucose	0	0.02	0	0.01	0	0.10	n	n				
D-Mannose	0	0	0	0	0	0.07	n	0.03				
D-Galactose	0	0.03	0	0.01	0	0.20	n	n				
D-Ribose	0	0.06	0	0.02	0	0.23	n	n				
D-Xylose	0	0.12	0	0.05	0.01	0.35	n	n				
D-Arabinose	0	0	0	0	0	0.05	n	n				
L-Arabinose	0	0.13	0	0.05	0	0.38	n	n				

*0.1 M-galactitol

tions, the respective products of these two reductions were presumably galactitol and L-arabinitol, though this assumption was not checked experimentally. D-lyxo-Hexulose, an expected oxidation product of galactitol, was not reduced either by NADH or by NADPH in the presence of extracts of *T. candida*.

Oxidation of ribitol by cell-free extracts. An apparent NAD-linked oxidation of ribitol by extracts from acetate-grown *Candida utilis* was traced to small amounts of ethanol (< 0.3 %, w/w) in ribitol recrystallized from ethanol. These extracts did not oxidize ribitol recrystallized from water. *C. utilis* extracts catalysed a very active NAD-linked oxidation of ethanol. No spurious activity with ribitol was observed with preparations from *Torulopsis candida*, which appeared to lack NAD- or NADP-linked ethanol dehydrogenase activity.

Table 6. *Rates of NAD reduction by crude extracts of Candida utilis (LTS 23) in the presence of various polyols or ethanol*

The yeast was grown as indicated on acetate, D-glucose or D-xylose, and the reaction mixtures were as described in Methods. Rates were estimated from ΔE_{340} between 15 and 45 sec. after addition of NAD⁺. Rates for the controls without substrate have been deducted from each observed rate. E_{340} readings were converted into NADH concentrations from $\epsilon_{mM} = 6.22$. For comparison, the final column gives the relative rates obtained with a partly purified enzyme from the same yeast by Chakravorty *et al.* (1962). Note: both preparations oxidized L-iditol.

Enzyme substrate	Specific activity of extracts (μ moles of polyol oxidized/mg. protein/min.)			Rates relative to that of D-glucitol			
	Growth substrate			Growth substrate			Purified enzyme
	Acetate	Glucose	Xylose	Acetate	Glucose	Xylose	
D-glucitol	0.058	0.061	0.82	100	100	100	100
Xylitol	0.070	0.047	0.75	121	77	92	115
D-arabinitol	0.063	0.061	0.19	109	100	23	8
D-mannitol	0.072	0.053	0.40	124	87	49	26*
Ribitol	0	.	0.17	0	.	20	26
L-arabinitol	.	.	0.027	.	.	3	1
Erythritol	.	.	0.018	.	.	2	1
Ethanol	4.4	2.2	2.3	7600	3600	280	

* Or 59 (Horecker, 1962a, Table 3).

Comparison of activities of extracts with those of whole organisms. Generally, yeasts that could respire any given polyol yielded extracts which oxidized that polyol: the only exception was that of *Torulopsis candida* which could utilize galactitol and L-arabinitol for growth but yielded extracts that were inactive towards galactitol and L-arabinitol. Extracts of erythritol-, but not succinate-, D-mannitol- or D-glucitol-grown *T. candida* oxidized erythritol (Table 5); this was true also for the oxidation of erythritol by intact organisms (Table 3).

There were a number of instances of a polyol oxidized by an extract of a yeast, but not utilized for growth by the corresponding intact organisms. Examples included *Candida utilis* (D-glucitol) and *Pichia membranaefaciens* (erythritol).

Products of polyol oxidation by cell-free extracts

The products of coenzyme-linked dehydrogenase activity of cell-free extracts were examined chromatographically and were identified as ketoses and aldoses, correspond-

Table 7. Results of chromatographic and colorimetric identification of products of polyol oxidation by crude extracts of three yeasts

The methods are described in the text. nr means no reaction, n means not done. ? indicates uncertainty owing to very weak reaction (low yield). Extracts of erythritol-grown *T. candida*, with NAD⁺, oxidized erythritol to glycero-tetrolulose.

Polyol oxidized	Co-enzyme	Product with extract from					
		<i>Torulopsis candida</i> (NCYC 576)		<i>Pichia delftensis</i> (LTS 24)		<i>Pichia membranefaciens</i> (NCYC 54).	
		Grown on	Grown on	Grown on	Grown on	Grown on	Grown on
D-Glucitol	NAD ⁺	Succinate	D-Glucitol	Succinate	D-Glucitol	Succinate	Fructose
		Fructose	Fructose + xylol-Hexulose	Fructose	Fructose + xylol-Hexulose	Fructose	Fructose
D-Mannitol	NADP ⁺	xylol-Hexulose	xylol-Hexulose + glucose	nr	nr	n	n
	NAD ⁺	nr	Fructose	Fructose	Fructose	n	n
	NADP ⁺	Fructose	Fructose + mannose	n	n	nr	nr
L-Iditol	NAD ⁺	xylol-Hexulose	n	n	n	n	n
Ribitol	NAD ⁺	erythro-Pentulose	n	nr	n	n	n
	NADP ⁺	nr	Ribose	nr	nr	nr	nr
Xylitol	NAD ⁺	threo-Pentulose	threo-Pentulose	n	n	n	n
	NADP ⁺	n	Xylose + threo-Pentulose	threo-Pentulose	threo-Pentulose + ? erythro-Pentulose	n	threo-Pentulose
D-Arabinitol	NAD ⁺	nr	threo-Pentulose	nr	threo-Pentulose	n	n
	NADP ⁺	threo-Pentulose	Arabinose + threo-Pentulose	n	glycero-Tetrolulose	n	glycero-tetrolulose
Erythritol	NAD ⁺	nr	nr	n	n	n	n

ing to the polyol oxidized (Table 7). The R_f values agreed with those of authentic substances run on the same chromatogram to within 3%. In certain experiments, two sugars were detected, suggesting that more than one dehydrogenase was acting on the polyol.

Fig. 3

Fig. 4

Fig. 3. Effect of pH on NAD: polyol oxidoreductase activity of crude extracts of succinate-grown *Torulopsis candida* (NCYC 576). Reaction mixtures as described in Methods, using 200 μmoles of buffer as follows: citrate/phosphate (pH 5.0–6.0), potassium phosphate (pH 6.1–7.5), tris/HCl (pH 7.6–8.6), glycine/NaOH (pH 8.8–10.5). Rates of oxidation and reduction were estimated from ΔE_{340} between 15 and 45 sec. after addition of coenzyme. Rates obtained without added substrate were deducted from each reading. Rates were calculated by converting E_{340} readings into NADH concentrations from $\epsilon_{\text{mM}} = 6.22$.

Fig. 4. The uptake of [U-¹⁴C]D-glucitol by intact cells of *Pichia delftensis* (LTS 24) and *Candida utilis* (LTS 23). LTS 24 was grown on D-glucitol and LTS 23 on D-xylose in the presence of 0.025 M-D-glucitol.

Entry of polyols into yeast cells

D-Glucitol: comparison of *Pichia delftensis* with *Candida utilis*. Intact *C. utilis* (LTS 23) did not use D-glucitol for growth (Table 1), though extracts from cells grown on D-xylose contained an enzyme which oxidized D-glucitol to D-fructose (cf. Chakravorty *et al.* 1962) and the yeast could use D-fructose for growth and for respiration. Hence exogenous D-glucitol probably does not penetrate intact cells of this strain (cf. Cirillo, 1961). This inference was confirmed when comparing the uptake of [U-¹⁴C]D-glucitol by *C. utilis* and by *Pichia delftensis* (which could use D-glucitol), assuming that the polyols are not modified before entering the yeast cells. Within 15 min. after the addition of labelled substrate, there was a much greater uptake by *P. delftensis* at 30° than at 0° (Fig. 4). For *C. utilis*, however, there was no significant difference between the radioactivity at 30° and at 0°, even after 45 min. incubation; the uptake at both temperatures by this yeast was lower than that of *P. delftensis* at 0°.

Erythritol: aerobic and anaerobic uptake by *Torulopsis candida*. In a similar experiment, the uptake of [U-¹⁴C]erythritol was studied with erythritol-grown *Torulopsis candida* (NCYC 576). The approximate rate of aerobic uptake was found to be 640

counts/sec./mg. dry wt./hr, equivalent to at least $0.69 \mu\text{mole}$ of erythritol/mg. dry wt./hr. This rate was of the same order as the rate of erythritol oxidation: $Q_{O_2} = 65$ (Table 3) $\equiv 0.64 \mu\text{mole}$ of erythritol/mg. dry wt./hr (assuming the complete oxidation of erythritol).

Entry of sugars into yeast cells has usually been measured either (i) with non-metabolizable substrates, for example the penetration of L-xylo-hexulose into *Saccharomyces cerevisiae* (e.g. Cirillo, 1961), or (ii) by using inhibitors of metabolism, such as iodoacetate (e.g. Burger, Hejmová & Kleinzeller, 1959). Since the specificity of inhibitors is often in doubt (cf. Davies, 1964) an advantageous technique is to study the entry of metabolites into a completely aerobic yeast under anaerobic conditions (Kotyč & Höfer, 1965). Yeasts do not appear to use polyols anaerobically, so the technique should be applicable to the study of polyol translocation with yeasts generally. Labelled erythritol entered the cells of *Torulopsis candida* under both aerobic and anaerobic conditions, though much more slowly anaerobically. (200 as against 640 counts/sec/mg. dry wt./hr). Further experiments with the same strain of *T. candida* have shown that D-glucitol, D-mannitol and erythritol are all concentrated by the cells (A. Kotyč & J. A. Barnett, unpublished observations). This 'uphill' transport of those polyols occurs even anaerobically or in the presence of 0.5 mM -iodoacetate, which stops oxygen uptake with these substrates and lowers the rate of endogenous respiration.

Pichia membranaefaciens and *Pichia delftensis*. In aerobic experiments such as those described above, succinate-grown *P. membranaefaciens* was found to be impermeable both to D-glucitol and to erythritol, and D-glucitol-grown *P. delftensis* to erythritol. In each case the yeast was grown in the presence of 0.025 M -substrate against which it was to be tested.

Number and identity of polyol dehydrogenases in crude extracts

Kinetic experiments. Activity against several polyols of certain extracts from *Torulopsis candida* suggested the presence of more than one NAD-linked polyol dehydrogenase in each extract. Tests for the presence of more than one polyol dehydrogenase in extracts of succinate-grown *T. candida* were done by adding two oxidizable polyols, of different configurations simultaneously (cf. Dixon & Webb, 1964). A mixture of D-glucitol and ribitol was oxidized a little more slowly ($0.057 \mu\text{mole}$ of NADH formed/min.) than D-glucitol alone ($0.062 \mu\text{mole/min.}$) Similarly, a mixture of D-fructose and L-xylo-hexulose was reduced more slowly ($0.111 \mu\text{mole}$ of NADH oxidized/min.) than D-fructose alone ($0.122 \mu\text{mole/min.}$). Since the experiment provided near saturation conditions for substrates, an additive effect would have been expected if two enzymes were present.

Separation of polyol dehydrogenases by gel electrophoresis

Enzymes of Torulopsis candida. Extracts of *T. candida* grown on succinate, D-mannitol, D-glucitol or erythritol, were subjected to starch-gel electrophoresis. The results of incubating the gels with different polyols and NAD^+ or NADP^+ are shown in Fig. 5. The polyol dehydrogenase activity was associated with nine distinct fractions (Fig. 5; Table 8), suggesting that there were nine distinct enzymes. These enzymes varied in specificity towards the polyols, and in coenzyme specificity. Also the presence or absence of some of the enzymes was affected by the carbon source on which the

yeast was grown. The enzymes have been numbered 1 to 9, no. 1 migrating farthest towards the anode during electrophoresis. Enzymes 1 to 5 were NAD-linked, 6 to 9 were NADP-linked.

Extracts of succinate-grown *Torulopsis candida* gave three bands of polyol dehydrogenase activity, numbered 1, 3 and 9 (Fig. 5; Table 8). Bands 1 and 3 were possibly isoenzymes since: (i) they are the only pair of all the nine bands which appear to be

Fig. 5. Location of NAD⁺- and NADP⁺-linked polyol dehydrogenase activity on starch gel after electrophoresis of crude extracts of *Torulopsis candida* (NCYC 576). See Methods for experimental details. Polyol dehydrogenase activity was located as formazan bands, in the presence of various polyols. The intensity of each band is represented by the degree of shading. The bands have been numbered in accordance with the scheme shown in Table 8.

identical in their specificity for polyols and coenzymes; and (ii) they are both constitutive. On the other hand, the relative intensities of the two bands varied with different substrates (Fig. 5). That bands 1 and 3 have the same characteristics fits the conclusion from the kinetic evidence, already discussed, that the succinate-grown material contained only a single NAD-linked polyol dehydrogenase.

The specificity of these two constitutive NAD-linked enzymes corresponded with that of L-iditol dehydrogenase (L-iditol: NAD oxidoreductase [EC 1.1.1.14] see Dixon & Webb (1964), Touster & Shaw (1962); and Smith (1962)), these enzymes oxidized ribitol to D-*erythro*-pentulose, xylitol to D-*threo*-pentulose, D-glucitol to D-fructose and L-iditol to L-*xylo*-hexulose. On the other hand, unlike the enzymes 1 and 3, L-iditol dehydrogenase also oxidizes D-mannitol, L-arabinitol and galactitol, though very slowly under most conditions of assay.

Table 8. Scheme for nine polyol dehydrogenases separated by starch-gel electrophoresis from crude extracts of *Torulopsis candida* (NCYC 576)

This table summarizes the information given in Fig. 5, the caption of which gives the methods used. The enzymes are arranged in the order they were detected on the gels, no. 1, farthest from the slot. +, substrate oxidized; -, substrate not oxidized. An asterisk indicates that the corresponding enzyme was detected in yeast grown on the carbon-source indicated. Enzymes 1-5 were NAD-linked; 6-9 were NADP-linked. None of the enzymes oxidized L-arabinitol or galactitol (the latter used at 0.1 M).

Direction of movement									
Slot	→ anode								
9	8	7	6	5	4	3	2	1	Enzyme no.
+	+	+	+	-	+	+	+	+	D-Glucitol
+	-	+	-	+	-	+	+	+	Xylitol
-	-	-	-	+	+	+	-	+	Ribitol
-	-	-	-	?	-	+	-	+	L-Iditol
+	-	-	-	-	-	-	+	-	D-Mannitol
+	-	-	-	-	-	-	+	-	D-Arabinitol
-	-	-	-	+	-	-	-	-	Erythritol
*	.	.	.	.	.	*	.	*	Succinate†
*	.	.	*	.	*	*	*	*	D-Glucitol
*	*	*	.	.	.	*	.	*	D-Mannitol
*	.	.	.	*	.	*	.	*	Erythritol
← NADP →				← NAD →					Coenzyme

† Enzymes found in the succinate-grown yeast were also present in that grown on polyols.

The constitutive, NADP-linked polyol dehydrogenase (band 9) oxidized D-glucitol, xylitol, D-mannitol and D-arabinitol, but not ribitol, L-iditol or erythritol. Enzyme 9 gave a single band, showing this specificity, both with the tris-DETPA discontinuous gel-buffer (pH 7.8) and with the phosphate buffer system (pH 7.0). In oxidizing xylitol, this enzyme differed from the NADP-linked D-mannitol dehydrogenase of *Acetobacter suboxydans* (Bygrave & Shaw, 1961; Kersters, Wood & De Ley, 1965).

The NADP⁺-linked polyol dehydrogenase activity of succinate- or erythritol-grown yeast was always associated with a single band (9), on both starch and polyacrylamide gels. However, the relative intensity of band 9 on different substrates varied with the source of extract; for example, the intensity with xylitol relative to that with D-glucitol, D-mannitol or D-arabinitol was greater for succinate-grown *Torulopsis candida* than that for D-mannitol-, D-glucitol- or erythritol-grown yeast (Fig. 5). This would not be expected if band 9 represented a single enzyme. Furthermore, it oxidized xylitol which does not possess the configuration common to D-glucitol, D-mannitol and D-arabinitol (*cis*-vicinal hydroxyls on carbon atoms 2 and 3, with the hydroxyl on carbon atom 4 in the *trans* position). Chromatographic evidence indicated that NADP⁺-linked activity of extracts from succinate-grown *Torulopsis candida* formed fructose from D-mannitol, *xylo*-hexulose from D-glucitol and *threo*-pentulose from

D-arabinitol. Such extracts, it is true, were capable of NADPH-linked reductions of L-arabinose, D-xylose, D-ribose, D-galactose and D-glucose (Table 5), but no other sign of these aldose-reductase activities was detected in extracts from the succinate-grown material. This apparent contradiction between NADP-linked oxidative and reductive behaviour (for example, L-arabinose and D-ribose were reduced with NADPH, but neither L-arabinitol nor ribitol was oxidized with NADP⁺ and extracts from succinate-grown *T. candida*) was not resolved.

The marked difference between the mannitol- and succinate-grown *Torulopsis candida* was the presence in the former of the NADP⁺-linked bands 7 and 8. Perhaps one or both of these bands represented some of the missing aldose reductase activity discussed above. They were not investigated.

The NAD⁺-linked enzymes 2 and 4 (Fig. 5; Table 8) appeared only in extracts of *Torulopsis candida* which were grown on D-glucitol. Enzyme 2 may be responsible for the NAD⁺-linked activity with D-mannitol and D-arabinitol, which was not found with succinate- or D-mannitol-grown material (Table 5). The polyol-specificity of enzyme 2 corresponded exactly with that of the NADP⁺-linked enzyme 9 (Table 8) which was discussed above.

The NADP⁺-linked enzyme 6, which appeared only to oxidize D-glucitol, differed from enzymes 7 and 8 of the mannitol-grown yeast. Electrophoresis of mixtures of extracts from mannitol- and glucitol-grown yeast produced all three bands separately, in relative positions and with specificities corresponding to those shown in Fig. 5.

Erythritol-grown yeast was the only material in which the oxidation of erythritol was observed. The pattern of specificity was such that this NAD⁺-linked enzyme (5) oxidized only the *meso*-polyols, i.e. xylitol, ribitol and erythritol; it was not tested against L-iditol.

Enzymes of Candida utilis. The NAD-linked polyol dehydrogenase activity of *C. utilis* (LTS 23) was difficult to resolve electrophoretically. The difficulties were mainly due (i) to the presence in the crude extracts of an NAD-linked ethanol dehydrogenase, (ii) to the presence of ethanol in the commercial starch preparation used for making the gel. After electrophoresis of extracts of *C. utilis*, starch-gel strips incubated with NAD⁺, PMS and NBT, but without substrate, developed about 20 formazon bands. When 0.1 M-ethanol was added to the incubation mixture, bands in corresponding positions appeared more rapidly and were darker than those that developed without added substrate. This failure of the negative controls with starch-gel electrophoresis of extracts of certain yeasts did not occur when polyacrylamide was used in place of starch. A comparable effect for the starch-gel electrophoresis of extracts of mammalian tissue has also been attributed to the presence of alcohol dehydrogenase (Shaw & Koen, 1965).

A commercial preparation of yeast alcohol dehydrogenase subjected to starch-gel electrophoresis gave bands which developed without the addition of ethanol to the incubation mixture; and these bands did not increase in number when ethanol was added. After starch-gel electrophoresis of liver alcohol dehydrogenase, C. J. R. Thorne (personal communication, 1966) also found strong bands without added substrate. He estimated enzymically that one batch of starch contained 3.6 g. ethanol/100 g. starch (cf. Beutler, 1967).

Polyacrylamide gels also presented some difficulty. Crude extracts of *Candida utilis* oxidized ribitol in the spectrophotometric assays (Table 6), but no activity against

ribitol was detected in polyacrylamide gels, either with tris-DETPA (pH 7.8) or with phosphate (pH 7.0) gel buffers, even if the gels were washed to remove catalyst and unpolymerized acrylamide (Tombs, 1965). These gels revealed: (i) a single band of NAD^+ -polyol dehydrogenase active against D-glucitol, D-mannitol, D-arabinitol and xylitol, and (ii) a band of ethanol dehydrogenase.

These two difficulties (the failure of the negative controls with starch and the apparent enzyme-lability in polyacrylamide) were side-stepped as follows. After only about 15 min. of incubation (before the bands evoked by ethanol appeared, but after those associated with the polyols had done so), the starch-gel strips were removed from the incubation-mixture and placed in 5% aqueous acetic acid. The result of this procedure can be seen in Fig. 6. The specificity of enzyme 1, oxidizing D-glucitol,

Fig. 6. Location of NAD^+ -linked polyol dehydrogenase activity on starch-gel after electrophoresis of crude extract of D-xylose-grown *Candida utilis* (LTS 23). Vertical starch-gel electrophoresis was run for 5 hr under the conditions described in Fig. 5, except that formazan development was for 15 min. only, and the coenzyme was NAD^+ only. The albumin marker was at about 10 cm. from the origin-slot of each strip in the direction of the anode.

D-mannitol, D-arabinitol and xylitol, was comparable with that of enzyme 2 of *Torulopsis candida* (see Table 8). Enzyme 3 showed a specificity similar to that of the L-iditol dehydrogenase-like enzyme (1 and 3) of *T. candida*.

Candida utilis enzyme 1 (Fig. 6) was detected after starch-gel electrophoresis of an extract from the same yeast grown on acetate. It was not practicable to identify other enzymes from this material owing to interference from a very active alcohol dehydrogenase (see Table 6).

Starch-gel electrophoresis of xylose-grown *Candida utilis* revealed a single band which appeared when the gel-strip was incubated with NADP^+ and D-glucitol, xylitol or L-arabinitol, but not with NADP^+ and D-mannitol or D-arabinitol. This band probably corresponds with the enzyme described by Scher & Horecker (1966a, b).

Enzymes of Pichia species. Only weak NAD^+ -linked polyol dehydrogenase activity was found on the starch-gels after electrophoresis of extracts of *P. delftensis* grown on D-glucitol or of *P. membranaefaciens* grown on succinate. Material from succinate-

grown organisms of both species yielded two or three rather indistinct bands with D-glucitol as substrate. D-glucitol-grown *P. delftensis* yielded one definite band with D-glucitol or D-mannitol, one with D-arabinitol only and another with D-mannitol only.

In general, there was a good agreement between the spectrophotometric observations, the results of starch-gel electrophoresis and the chromatographic analysis of the products of polyol oxidation. These results are considered together in the Discussion.

DISCUSSION

Identity of polyol dehydrogenases

Fifteen distinct polyol dehydrogenases have been described above: eight for *Torulopsis candida*, four for *Candida utilis* and three for *Pichia delftensis*. These enzymes varied in their substrate-, coenzyme-, and inducer-specificities. However, identity with a previously reported enzyme has been suggested only for the constitutive NAD-linked dehydrogenase of *T. candida*, corresponding with enzyme 3 of *C. utilis*. Too few substrates were used for identifying these enzymes; and, furthermore, the presence of more than one polyol dehydrogenase in the crude extracts usually made impracticable the measurement of relative rates of activity of a single enzyme. Attempts to elute the enzymes from the gels failed.

Polyol dehydrogenases of Torulopsis candida

Extracts of succinate-grown yeast oxidized D-glucitol to fructose in the presence of NAD⁺ and to xyl⁻hexulose with NADP⁺. From the configuration of D-glucitol, the two products were presumably D-fructose and L-xyl⁻hexulose (though the configurations were not checked experimentally); both these hexuloses were reduced by the extracts (Table 5).

The only constitutive dehydrogenase found to oxidize D-mannitol was the NADP-linked enzyme 9. Extract of succinate-grown yeast, with NADP⁺, oxidized D-mannitol to D(?)⁻fructose only. Probably, therefore, enzyme 9 catalyzed the above oxidation-reduction. L-Iditol, on the other hand, was oxidized only by enzymes 1 and 3; this polyol, when incubated with crude extracts in the presence of NAD⁺, formed L(?)⁻xyl⁻hexulose. Similarly, NAD⁺-enzymes 1 and 3 appeared to oxidize ribitol to erythro⁻pentulose. Since the specificity of this enzyme seemed to correspond to that of L-iditol dehydrogenase, the erythro⁻pentulose probably had the D-configuration (see Touster & Shaw, 1962; Smith, 1962). Similarly, xylitol was probably oxidized to D-threo⁻pentulose by enzymes 1 and 3 and to L-threo⁻pentulose by 9.

With D-glucitol-grown *Torulopsis candida*, more than one enzyme specific for each coenzyme was present (Table 8) and, correspondingly, more than one polyol oxidation product was formed (Table 7).

The failure to detect the oxidation of both galactitol and L-arabinitol, despite evidence of their formation from corresponding aldoses, may have been because the affinity of the appropriate dehydrogenase for these polyols was very low, or the experimental conditions (e.g. pH) were unsuitable for this reaction. However, the catabolism of galactitol and L-arabinitol was not investigated further. None the less, it is worth drawing attention to additional evidence for the association between the catabolism of galactitol and of L-arabinitol. All the yeasts which grew on L-arabinitol used galactitol too (Table 1). This was not true for D-arabinitol. Possibly, therefore,

an NADP-linked aldose reductase may initiate the catabolism of galactitol and L-arabinitol; and this enzyme may be specific for polyols of the L-*threo*-configuration adjacent to the primary alcohol group, as Shaw (1956, 1962) found for the ketose-forming galactitol: NAD oxidoreductase of a pseudomonad. Alternatively, the galactitol-specific enzyme of *Torulopsis candida* may prove comparable with the NADP-linked (aldose-forming) polyol dehydrogenase of *Candida utilis* (Scher & Horecker, 1966*a, b*) or even with that of *Candida albicans* (Veiga, Bacila & Horecker, 1960), though the latter enzyme was not tested against galactitol or galactose.

Where two sugars were detected as products of activity of cell-free extracts, these sugars are interpreted here as direct products of polyol oxidation, though they could have been formed by the action of isomerases. This suggestion applies most obviously to the mixture of mannose and fructose found after incubation of an extract of D-glucitol-grown *Torulopsis candida* with D-mannitol and NADP⁺. The mannose could have been formed from fructose by the action of a mannose isomerase (Palleroni & Doudoroff, 1956). Similarly, a xylose isomerase (Slein, 1962) could have formed D-xylose from D-*threo*-pentulose. Although such possibilities must be borne in mind, extracts of D-glucitol-grown yeast showed high aldose-reductase activity (Table 5) and controls incubated without coenzyme formed no sugars.

Table 9. Scheme for specificities of four polyol dehydrogenases of *Torulopsis candida* (NCYC 576)

Data from Tables 5, 7 and 8. The basis of this scheme is explained in the text. The enzyme numbers are those used in Table 8.

Polyol oxidized	Products with enzymes from yeast			
	Succinate-grown		D-Glucitol-grown	
	Enzymes 1 and 3 (NAD ⁺)	Enzyme 9 (NADP ⁺)	Enzyme 2 (NAD ⁺)	Enzyme 6* (NADP ⁺)
D-Glucitol	D-Fructose	L-xylo-Hexulose	D-Fructose	D-Glucose
D-Mannitol	None	None	None	D-Mannose
L-Iditol	L-xylo-Hexulose	None	None	None
Ribitol	D-erythro-Pentulose	None	None	D-Ribose
Xylitol	D-threo-Pentulose	L-threo-Pentulose	None	D-Xylose
D-Arabinitol	None	D-threo-Pentulose	None	D-Arabinose

* The specificities shown for enzyme no. 6 were not substantiated by the electrophoretic results (Table 8). N.B. the D- and L-forms were not identified experimentally (see text).

Table 9 summarizes the above and additional inferences on the specificities of the constitutive enzymes, from succinate-grown *Torulopsis candida* (1, 3 and 9) as well as two enzymes found in D-glucitol-grown yeast (2, 6). Extracts from D-glucitol-grown *T. candida* in the presence of NADP⁺ formed certain aldoses in addition to those formed by succinate-grown yeast (Table 7), and only one additional NADP⁺-linked enzyme (6) was detected on the gels for D-glucitol-grown material (Table 8). Although it appeared to react with D-glucitol only, the aldose-producing activity has been tentatively attributed to enzyme 6 (Table 9), assuming that its reactions with D-mannitol, ribitol, xylitol and D-arabinitol were too weak to detect on the gels. None of the other enzymes can be considered in this way, because the products with extracts from yeast grown on other substrates were not examined chromatographically.

NAD-linked polyol dehydrogenases of Candida utilis

In the present work, three different polyol dehydrogenases were separated from the crude extracts of *Candida utilis* by starch-gel electrophoresis. The purified preparation of Chakravorty *et al.* (1962) probably contained enzyme 1 (Fig. 6), since it oxidized mannitol. Since the preparation also oxidized L-iditol and ribitol, it probably contained also at least one of the other enzymes. Alternatively, the oxidation of the last two polyols may have been only apparent. The reduction of NAD⁺ could have been due, not to the oxidation of ribitol or L-iditol, but to the presence of both (i) NAD-linked alcohol dehydrogenase in the purified preparation and (ii) traces of ethanol present in recrystallized polyols, as was experienced in the present work with crude extracts of acetate-grown *C. utilis*.

Although eight separate polyol dehydrogenases were detected by starch-gel electrophoresis in extracts of *Torulopsis candida*, and only four such enzymes in *Candida utilis*, the effective difference between these yeasts in their abilities to use polyols is probably one of penetration: that is to say, polyols do not enter the cells of this strain of *C. utilis*. As can be seen from the rates shown in Table 5, the specific activities of the NAD⁺-linked dehydrogenases against a number of the polyols were of the same order for the crude extracts of both yeasts. Most of the polyol dehydrogenase activity of *Pichia delftensis* seemed to be NAD⁺- rather than NADP⁺-linked (Table 5). Further the NAD⁺-linked activity was about 100 times greater in D-glucitol- than in succinate-grown *P. delftensis*; that of the latter was comparable to the activity of succinate-grown *P. membranaefaciens*.

As for *Candida utilis*, certain polyols were oxidized at appreciable rates by extracts of these yeasts, although the same polyols were not respired by the corresponding intact cells. Thus extracts of D-glucitol-grown *Pichia delftensis* oxidized xylitol and erythritol, and extracts of succinate-grown *P. membranaefaciens* oxidized ribitol and erythritol (Table 5); whereas corresponding intact cells did not respire these substrates. Hence, on this evidence alone, it seemed likely that permeability barriers would be found to be deciding factors of utilization or non-utilization of most of the polyols by these yeasts. This suggestion was corroborated by the results of experiments with labelled substrates. Probably, therefore, *P. delftensis* and *P. membranaefaciens* are distinguished from each other by some polyol translocation mechanism (or mechanisms) present in the first species and not in the second.

Only in the case of erythritol for *Torulopsis candida* was the presence and absence of an enzyme clearly associated with the capacity of the yeast to use the enzyme's substrate. Most of the evidence in this study has been of an abundance of polyol dehydrogenase activity in extracts of the yeasts (mainly NAD⁺-linked and ketose producing) whether or not the intact yeast could use the polyols which its enzymes oxidized. Active NADP⁺-linked enzymes were also found particularly for the oxidation of mannitol by *T. candida*. Scher & Horecker (1966*a, b*) have described the NADP-linked polyol dehydrogenase of *Candida utilis*.

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REFERENCES

- ARCUS, A. C. & EDSON, N. L. (1956). Polyol dehydrogenases. 2. The polyol dehydrogenases of *Acetobacter suboxydans* and *Candida utilis*. *Biochem. J.* **64**, 385.
- BARNETT, J. A. (1968). Biochemical differentiation of taxa. In *The Fungi*. Ed. by G. C. Ainsworth and A. S. Sussman, vol. III. New York: Academic Press.
- BARNETT, J. A. & INGRAM, M. (1955). Technique in the study of yeast assimilation reactions. *J. appl. Bact.* **18**, 131.
- BARNETT, J. A. & KORNBERG, H. L. (1960). The utilization by yeasts of acids of the tricarboxylic acid cycle. *J. gen. Microbiol.* **23**, 65.
- BEECH, F. W. (1965). *Pichia delftensis* sp.n. *Antonie van Leeuwenhoek* **31**, 81.
- BEUTLER, E. (1968). 'Galactose dehydrogenase', 'nothing dehydrogenase', and alcohol dehydrogenase: interrelation. *Science, N.Y.* **156**, 1516.
- BURGER, M., HEJMOVÁ, L. & KLEINZELLER, A. (1959). Transport of some mono- and di-saccharides into yeast cells. *Biochem. J.* **71**, 233.
- BYGRAVE, F. L. & SHAW, D. R. D. (1961). Separation of the polyol dehydrogenases of *Acetobacter suboxydans*. *Proc. Univ. Otago med. Sch.* **39**, 15.
- CHAKRAVORTY, M. & HORECKER, B. L. (1966). Polyol dehydrogenases of *Candida utilis* I. DPN-linked dehydrogenase. *Meth. Enzymol.* **9**, 163.
- CHAKRAVORTY, M., VEIGA, L. A., BACILA, M. & HORECKER, B. L. (1962). Pentose metabolism in *Candida*. II. The diphosphopyridine nucleotide-specific polyol dehydrogenase of *Candida utilis*. *J. biol. Chem.* **237**, 1014.
- CIRILLO, V. P. (1961). Sugar transport in micro-organisms. *A. Rev. Microbiol.* **15**, 197.
- CIRILLO, V. P. & WILKINS, P. O. (1964). Use of uranyl ion in membrane transport studies. *J. Bact.* **87**, 232.
- DATTA, S. P. & GRZYBOWSKI, A. K. (1961). pH and acid-base equilibria. In *Biochemist's Handbook*. Ed. by C. Long, p. 22. London: Spon.
- DAVIES, R. (1964). Lactose utilization and hydrolysis in *Saccharomyces fragilis*. *J. gen. Microbiol.* **37**, 81.
- DISCHE, Z. & BORENFREUND, E. (1951). A new spectrophotometric method for the detection and determination of keto sugars and trioses. *J. biol. Chem.* **192**, 583.
- DISCHE, Z. & DEVI, A. (1960). A new colorimetric method for the determination of ketohexoses in presence of aldoses, ketoheptoses and ketopentoses. *Biochim. biophys. Acta* **39**, 140.
- DIXON, M. & WEBB, E. C. (1964). *Enzymes*, 2nd ed. London: Longmans Green.
- EDSON, N. L. (1953). The metabolism of the sugar alcohols. *Aust. N.Z. Ass. Adv. Sci., Rep. 29th Meeting, Sydney*, 1952, **29**, 281.
- FINE, I. H. & COSTELLO, L. A. (1963). The use of starch electrophoresis in dehydrogenase studies. *Meth. Enzymol.* **6**, 958.
- FOSSITT, D., MORTLOCK, R. P., ANDERSON, R. L. & WOOD, W. A. (1964). Pathways of L-arabitol and xylitol metabolism in *Aerobacter aerogenes*. *J. biol. Chem.* **239**, 2110.
- GOMORI, G. (1955). Preparation of buffers for use in enzyme studies. *Meth. Enzymol.* **1**, 138.
- GORDON, H. T., THORNBURG, W. & WERUM, L. N. (1956). Rapid paper chromatography of carbohydrates and related compounds. *Analyt. Chem.* **28**, 849.
- HARRIS, G. & THOMPSON, C. C. (1961). The uptake of nutrients by yeasts III. The maltose permease of a brewing yeast. *Biochim. biophys. Acta* **52**, 176.
- HAYWARD, L. D. & WRIGHT, I. G. (1963). Xylitol pentanitrate. In *Meth. Carbohydrate Chem.* **2**, 258.
- HOLLMANN, S. & TOUSTER, O. (1964). *Non-Glycolytic Pathways of Metabolism of Glucose*. New York: Academic Press.
- HORECKER, B. L. (1961). Alternative pathways of carbohydrate metabolism in relation to evolutionary development. *Int. Congr. Biochem.* **3**, 86.
- HORECKER, B. L. (1962a). *Pentose Metabolism in Bacteria*. New York: Wiley.
- HORECKER, B. L. (1962b). Interdependent pathways of carbohydrate metabolism. *Harvey Lect.* **57**, 35.
- KAY, D. & FIELDS, P. (1950). The calcium requirement of a typhoid bacteriophage. *Br. J. exp. Path.* **31**, 338.
- KERSTERS, K., WOOD, W. A. & DE LEY, J. (1965). Polyol dehydrogenases of *Gluconobacter oxydans*. *J. biol. Chem.* **240**, 965.

- KIRSOP, B. (1954). The National Collection of Yeast Cultures. *J. Inst. Brew.* **60**, 210.
- KOTYK, A. & HÖFER, M. (1965). Uphill transport of sugars in the yeast *Rhodotorulla gracilis*. *Biochim. biophys. Acta* **102**, 410.
- KOTYK, A. & KLEINZELLER, A. (1963). Transport of D-xylose and sugar space in baker's yeast. *Folia microbiol.* **8**, 156.
- KREGER-VAN RIJ, N. J. W. (1964). *A Taxonomic Study of the Yeast Genera Endomycopsis, Pichia and Debaryomyces*. Thesis. University of Leiden.
- KUDRIAEV, V. I. (1954). *The Systematics of Yeasts*. Moscow: Academy of Sciences. (In Russian.) Translated edition: Kudrjawzew, W. I. (1960), *Die Systematik der Hefen*. Berlin: Akademie-Verlag.
- LAYNE, E. (1957). Spectrophotometric and turbidimetric methods for measuring proteins. *Meth. Enzymol.* **3**, 447.
- LEWIS, D. H. & SMITH, D. C. (1967*a*). Sugar alcohols (polyols) in fungi and green plants. I. Distribution, physiology and metabolism. *New Phytol.* **66**, 143.
- LEWIS, D. H. & SMITH, D. C. (1967*b*). Sugar alcohols (polyols) in fungi and green plants II. Methods of detection and quantitative estimation in plant extracts. *New Phytol.* **66**, 185.
- LODDER, J. & KREGER-VAN RIJ, N. J. W. (1952). *The Yeasts. A Taxonomic Study*. Amsterdam: North Holland Publishing Co.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265.
- LUND, B. M. (1965). A comparison by the use of gel electrophoresis of soluble protein components and esterase enzymes of some Group D Streptococci. *J. gen. Microbiol.* **40**, 413.
- MACQUILLAN, A. M. & HALVORSON, H. O. (1962). Physiological changes occurring in yeast undergoing glucose repression. *J. Bact.* **84**, 31.
- MAGASANIK, B. (1957). Nutrition of bacteria and fungi. *A. Rev. Microbiol.* **11**, 221.
- MITCHELL, P. (1949). A new technique for stirred aerated culture. *Nature, Lond.* **164**, 846.
- MORTLOCK, R. P. & WOOD, W. A. (1964). Metabolism of pentoses and pentitols by *Aerobacter aerogenes*. I. Demonstration of pentose isomerase, pentulokinase and pentitol dehydrogenase enzyme families. *J. Bact.* **88**, 838.
- NOSSAL, P. M. (1953). A mechanical cell disintegrator. *Aust. J. exp. Biol. med. Sci.* **31**, 583.
- PALLERONI, N. J. & DOUDOROFF, M. (1956). Mannose isomerase of *Pseudomonas saccharophila*. *J. biol. Chem.* **218**, 535.
- PONCET, S. & ARPIN, M. (1965). Les *Candida* sans pouvoir fermentaire (Cryptococcacées). *Antonie van Leeuwenhoek* **31**, 433.
- POULIK, M. D. (1957). Starch-gel electrophoresis in a discontinuous system of buffers. *Nature, Lond.* **180**, 1477.
- PRICE, V. E. & LEVINTOW, L. (1952). Sodium pyruvate. In *Biochemical Preparations*. Ed. by E. G. Ball, vol. II, p. 22. New York: Wiley.
- RAYMOND, S. (1964). Acrylamide gel electrophoresis. *Ann. N.Y. Acad. Sci.* **121**, 350.
- SCHER, B. M. & HORECKER, B. L. (1966*a*). Pentose metabolism in *Candida* III. The triphosphopyridine nucleotide-specific polyol dehydrogenase of *Candida utilis*. *Archs Biochem. Biophys.* **116**, 117.
- SCHER, B. M. & HORECKER, B. L. (1966*b*). Polyol dehydrogenases of *Candida utilis*. II. TPN-linked dehydrogenase. In *Meth. Enzymol.* **9**, 166.
- SCHMIDT, O. T. & TREIBER, R. (1933). D-Xyloketose. *Chem. Ber.* **66**, 1765.
- SCOPES, R. K. (1963). Starch-gel electrophoresis of pig serum proteins. *Nature, Lond.* **197**, 1201.
- SCOPES, R. K. (1964). The influence of post-mortem conditions on the solubilities of muscle proteins. *Biochem. J.* **91**, 201.
- SHAW, C. R. & KOEN, A. L. (1965). On the identity of 'nothing dehydrogenase'. *J. Histochem. Cytochem.* **13**, 431.
- SHAW, D. R. D. (1956). Polyol dehydrogenases. 3. Galactitol dehydrogenase and D-iditol dehydrogenase. *Biochem. J.* **64**, 394.
- SHAW, D. R. D. (1962). Galactitol dehydrogenase. *Meth. Enzymol.* **5**, 323.
- SLEIN, M. W. (1962). Xylose isomerase. *Meth. Enzymol.* **5**, 347.
- SMITH, M. G. (1962). Polyol dehydrogenases. 4. Crystallization of the L-iditol dehydrogenase of sheep liver. *Biochem. J.* **83**, 135.

- SMITHIES, O. (1959). An improved procedure for starch-gel electrophoresis: further variations in the serum proteins of normal individuals. *Biochem. J.* **71**, 585.
- STRAIN, H. H. (1934). D-Sorbitol: a new source, method of isolation, properties and derivatives. *J. Am. chem. Soc.* **56**, 1756.
- STRAIN, H. H. (1937). Sources of *d*-sorbitol. *J. Am. chem. Soc.* **59**, 2264.
- TOMBS, M. P. (1965). The interpretation of gel electrophoresis. *Analyt. Biochem.* **13**, 121.
- Topley & Wilson's Principles of Bacteriology and Immunology* (1964). 5th ed. Revised by G. S. Wilson and A. A. Miles, vol. 1. London: Arnold.
- TOUSTER, O. & SHAW, D. R. D. (1962). Biochemistry of the acyclic polyols. *Physiol. Rev.* **42**, 181.
- VEIGA, L. A., BACILA, M. & HORECKER, B. L. (1960). Pentose metabolism in *Candida albicans*. I. The reduction of D-xylose and L-arabinose. *Biochem. biophys. Res. Commun.* **2**, 440.
- WICKERHAM, L. J. (1951). Taxonomy of yeasts. *Tech. Bull. U.S. Dep. Agric.* no. 1029.
- WICKERHAM, L. J. & BURTON, K. A. (1948). Carbon assimilation tests for the classification of yeasts. *J. Bact.* **56**, 363.
- WOLFE, J. B. & KAPLAN, N. O. (1956). D-Mannitol 1-phosphate dehydrogenase from *Escherichia coli*. *J. biol. Chem.* **218**, 849.
- WOLFROM, M. L. & THOMPSON, A. (1963). Reduction with sodium borohydride. In *Meth. Carbohydrate Chem.* **2**, 65.