Polyol Dehydrogenases
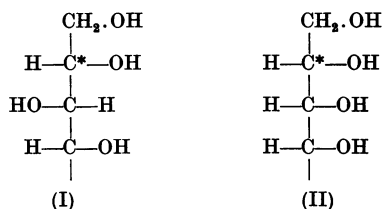
2. THE POLYOL DEHYDROGENASES OF *ACETOBACTER SUBOXYDANS* AND *CANDIDA UTILIS**

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In the first communication of this series it was shown that the polyol dehydrogenase of rat liver reacts specifically with diphosphopyridine nucleotide and polyhydric alcohols (pentitols, hexitols, heptitols) which contain configuration (I) or (II) (McCorkindale & Edson, 1954).

$$
\begin{array}{cc}
CH_2.OH & CH_2.OH \\
| & | \\
H—C^*—OH & H—C^*—OH \\
| & | \\
HO—C—H & H—C—OH \\
| & | \\
H—C—OH & H—C—OH \\
| & | \\
(I) & (II)
\end{array}
$$

* Site of oxidation.

Pending critical tests of homogeneity on purified material, it has been assumed that the rat-liver preparation contains a single enzyme (L-iditol dehydrogenase) possessing a specificity rule different from the Bertrand–Hudson rule, which applies to cultures of *Acetobacter*.

Bertrand (1898, 1904) discovered the selective oxidation of polyols in proliferating cells of *Acetobacter xylinum* and enunciated the well-known rule that was confirmed and extended by later workers who preferred to study *Acetobacter suboxydans* (Kluyver & de Leeuw, 1924; Reichstein, 1934; Steiger & Reichstein, 1935; Hann, Tilden & Hudson, 1938; Fulmer & Underkofler, 1947; Bernhauer, 1938, 1950; Stewart, Richtmyer & Hudson, 1949, 1952; Totten & Lardy, 1949; Bollenback & Underkofler, 1950; Richtmyer, Stewart & Hudson, 1950; Pratt, Richtmyer & Hudson, 1952).

The contrast between the specificity of the rat-liver enzyme and the behaviour of *Acetobacter* (Edson, 1953; McCorkindale & Edson, 1954) makes it desirable to study the oxidation of polyhydric alcohols in cell-free extracts of the bacteria. This communication shows that *A. suboxydans* contains at least two polyol dehydrogenases, one of which is responsible for the reactions described by the

Bertrand–Hudson rule; the other exhibits activity resembling that of an extract made from *Candida utilis*.

MATERIALS AND METHODS

Organisms. Acetobacter suboxydans, no. 621, was obtained from the American Type Culture Collection, Washington, D.C. It was grown at 30° for 3 days in a yeast-water medium to which 0·3 % (w/v) of KH_2PO_4 and 2 % (w/v) of glycerol were added (pH about 5·5). The cultures (100 ml. of medium in 500 ml. conical flasks) were aerated by continuous shaking. Yeast water was prepared by boiling 1 lb. of baker's compressed yeast with 400 ml. of water for 30 min., allowing the yeast to settle overnight, filtering the supernatant through kieselguhr and making the volume up to 5 l. with water.

Candida utilis (strain Y 90/77) was obtained through the courtesy of the Northern Regional Research Laboratories, Department of Agriculture, U.S.A. The stock culture was maintained on malt-agar slopes. For experiments the organism was transferred either to the glucose-containing basal medium of Schultz & Atkin (1947) or to a medium having the following composition: 2·0 g. of KH_2PO_4, 0·25 g. of anhydrous $CaCl_2$, 0·5 g. of $MgSO_4,7H_2O$, 4·5 g. of $(NH_4)_2SO_4$, 20 g. of mannitol and tap water to 1 l. The cultures were incubated at 30° for 2 days and continuously shaken.

Cell-free extracts. The cells of *A. suboxydans* were harvested and washed three times with water by centrifuging. Washed cells (5–10 g.) were suspended in sufficient 0·01 M-Na_2HPO_4 solution to make a total volume of 25 ml., and disrupted by treating for 40 min. in a Raytheon 9 kcyc./sec. sonic disintegrator. The cup assembly of the apparatus was set up in a cold room at 2–3° and the internal temperature of the cup kept at 0–1° by circulation of ice-water (1·8 l./min.). The extract was clarified by centrifuging at 20 000 g for 30 min. in a centrifuge operating at −5°, and dialysed against water for 12 hr. at 2°. The extracts were clear red-brown solutions, opalescent in reflected light. Examination with a direct-vision spectroscope revealed cytochrome absorption bands centred approximately at 527 and 554 mμ., the latter band being narrower and more intense. The full enzymic activities are retained for several weeks if the extract is frozen.

The cells of *C. utilis* were harvested and washed three times with 0·9 % (w/v) NaCl solution. Extracts were prepared either by hand-grinding 1 g. of wet cells and 2 g. of powdered Pyrex glass moistened with 0·1 M-Na_2HPO_4 to give a thick paste and adjusting to 10 ml. with the same phosphate solution, or by autolysis of vacuum-dried cells (1 g. of dry cells/5 ml. of water at 38° for 3 hr.). The crude extracts were clarified by centrifuging and dialysed against water for 24 hr. at 2°.

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* Part 1: McCorkindale & Edson (1954).

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Polyols and sugars. D- and L-Iditol and L-rhamnitol (gifts) were prepared by Dr D. R. D. Shaw. The sources of the other polyols and of the sugars used in this work are acknowledged in the first paper (McCorkindale & Edson, 1954). ·

Coenzymes. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) were the chromatographically purified products of the Sigma Chemical Co., St Louis, U.S.A.; reduced diphosphopyridine nucleotide (DPNH) was the product of the same firm.

Analytical methods

The analytical methods were essentially the same as those used by McCorkindale & Edson (1954). Ketoses, however, were determined by Kulka's (1956) modification of the method of Bacon & Bell (1948) after deproteinization and, when necessary, removal of methylene blue. Fructose and sorbose were determined in mixtures by measuring the total ketose in a portion of the original solution, the fructose was destroyed by fermentation with baker's yeast (10 %, w/v) for 4 hr. at 30°, the yeast was removed by centrifuging, and the residual ketose estimated. Thus the fructose was determined by difference. A water blank containing the same quantity of yeast corrected for the small amount of resorcinol-reacting material which is extracted from the yeast during the fermentation (0·16 μmole as fructose).

Preparation of phenylosazones

The preparations were carried out as before (McCorkindale & Edson, 1954). The phenylosazone of sorbose was amorphous when prepared by this method. It was crystallized by dissolving in water at 100° and evaporating until a faint cloudiness appeared. The container was stoppered, immersed in a water bath at 100° for 0·5 hr. and the bath allowed to cool slowly to room temperature.

RESULTS

Oxidation of mannitol and sorbitol by extracts of Acetobacter suboxydans

At pH 8 dialysed extracts of *A. suboxydans* reduced methylene blue anaerobically in the presence of added DPN and either mannitol or sorbitol. When methylene blue was omitted and the reactions were carried out aerobically, O_2 was consumed and the products of oxidation accumulated. Mannitol gave fructose as expected, but sorbitol also formed fructose. This was surprising because *A. suboxydans* grown in yeast-water media at pH 5–6·8 oxidizes sorbitol to sorbose in a yield greater than 90 % (Fulmer & Underkofler, 1947). At pH 5·5 the extracts did not reduce methylene blue anaerobically on addition of polyols and DPN. Aerobically with methylene blue omitted the enzyme system, like the intact bacteria, consumed O_2, converting mannitol into fructose and sorbitol into sorbose.

The behaviour of the bacterial extract is explained by the experiments illustrated in Figs. 1 and 2. Oxygen consumption and simultaneous ketose formation were measured at pH values from 5·6 to

7·9. The conversion of mannitol into fructose occurs throughout this range of pH but the rate of oxidation exhibits a minimum about pH 6·5. The rate of oxidation of sorbitol also passes through a minimum (pH 6·4); at pH 7·1–7·9 the product is entirely fructose, whereas at pH 5·6 it is almost entirely sorbose. At intermediate pH values the product is a mixture of the two ketoses in proportions which depend on the pH. At all pH values the O_2 uptake and the ketose formation correspond to the stoicheiometry of the reaction:

$$polyol + 0·5O_2 = ketose + H_2O.$$
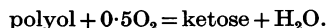
These experiments indicated that extracts of *A. suboxydans* contain two distinct polyol dehydrogenases, one most active about pH 5·5 ('acid' enzyme) and the other about pH 8 ('alkaline'
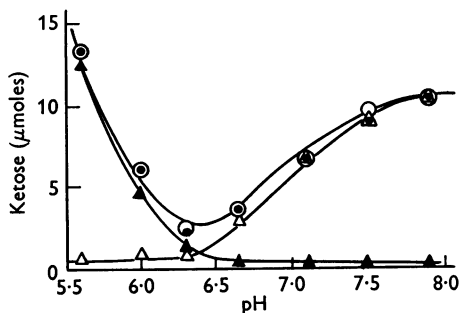

Fig. 1. Effect of pH on the oxidation of sorbitol in dialysed extracts of *A. suboxydans*. Each Warburg flask contained 0·5 ml. of dialysed bacterial extract, 150 μmoles of potassium phosphate buffer, 0·4 μmole of DPN and 150 μmoles of sorbitol (added from side bulb after equilibration). Volume, 2·7 ml. Gas, air. Temp., 25°. Time, 1 hr. △, Fructose (analysis); ▲, sorbose (analysis); ●, total ketose (analysis); ○, total ketose (calculated from O_2 uptake on the basis 0·5 μmole of O_2 consumed = 1·0 μmole of ketose formed).
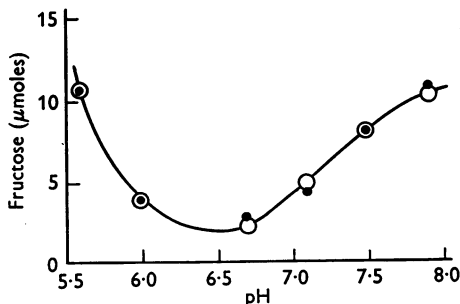

Fig. 2. Effect of pH on the oxidation of mannitol in dialysed extracts of *A. suboxydans*. Substrate, 150 μmoles of mannitol. Conditions otherwise the same as in Fig. 1. ●, Fructose (analysis); ○, fructose (calculated from O_2 uptake as in Fig. 1).

enzyme). This view was confirmed by experiments (Table 1) which show that the 'alkaline' enzyme requires added DPN as a cofactor but the 'acid' enzyme does not.

Partial separation of the 'acid' and 'alkaline' enzymes

After a bacterial extract had been dialysed for 1 week and repeatedly frozen and thawed the 'acid' enzyme separated in a brown flocculent precipitate. The supernatant containing the 'alkaline' enzyme no longer took up O_2 at pH 8 in the presence of polyols and DPN unless methylene blue was added. The natural hydrogen carrier is associated with the particles containing the 'acid' enzyme. The

enzymes were partially separated in the following ways:

(1) An effective method, which was not available as a routine, was centrifuging of freshly prepared bacterial extract for 2 hr. in a Spinco preparative centrifuge at 60 000 g. The 'acid' enzyme was largely in the residue, which formed a red gel, and most of the 'alkaline' enzyme in the straw-coloured supernatant (Table 2).

(2) A modification of the extraction procedure gave a primary separation. The sonic disintegration was performed with the usual proportions of bacteria in 0·06 M sodium acetate solution instead of in phosphate solution, and the extract clarified as usual. The extract was then frozen, thawed and centrifuged repeatedly until the supernatant was quite clear. The residue was suspended in 0·06 M sodium acetate solution and washed several times with this solution. The 'acid' enzyme was in the precipitate and the 'alkaline' enzyme in the supernatant (Table 2).

(3) The two enzymes are separated less effectively by precipitation with ammonium sulphate at 0°. Addition of solid ammonium sulphate (6·25 g.) to 25 ml. of bacterial extract freshly prepared by sonic disintegration in 0·01 M-Na_2HPO_4 solution gave a brown precipitate which was separated by centrifuging, dispersed in 25 ml. of water and dialysed overnight (fraction P). Ammonium sulphate (2·5 g.) was dissolved in the supernatant and the resulting precipitate treated in the same way (fraction S). The activities of these fractions towards mannitol (Table 2) show that the 'acid'

Table 1. *Effect of DPN on the oxidation of mannitol and sorbitol by an extract of* A. suboxydans

Each Warburg flask contained 0·5 ml. of dialysed bacterial extract, 15 μmoles of KH_2PO_4 (to give pH 5) or 15 μmoles of Na_2HPO_4 (to give pH 8), 0·4 μmole of DPN where indicated, and 150 μmoles of substrate (added from the side bulb after equilibration). Volume, 2·5 ml. Temp., 25°. Gas, air. Time, 1 hr. Inseal, 0·2 ml. of 10% (w/v) KOH.

		O_2 uptake (μmoles)	
pH	DPN	Mannitol	Sorbitol
5	Absent	33·6	38·7
5	Present	32·4	37·6
8	Absent	0·4	0·9
8	Present	5·5	6·9

Table 2. *Partial separation of the* '*acid*' *and* '*alkaline*' *enzyme present in extracts of* A. suboxydans

Each Warburg flask contained 0·5–1·0 ml. of enzyme preparation, buffer solution and 150 μmoles of mannitol. In the absence of substrate O_2 uptake was negligible in all preparations. In the flasks containing alkaline-buffer solutions 0·4 μmole of DPN and 1·6 μmoles of methylene blue were also present. In experiments with the ammonium sulphate preparation 3 μmoles of $CaCl_2$ were added. Volume, 2·8 ml. Gas, air. Temp., 25°. Time, 1 hr. Inseal, 0·2 ml. of 10% (w/v) KOH.

Enzyme preparation	Buffer	pH	O_2 uptake (μmoles)
Centrifuging (60 000 g):			
Precipitate	Phosphate, 100 μmoles	5·0	3·3
		7·8	0·8
Supernatant		5·0	0·3
		7·8	9·3
Sodium acetate procedure:			
Precipitate	Phosphate, 15 μmoles	5·6	6·5
		7·5	0·1
Supernatant		5·6	0·4
		7·5	1·4
Ammonium sulphate procedure:			
Precipitate (P)	Tris–succinic* acid, 1·0 m-mole	4·7	7·6
		7·9	5·7
Supernatant (S)		4·7	0·3
		7·9	3·8

* This buffer was made by mixing appropriate volumes of 0·2 M aminotrishydroxymethylmethane (tris) solution and 0·1 M succinic acid solution.

enzyme is almost entirely in fraction P, but two-thirds of the 'alkaline' enzyme remains in fraction P.

Properties of the 'acid' enzyme

In some experiments partially separated 'acid' enzyme was used; in others the unfractionated bacterial extract was examined at pH 5 to eliminate the activity of the 'alkaline' enzyme. The preparations always contained the red-brown pigment showing cytochrome absorption bands.

Solubility. Although the subcellular particles containing the 'acid' enzyme are well dispersed by sonic treatment, they are flocculated by the operations described in the preceding section. Since they are aggregated at pH 5–6 the properties of the enzyme were studied in a suspension of extremely fine particles which do not sediment at centrifugal speeds up to 4000 rev./min. for 30 min. They are redispersed in solutions at pH 8. Dispersion with commercial detergents and sodium deoxycholate (0·2 %) was successful at lower pH values, but the enzyme was inactivated.

Effect of pH. The curve relating activity to pH (Fig. 3) shows an optimum at pH 5·4 in the buffer used. Citric acid buffer was precluded because citrate inactivated the enzyme.

Substrate concentration. The effect of mannitol concentration on the rate of O_2 uptake at 25° was studied with a 'sodium acetate preparation' containing 0·017M-KH_2PO_4 (pH 5). A plot of S/v against S by the method of Lineweaver & Burk (1934) gave a linear graph from which K_m was found to be $3·4 \times 10^{-2}$M.

Effect of HCN. Oxygen uptake at 25° and pH 5 (0·005M-KH_2PO_4) in the presence of mannitol (150 μmoles) was almost completely inhibited by 10^{-3}M-HCN in all preparations. Since anaerobic oxidation of mannitol with phenol blue or ferri-

cyanide as oxidant was not inhibited, the locus of cyanide inhibition is likely to be the cytochrome carrier.

Anaerobic reduction of ferricyanide and dyes. On addition of mannitol (150 μmoles) the dialysed bacterial extract reduced ferricyanide and several redox indicators (10^{-4}M), e.g. phenol blue (0·3 min.), phenolindo-2:6-dichlorophenol (6 min.) and *o*-chlorophenolindo-2:6-dichlorophenol (8 min.). The approximate visual reduction times are given in brackets. Methylene blue, pyocyanine and brilliant cresyl blue were not reduced. Reduction of phenol blue or ferricyanide can be used under anaerobic conditions, or in the presence of cyanide, to determine the activity of the dehydrogenase by measuring decrease in optical density at 655 and 420 mμ. respectively (Fig. 4).

Cofactors. Although the addition of DPN does not increase the activity of the 'acid' enzyme (Table 1) it is possible that the coenzyme is firmly bound. A requirement for DPN or TPN was not induced by dialysis against water (6 days at 2°), 0·01M-Na_2HPO_4 solution or 0·01M acetate buffer, pH 4·5 (48 hr. at 2°), or by treatment with charcoal (Norit A, Pfanstiehl Chemical Co., U.S.A.) according to Velick, Hayes & Harting (1953). The absorption
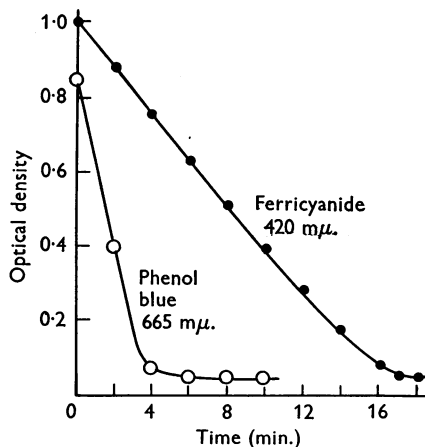
Fig. 4. Reduction of ferricyanide and phenol blue by the 'acid' enzyme of *A. suboxydans*. The cuvette containing potassium ferricyanide (3·0 μmoles) also contained 0·13 ml. of dialysed bacterial extract, 75 μmoles of succinic acid-Na_2HPO_4 buffer, pH 5·2, 150 μmoles of mannitol and 30 μmoles of KCN (to prevent reoxidation of the carrier system); volume, 3·0 ml.; temp., 20°. The cuvette containing phenol blue (1·0 μmole) also contained 0·2 ml. of bacterial extract (tenfold dilution of the original dialysed extract), 18 μmoles of KH_2PO_4 (to give pH 5) and 150 μmoles of mannitol; nitrogen was bubbled through the reaction mixture before the substrate was added; volume, 3·0 ml.; temp., 20°. In the blank cuvettes water was substituted for the oxidant in both. There was no reduction in the absence of substrate.
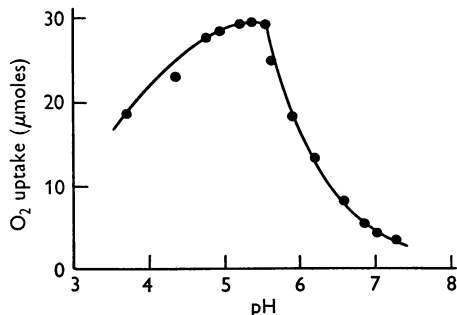
Fig. 3. Effect of pH on the activity of the 'acid' enzyme from *A. suboxydans*. Each Warburg flask contained 1·0 ml. of dialysed bacterial extract, 25 μmoles of succinic acid-Na_2HPO_4 buffer and 150 μmoles of mannitol (added from the side bulb after equilibration). Volume, 2·5 ml. Gas, air. Temp., 25°. Time, 1 hr.

spectrum of enzyme preparations did not show increased absorption in the wavelength range 300–400 mμ. on addition of mannitol anaerobically. Although these observations do not exclude completely the participation of DPN in the dehydrogenation, they make it unlikely.

Attempts to separate the polyol dehydrogenase from the cytochrome and other constituents of the active particles by treatment with dilute alkali (pH 9·5) or with acetone or butanol (Morton, 1950) were unsuccessful. The activity was tested by following the reduction of phenol blue or ferricyanide spectrophotometrically.

When the bacterial extract (50 ml.) was treated with sodium ethylenediaminetetraacetate (EDTA) (50 μmoles/ml. of extract adjusted to pH 7·1) and then dialysed to remove EDTA–metal complexes, the power to consume O_2 in the presence of mannitol or sorbitol was lost. Oxygen consumption was restored by addition of Ca^{2+} and Mg^{2+} ions. Treatment with both EDTA and ammonium sulphate induced a further requirement. EDTA (2·5 mM) was added to 50 ml. of fresh bacterial extract (pH 7·1) and the mixture dialysed against water at 2° for 3 days. Then ammonium sulphate (250 mg./ml.) was dissolved in the extract and the pH brought to 9·0 with KOH. The precipitate was washed twice in ammonium sulphate solution (250 mg./ml., pH adjusted to 9), suspended in water and dialysed overnight. The activity, partly restored by addition of calcium and magnesium, was enhanced by another factor (Table 3). This 'activator' was prepared by heating (100° for 5 min.) a portion of the same bacterial extract which had been treated with EDTA and dialysed, but not treated with ammonium

Table 3. *Effect of calcium and magnesium ions and of an 'activator' on a preparation of the 'acid' enzyme previously treated with EDTA and ammonium sulphate*

Each Warburg flask contained 0·5 ml. of dialysed 'acid' enzyme preparation which had been treated with EDTA and ammonium sulphate (see text), 100 μmoles of tris-succinic acid buffer, pH 5·0, and 150 μmoles of mannitol (added from the side bulb after equilibration). Volume, 2·2 ml. Gas, air. Temp., 30°. Time, 1 hr. Inseal, 0·2 ml. of 10% (w/v) KOH.

Additions Ca²⁺ (10 µmoles)	Mg²⁺ (10 µmoles)	'Activator'* (0·5 ml. of solution)	O₂ uptake (µmoles)
–	–	–	0·1
–	–	+	0·1
+	+	–	0·5
+	–	+	3·4
–	+	+	8·3
+	+	+	11·1

* See text for the preparation of the 'activator' solution. Note: the original extract (0·5 ml.) consumed 20·5 μmoles of O_2 under the same conditions.

sulphate. The denatured protein was removed by filtration. While investigating the oxidation of cyclitols in extracts of *A. suboxydans* Franzl & Chargaff (1951) observed a similar effect due to a heat-stable factor present in the cells. The ions and the unidentified factor which restore the activity (measured manometrically or by anaerobic reduction of ferricyanide) may be involved in some part of the hydrogen- or electron-transporting mechanism other than the polyol dehydrogenase.

Cytochrome. The 'acid' enzyme preparations assume a pink tinge on addition of dithionite or an oxidizable polyol. The absorption spectrum of the oxidized pigment obtained by aeration shows a high maximum at 410 mμ., and the reduced form obtained by addition of 150 μmoles of mannitol has the high maximum at 420 mμ. and low maxima at 520 and 550 mμ. An approximate difference spectrum plotted in a Beckman spectrophotometer with the oxidized form of the pigment in the reference cell showed maxima at 420, 520 and 550 mμ. and minima at 405, 450 and 525 mμ. These characteristics correspond fairly well to the accurate steady state values which Smith (1954a, b) has determined in cell suspensions of *A. suboxydans*. Smith (1954b) considers that the pigment of *A. suboxydans* is a unique bacterial cytochrome.

Specificity of the 'acid' enzyme

Table 4 shows the specificity of the enzyme towards polyols. Erythritol, ribitol, D-arabitol, mannitol, sorbitol and 6-deoxy-L-gulitol are oxidized rapidly; xylitol, allitol, D-talitol, volemitol, perseitol and β-sedoheptitol are oxidized slowly; L-arabitol, D-gulitol, dulcitol, L-*gulo*-D-*gala*-heptitol and the cyclitols are not oxidized. With the exception of the negative response to the cyclitols, these findings agree with the known behaviour of *A. suboxydans* in cultures. When oxidation occurred the product was a reducing substance giving a positive resorcinol test. Phenylosazones were prepared from a few of the products (Table 4). Although the 'acid' enzyme may exist in different states of aggregation in the preparations described, the specificity of all preparations was the same.

The unfractionated bacterial extract has the ability to oxidize glycerol (cf. Hauge, King & Cheldelin, 1955), ethanol and *n*-, *sec.*- and *iso*-butanol. There was no increase of O_2 uptake in the presence of *tert.*-butanol, fructose, sorbose or succinate (Table 5).

Properties of the 'alkaline' enzyme

In some experiments the partially separated enzyme was used; in others the unfractionated bacterial extract was employed at pH 8, which is outside the active range of the 'acid' enzyme.

Table 4. *Substrate specificity of the 'acid' enzyme of* A. suboxydans

Each Warburg flask contained 1·0 ml. of enzyme (ammonium sulphate preparation), 100 μmoles of tris–succinic acid buffer, pH 5·0, 10 μmoles (each) of $CaCl_2$ and $MgCl_2$, 0·5 ml. of heated (100° for 5 min.) fresh bacterial extract and substrate. Volume, 2·2 ml. Gas, air. Temp., 30°. Time, 1 hr. Inseal, 0·2 ml. of 10 % (w/v) KOH.

Substrate	Quantity (μmoles)	O_2 uptake (μmoles)	Reduction test	Resorcinol test (Kulka, 1956)	Phenylosazone
None	—	0·0	Negative	Negative	Negative
Erythritol	150	17·3	Positive	Ketotetrose	—
Ribitol	150	6·2	Positive	Ketopentose	—
D-Arabitol	150	15·0	Positive	Ketopentose	—
L-Arabitol	150	0·0	Negative	Negative	—
Xylitol	150	0·4	Positive	Ketopentose	—
Allitol	40	1·4	Positive	Ketohexose	Allosazone, m.p. 159–161°
D-Mannitol	150	8·5	Positive	Ketohexose	Glucosazone, m.p. 208° (decomp.)
D-Gulitol	40	0·0	Negative	Negative	—
Sorbitol (L-gulitol)	150	6·4	Positive	Ketohexose	Idosazone, m.p. 160–164°
Dulcitol (galactitol)	150	0·0	Negative	Negative	—
D-Talitol	40	1·8	Positive	Ketohexose	—
6-Deoxy-L-gulitol	40	7·4	Positive	Deoxyketohexose	6-Deoxyidosazone, m.p. 168–172°
Perseitol (D-*manno*-D-*gala*-heptitol)	40	1·9	Positive	Ketoheptose	Perseulosazone, m.p. 194–197° (decomp.)
Volemitol (D-*manno*-D-*talo*-heptitol)	40	1·4	Positive	Ketoheptose	—
β-Sedoheptitol (L-*gulo*-D-*talo*-heptitol)	40	0·4	Positive	Ketoheptose	—
L-*gulo*-D-*gala*-Heptitol	40	0·0	Negative	Negative	—
meso-Inositol	150	0·0	—	Negative	—
(+)-Inositol	30	0·0	—	Negative	—
(−)-Inositol	30	0·0	—	Negative	—

Note: activity could not always be tested at high substrate concentrations because the supply of certain polyols was strictly limited. This applies also to Tables 7 and 9.

Table 5. *Oxidation of glycerol and butanol by an extract of* A. suboxydans

Each Warburg flask contained 0·2 ml. of dialysed bacterial extract, 10 μmoles of 0·1 M succinic acid–Na_2HPO_4 buffer, pH 5·0, and 150 μmoles of substrate (added from the side bulb after equilibration). Volume, 1·0 ml. Gas, air. Temp., 25°. Time, 1 hr. Inseal, 0·2 ml. of 10 % (w/v) KOH.

Substrate	O_2 uptake (μmoles)
None	0·0
Glycerol	7·2
n-Butanol	5·1
sec.-Butanol	10·0
*iso*Butanol	3·0
tert.-Butanol	0·0
Ethanol	4·6

Table 6. *Codehydrogenase specificity of the 'alkaline' enzyme*

Each Warburg flask contained 0·5 ml. of dialysed bacterial extract, 15 μmoles of sodium phosphate buffer, pH 8·0, 0·4 μmole of coenzyme and 150 μmoles of substrate. Volume, 2·7 ml. Gas, air. Temp., 25°. Time, 1 hr. Inseal, 0·2 ml. of 10 % (w/v) KOH.

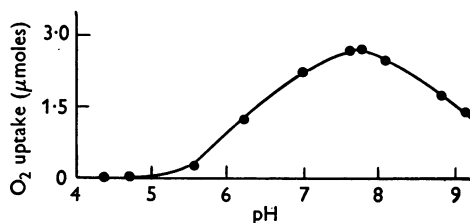| Coenzyme	O_2 uptake (μmoles) Mannitol	Sorbitol
None	0·4	0·9
DPN	5·5	6·9
TPN	0·8	1·3

Fig. 5. Effect of pH on the activity of the 'alkaline' enzyme from *A. suboxydans*. Each Warburg flask contained 1·0 ml. of 'alkaline' enzyme solution (sodium acetate preparation), 20 μmoles of succinic acid–K_3PO_4 buffer, 0·4 μmole of DPN, 1·6 μmoles of methylene blue and 150 μmoles of mannitol (added from the side bulb after equilibration). Volume, 2·8 ml. Gas, air. Temp., 25°. Time, 1 hr.

Requirement for DPN and a hydrogen carrier. At pH 8 the dialysed unfractionated extract takes up O_2 in the presence of mannitol or sorbitol if DPN is added (Table 1). When TPN is substituted for DPN the activity is slight (Table 6). After removal of the 'acid' enzyme and the associated carrier the 'alkaline' enzyme does not promote O_2 consumption until methylene blue (optimum concentration 10^{-4} M) is added.

Effect of pH. The pH–activity curve shows an optimum at pH 7·8 in the buffer used (Fig. 5).

Effect of HCN. The aerobic oxidation of polyols in the unfractionated extract at pH 7·8 is strongly inhibited by HCN (10^{-3}M), but O_2 uptake is not reduced by cyanide when the same oxidations are carried out with methylene blue as hydrogen carrier.

Substrate concentration. The rate of reaction catalysed by the 'alkaline' enzyme increases with substrate concentration to attain a maximum in the presence of approximately 0·15M substrate, but the Lineweaver–Burk plot is not linear with mannitol or sorbitol.

Substrate specificity of the 'alkaline' enzyme

Table 7 shows the activity of the 'alkaline' enzyme towards polyols measured by the rate of reduction of DPN. Xylitol, mannitol, sorbitol, β-sedoheptitol and L-*gulo*-D-*gala*-heptitol are oxidized rapidly; D- and L-arabitol, D-talitol, L-iditol and volemitol are oxidized slowly. Erythritol, ribitol, D-gulitol, dulcitol, D-iditol, L-rhamnitol, perseitol and the cyclitols are not oxidized. The products of oxidation accumulating in aerobic experiments were reducing substances giving positive resorcinol tests. The ketose obtained from mannitol or sorbitol gave typical phenylglucosazone crystals, m.p. 202–206° (decomp.). A manometric experiment with a sodium acetate preparation of the enzyme (2·0 ml. in 0·05M phosphate buffer, pH 7·8) showed no O_2 uptake in the presence of DPN (0·4 μmole), methylene blue (1·6 μmoles) and allitol, glycerol, ethanol, fructose or sorbose (150 μmoles).

The product of sorbitol oxidation was analysed chromatographically. Each of two Warburg flasks contained in 2·9 ml. of solution 2·0 ml. of 'alkaline' enzyme (ammonium sulphate preparation), 300 μmoles of sodium phosphate buffer, pH 7·8, 1·0 μmole of DPN, 1·6 μmoles of methylene blue and 600 μmoles of sorbitol. To one flask 60 μmoles of sorbose was also added. Oxygen uptake at 30° was 22 μmoles/6 hr. in each flask. The contents of the flasks were passed successively through columns of Amberlite IR-120 (H^+ form) and Amberlite IR-4B (HCO_3^- form) to remove protein, methylene blue and ions. The eluates were evaporated to dryness *in vacuo* at room temperature, the residues taken up in 0·2 ml. of water and subjected to descending paper chromatography at 25° with a solvent composed of water-saturated phenol containing 0·04% of 8-hydroxyquinoline (Block, 1950). The chromatograms were sprayed with an orcinol reagent (Bevenue & Williams, 1951), heated at 110° for 10 min. and the spots examined under ultraviolet light. The product from the flask which initially contained sorbitol gave a single spot with the same R_F value (0·52) as authentic fructose run concurrently, and the product from the flask which originally contained sorbitol and sorbose gave two

well-separated spots with the same R_F values as the reference spots due to authentic specimens of fructose (0·52) and sorbose (0·41) run concurrently.

Since sorbose is not oxidized, this experiment shows that the 'alkaline' enzyme converts sorbitol into fructose without forming a trace of sorbose detectable by the method used.

Reversibility. The oxidation of DPNH by D-xylulose, fructose, sorbose and perseulose is illustrated in Table 7 (cf. L-iditol dehydrogenase, McCorkindale & Edson, 1954).

Possibility of the 'alkaline' enzyme being an artifact. Since the 'alkaline' enzyme possessed mannitol-oxidizing power, it seemed possible that this enzyme might be a fragment which had been derived from the 'acid' enzyme during sonic treatment and

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Table 7. *Substrate specificity of the 'alkaline' enzyme of* A. suboxydans

Each cuvette contained 0·5 ml. of 'alkaline' enzyme solution (ammonium sulphate preparation), 460 μmoles of tris–succinic acid buffer, pH 7·8, 0·4 μmole of DPN or 0·01 μmole of DPNH and substrate as indicated. Volume, 3·0 ml. Temp., 20°.

Substrate	Quantity (μmoles)	Optical density (340 mμ.) Increase/9 min.
With DPN		
None	—	0·000
Erythritol	180	0·000
Ribitol	120	0·004
D-Arabitol	120	0·033
L-Arabitol	120	0·034
Xylitol	10	0·303
Mannitol	10	0·176
D-Gulitol	25	0·001
Sorbitol (L-gulitol)	10	0·184
Dulcitol	100	0·000
D-Talitol	200	0·039
D-Iditol	10	0·006
L-Iditol	12	0·079
L-Rhamnitol	100	0·007
Volemitol	20	0·056
Perseitol	30	0·000
β-Sedoheptitol	35	0·138
L-*gulo*-D-*gala*-Heptitol	30	0·163
meso-Inositol	100	0·001
(+)-Inositol	10	0·003
(−)-Inositol	10	0·005

		Initial decrease/min.
With DPNH		
None	—	0·003
D-Xylulose	10	0·170
D-Fructose	10	0·030
L-Sorbose	10	0·005
D-Tagatose	150	0·003
D-Mannoheptulose	10	0·003
D-Glucoheptulose	10	0·003
Perseulose	10	0·007

		Decrease/15 min.
None	—	0·044
L-Sorbose	10	0·075
Perseulose	10	0·105

had acquired a greater range of polyol-oxidizing power in the process. To test this possibility 'acid' enzyme that had been freed of 'alkaline' enzyme by sodium acetate treatment (see earlier) was subjected a second time to the action of the sonic disintegrator. The 'acid' enzyme preparation was also digested with various concentrations of trypsin at 25° for 1 hr. The 'alkaline' enzyme activity was not produced in any of these experiments.

'Alkaline' enzyme in the intact cells of Acetobacter suboxydans

Although *A. suboxydans* is normally cultivated in acid media and does not grow at pH 7·8, washed cells suspended in 0·01 M phosphate buffer, pH 7·5, consumed O_2 rapidly at 25° in the presence of sorbitol (150 μmoles). In the absence of substrate there was no O_2 uptake. During the oxidation of sorbitol 38 μmoles of O_2 were consumed in 1 hr. and 11·7 μmoles of fructose and 18·9 μmoles of sorbose accumulated in the medium. The experiment appears to show that both the 'alkaline' and 'acid' enzyme are functioning in the cells, the action of the latter predominating, although the external pH is higher than the pH at which the extracted 'acid' enzyme ceases to oxidize sorbitol to sorbose. If these facts are to be explained in terms of enzymic properties, it would seem that the cells are able actively to maintain an internal H^+ ion concentration exceeding that of the external medium. Alternatively, they may transform fructose into sorbose.

Polyol dehydrogenase activities of Candida utilis

Mannitol and sorbitol, but not dulcitol (Table 8), are oxidized by a washed suspension of *C. utilis*, the cells of which were grown in the glucose-containing medium of Schultz & Atkin (1947). The same effects are observed in suspensions of cells grown in a mannitol medium (see Methods). Ketoses do not accumulate in the suspending medium.

Dialysed cell-free extracts of *C. utilis* reduce DPN in the presence of polyols, the optimum pH for the reaction with mannitol being 9·4. The specificity of this polyol dehydrogenase preparation is illustrated in Table 9. Mannitol, sorbitol, xylitol and L-rhamnitol are oxidized rapidly; D-arabitol is oxidized slowly; erythritol, ribitol, L-arabitol, D- and L-iditol, dulcitol, *meso*-inositol and glycerol do not reduce DPN significantly at the concentrations used. Table 9 also contains a demonstration of reversibility: fructose oxidizes DPNH rapidly, sorbose slowly. Tagatose does not react.

Dialysed extracts of *C. utilis* do not consume O_2 in the presence of polyols unless DPN and methylene blue are added to the reaction mixture. The products accumulating are reducing substances which give ketose colour reactions with resorcinol. The product

of mannitol oxidation gave typical phenylglucosazone crystals, m.p. 208–209° (decomp.). The products of sorbitol oxidation were obtained from a Warburg flask in which 3 ml. of *C. utilis* extract was shaken at 30° in air with 300 μmoles of phosphate buffer, pH 7·8, 0·4 μmole of DPN, 1·6 μmoles of methylene blue and 500 μmoles of sorbitol. The O_2 uptake was 9·8 μmoles/12 hr. in the presence of sorbitol and negligible in its absence. At the end of the experiment the flask contents were deproteinized, concentrated by the method described for a similar experiment with the 'alkaline' enzyme of *A. suboxydans* (see above) and subjected to the same chromatographic analysis. The chromatogram showed two well-separated spots corresponding in colour and R_F values to those obtained from a

Table 8. Oxidation of hexitols by a washed suspension of C. utilis

Each Warburg flask contained 15 mg. of washed cells in 2·0 ml. of 0·9 % (w/v) sodium chloride solution, 200 μmoles of sodium phosphate buffer, pH 6·8, and substrate (added from the side bulb after equilibration). Volume, 2·4 ml. Gas, air. Temp., 30°. Time, 1·5 hr. Inseal, 0·2 ml. of 2N-NaOH.

Substrate	O_2 uptake (μmoles)
None	10·5
Mannitol, 200 μmoles	13·8
Sorbitol, 200 μmoles	11·7
Dulcitol, 180 μmoles	10·6

Table 9. Reversible DPN-linked polyol dehydrogenase activity in extracts of C. utilis

Each cuvette contained 1·0 ml. of *C. utilis* extract, 300 μmoles of glycine buffer, pH 9·0, either 0·4 μmole of DPN or 0·01 μmole of DPNH and substrate as indicated. Volume, 3·0 ml. Temp., 20°.

Substrate	Quantity (μmoles)	Change in optical density (340 mμ.)/9 min.
With DPN		
None	—	+0·009
Erythritol	200	+0·010
Xylitol	150	+0·140
Ribitol	300	+0·013
D-Arabitol	150	+0·095
L-Arabitol	300	+0·008
Mannitol	300	+0·172
Sorbitol	300	+0·150
D-Iditol	10	+0·009
L-Iditol	55	+0·008
Dulcitol	300	+0·013
L-Rhamnitol	300	+0·155
Glycerol	300	+0·008
meso-Inositol	300	+0·020
With DPNH		
None	—	−0·038
D-Fructose	300	−0·101
L-Sorbose	300	−0·052
D-Tagatose	300	−0·038

reference mixture of fructose and sorbose in equal proportions. The relative intensities of the spots indicated that fructose was the main product of oxidation. The formation of fructose *and* sorbose from sorbitol in *C. utilis* extract at pH 7·8 contrasts with the formation of fructose alone by the 'alkaline' enzyme of *A. suboxydans* under very similar conditions.

DISCUSSION

The first step in this study of the metabolism of polyhydric alcohols was the construction of a simple hypothesis to explain their occurrence in plants and their metabolism in animals and micro-organisms (Edson, 1953). The hypothesis assumed that a general reaction, polyol + DPN^+ ⇌ ketose + $DPNH + H^+$, was catalysed by three types of stereo-specific polyol dehydrogenases (L-iditol, D-mannitol and galactitol dehydrogenases); and it was based largely on the properties of rat-liver polyol dehydrogenase (Blakley, 1951; McCorkindale & Edson, 1954) and on the assumption that the behaviour of *A. suboxydans* in culture is due to the possession of D-mannitol dehydrogenase. Shaw (1954, 1956) has since demonstrated the existence of galactitol dehydrogenase in micro-organisms.

An examination of cell-free extracts from *A. suboxydans* has shown that the original hypothesis will not account for all the phenomena. The extracts contain at least two polyol dehydrogenase activities having different substrate specificities and pH optima, and apparently different cofactor requirements. The enzyme with a pH optimum about 5·5 is found in subcellular particles which can be sedimented by high-speed centrifuging. The particles also contain the cytochrome pigment peculiar to the organism. The methods used have failed to extract a soluble polyol dehydrogenase from the particles and to establish a requirement for DPN or TPN. The substrate specificity (Table 4) can be described by the Bertrand–Hudson rule (e.g. mannitol is oxidized to fructose and sorbitol to sorbose), and the enzyme is most active within the pH range that is optimum for the growth of the organism. It is proposed to call this enzyme the *Bertrand–Hudson enzyme* or *cytochrome-linked* D-*mannitol dehydrogenase* without prejudice to the possible intermediate links. Reversibility of its action has not been investigated. Although the polyol dehydrogenase activity is well defined, the complexity of the 'acid' enzyme preparation is revealed in its other enzymic properties (Table 5) such as the ability to oxidize glycerol (cf. King & Cheldelin, 1954), ethanol and three butanol isomers.

The second polyol dehydrogenase ('alkaline' enzyme) is soluble, and has a pH optimum about 7·8, a requirement for DPN and a different sub-strate specificity (Table 7). Mannitol is oxidized to fructose according to the Bertrand–Hudson rule, whereas the oxidation of sorbitol to fructose follows the rule for L-iditol dehydrogenase; and the failure to oxidize dulcitol excludes an application of the rule for galactitol dehydrogenase (Shaw, 1956). There is no configuration common to all the sub-strates oxidized and absent from all those not oxidized. While it is possible that the 'alkaline' enzyme is a single polyol dehydrogenase, the sub-strate specificity of which cannot be described by a simple rule, it is also possible that the preparations contain more than one species of DPN-linked dehydrogenase. A plausible but not entirely satisfactory explanation is the combination of a stable DPN-linked mannitol dehydrogenase with an enzyme of the L-iditol dehydrogenase type.

The DPN-linked polyol dehydrogenase activity found in extracts of *C. utilis* (Table 9) resembles that of the 'alkaline' enzyme from *A. suboxydans* but is not identical. The important common features are the oxidation of mannitol to fructose, the vigorous attack on xylitol and the failure to oxidize dulcitol. Sorbitol oxidation reveals a difference, since the product formed by the *C. utilis* extract is a mixture of fructose and sorbose. This fact suggests that extracts of *C. utilis* contain more than one DPN-linked polyol dehydrogenase.

The DPN-linked polyol dehydrogenases occurring in *A. suboxydans* and *C. utilis* differ from other members of this class (L-iditol dehydrogenase, galactitol dehydrogenase) in possessing the ability to attack mannitol vigorously. Mannitol dehydrogenase requiring DPN was discovered by Müller (1937) in cell-free extracts of brewer's yeast and distinguished from alcohol dehydrogenase. Regardless of the number of DPN-linked polyol dehydrogenases which may be responsible for the observed oxidations (Tables 4, 7 and 9), it is proposed to call the soluble enzyme which attacks mannitol *DPN-linked* D-*mannitol dehydrogenase*. This enzymic activity is widely distributed (McCorkindale, 1953; Edson, 1953; Shaw, 1956).

SUMMARY

1. Extracts of *Acetobacter suboxydans* contain a particulate dehydrogenase which catalyses the oxidation of polyols at pH 5 without addition of diphosphopyridine nucleotide (DPN). The substrate specificity corresponds to the Bertrand–Hudson rule. The enzyme is associated with particles containing cytochrome and is named cytochrome-linked D-mannitol dehydrogenase.

2. Extracts of *A. suboxydans* also contain soluble DPN-linked polyol dehydrogenase activity best developed about pH 8. The activity can be dissociated from the particles containing cytochrome.

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.

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Polyol Dehydrogenases

3. GALACTITOL DEHYDROGENASE AND D-IDITOL DEHYDROGENASE

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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