Biochem. J. (1974) **143**, 341–352
Printed in Great Britain

A Comparison of Wild-Type and Mutant Ribitol Dehydrogenases from *Klebsiella aerogenes*

By BRUCE D. BURLEIGH, Jr.,* PETER W. J. RIGBY† and BRIAN S. HARTLEY‡
Medical Research Council Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

(*Received* 20 *May* 1974)

A ribitol dehydrogenase (ribitol–NAD^+ oxidoreductase, EC. 1.1.1.56) having increased specificity and catalytic efficiency toward xylitol was isolated from mutant strains of *Klebsiella aerogenes*, which were selected for increased growth rate on xylitol over the ribitol dehydrogenase constitutive wild-type organism. 2. The mutant enzyme was purified to homogeneity and its general characteristics were compared with those of the previously purified wild-type enzyme. 3. Initial-velocity steady-state kinetic parameters were determined for both wild-type and mutant enzymes and the results compared. 4. The results are interpreted in terms of a model in which the mutant enzyme results from a small change of amino acid sequence, which affects both the stability and conformational equilibria of the molecule.

Klebsiella aerogenes grows on ribitol or D-arabitol as sole carbon and energy source, but not on xylitol or L-arabitol. Specific inducible dehydrogenases exist for ribitol and D-arabitol, but not for the other pentitols (Mortlock *et al.*, 1965). However, mutants that are constitutive for ribitol dehydrogenase (ribitol–NAD^+ oxidoreductase, EC 1.1.1.56) grow weakly on xylitol by using a side-specificity of this enzyme (Lerner *et al.*, 1964). The apparent K_m for xylitol at 1mM-NAD^+ is around 1M compared with 1mM for ribitol. Hence this enzyme can be considered a bad 'xylitol dehydrogenase', which limits the growth of the organism on this substrate (Wu *et al.*, 1968).

If the organism is grown on xylitol in continuous culture, the steady-state biomass within the chemostat vessel is controlled by the intracellular activity of this enzyme. Mutational improvements in the activity of the enzyme towards xylitol allow takeover by the 'evolvant' from the ancestral strain and are signalled by increases in the steady-state biomass within the vessel. We have described elsewhere a study of the evolution of the organism under this selective pressure (Hartley *et al.*, 1972; Hartley, 1974; Rigby *et al.*, 1974).

We have previously discussed the purification and some of the properties of the ribitol dehydrogenase-A from our ancestral strain of *Klebsiella aerogenes* (strain A) (Taylor *et al.*, 1974). The present paper reports the detailed steady-state kinetics of this enzyme with its 'natural' substrate, ribitol, and with two 'unnatural' substrates, xylitol and L-arabitol.

A mutant strain of *K. aerogenes* (strain B), isolated by Wu *et al.* (1968), contained a mutant ribitol dehydrogenase with increased activity towards xylitol. We have purified this enzyme (ribitol dehydrogenase-B) to homogeneity, both from strain B and from a derivative strain, BN11, that we have selected by growing mutagenized cultures of strain B on xylitol in a chemostat. This latter strain produces large amounts of ribitol dehydrogenase-B (Hartley, 1974). We compared the steady-state kinetics of this enzyme with those of ribitol dehydrogenase-A in order to determine which parameters reflect the increased xylitol activity and how the activities towards ribitol and L-arabitol are affected by the adaptation to a new substrate.

Materials and Methods

Reagents

The pentitol substrates were BDH reagent grade (BDH Chemicals Ltd., Poole, Dorset, U.K.), and were recrystallized from water solutions and dried to constant weight over P_2O_5 in a vacuum desiccator. NAD^+ [Boehringer Corp. (London) Ltd., London W.5, U.K.] was purified by the method of Dalziel (1963) and stored frozen in 0.1M-sodium–potassium phosphate buffer solution, pH7.0, for no longer than 2 weeks before use. BDH Aristar $(NH_4)_2SO_4$ was used in the enzyme purifications and

* Present address: Department of Biochemistry, The University of Texas M.D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston, Tex. 77025, U.S.A.

† Present address: Department of Biochemistry, Stanford University, Stanford, Calif. 94305, U.S.A.

‡ To whom reprint requests should be addressed, at the Department of Biochemistry, Imperial College, London SW7 2AZ, U.K.

BDH Aristar urea was used in the thiol titrations; other chemicals used were BDH AnalaR or other standard laboratory-reagent grade. Chemicals used in the polyacrylamide-gel-electrophoresis procedures were purchased from the Eastman Kodak Company, Eastman Organic Chemicals, Rochester, N.Y. 14650, U.S.A., as specifically prepared for that purpose.

Assay for ribitol dehydrogenase

Ribitol dehydrogenase activity was measured as the increase in E_{340} owing to the formation of NADH, by using a Gilford 2000 recording spectrophotometer. The assay mixtures had a total volume of either 1.0 or 3.0ml, and the measurements were made at 28°C in quartz cuvettes with a 1cm light-path. The mixtures contained 50mM-D-ribitol, 0.83mM-NAD^+ and 100mM-sodium–potassium phosphate, pH7.0. The unit of ribitol dehydrogenase activity under these conditions is the formation of 1.0μmol of NADH/min.

Assay for NAD^+

The NAD^+ content of purified NAD preparations was measured spectrophotometrically ($\varepsilon_{260} = 1.76 \times 10^4 M^{-1} \cdot cm^{-1}$) and by determining its quantitative reduction to NADH by glyceraldehyde phosphate dehydrogenase. In the latter case, samples of NAD^+ were added to 1cm cuvettes containing 3ml of 0.5mM-glyceraldehyde 3-phosphate, 5mM-sodium arsenate, 0.5μg of glyceraldehyde 3-phosphate dehydrogenase (Boehringer), 50mM-sodium pyrophosphate, pH8.5, at 28°C. This assay was linear with NAD^+ amounts up to about 0.15μmol, calculated by using $\varepsilon_{340} = 6.25 \times 10^3 M^{-1} \cdot cm^{-1}$. Only those preparations in which 'reducible NAD^+' was greater than 95% of 'total NAD^+' (calculated from ε_{260}) were used for kinetic studies.

Amino acid analyses and protein determination

Amino acid analyses were performed on a Locarte automatic amino acid analyser by using a single-column system. Samples were prepared as a routine by dialysing a portion of the protein against water, freeze-drying and hydrolysing the resulting product with 0.5ml of 6M-HCl at 105°C for 48h in an evacuated, sealed ignition tube. The HCl solution was thoroughly degassed under vacuum before sealing, and 10μl of 0.1M-phenol was added as a routine to protect the tyrosine from oxidation. In cases where the amino acid analysis was used as a protein assay, as with the thiol titrations, a portion of the solution was transferred directly to the hydrolysis tube without dialysis against water. The small amounts of buffer compounds thus present did not significantly affect the results. Protein determinations other than those by direct amino acid analysis were done by the method of Lowry *et al.* (1951) as modified by Miller (1959).

Purification of ribitol dehydrogenase-A from strain A

The enzyme was purified from cells grown in multiple 10-litre batch cultures of high enzyme-producing strains (strains A11 or A211 of Rigby *et al.*, 1974) by the method of Taylor *et al.* (1974).

Purification of ribitol dehydrogenase-B from strain B

This strain was an autotrophic revertant (*arg*$^+$, *gua*$^+$) of strain X2 of Wu *et al.* (1968), which was constitutive for ribitol dehydrogenase-B. Cells were grown, harvested and frozen as described by Taylor *et al.* (1974). All subsequent operations were carried out at 4°C in potassium phosphate buffers, pH7.0, containing 10mM-2-mercaptoethanol unless otherwise stated.

Frozen cells (1kg) were thawed into 2 litres of 10mM buffer, and the suspension was disrupted by two consecutive passes through a French pressure cell (Aminco) at 60–100MPa (600–1000kg/cm^2), into a vessel cooled at 0°C. The pressate was diluted with 2 litres of the above buffer and centrifuged at 23000*g* for 30min to remove debris. The supernatant plus wash was diluted with buffer to 6 litres (15mg of protein/ml), and 600ml of protamine sulphate (40mg/ml) was added with stirring. After 1h the precipitated nucleic acids were removed by centrifugation at 23000*g*.

$(NH_4)_2SO_4$ (1360g) was added slowly to the stirred supernatant (6 litres) to give 40% saturation. After 1h the precipitate was removed by centrifugation at 23000*g* for 30min. It contained 39% (2230units) of the ribitol dehydrogenase activity in the initial extract (5690units). The supernatant (6355ml) was adjusted to 55% saturation with $(NH_4)_2SO_4$ (590g). The resulting 40–55%-satd.-$(NH_4)_2SO_4$ precipitate was collected as above and found to contain 290% (16390 enzyme units) of the initial extract activity. A similar activation of the enzyme or removal of inhibitors was regularly observed at this step in pilot purifications of this enzyme, but never during purifications of ribitol dehydrogenase-A. Because of this activation step, calculations of yield and purification from the initial extract are meaningless.

The 40–55%-satd.-$(NH_4)_2SO_4$ precipitate was dissolved in 250ml of buffer containing 0.1mM-NAD^+, dialysed three times against 4 litres of the same buffer, and centrifuged at 23000*g* for 30min to remove an inactive flocculent precipitate. It was then loaded on to a column (40cm×4cm diam.) of Whatman DE 52 equilibrated with 10mM-buffer and eluted at 60ml/h with 20mM buffer (which removed inactive protein) followed by a step of 100mM buffer, which eluted most of the ribitol dehydrogenase activity. The active fractions (840ml) were concen-

Table 1. *Purification of ribitol dehydrogenase-B from 1 kg of Klebsiella aerogenes strain B cells*

For full details see the text.

Purification step	Total protein (g)	10^{-3} × Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	66	5.69	0.09	100
40–55%-satd.-$(NH_4)_2SO_4$ precipitate	24	14.16	0.59	50
DE-52 (phosphate-step elution)	1.64	8.56	5.2	30
DE-52 (phosphate-gradient elution)	0.50	4.37	8.7	15
Sephadex G-200	0.35	3.50	10.0	12
Preparative gel electrophoresis	0.17	2.58	15.4	9
Dialysis and centrifugation	0.13	5.67	43.6	20

trated to 155ml in an Amicon Diaflo apparatus with a UM-10 membrane, and diluted with water to 310ml to lower the ionic strength.

This active fraction was then rechromatographed on a column (60cm×3cm diam.) of DE52 equilibrated with 10mM buffer, eluted at 17ml/h with a linear gradient of 4 litres of 20–100mM buffer. The active fractions (600ml) were concentrated by ultrafiltration to 20ml and chromatographed at 12ml/h on a column (55cm×3.0cm diam.) of Sephadex G-200 in 100mM buffer containing 0.2mM-NAD^+. The active fractions were pooled and concentrated to 40ml by ultracentrifugation. NAD^+ was added to 5mM and the preparation was stored at 6°C for 24h. Analytical gel electrophoresis revealed the need for further purification.

Final purification was achieved by preparative polyacrylamide-gel electrophoresis in a Buchler Polyprep apparatus by using a 7% (w/v) gel (10cm) and a 5mM-Tris–37.5mM-glycine buffer, pH8.9. Glycerol (5ml) was added to 35ml of the above sample which was made 10mM in 2-mercaptoethanol before application to the gel column. The lower membrane compartment was eluted at 45ml/h with 0.1M-Tris–HCl–1mM-NAD^+–10mM-2-mercaptoethanol, pH8.1, the E_{280} in the effluent being monitored with an LKB Uvicord instrument. Fractions with ribitol dehydrogenase activity were pooled and concentrated by ultrafiltration. The purification is summarized in Table 1.

Purification of ribitol dehydrogenase-B from strain BN11

This strain, which produces about eight times more enzyme than strain B, was obtained by mild nitrosoguanidine mutagenesis of strain B followed by growth in a chemostat on xylitol (Hartley, 1974; Rigby *et al.*, 1974). Enzyme-purification steps were essentially the same as reported up to and including the DE-52 phosphate gradient step, except that 0.1mM-phenylmethanesulphonyl fluoride was included in all buffers. The active fractions from this were concentrated by ultracentrifugation and applied to a column (60cm×3.0cm diam.) of Sephadex G-150 equilibrated and eluted with 0.1M-Tris–HCl–10mM-2-mercaptoethanol–0.1mM-phenylmethanesulphonyl fluoride, pH7.5. The active fractions were pooled, concentrated by ultrafiltration and stored at 2°C after adding NAD^+ to 5mM. The purification is summarized in Table 2.

Polyacrylamide-gel electrophoresis

Samples of native enzymes (20–50μg) in 50μl of 0.1M-sodium phosphate–1M-glycerol, pH7, containing 5μl of 0.1% (w/v) Bromophenol Blue were applied to gels (1cm of 3% stacking gel and 6cm of 7.5% separating gel) in tubes (8cm×0.5cm internal diameter) run in the discontinuous Tris–HCl:Tris–glycine buffer system of Davis (1964). Electrophoresis was carried out at currents less than 2mA/tube. When the tracking dye reached the end of the gel, the contents were removed and stained either for protein or for ribitol dehydrogenase activity. For protein staining, the gel was immersed overnight in 1% (w/v) Naphthalene Black in 7% (v/v) acetic acid and destained electrophoretically in 7% (v/v) acetic acid. The activity stain is described by Taylor *et al.* (1974).

Electrophoresis in sodium dodecyl sulphate was carried out as described by Laemmli (1970) or in a slab-gel apparatus (Glenco Scientific Inc., Houston, Tex., U.S.A.) or by a modification of Laemmli's (1970) technique. In the last case the samples were applied in twelve channels in a 3% (w/v) polyacrylamide stacking gel moulded into a 10% separating gel (15cm×15cm×0.15cm) and run at 25mA for 5.5h. The slabs were stained with Coomassie Brilliant Blue R-250 and mobilities relative to the tracking dye, Bromophenol Blue, were calculated as described by Weber & Osborn (1969).

Titration of thiol groups with 5,5'-dithiobis-(2-nitrobenzoic acid)

The enzyme samples used were preincubated at 28°C for 1h in the presence of 10mM-mercaptoethanol–0.1mM-EDTA–0.1M-Tris–HCl, pH8.0. This treatment did not affect the activity of either enzyme. The samples were then freed of excess of thiol by gel filtration through small columns (8cm×1cm diam.) of Sephadex eluted with the same buffer minus

2-mercaptoethanol immediately before the titrations. The buffers used throughout the experiment were degassed under aspirator vacuum and saturated with O_2-free N_2. Enzyme concentrations were determined by amino acid analysis of portions of the samples used.

In the titrations of the native enzymes, the enzyme samples (20.5 and 29.8nmol of subunit of ribitol dehydrogenase-A and -B respectively) were pre-incubated at 28°C in 3ml quartz cuvettes in an initial volume of 2.5ml containing 0.1mM-EDTA–0.1M-Tris–HCl, pH8.0, for 10min. Batches (20μl) of 0.5mM-5,5′-dithiobis-(2-nitrobenzoic acid) in 0.1M-Tris–HCl, pH8.0, were added sequentially and the E_{412} was measured 10min after each addition against a blank containing all the same components except enzyme. The catalytic activity was measured after each addition by taking a 5μl sample for assay; it remained constant at the initial value until about 2 thiol groups/tetramer had reacted, after which it fell rapidly to zero for both enzymes.

The thiol titrations in the presence of urea were done in a similar manner, except that 8.0M-urea was included in the cuvette solutions; 17.1 and 14.9nmol of subunit of ribitol dehydrogenase-A and -B respectively were used for these titrations. Both enzymes were inactive in the urea solutions. Final readings were made 1h after the last addition of reagent and were stable within a reasonable period, corresponding to a reaction of 3.7 groups/tetramer in native ribitol dehydrogenase-A and 6.7 groups/tetramer in native ribitol dehydrogenase-B. In 8M-urea, as expected from the amino acid analyses, 7.7 groups in the A enzyme and 8.3 groups in the B enzyme reacted rapidly with low concentrations of the reagent.

Treatment of kinetic data

All rate measurements were expressed as absolute velocities, which were normalized with respect to amount of active enzyme from the concomitant standard ribitol assays by using maximum specific activities of 91units/mg of ribitol dehydrogenase-A and 44units/mg of ribitol dehydrogenase-B. Preliminary manual plots of the data appeared to fit the forward reaction equation (eqn. 1) for a ternary-complex mechanism (Cleland, 1963*a*,*b*), as is the case for most nicotinamide nucleotide-linked dehydrogenases:

$$v_i = \frac{V_f}{1 + \frac{K_P}{[P]} + \frac{K_{NAD}}{[NAD^+]} + \frac{K_{P,NAD}}{[P][NAD^+]}} \quad (1)$$

K_P and K_{NAD} are the binary-complex saturation constants for the penitol (P) and nicotinamide nucleotide (NAD^+) substrates, respectively, $K_{P,NAD}$ is the ternary-complex constant, v_i is the (measured) initial velocity, and V_f is the maximum forward velocity, which is normalized with respect to enzyme concentration. At fixed NAD^+ concentrations, eqn. 1 can conveniently be expressed as:

$$\frac{1}{v_i} = \frac{K_{app.,P}}{V_{app.,P}} \cdot \frac{1}{[P]} + \frac{1}{V_{app.,P}} \quad (2)$$

where

$$\frac{1}{V_{app.,P}} = \frac{K_{NAD}}{V_f} \cdot \frac{1}{[NAD^+]} + \frac{1}{V_f} \quad (3)$$

and

$$\frac{K_{app.,P}}{V_{app.,P}} = \frac{K_{P,NAD}}{V_f} \cdot \frac{1}{[NAD^+]} + \frac{K_P}{V_f} \quad (4)$$

Similar equations can be generated for various NAD^+ concentrations at fixed pentitol concentrations.

To obtain estimates of the various kinetic constants the data were plotted manually in primary plots (eqn. 2) and secondary plots (eqns. 3 and 4) as suggested by Florini & Vestling (1957). Up to 10% (the worst nine of 96 measurements, counting duplicate measurements of each point) of the measurements in each experiment, as judged from the manual plots, were discarded, and the remaining measurements were fitted to the rectangular hyperbola (eqn. 5) by using the LABCAL program devised by Furlong (1972). This program uses weighted averaging methods which give greater weight to the middle concentration range of measurements, where inherent errors in the measurements are least.

$$(V_{app.,P} - v_i)(K_{app.,P} + [P]) = V_{app.,P} \cdot K_{app.,P} \quad (5)$$

and

$$(V_{app.,NAD} - v_i)(K_{app.,NAD} + [NAD^+]) = V_{app.,NAD} \cdot K_{app.,NAD}$$

The primary constants thus obtained were analysed according to eqns. 3 and 4 in double-reciprocal plots against their corresponding substrate concentrations by linear regression analysis, by using an Olivetti programmable calculator, to obtain estimates of the true K_P, K_{NAD} and V_f as expressed in eqn. 1. Estimates of the ternary-complex constant $K_{P,NAD}$, were calculated from the abscissae of the computer-derived primary-plot intersection points at fixed $[NAD^+]$ (Abscissa$_1$) or at fixed [P] (Abscissa$_2$) by eqn. 6 (Florini & Vestling, 1957):

$$K_{P,NAD} = \frac{-K_{NAD}}{\text{Abscissa}_1} = \frac{-K_P}{\text{Abscissa}_2} \quad (6)$$

In cases where these intersection points differed significantly, the average value of the abscissae was used.

Experimental

Purification and properties of ribitol dehydrogenase-B

The purification of the enzyme from strain B is summarized in Table 1. As discussed in the Materials

Table 2. *Purification of ribitol dehydrogenase-B from 1 kg of Klebsiella aerogenes strain BN11 cells*

For full details see the text.

Purification step	Total protein (g)	10^{-3} × Total activity (units)	Specific activity (units/mg)	Purification	Yield (%)
Crude extract	131	360	2.8	1.0	100
40–55%-satd.-$(NH_4)_2SO_4$ precipitate	62	190	3.0	1.1	52
DE-52 (phosphate step elution)	7.4	135	18.4	5.6	37
DE-52 (phosphate-gradient elution)	2.8	40	13.7	4.9	11
Sephadex G-150	1.0	25	25.2	9.0	7

and Methods section, the apparent activation in the 40–55%-satd.-$(NH_4)_2SO_4$ precipitate makes calculations of yields relative to the crude extract rather meaningless, but a yield of 50% has been guessed at this step for comparisons with Table 2.

After the preparative polyacrylamide-gel-electrophoresis step, the enzyme appeared 'pure' by the criterion of analytical gel electrophoresis, giving only a closely spaced triplet of bands each of which stained for ribitol dehydrogenase activity (see below). However, a yellow-orange pigment was present which was removed by overnight dialysis at 2°C against 0.1M-sodium phosphate–5mM-NAD–10mM-2-mercaptoethanol, pH7.0. Analytical gel electrophoresis showed an unchanged triplet of active bands, but the specific enzyme activity had greatly increased (Table 1). Further incubation of the enzyme with ribitol, NAD^+ or 2-mercaptoethanol in various combinations did not change the specific ribitol dehydrogenase activity.

A much larger amount of enzyme was obtained from the 'superproducer' strain BN11 (Table 2). This enzyme gave only a single band on analytical gel electrophoresis, since phenylmethanesulphonyl fluoride was present throughout the purification. However, the specific enzyme activity was lower than that for the previous preparation and further purification steps or incubation with ligands or thiols failed to increase it.

Stability and gel electrophoresis of ribitol dehydrogenase-A and -B

The mutant B enzyme was found to be much less stable than the wild-type A enzyme. Freezing and thawing of both enzymes in phosphate buffer caused 30–40% inactivation of the mutant enzyme, but only 5–8% for the wild-type. Ribitol dehydrogenase-A can be stored frozen for months without any greater loss of activity, but the B enzyme was inactive after 2 months of such storage. Storage in Tris buffers showed no change relative to phosphate buffers. When both enzymes were stored at 2°C under similar conditions (10mg of protein/ml–0.1M-Tris–HCl–10mM-2-mercaptoethanol–1mM-NAD^+–0.1mM-phenylmethanesulphonyl fluoride, pH7.0),

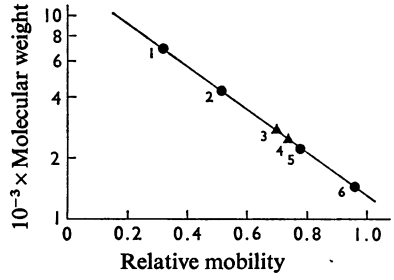

Fig. 1. *Molecular weight of ribitol dehydrogenase-A and -B by slab-gel electrophoresis in sodium dodecyl sulphate*

Mobilities of the enzymes (4, ribitol dehydrogenase-A; 3, ribitol dehydrogenase-B) and of marker proteins: bovine serum albumin (1), ovalbumin (2), bovine trypsin (5) and ribonuclease (6) are expressed relative to that of Bromophenol Blue.

the B enzyme showed a linear decay of activity corresponding to a half-life of 10 days, compared with 70 days for the A enzyme. Storage at −20°C in 0.1M-sodium phosphate–5mM-NAD^+–10mM-2-mercaptoethanol–10% (w/v) glycerol, pH7.0, was found to be optimal, but even so the enzyme was completely inactive after 3 months.

The triplet of active bands observed after gel electrophoresis of the B enzyme had already been observed in several preparations of the A enzyme (Taylor *et al.*, 1974). When such preparations of 'triplified' A enzyme and B enzyme were mixed and run on the same gel, no significant difference in their mobilities could be detected (0.37, 0.40 and 0.44 relative to the Bromophenol Blue tracking dye). In crude extracts (with the activity strain) and in freshly purified preparations from strains A and BN11, the slower band (mobility 0.37) was the stronger and sometimes the only band, with more of the two faster bands appearing on aging of the preparation or as the purification proceeded. It was therefore likely that the phenomenon was due to 'nicking' of the native structure by proteolytic enzyme, and that inhibitors of serine proteinases such as phenylmethanesulphonyl fluoride would prevent this.

Table 3. *Amino acid analysis of carboxymethylated ribitol dehydrogenase-B*

Best values for threonine and serine are obtained by extrapolation to zero time and those for valine and isoleucine are maximum values found in the time-course of hydrolysis. The suggested value for aspartic acid allows for some contamination by methionine sulphoxide. The analysis of ribitol dehydrogenase-A is from Taylor *et al.* (1974). Abbreviation: CmCys, carboxymethylcysteine.

Amino acid	Carboxymethylated ribitol dehydrogenase-B (residues/27000 daltons)					Carboxymethylated ribitol dehydrogenase-A (residues/27000 daltons)
	Hydrolysis time					
	12h	24h	72h	96h	Best value	
CmCys	1.1	1.4	1.5	1.5	2	2
Asp	25.6	25.2	26.0	25.4	25	24
Thr	10.3	10.0	9.6	9.4	11	11
Ser	13.5	12.6	11.3	11.1	14	15
Glu	21.7	21.4	21.7	21.6	22	21
Pro	10.9	10.8	11.7	11.7	11	20
Gly	19.4	19.6	19.7	19.7	20	20
Ala	30.0	30.0	30.0	30.0	30	29
Val	23.3	24.6	29.6	28.4	31	33
Met	5.2	5.2	5.2	5.2	6	6
Ile	11.3	12.9	14.6	14.4	15	15
Leu	26.8	26.9	27.8	26.8	27	27
Tyr	2.7	3.0	3.1	3.2	3	3
Phe	6.6	6.7	6.9	6.9	7	7
Lys	8.7	8.7	9.2	8.6	9	10
His	4.2	4.3	4.5	4.0	5	5
Arg	10.2	10.4	11.3	10.5	11	11
Trp	—	—	—	—	—	3

For this reason, 0.1mM-phenylmethanesulphonyl fluoride has been included in all subsequent purifications of ribitol dehydrogenase, and 'triplification' has seldom been observed since.

Molecular weight and amino acid analysis

Gel electrophoresis of ribitol dehydrogenase-A or -B together with known protein markers on slabs of polyacrylamide in the presence of sodium dodecyl sulphate gave apparent molecular weights for the monomers of 25000 for the A enzyme and 27000 for the B enzyme (Fig. 1). When the two enzymes are mixed they clearly separate on these slab gels.

However, we are not at present prepared to conclude that ribitol dehydrogenase-B is larger than ribitol dehydrogenase-A. From amino acid analysis and peptide 'maps', equilibrium sedimentation of the native holoenzyme or of the subunit in 5M-guanidinium chloride and Sephadex-gel filtration of the holoenzyme, Taylor *et al.* (1974) concluded that the molecular weight of the A enzyme was 27000. The complete amino acid sequence of the A enzyme has subsequently been determined (C. H. Moore, S. S. Taylor, M. J. Smith & B. S. Hartley, unpublished work) giving a mol.wt. of 26630 for the monomeric chain. Taylor *et al.* (1974) also observed that the A enzyme gave an anomalously low mol.wt. of 25000 on polyacrylamide-gel electrophoresis in sodium dodecyl sulphate, but we cannot assume that the B enzyme must be equally anomalous. There is no significant difference between the amino acid analyses of the A and B enzymes compared on the assumption of mol.wt. 27000 for both (Table 3) and the fragments produced by CNBr cleavage of both enzymes give identical patterns after slab-gel electrophoresis in the presence of sodium dodecyl sulphate. For these reasons, a mol.wt. of 27000 has been assumed for the monomer of both enzymes throughout this paper. It will be interesting to know the sequence changes in the B enzyme that are responsible for suppressing its anomalous behaviour on gel electrophoresis in sodium dodecyl sulphate.

Thiol groups of wild-type and mutant enzymes

Fig. 2 shows the results of titration of the thiol groups of both enzymes with 5,5'-dithiobis-(2-nitrobenzoic acid). In 8M-urea, both thiol groups in each subunit are completely exchanged at a 5,5'-dithiobis-(2-nitrobenzoic acid) concentration of 40μM, 1.93 groups/monomer for ribitol dehydrogenase-A and 2.07 groups/monomer for ribitol dehydrogenase-B. In native A enzyme only two of the eight thiol groups in the tetramer have exchanged at 40μM-5,5'-dithiobis-(2-nitrobenzoic acid), and there was no change in enzyme activity up to this point. A further two groups

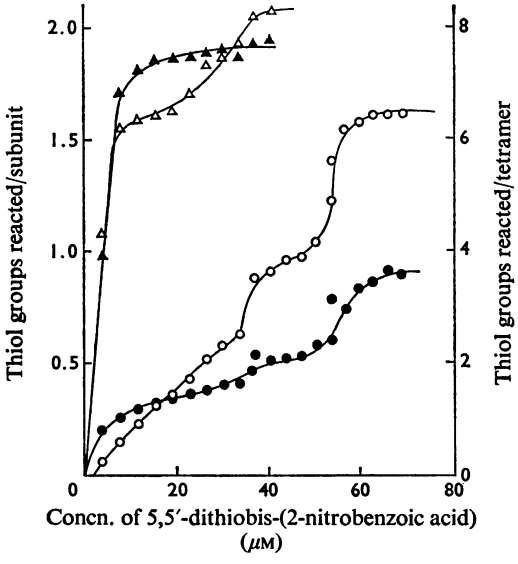

Fig. 2. *Titration of ribitol dehydrogenase-A and -B with 5,5'-dithiobis-(2-nitrobenzoic acid) in the presence or absence of* 8M-*urea*

For the native enzymes, the number of thiol groups that have reacted per tetramer (110000daltons) of ribitol dehydrogenase-A (●; initial concentration 2.05μM, final 1.78μM) or ribitol dehydrogenase-B (○; initial concn. 2.97μM, final 2.58μM) are plotted against the concentration of 5,5'-dithiobis-(2-nitrobenzoic acid) added. In 8M-urea, the number of thiol groups/subunit (27000daltons) of ribitol dehydrogenase-A (▲; initial concn. 1.71μM, final 1.58μM) or ribitol dehydrogenase-B (△, initial concn. 1.49μM, final 1.38μM) are shown.

seem to exchange in the range 40–70μM-5,5'dithiobis-(2-nitrobenzoic acid) and the enzyme activity falls to zero in the process. In native B enzyme, two groups/tetramer can be titrated without loss of activity in the range 0–30μM-5,5'-dithiobis-(2-nitrobenzoic acid), and the curves suggest titration of further pairs of groups in a stepwise fashion up to a maximum of 6.4 groups/tetramer at 70μM-5,5'-dithiobis-(2-nitrobenzoic acid).

Steady-state kinetics

The extent of selective advantage for growth on xylitol of the mutant enzyme ribitol dehydrogenase-B over the wild-type enzyme ribitol dehydrogenase-A can be expressed in terms of its steady-state kinetic parameters for xylitol as substrate. Fig. 3 shows the fit of the experimental points to the computed slopes and Fig. 4 shows the secondary plots of the slopes and intercepts from which the constants in Table 4 were calculated.

It is pertinent to ask whether the improvement of the B enzyme relative to the wild-type enzyme towards xylitol as substrate has been at the expense of its activity towards the 'natural' substrate ribitol? Fig. 5 shows the primary plots and Fig. 6 the secondary plots for the activity of both enzymes towards ribitol.

L-Arabitol is another 'unnatural' substrate for the wild-type enzyme. We can ask to what extent the improvement in xylitol specificity that has been selected in strain B of *Klebsiella aerogenes* is reflected in the activity of the B-enzyme towards a non-selected 'unnatural' substrate? Fig. 7 shows the primary data for both enzymes towards L-arabitol, and the secondary plots (Fig. 8) were used to calculate the constants shown in Table 4.

It may be noted in Table 4 that the saturation constants of both ribitol dehydrogenase-A and -B for xylitol, and to a lesser extent L-arabitol, are much higher than the concentrations of pentitol substrate, which are likely to be found under normal physiological conditions *in vivo*. It is therefore desirable, if these parameters are to have biological meaning as a measure of evolutionary adaptation, to examine the saturation behaviour at lower xylitol concentrations and show that it is consistent with the kinetic parameters which were determined, of necessity for accurate measurement, over a higher concentration range which spanned the K_P values. Figs. 9(*a*) and 9(*b*) are primary and secondary plots of data obtained from 15mM- to 200mM-xylitol for ribitol dehydrogenase-A; these plots indicate clearly that the kinetic behaviour of the enzyme at the lower, more physiological xylitol concentrations is consistent with that at the higher concentrations from which the parameters in Table 4 were determined. Similar studies with equivalent results were done with ribitol dehydrogenase-B.

Discussion

This work is part of a major study to monitor the evolutionary steps that occur in an enzyme that is under selective pressure to change its specificity (Hartley *et al.*, 1972; Hartley, 1974; Rigby *et al.*, 1974). At spontaneous mutation rates or under conditions of mild mutagenesis, the response of *Klebsiella aerogenes* to growth on xylitol in a chemostat, in which the activity of ribitol dehydrogenase is growth-limiting, is to select mutants that make large quantities of wild-type enzyme (Rigby *et al.*, 1974). These events are frequently due to multiplication of the structural gene for the wild-type enzyme. Under severe mutagenesis, however, evolutionary steps that produce enzymes with improved specificity for xylitol predominate (Wu *et al.*, 1968; J. M. Dothie & B. S. Hartley, unpublished work).

In order to describe and distinguish the enzymic properties of these 'evolvant' enzymes, a careful

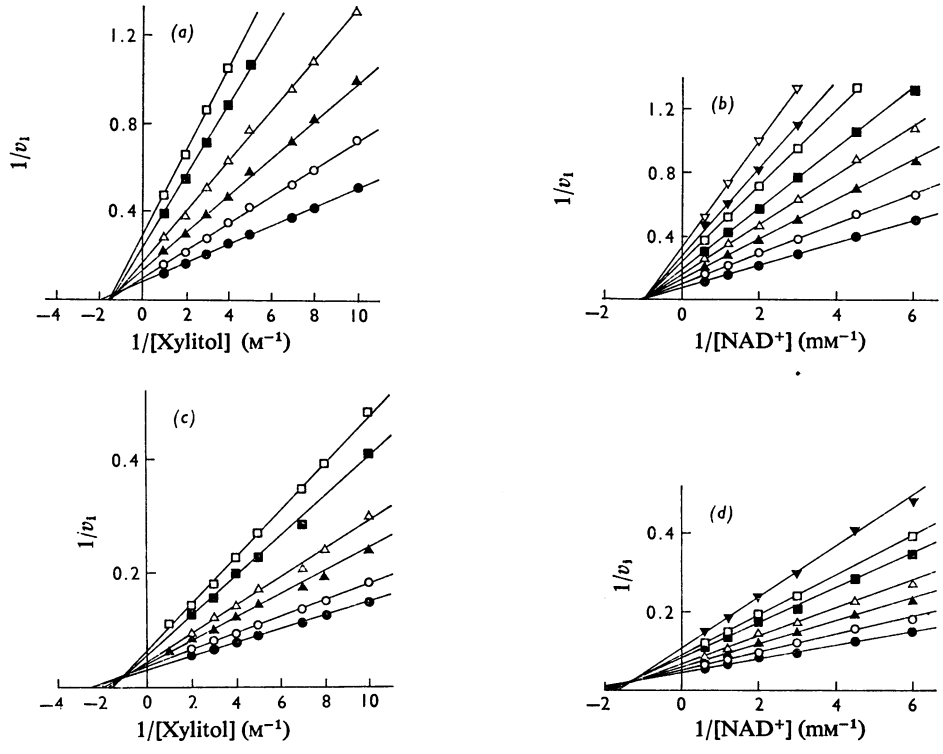

Fig. 3. *Initial velocities for ribitol dehydrogenase-A and -B with xylitol*

For details see the text. Units of v_i are μmol of NADH/min (at 28°C, pH 7.0) per mg of enzyme. (*a*) Ribitol dehydrogenase-A: [NAD$^+$] (mM): ●, 1.67; ○, 0.833; ▲, 0.500; △, 0.333; ■, 0.222; □, 0.167. (*b*) Ribitol dehydrogenase-A: [xylitol] (mM): ●, 1000; ○, 500; ▲, 333; △, 250; ■, 200; □, 143; ▼, 125; △, 100. (*c*) Ribitol dehydrogenase-B: [NAD$^+$] (mM): ●, 1.67; ○, 0.833; ▲, 0.500; △, 0.333; ■, 0.222; □, 0.167. (*d*) Ribitol dehydrogenase-B: [xylitol] (mM): ●, 500; ○, 333; ▲, 250; △, 200; ■, 143; □, 125; ▼, 100.

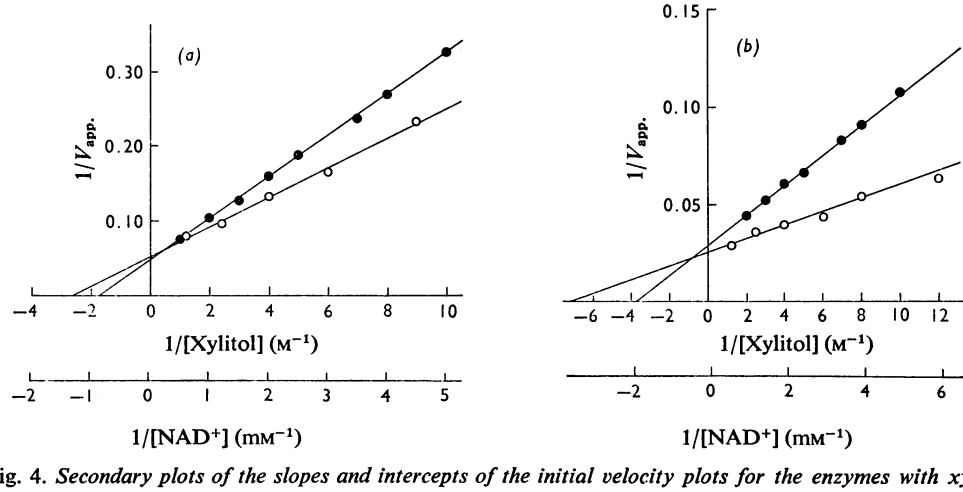

Fig. 4. *Secondary plots of the slopes and intercepts of the initial velocity plots for the enzymes with xylitol*

For details see the text. Units of $V_{app.}$ are μmol of NADH/min (at 28°C, pH 7.0) per mg of enzyme. (*a*) Ribitol dehydrogenase-A; (*b*) ribitol dehydrogenase-B. ●, Xylitol; ○, NAD$^+$.

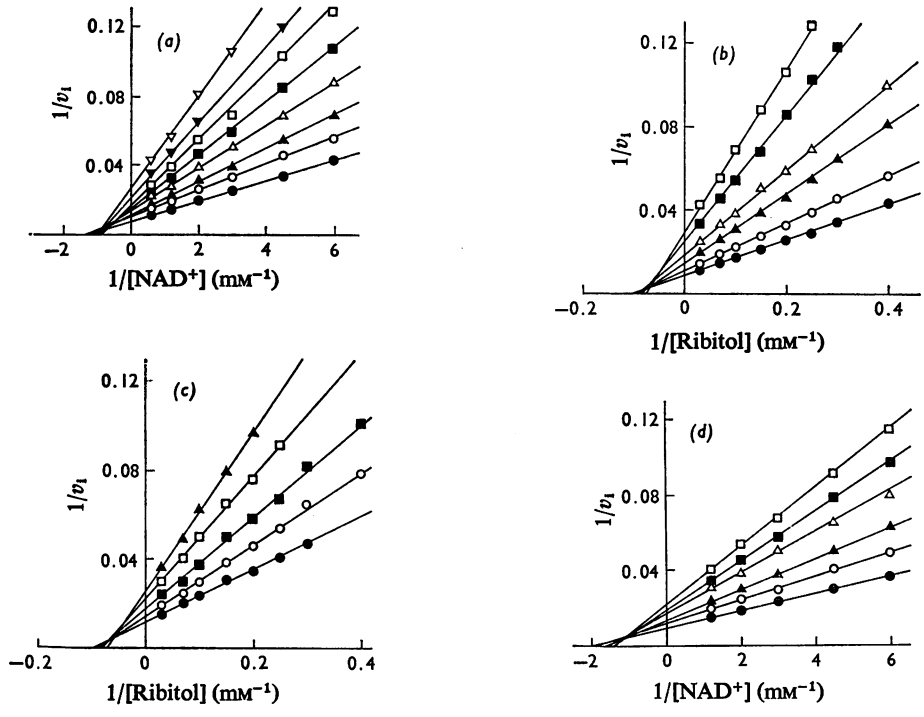

Fig. 5. *Initial velocities for ribitol dehydrogenase-A and -B with ribitol*

For details see the text. Units of v_1 are μmol of NADH/min (at 28°C, pH 7.0) per mg of enzyme. (*a*) Ribitol dehydrogenase-A: [NAD^+] (mM): ●, 1.67; ○, 0.833; ▲, 0.500; △, 0.333; ■, 0.222; □, 0.167. (*b*) Ribitol dehydrogenase-A: [Ribitol] (mM): ●, 33.3; ○, 14.3; ▲, 10.0; △, 6.67; ■, 5.00; □, 4.00; ▼, 3.33; ▽, 2.50. (*c*) Ribitol dehydrogenase-B: [NAD^+] (mM): ●, 0.833; ○, 0.500; ■, 0.333; □, 0.222; ▲, 0.167. (*d*) Ribitol dehydrogenase-B; [Ribitol] (mM): ●, 33.3; ○, 14.3; ▲, 10.0; △, 6.67; ■, 5.00; □, 4.00.

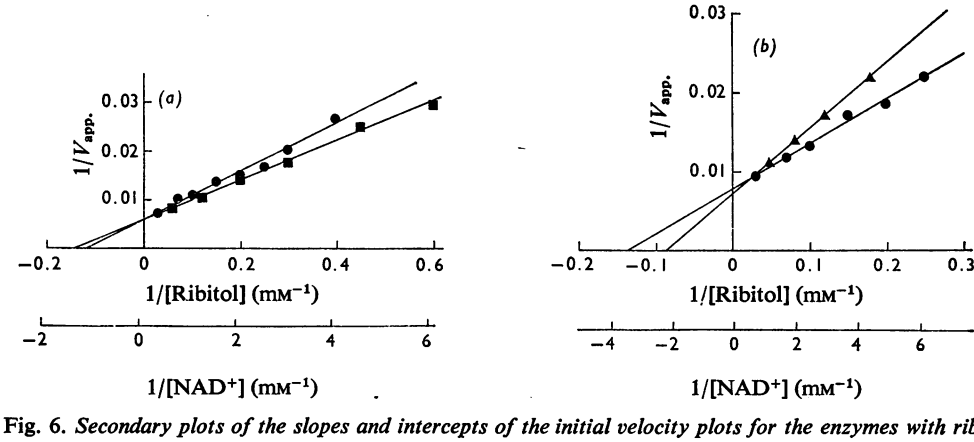

Fig. 6. *Secondary plots of the slopes and intercepts of the initial velocity plots for the enzymes with ribitol*

For details see the text. Unit sof $V_{app.}$ are μmol of NADH/min (at 28°C, pH 7.0) per mg of enzyme. (*a*) Ribitol dehydrogenase-A; (*b*) ribitol dehydrogenase-B. ●, Ribitol; ■, NAD^+.

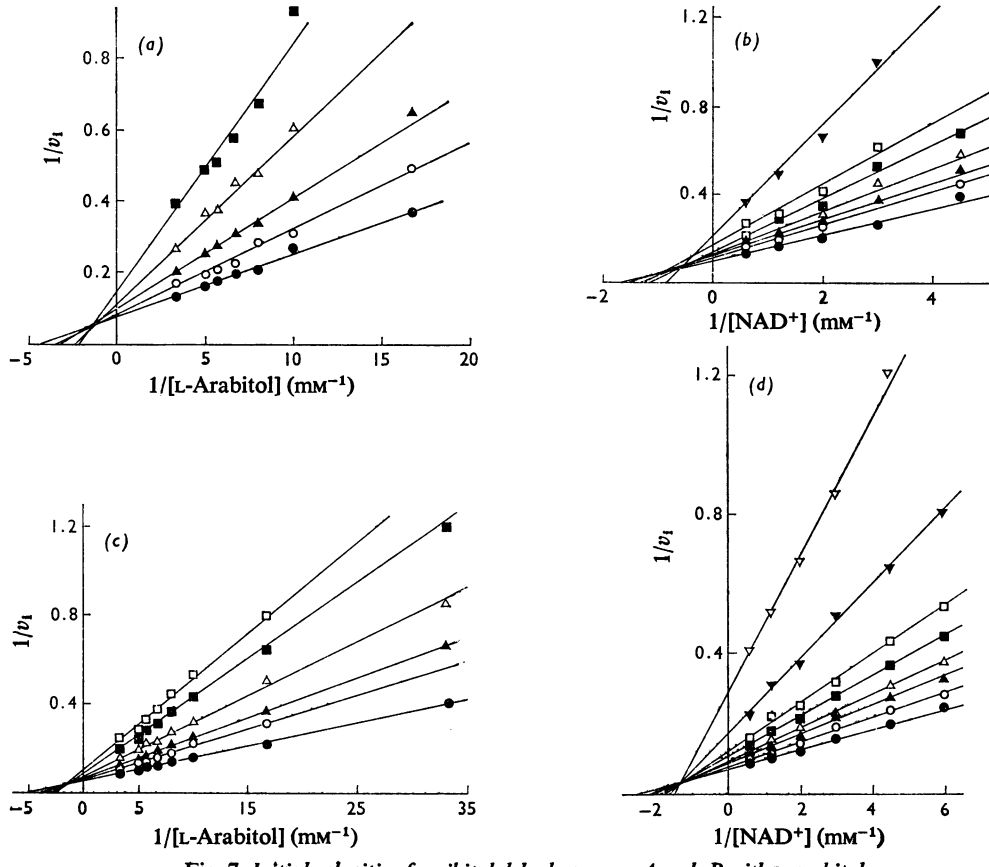

Fig. 7. *Initial velocities for ribitol dehydrogenase-A and -B with* L-*arabitol*

For details see the text. Units of v_1 are μmol of NADH/min (at 28°C, pH 7.0) per mg of enzyme. (*a*) Ribitol dehydrogenase-A: [NAD$^+$] (mM): ●, 1.67; ○, 0.833; ▲, 0.500; △, 0.333; ■, 0.222. (*b*) Ribitol dehydrogenase-A: [L-Arabitol] (mM): ●, 300; ○, 200; ▲, 175; △, 150; ■, 125; □, 100; ▼, 60. (*c*) Ribitol dehydrogenase-B: [NAD$^+$] (mM): ●, 1.67; ○, 0.833; ▲, 0.500; △, 0.333; ■, 0.222; □, 0.167. (*d*) Ribitol dehydrogenase-B: [L-Arabitol] (mM): ●, 300; ○, 200; ▲, 175; △, 150; ■, 125; □, 100; ▼, 60; ▽, 30.

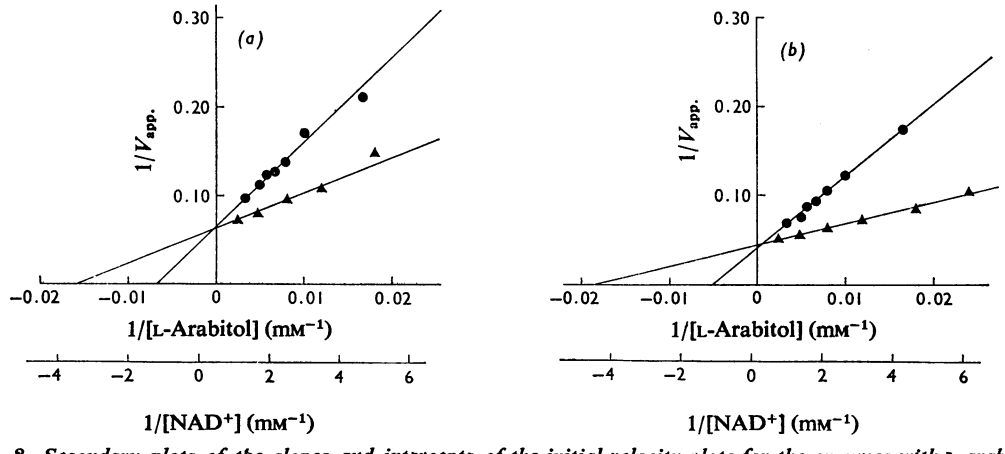

Fig. 8. *Secondary plots of the slopes and intercepts of the initial velocity plots for the enzymes with* L-*arabitol*

For details see the text. Units of $V_{app.}$ are μmol of NADH/min (at 28°C, pH 7.0) per mg of enzyme. (*a*) Ribitol dehydrogenase-A; (*b*) ribitol dehydrogenase-B. ●, L-Arabitol; ▲, NAD$^+$.

study of their steady-state kinetic parameters is necessary. For this purpose the enzymes must be purified to homogeneity. Convenient methods have been described for the wild-type enzyme (Taylor *et al.*, 1974), and purification of a mutant enzyme with increased xylitol activity is described above. Ribitol dehydrogenase-B proved to be much less stable than ribitol dehydrogenase-A, so the maximum velocities quoted in Table 4 are likely to be the minimum values.

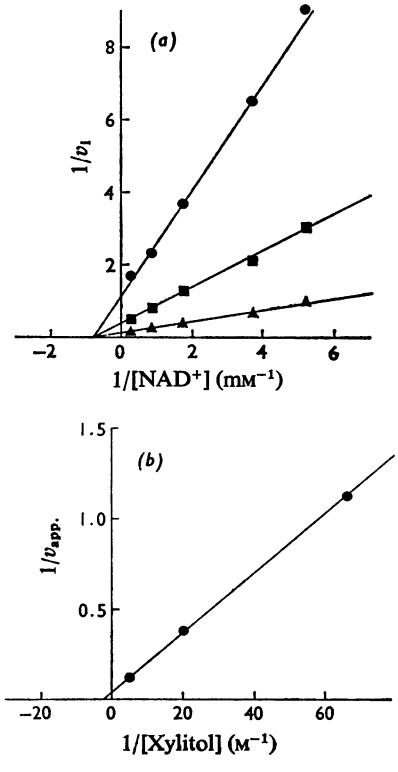

Fig. 9. *Kinetics of ribitol dehydrogenase-A at low concentrations of xylitol*

For details see the text. (*a*) Initial velocities Units of v_i are μmol of NADH/min (at 28°C, pH7.0) per mg of enzyme: [xylitol] (mM): 15 (●), 50 (■), and 200 (▲). (*b*) Secondary plot of the slopes and intercepts for the initial-velocity plot of Fig. 9(*a*).

The maximum velocity of the wild-type enzyme on xylitol is only 12% of that on ribitol, but in the mutant enzyme the maximum velocity for xylitol has doubled without any significant loss of activity for ribitol. For the other 'unnatural' substrate, L-arabitol, the catalytic activity of the wild-type enzyme is only 9% of that for ribitol, but selection for xylitol activity has significantly increased the activity for this non-selected 'unnatural' substrate in the mutant enzyme.

A large improvement in binding of xylitol to the mutant enzyme is also observed. There is a 2–4-fold increase in affinity of xylitol for the enzyme–NAD^+ complex (K_P) or of NAD^+ to the enzyme–pentitol complex (K_{NAD}), and the ternary-complex constant ($K_{P,NAD}$) reflects a similar increase in affinity. Yet once again there appears to be a negligible decrease in affinity of ribitol for the B enzyme, and the binding constants for L-arabitol show negligible change.

We tentatively interpret these changes in terms of a model (Fig. 10) in which ribitol, xylitol and L-arabitol bind to slightly different conformations of the ribitol dehydrogenase subunits. In the wild-type enzyme, ribitol dehydrogenase-A, the equilibria would be predominantly in favour of the ribitol conformation (Rb), but the mutation in ribitol dehydrogenase-B would slightly favour the xylitol conformation (Xy) without significantly affecting the proportions of Rb- and Ab-conformations in solution.

The models in Fig. 10 depict asymmetric tetramers of identical subunits. We lean to this hypothesis in order to explain the phenomenon of 'triplet' bands on gel electrophoresis caused by proteolysis of both A- and B-enzymes. If identical proteolytic cleavages could occur in each subunit of the tetramer, we would expect five bands rather than the three invariably observed, whereas if only two subunits are sus-

Table 4. *Steady-state kinetic constants for ribitol dehydrogenase-A and -B with pentitols at 28°C, pH7.0*

The constants in eqn. 1 are derived as described in the Materials and Methods section. k is derived from V_f by assuming mol.wt. 110000 for the tetramer. V_B/V_A is the relative velocity of the B enzyme compared with the A enzyme at 1 mM-NAD^+ and (*a*) 5 mM-pentitol or (*b*) 500 mM-pentitol.

Substrate	Enzyme	K_P (mM)	K_{NAD} (mM)	$K_{P,NAD}$ (mM²)	V_f (units/mg)	k (s^{-1})	V_B/V_A (*a*)	V_B/V_A (*b*)
Ribitol	A	8.0	0.66	10.4	166	299	—	—
	B	7.2	0.37	5.9	127	299	1.0	0.9
Xylitol	A	579	0.76	612	20.1	36	—	—
	B	273	0.23	202	36.1	65	4.5	3.4
L-Arabitol	A	147	0.25	166	15.6	28	—	—
	B	170	0.21	140	22.5	41	1.5	1.5

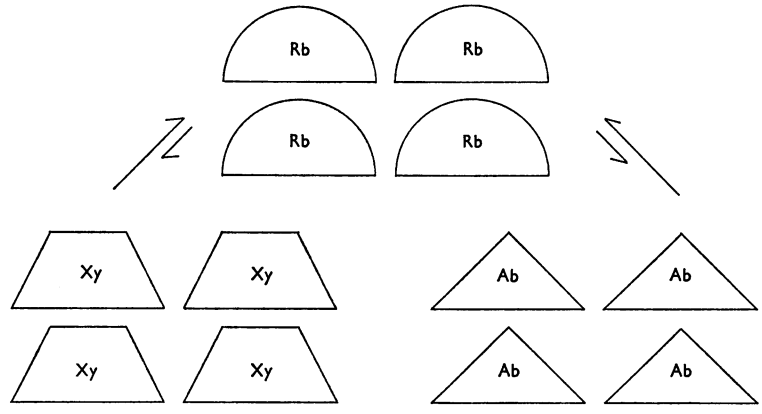

Fig. 10. *Possible conformations of ribitol dehydrogenase*

Conformation Rb is assumed to react with ribitol, Xy with xylitol and Ab with L-arabitol.

ceptible to proteolytic 'nicking' only three products could result. This hypothesis also explains the thiol reactivity of native ribitol dehydrogenase-A. Two thiol groups/tetramer exchange readily at low concentrations of disulphide reagent without loss of activity, but higher concentrations are necessary to exchange a further pair, and inactivation results. The remaining four thiol groups are inaccessible to disulphide exchange. The mutant enzyme, ribitol dehydrogenase-B, shows analogous pair-wise reactivity of its eight thiol groups.

The biological advantage of a mutant strain such as strain B over the ancestral strain A grown on xylitol in a chemostat depends on the intracellular concentrations of enzyme, NAD^+ and substrate under actual growth conditions. The enzyme activities can readily be assayed in extracts of the chemostat cultures, but substrate and NAD^+ concentrations are less easy to define. A reasonable estimate for the NAD^+ concentration is 1mM (Park, 1973), but the intracellular xylitol concentration will depend on whether the permease (which is not rate-limiting) catalyses passive or active transport. In the former case, the intracellular xylitol concentration would be of the same order as the steady-state extracellular concentration (approx. 5mM). With active transport, the intracellular xylitol concentration could rise to much higher values, but these are very unlikely to be higher than 500mM. Table 4 shows calculations of the 'intracellular' velocities of ribitol dehydrogenase-B relative to that of ribitol dehydrogenase-A under each of these extreme assumptions. It can be seen that the intracellular pentitol concentration has very little effect on the relative biological value of the B enzyme over the A enzyme. There is a 3–4-fold improvement on xylitol without any loss of activity with ribitol or L-arabitol.

References

Cleland, W. W. (1963*a*) *Biochim. Biophys. Acta* **67**, 104–172
Cleland, W. W. (1963*b*) *Biochim. Biophys. Acta* **67**, 173–187
Dalziel, K. (1963) *J. Biol. Chem.* **238**, 1538–1543
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Florini, J. R. & Vestling, C. S. (1957) *Biochim. Biophys. Acta* **25**, 575–578
Furlong, N. B. (1972) *Tex. Rept. Biol. Med.* **30**, 109–123
Hartley, B. S. (1974) in *Evolution in the Microbial World* (Carlile, M. J. & Skehel, J. J., eds.), pp. 151–182, Cambridge University Press, London
Hartley, B. S., Burleigh, B. D., Midwinter, G. G., Moore, C. H., Morris, H. R., Rigby, P. W. J., Smith, M. J. & Taylor, S. S. (1972) *FEBS Symp.* **29**, 151–176
Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
Lerner, S. A., Wu, T. T. & Lin, E. C. C. (1964) *Science* **146**, 1313–1315
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Miller, G. L. (1959) *Anal. Chem.* **31**, 964
Mortlock, R. P., Fossitt, D. D. & Wood, W. A. (1965) *Proc. Nat. Acad. Sci. U.S.* **54**, 572–579
Rigby, P. W. J., Burleigh, B. D. & Hartley, B. S. (1974) *Nature* (London) *in the press*
Park, J. H. (1973) *J. Biol. Chem.* **248**, 5562–5571
Taylor, S. S., Rigby, P. W. J. & Hartley, B. S. (1974) *Biochem. J.* **141**, 693–700
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
Wu, T. T., Lin, E. C. C. & Tanaka, S. (1968) *J. Bacteriol.* **96**, 447–456