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Purification and properties of D-ribulokinase and D-xylulokinase from *Klebsiella aerogenes*

Michael S. NEUBERGER,*‡ Brian S. HARTLEY* and John E. WALKER†
**Department of Biochemistry, Imperial College of Science and Technology, London SW7 2AZ, U.K., and*
†*M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.*

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The D-ribulokinase and D-xylulokinase of *Klebsiella aerogenes* were purified to homogeneity from *Escherichia coli* K12 construct strains that synthesized these enzymes constitutively. The D-ribulokinase, which is encoded in the ribitol operon, is active as a dimer of 60000 subunit mol.wt., whereas the D-xylulokinase, which is encoded in the D-arabitol operon, is active as a dimer of 54000 subunit mol.wt. The amino acid compositions and *N*-terminal sequences of both pentulokinases are reported. The $K_{app.}$ values of the enzymes for their D-pentulose substrates were determined, and the D-ribulokinase was shown to have a low-affinity side-specificity for ribitol and D-arabitol. These results are discussed in the context of the evolution of the *Klebsiella aerogenes* pentitol operons.

Klebsiella aerogenes can use each of ribitol and D-arabitol as sole sources of carbon and energy. For each of these two pentitols, the organism possesses an operon encoding a pentitol dehydrogenase and a D-pentulokinase. The genes of the ribitol operon (*rbt*) and D-arabitol operon (*dal*) are clustered on the bacterial chromosome (Charnetzky & Mortlock, 1974). This tight clustering of the operons, the similarity of the catabolic pathways and the demonstration that the two operons are transcribed in a bipolar manner (Neuberger & Hartley, 1979) have prompted speculation that the operons arose from a common ancestor by a process of invert gene duplication followed by sequence divergence. The existence of a repeated sequence in the region of the genes of the pentitol operons (Neuberger & Hartley, 1979) lends support to such speculations.

A comparison of the enzymes of the *rbt* and *dal* operons might reveal the existence of any homology between the two sets of catabolic enzymes. Purified ribitol dehydrogenase shows no immunological cross-reaction with purified D-arabitol dehydrogenase (Neuberger *et al.*, 1979); this, however, does not preclude the possibility that the two proteins contain regions of homologous sequence. The complete amino acid sequence of ribitol dehydrogenase has been determined by classical techniques (C. H. Moore, S. S. Taylor, M. J. Smith & B. S. Hartley, unpublished work), but, since the genes of the pentitol operons have now been recombined into the genome of bacteriophage λ (Neuberger & Hartley, 1979), we hope to obtain the amino acid sequences of D-arabitol dehydrogenase, D-ribulokinase and D-xylulokinase from DNA sequence analysis. In the present paper we report the *N*-terminal amino acid sequences of D-ribulokinase and D-xylulokinase, which should enable us to locate the genes coding for the D-pentulokinases. The molecular weights and the amino acid compositions of the pentulokinases were also estimated: these parameters will be used to check the amino acid sequences deduced from the investigations into the DNA. The *N*-terminal sequence of D-arabitol dehydrogenase and of several peptides derived from it have already been determined (M. S. Neubergber, A. Dell, J. E. Walker & B. S. Hartley, unpublished work).

Scangos & Reiner (1978*b*, 1979) have made predictions concerning the substrate specificities of the pentulokinases from *Escherichia coli* C, based on the genetic demonstration that constitutive synthesis of the pentulokinases results in sensitivity to certain polyols. In particular, these authors have proposed that the D-ribulokinase can phosphorylate ribitol and that the D-xylulokinase can phosphorylate D-arabitol, and that these side-specificities might be signs that, in each pentitol operon, the kinase and dehydrogenase genes have evolved from a common ancestor by gene duplication. Such a model follows the classic hypothesis of Horowitz (1945) for the evolution of metabolic pathways. Since *E coli* C is closely related to *K. aerogenes*, it was decided to

Abbreviation used: SDS, sodium dodecyl sulphate.

‡ Present address: M.R.C. Laboratory of Molecular Biology, Hills Road, Cambridge CB2 2QH, U.K.

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investigate the substrate specificities of the purified *K. aerogenes* pentulokinases; the results obtained are presented below.

A further interest in the evolution of the pentulokinases comes from the demonstration by Wilson & Mortlock (1973) that *K. aerogenes* possesses two genes encoding D-xylulokinase. Apart from the D-xylulokinase encoded in the *dal* operon (which is the subject of the work described in the present paper), there is a second gene encoding a D-xylulokinase in the D-xylose operon. Wilson & Mortlock (1973) have shown that the D-xylulokinases are of similar native molecular weight and have similar electrophoretic mobility in native polyacrylamide gel, although the *dal* operon D-xylulokinase, unlike the enzyme encoded in the D-xylose operon, is cold-sensitive. It will clearly be of interest to discover whether the two D-xylulokinases show homology in their amino acid sequences.

Purification procedures for both D-ribulokinase and D-xylulokinase have been described previously (Mortlock *et al.*, 1965; Wilson & Mortlock, 1973), although in neither case was homogeneity of the final product demonstrated.

Experimental

Materials

Bacterial strains and bacteriophage. E. coli K12 strain L250 (HfrPO13 *thi leu lacZ str*^{r} *mtlA mtlD gutA*) was obtained from Dr. J. Lengeler (University of Regensburg, Regensburg, West Germany) and strain CSH62 (HfrH *thi*) was from Cold Spring Harbor Laboratory (Cold Spring Harbor, NY, U.S.A.). Bacteriophage λ627 [full genotype; *λcI857 S7 nin* 5Δ(*srI* λ*1–2*) *srIλ4*° *srIλ5*°] was a gift from Dr. N. E. Murray (University of Edinburgh, Edinburgh, Scotland, U.K.). Bacteriophage *λprbt-101* (full genotype; *λcI857 S7 nin 5 srIλ4*° *srIλ5*° *rbt-101 rbtD*$^{+}$ *rbtK*$^{+}$) is derived from bacteriophage λ627 and is a specialized transducing bacteriophage that carries the ribitol operon (*rbt*) of *K. aerogenes*; *rbt-101* indicates a mutation causing constitutive *rbt* operon expression. Bacteriophage *λprbt-101 dal*$^{+}$ (full genotype: *λcI857 S7 nin5 srIλ4*° *srIλ5*° *rbt-101 rbtD*$^{+}$ *rbtK*$^{+}$ *dal*$^{+}$) is a derivative of bacteriophage *λprbt-101* that carries an inducible (wild-type) *K. aerogenes* D-arabitol operon (*dal*) as well as a constitutive *rbt* operon (Neuberger & Hartley, 1979).

Bacteriophage *λprbt-101 dal-201* is a derivative of bacteriophage *λprbt-101 dal*$^{+}$ that expresses the *dal* operon constitutively; *E. coli* K12 strain NC260 is a putative *λprbt-101 dal-201* dilysogen of strain L250 (Neuberger *et al.*, 1979). Strain NC63 is a λ627 lysogen of strain CSH62, and strain NC621 is a *λprbt-101* monolysogen of strain CSH62. Strain NC629 is a *λprbt-101* polylysogen of strain CSH62 and was isolated as described below under 'Methods'.

Reagents. Iodo[2-^{14}C]acetic acid (specific radioactivity 57 mCi/mmol) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Pentitols were bought from Cambrian Chemicals (Croydon, Surrey, U.K.). D-Ribulose, ATP, ADP, glucose 1-phosphate, phosphoenolpyruvate, NADH, bovine serum albumin, rabbit muscle L-lactate dehydrogenase (type I; 67 units of lactate dehydrogenase/mg, 85 units of pyruvate kinase/mg), rabbit muscle L-lactate dehydrogenase (type II; substantially pyruvate kinase-free), pig heart fumarase, *E. coli* alkaline phosphatase, ovalbumin, rabbit muscle pyruvate kinase and rabbit muscle aldolase were obtained from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Methionyl-tRNA synthetase and isoleucyl-tRNA synthetase from *E. coli* were gifts from Dr. C. J. Bruton (Imperial College) and yeast hexokinase was a gift from Mr. A. Gray (Imperial College). Ribitol dehydrogenase and D-arabitol dehydrogenase were purified as described previously (Taylor *et al.*, 1974; Neuberger *et al.*, 1979). D-Xylulose was a gift from Dr. S. Bungard (I.C.I. Agricultural Division, Billingham, Cleveland, U.K.). Ion-exchange resins DEAE-cellulose (DE-52), CM-cellulose (CM-52) and phosphocellulose (P-11) were bought from Whatman (Maidstone, Kent, U.K.); phosphocellulose was pre-cycled as described by Clarke & Hartley (1979). Hydroxypatite, prepared by the method of Atkinson *et al.* (1973), was a gift from Dr. C. J. Bruton. Hydrophobic chromatography medium ω-aminobutyl-agarose was bought from Miles Laboratories (Stoke Poges, Berks., U.K.), and Ultrogel AcA-34 was obtained from LKB (Selsdon, Surrey, U.K.). Guanidinium chloride ('ultra pure') was bought from Schwartz/Mann (Orangeburg, NY, U.S.A.), and activated charcoal (Norit OL) was obtained from Hopkin and Williams (Chadwell Heath, Essex, U.K.). Casein hydrolysate (not vitamin-free) and AristaR-grade $(NH_4)_2SO_4$ were obtained from BDH Chemicals (Poole, Dorset, U.K.).

Methods

Microbiological techniques. E. coli K12 strain NC629 was constructed by infecting strain CSH62 with bacteriophage *λprbt-101* and selecting for a derivative growing fast on minimal xylitol plates, as described previously (Neuberger & Hartley, 1979). CH plates contained 5 g of casein hydrolysate/l and 15 g of agar/l; polyols were added, if required, to a final concentration of 10 mM.

Enzyme assays. For the pyruvate kinase/lactate dehydrogenase coupled assay of pentulokinase activity, the oxidation of NADH was monitored by following the decrease in absorbance at 340 nm in a

Gilford 252 recording spectrophotometer with a Unicam SP.500 monochromator. Assays were performed in 50mM-Tris/HCl (pH7.9)/mM-KCl/1mM-EDTA/5mM-$MgCl_2$/0.5mM-ATP/1mM-phosphoenolpyruvate/1mM-dithiothreitol/0.33mM-NADH/1mM-D-pentulose/rabbit muscle lactate dehydrogenase (1.2 units/ml) (Sigma type I; containing pyruvate kinase activity) at 28°C. One unit of pentulokinase activity catalyses the oxidation of 1μmol of NADH/min in the above assay system.

For the cysteine/carbazole pentulokinase assay, a sample of enzyme (volume less than 10μl) was added to 0.2ml of 50mM-Tris/HCl (pH7.9)/20mM-magnesium acetate/10mM-KF/12mM-ATP/3mM-D-pentulose/10mM-2-mercaptoethanol that had been preincubated at 37°C. The reaction proceeded at 37°C for 20min, at which time 0.4ml of 5% (w/v) $ZnSO_4,7H_2O$ and 0.4ml of 0.15mM-$Ba(OH)_2$ were added to the reactions and the samples were centrifuged for 3min in a Beckman Microfuge B centrifuge. A sample (0.5ml) of supernatant was removed, mixed with 0.1ml of 1.5% (w/v) cysteine hydrochloride, 3ml of 12M-H_2SO_4 and 0.1ml of ethanolic 0.12% (w/v) carbazole. After 30min at room temperature (22°C), the absorbance was read at 560nm.

Ribitol dehydrogenase and D-arabitol dehydrogenase were assayed as described previously (Neuberger *et al.*, 1979). Alkaline phosphatase was assayed as described in the *Worthington Enzyme Manual* (1972), and yeast hexokinase was assayed in the pyruvate kinase/lactate dehydrogenase coupled assay system described above, with D-glucose substituted for D-pentulose. Fumarase was assayed by adding the enzyme sample to 50mM-L-malic acid in 100mM-potassium phosphate buffer, pH7.6, and following the increase in absorbance at 240nm; lactate dehydrogenase activity was determined by following the decrease in absorbance at 340nm after adding the enzyme to 100mM-Tris/HCl (pH7.9)/33mM-pyruvate/0.33mM-NADH. Pyruvate kinase was assayed by following the decrease in absorbance at 340nm after the addition of enzyme to 50mM-Tris/HCl (pH7.5)/80mM-KCl/10mM-$MgCl_2$/1mM-phosphoenolpyruvate/0.3mM-ADP/0.33mM-NADH/lactate dehydrogenase (20 units/ml) (Sigma type II).

Protein determination. Protein was measured by a modified Lowry procedure (Miller, 1959), with bovine serum albumin as a standard. For the specific activities of the purified enzymes, the protein concentration was determined by weighing salt-free freeze-dried samples.

Purification of D-*ribulokinase.* A culture of *E. coli* K12 strain NC629 (60 litres) was grown in the Imperial College Pilot Plant at 32°C on a casein-hydrolysate/salts medium (Neuberger *et al.*, 1979). When the culture reached late exponential phase, the cells were spun down in a Sharples (model AS-6) continuous-flow centrifuge and the cell paste was stored at −20°C. Unless otherwise stated, all steps in the purification were performed at 4°C in de-aerated potassium phosphate buffers supplemented to 2mM in $MgCl_2$, 0.2mM in EDTA, 10mM in 2-mercaptoethanol and 0.1mM in phenylmethanesulphonyl fluoride. Unless otherwise indicated, all buffers were at pH7.0. Five 60g batches of frozen paste were thawed into 5 × 75ml of 10mM-buffer/1mM-dithiothreitol, and the cells were ruptured by ultrasonication with a Dawe Soniprobe at setting 6, tuned to give maximum amplitude, for a total of 6min in 1.5min bursts with 12min pauses between bursts. Cell debris was removed by centrifugation at 23000*g* for 20min and at 40000*g* for 2 × 20min in a Sorvall RC-5 centrifuge. The cell-free extract was then centrifuged in the Ti45 head of a Beckman L5-65 ultracentrifuge at 100000*g* for 90min to pellet the ribosomes.

The enzyme was purified from the 100000*g* supernatant by DEAE-cellulose chromatography. The 100000*g* supernatant (volume 383ml) was adjusted to a conductivity of 2.4mS by the addition of 10mM-2-mercaptoethanol/0.1mM-phenylmethanesulphonyl fluoride/1mM-$MgCl_2$ and the pH then adjusted back to 7.0 with KOH. The diluted 100000*g* supernatant (volume 1400ml) was applied to a column (11cm × 3.5cm diam.) of DEAE-cellulose (Whatman DE-52) equilibrated in 10mM-buffer. After the column had been washed with 10mM-buffer until no more protein emerged, the enzyme was eluted with an 800ml linear gradient from 10mM- to 180mM-buffer applied at a flow rate of 40ml/h. Active fractions, which were eluted at a conductivity of 4–10.5mS, were pooled (volume 340ml) and concentrated by ultrafiltration to a volume of 40ml in an Amicon pressure cell with a PM10 membrane at a pressure of 0.2MPa.

The sample was applied to a column (85cm × 5cm diam.) of Ultrogel AcA-34 equilibrated in 100mM-buffer and eluted by downward flow at 80ml/h. Active fractions were pooled, concentrated to a volume of 35ml by ultrafiltration and dialysed exhaustively against 10mM-buffer.

The sample was then applied at a rate of 5ml/h to a column (4cm × 2.5cm diam) of hydroxyapatite equilibrated in 10mM-buffer; the column was washed with 100ml of 10mM-buffer. Enzyme activity was located only in the flow-through and wash, no further activity being eluted by washing the column with 100mM-buffer. The flow-through and wash were combined and concentrated to a volume of 30ml by ultrafiltration.

The concentrated hydroxyapatite pool was dialysed against 10mM-sodium acetate (pH5.5)/2mM-$MgCl_2$ / 0.1mM-EDTA / 10mM-2-mercaptoethanol / 0.1mM-phenylmethanesulphonyl fluoride.

Centrifugation (at 15000*g* for 15min) was necessary to remove a precipitate, which did not contain active enzyme. The sample was applied to a column (5cm × 2.5cm diam.) of CM-cellulose (Whatman CM-52) equilibrated in the 10mM-sodium acetate buffer described above. The enzyme did not bind to the resin; therefore the flow-through and wash were combined and then adjusted to pH7.0 by the addition of 1M-potassium phosphate buffer, pH7.0, to a final concentration of 60mM, and the sample was concentrated to a volume of 40ml by ultrafiltration.

After dialysis against 10mM-buffer, pH6.5, the sample was applied to a column (5cm × 2.5cm diam.) of phosphocellulose (Whatman P-11) equilibrated in 10mM-buffer, pH6.5. The enzyme did not bind to the resin, so the flow-through and a 10mM-buffer wash were combined and concentrated by ultrafiltration to a volume of 33ml.

The enzyme was purified from the phosphocellulose pool by $(NH_4)_2SO_4$ fractionation. The sample was slowly adjusted to 35% saturation with $(NH_4)_2SO_4$, stirred for 20min and centrifuged for 20min at 23000*g*. The supernatant was adjusted to 43% saturation with $(NH_4)_2SO_4$, stirred for 20min and centrifuged as before. The precipitate was dissolved in 50mM-buffer, dialysed against 50mM-buffer in 50% (v/v) glycerol and stored at −20°C.

Purification of D-*xylulokinase.* A 100000*g* supernatant was made from a cell-free extract of *E. coli* K12 strain NC260 as described for the purification of D-ribulokinase, except that ultrasonication and centrifugation were performed at 18°C. All subsequent steps in the purification were performed at room temperature with potassium phosphate buffers, pH7.0, supplemented to 2mM in $MgCl_2$, 0.2mM in EDTA, 10mM in 2-mercaptoethanol and 0.1mM in phenylmethanesulphonyl fluoride.

The 100000*g* supernatant (volume 320ml) was diluted with 2mM-$MgCl_2$/0.2mM-EDTA/10mM-2-mercaptoethanol/0.1mM-phenylmethanesulphonyl fluoride to a conductivity of 2.3mS and concentrated by ultrafiltration to a volume of 250ml. The sample was applied to a column (11cm × 3.5cm diam.) of DEAE-cellulose (Whatman DE-52) equilibrated in 10mM-buffer, and the column was washed with 10mM-buffer until no more protein emerged. The enzyme was eluted with a linear gradient of 800ml from 10mM- to 150mM-buffer applied at a flow rate of 65ml/h. Active fractions, which were eluted at a conductivity of 7–10mS, were pooled and concentrated to a volume of 41ml by ultrafiltration.

The sample was applied to a column (88cm × 5cm diam.) of Ultrogel AcA-34 equilibrated in 100mM-buffer and eluted by downward flow at 80ml/h. Active fractions were pooled, diluted to a conductivity of 1mS with 2mM-$MgCl_2$/0.2mM-EDTA/10mM-2-mercaptoethanol/0.1mM-phenylmethanesulphonyl fluoride and concentrated to a volume of 50ml by ultrafiltration.

The sample was loaded on to a column (12cm × 2.3cm diam.) of ω-aminobutyl-agarose equilibrated in 5mM-buffer. After the column had been washed with 200ml of 5mM-buffer, the enzyme was eluted at a flow rate of 40ml/h with a linear gradient of 500ml of 5mM- to 80mM-buffer. Active fractions, which were eluted at a conductivity of 1.8–3.5mS, were pooled.

The ω-aminobutyl-agarose pool (volume 92ml, conductivity 2.6mS) was loaded on to a column (2.3cm × 2.5cm diam.) of hydroxyapatite in 10mM-buffer. After the column had been washed with 10mM-buffer until no more protein was eluted, the enzyme was eluted with a linear gradient of 100ml from 10mM- to 150mM-buffer applied at a flow rate of 20ml/h. Active fractions, which were eluted at a conductivity of 3–6mS, were pooled (volume 18.4ml) and enzyme was purified from the pool by $(NH_4)_2SO_4$ fractionation.

$(NH_4)_2SO_4$ fractionation was performed at room temperature, but otherwise as described for D-ribulokinase. The 35–43%-saturation fraction, which contained the enzyme, was dissolved in 50mM-buffer.

Polyacrylamide-gel electrophoresis. Electrophoresis in polyacrylamide slab gels both under native conditions and in the presence of SDS was performed as described previously (Neuberger *et al.*, 1979).

Staining for D-*ribulokinase activity.* Native polyacrylamide gels were stained for D-ribulokinase activity by using a procedure based on that described for pyruvate kinase by Imamura & Tanaka (1972). After electrophoresis, a mixture containing 15ml of 50mM-Tris/HCl (pH7.5)/100mM-KCl / 10mM-$MgCl_2$ / 2.5mM-phosphoenolpyruvate/2mM-ATP/3mM-NADH/3mM-D-ribulose, prewarmed to 37°C, 3.5ml of molten 3.5% (w/v) agarose and 25 units of lactate dehydrogenase (Sigma type I) was poured onto the gel and allowed to solidify. After 15min at 37°C, the gel was viewed under u.v. light. Areas in which NADH had been oxidized appeared dark, owing to loss of fluorescence. Both ribitol dehydrogenase and D-ribulokinase give rise to bands in this system. The bands are broader than those given by the Nitro Blue Tetrazolium linked stain for pentitol dehydrogenases (Neuberger *et al.*, 1979), owing to the diffusion of NADH.

Performic acid oxidation. The procedure is based on that described by Hirs (1956). To 1ml of formic acid was added 50μl of '100-volume' H_2O_2 solution, and the mixture was left at room temperature for 2h to allow the generation of performic acid. A sample of protein at 1mg/ml in methanol/formic acid (1:4, v/v) was mixed with an equal volume of

the performic acid, and the mixture was incubated for 2.5h at between −15°C and −5°C. After addition of 12vol. of water, the sample was freeze-dried.

Carboxymethylation. A sample (17.3mg) of salt-free freeze-dried D-ribulokinase was dissolved in 2ml of degassed 200mM-Tris/HCl/6M-guanidinium chloride, pH8.2. The solution was mixed with 49μl of 20mM-dithiothreitol in the same buffer and incubated at 37°C for 3.5h, before the addition of 144μCi of iodo[2-^{14}C]acetic acid (specific radioactivity 5μCi/μmol). After incubation for 30min at 37°C, 29μmol of unlabelled iodoacetic acid was added to the mixture and, after a further 30min, carboxymethylation of the protein was quenched by the addition of 12μl of 2-mercaptoethanol, followed by a final incubation of 60min at 37°C. The sample was dialysed over 40h against 2 × 500ml of 15% (v/v) formic acid, freeze-dried and stored at −20°C.

Amino acid analysis. This was performed as described previously (Neuberger *et al.*, 1979), except that the samples of native D-ribulokinase were analysed on a Rank–Hilger Chromaspek instrument.

N-Terminal sequence analysis. Amino acid sequences in the *N*-terminal regions of *S*-carboxymethyl-D-ribulokinase and unmodified D-xylulokinase were determined with the aid of a Beckman 890B sequencer, Polybrene being used as a carrier. Details of procedures used in sequence analysis and in the identification of phenylthiohydantoins are described elsewhere (Walker *et al.*, 1980).

Purification and analysis of pentitol phosphates. Purified D-ribulokinase (150 units) was incubated for 70h at 37°C in 1.4ml of 200mM-ammonium acetate (pH8.0) / 100mM-pentitol (ribitol or D-arabitol)/100mM-ATP/20mM-$MgCl_2$/10mM-2-mercaptoethanol/0.1mM-phenylmethanesulphonyl fluoride. The reaction mixtures were freeze-dried, redissolved in 0.4ml of water and treated with activated charcoal to remove adenine nucleotides by adsorption. Samples were then applied to Whatman 3MM paper, with glucose 1-phosphate and ribitol as markers, and subjected to electrophoresis for 40min at 3kV at pH6.5 in the pyridine/acetic acid system described by Ambler (1963). The paper was dried and cut into strips, and some of the strips were stained for sugar by spraying with 20mM-potassium periodate, drying, dipping in $AgNO_3$ solution (made by mixing 5ml of saturated aqueous $AgNO_3$ with 1 litre of acetone and adding just enough water to redissolve the $AgNO_3$), drying and dipping in ethanolic NaOH (made by mixing 10ml of 10M-NaOH with 200ml of ethanol). The pentitol phosphates were eluted from appropriate areas of unstained strips of the electropherogram with degassed de-ionized water and freeze-dried.

Field-desorption mass spectra of the purified pentitol phosphates were obtained by Dr. Anne Dell (Imperial College) with a dual field-desorption/electron-impact source on a KRATOS MS50 mass spectrometer. The spectra were counted by comparison with the electron-impact spectrum of Fomblin (Henning & Lotz, 1977). Desorption of the pentitol phosphates occurred at a wire current of 20–23mA.

Results and discussion

Enzyme-superproducing strains

To facilitate enzyme purification, mutants were used that constitutively synthesized elevated amounts of the pentulokinases. The dehydrogenase and pentulokinase of each pentitol operon are expressed co-ordinately; thus mutants constitutive for pentitol dehydrogenase synthesis also synthesize the corresponding pentulokinase constitutively. Ribitol dehydrogenase has a side-specificity for xylitol, and D-arabitol dehydrogenase has a side-specificity for D-mannitol; these side-specificities have previously been exploited for the isolation of *rbt*-constitutive and *dal*-constitutive mutants (Wu *et al.*, 1968; Neuberger *et al.*, 1979). The ribitol dehydrogenase specific activity of λ *prbt-101* lysogens of *E. coli* K12 limits their rates of growth on xylitol; mutants growing faster on xylitol constitutively synthesize elevated quantities of ribitol dehydrogenase. Such mutants have been found to arise at high frequency from λ*prbt-101* lysogens, as increased *rbt* gene dosage is easily achieved by the presence of multiple prophage copies (Neuberger & Hartley, 1979). Polylysogens selected for fast growth on xylitol will contain several copies of the entire *rbt* operon and are therefore expected to synthesize elevated quantities of D-ribulokinase.

E. coli K12 strain NC629, which harbours multiple λ*prbt-101* prophages, was used for purification of D-ribulokinase. For the purification of the *dal* operon D-xylulokinase, *E. coli* strain NC260 was used; this strain harbours multiple copies of a λ*prbt-101 dal-201* prophage, which effects constitutive expression of both the *rbt* and *dal* operons (Neuberger *et al.*, 1979).

The use of a *dal*-constitutive mutant for the isolation of the D-xylulokinase has the important advantage that, after growth in the absence of exogenous D-arabitol and D-xylose, the cells contain only the D-xylulokinase activity encoded in the *dal* operon. Growth in the presence of D-arabitol (which is required for induction of the wild-type *dal* operon) results in the synthesis of the D-xylulokinase encoded in the D-xylose operon in addition to that encoded in the D-arabitol operon; separation of the two enzymes is not easily achieved (Wilson & Mortlock, 1973).

Evaluation of assay methods

The strategy of enzyme purification was con-

siderably influenced by the kinase assay procedures available. Three types of assay have been described. The generalized kinase assay in which the formation of ADP by the kinase is coupled to oxidation of NADH through the addition of phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase has been widely used (Anderson & Wood, 1962). This assay is easy to perform but is highly sensitive to interference by ATPases, NADH oxidase and NADH-linked reductases capable of acting on substrates present in the assay mix. With the pentulokinases, the presence of large amounts of the pentitol dehydrogenases in the cell extracts causes particularly severe interference, and the pyruvate kinase/lactate dehydrogenase coupled assay was therefore only used once substantial separation of the pentitol dehydrogenase and pentulokinase had been achieved. There is no problem of ribitol hydrogenase interference in the D-ribulokinase assay we have described previously (Neuberger & Hartley, 1979), in which [1-^{3}H]ribulose is generated in the assay mixture by the action of ribitol dehydrogenase on [1-^{3}H]ribitol, and the [1-^{3}H]ribulose 5-phosphate produced is precipitated as the barium salt and its radioactivity counted. A similar assay has been devised for D-xylulokinase in which [U-^{14}C]xylulose is generated by the action of *Arthrobacter* xylose isomerase on D-[U-^{14}C]xylose (C. A. Smith, personal communication). However, these methods are laborious to perform and are not suitable for assaying large numbers of samples. For the early stages of enzyme purification, a semiquantitative assay was used in which, after incubation of the enzyme with pentulose and ATP, the pentulose phosphate produced was precipitated as the barium salt and the remaining pentulose in the supernatant was estimated by the cysteine/carbazole reaction. Although this assay is sensitive and easy to perform, its semiquantitative nature renders it more suitable for the location of activity eluted from a column than for use with batch purification procedures. Therefore DEAE-cellulose chromatography was used as the first purification stage for both kinases in preference to $(NH_4)_2SO_4$ fractionation.

The purification procedures described yielded

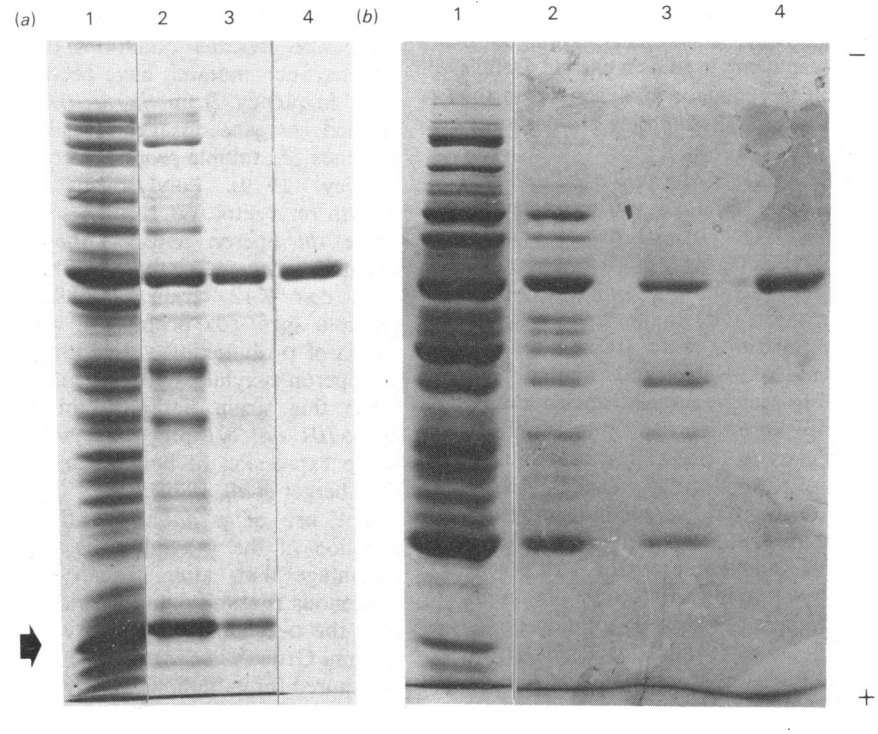


Fig. 1. *SDS/polyacrylamide-gel electrophoresis of fractions during the purifications of the pentulokinases*
The following fractions were examined on a 9% (w/v) slab gel as described under 'Methods' in the Experimental section. (*a*) D-Ribulokinase purification: lane 1, 100000 *g* supernatant; lane 2, Ultrogel AcA-34 eluate; lane 3, hydroxyapatite eluate; lane 4, 35–43%-satn.-$(NH_4)_2SO_4$ fraction. (*b*) D-Xylulokinase purification: lane, 1, DE-52 DEAE-cellulose eluate; lane 2, ω-aminobutyl-agarose eluate; lane 3, hydroxyapatite eluate; lane 4, 35–43%-satn.-$(NH_4)_2SO_4$ fraction. Each fraction contained about 40μg of pentulokinase. The position of ribitol dehydrogenase on gel (*a*) is indicated with an arrow.

enough of the purified enzymes to allow determination of the chemical and kinetic parameters described below. It should, however, be noted that the purification procedures described have not been optimized.

Purification of D-*ribulokinase*

The purification of the enzyme is described under 'Methods' in the Experimental section, and the purification achieved at some of the steps is illustrated by SDS/polyacrylamide-gel electrophoresis (Fig. 1). It can be seen that the enzyme constitutes one of the major proteins present in the cell-free extract of strain NC629 (Fig. 1); the purification yielded 81 mg of enzyme from 300 g of cell paste. Substantial separation of ribitol dehydrogenase and D-ribulokinase was achieved only at the second DEAE-cellulose chromatography; ribitol dehydrogenase was eluted between conductivities of 5 mS and 8 mS, whereas D-ribulokinase was eluted between 8 mS and 11 mS. This difference in the binding of the two enzymes to DEAE-cellulose correlates well with their different mobilities in native polyacrylamide-gel-electrophoresis in the buffer system of Ornstein & Davis (1964), where ribitol dehydrogenase has a mobility relative to the Bromophenol Blue dye front in 7.5% (w/v) polyacrylamide gels of 0.35 and D-ribulokinase has a mobility of 0.48.

The purified enzyme was homogeneous as judged by SDS/polyacrylamide-gel electrophoresis, electrophoresis in native polyacrylamide gels and *N*-terminal sequence analysis (see below). After electrophoresis in native polyacrylamide gel, activity staining revealed a unique band of D-ribulokinase activity that was of the same mobility as the sole protein-staining species. The specific activity of the purified enzyme in the pyruvate kinase/lactate dehydrogenase coupled assay system was 51 units/mg.

The purified enzyme was dialysed against 50 mM-buffer in 50% (v/v) glycerol and stored in the dark at −20°C. Stored in this way, the enzyme lost less than 5% of its activity in one month.

Purification of D-*xylulokinase*

Because the D-xylulokinase activity encoded in the *dal* operon had been reported to be cold-labile (Wilson & Mortlock, 1973), the enzyme was purified at room temperature. The purification was therefore performed as rapidly as possible to minimize oxidation and proteolytic cleavage of the enzyme. Owing to the large difference in their native molecular weights, complete separation of D-xylulokinase and D-arabitol dehydrogenase was achieved at the gel-filtration step. Therefore the D-xylulokinase activity in the subsequent stages of the purification was determined by using the pyruvate kinase/lactate dehydrogenase coupled assay (Table 1). The purified enzyme was homogeneous as judged by SDS/polyacrylamide-gel electrophoresis (Fig. 1) and *N*-terminal sequence analysis (see below); it had a specific activity in the pyruvate kinase/lactate dehydrogenase coupled assay system of 84 units/mg.

After incubation of the purified enzyme for 18 h at 4°C in 100 mM-potassium phosphate, pH 7.0/2 mM-$MgCl_2$ / 0.1 mM-EDTA / 0.1 mM-phenylmethanesulphonyl fluoride/10 mM-2-mercaptoethanol at a concentration of 1 mg/ml, the activity had decreased to 10%. This cold-inactivation was only partially reversed by incubation of the cold-inactivated enzyme at 37°C. After 2 h incubation at 37°C of the cold-inactivated enzyme, the activity increased to 23% of the original value. The efficacy of the 37°C re-activation was not increased by addition of ATP to the enzyme to a concentration of 0.5 mM.

One might reasonably speculate that the cold-sensitivity of the enzyme reflects the entropic importance of hydrophobic interactions in the association between subunits. The cold-sensitivity of the *E. coli* prolyl-tRNA synthetase has been shown to be due to temperature-dependent dissociation

Table 1. *Purification of* D-*xylulokinase from 300 g of E. coli K12 strain-NC260 cells*

Experimental details are given in the text. Activity in the 100000*g* supernatant and the DE-52 DEAE-cellulose pool was not determined because the presence of D-arabitol dehydrogenase caused gross interference in the enzyme assay; purification and yield in the subsequent stages are expressed relative to the activity present in the Ultrogel AcA-34 pool. Protein was determined by the modified Lowry procedure, except in the final $(NH_4)_2SO_4$ fraction, where protein concentration was determined by weighing salt-free freeze-dried samples.

Fraction	Total protein (g)	Total activity (units)	Specific activity (units/mg)	Relative purification	Yield (%)
100000*g* supernatant	8.2	—	—	—	—
DE-52 DEAE-cellulose pool	0.62	—	—	—	—
Ultrogel AcA-34 pool	0.29	950	3.3	1	100
ω-Aminobutyl-agarose pool	0.12	650	5.4	1.6	68
Hydroxyapatite pool	0.016	470	29	8.8	49
35–43%-satn.-$(NH_4)_2SO_4$ fraction	0.0051	430	84	25	45

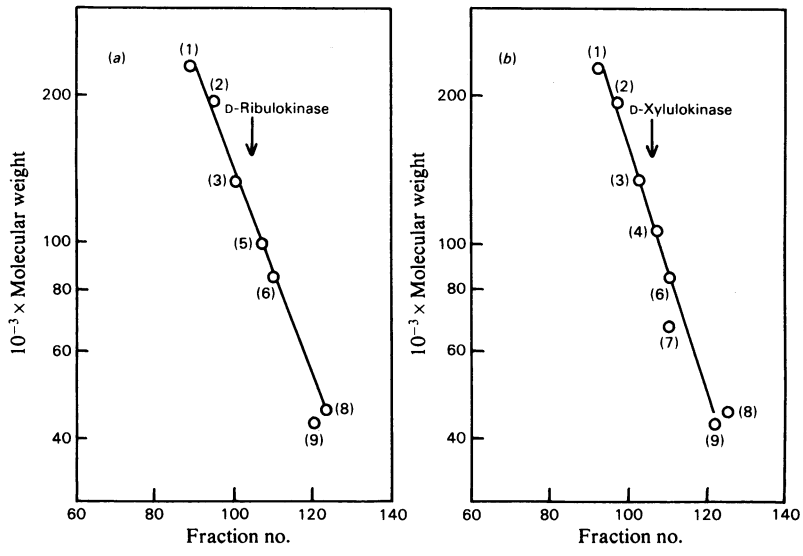


Fig. 2. *Molecular weights of native* D-*ribulokinase and* D-*xylulokinase by gel filtration*

Samples (5ml) containing the pentulokinase and molecular-weight markers were loaded on to Ultrogel AcA-34 columns (82cm × 5cm diam.) and eluted with 100mM-buffer at 80ml/h. Fractions (10ml) were collected from the time of sample application. The eluted enzymes were located by assay and bovine serum albumin and ovalbumin by measuring the A_{280} of the fractions. (*a*) Molecular weight of D-ribulokinase; the column was run at 4°C. (*b*) Molecular weight of D-xylulokinase; the column was run at room temperature. Molecular-weight markers were: rabbit muscle pyruvate kinase (1) (mol.wt. 237000); pig heart fumarase (2) (mol.wt. 204000); bovine heart lactate dehydrogenase (3) (mol.wt. 136700); *K. aerogenes* ribitol dehydrogenase (4) (mol.wt. 108000); yeast hexokinase (5) (mol.wt. 102000); *E. coli* alkaline phosphatase (6) (mol.wt. 86000); bovine serum albumin (7) (mol.wt. 68000); *K. aerogenes* D-arabitol dehydrogenase (8) (mol.wt. 46000); ovalbumin (9) (mol.wt. 43250).

(Lee & Muench, 1969). It is therefore worth noting that the 37°C re-activation of pre-cooled D-xylulokinase was not inhibited by the addition of bovine serum albumin to 5mg/ml.

Freshly purified enzyme was used for the determination of the native molecular weight and kinetic parameters. The remainder of the enzyme was stored salt-free and freeze-dried at −20°C.

Molecular weights

The native molecular weights of the purified enzymes were determined by gel filtration in an Ultrogel AcA-34 column with protein markers of known molecular weights (Fig. 2). The elution positions of the enzymes gave an estimate of approx. 112000 for the molecular weight of D-ribulokinase and approx. 110000 for D-xylulokinase.

SDS/polyacrylamide-gel electrophoresis of the enzymes with proteins of known subunit molecular weights gave values of 60000 for the subunit molecular weight of D-ribulokinase and 54000 for the subunit molecular weight of D-xylulokinase (Fig. 3).

As both the purified enzymes gave a single band in SDS/polyacrylamide-gel electrophoresis and each yielded a unique *N*-terminal sequence, both the kinases must be dimers, each being composed of a single subunit type.

Amino acid compositions and N-terminal sequences

The results obtained from analysis of the products of acid hydrolysis of the two pentulokinases are presented in Table 2. The numbers of residues per subunit were calculated assuming a subunit molecular weight of 60000 for D-ribulokinase and one of 54000 for D-xylulokinase. Tryptophan was estimated spectrophotometrically by the method of Beaven & Holiday (1952), and cysteic acid was estimated from protein that had been oxidized with performic acid.

The *N*-terminal sequences of the pentulokinases were determined as described under 'Methods' in the Experimental section, and the results are presented in Fig. 4.

Substrate specificities

The kinetic constants of D-xylulokinase for D-xylulose and of D-ribulokinase for D-ribulose at an ATP concentration of 0.5mM were determined by using the pyruvate kinase/lactate dehydrogenase

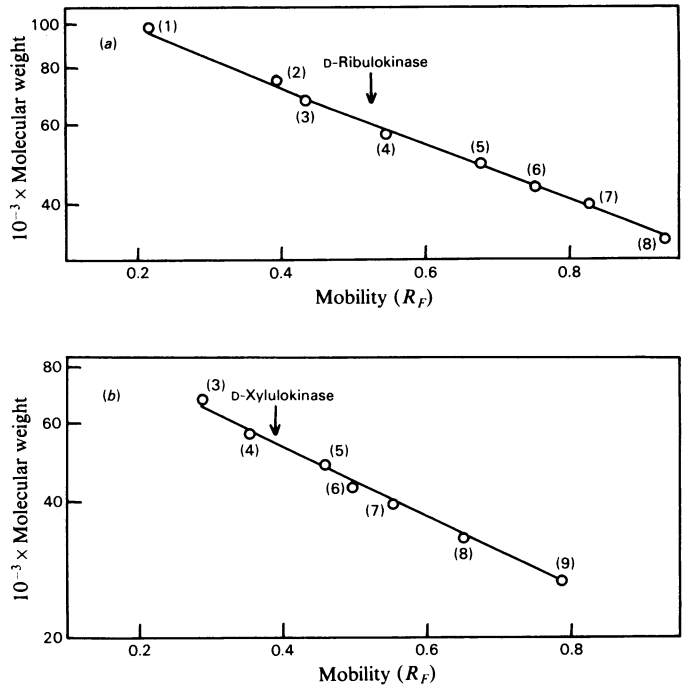


Fig. 3. *Subunit molecular weights of* D-*ribulokinase and* D-*xylulokinase*

Electrophoresis was performed in 8% (w/v) (*a*) or 9% (w/v) (*b*) polyacrylamide gels containing 0.1% (w/v) SDS. Protein markers were: *E. coli* isoleucyl-tRNA synthetase (1) (mol.wt. 101000); *E. coli* methionyl-tRNA synthetase (2) (mol.wt. 75000); bovine serum albumin (3) (mol.wt. 68000); rabbit muscle pyruvate kinase (4) (mol.wt. 57000); pig heart fumarase (5) (mol.wt. 49000); ovalbumin (6) (mol.wt. 43250); rabbit muscle aldolase (7) (mol.wt. 40000); bovine heart lactate dehydrogenase (8) (mol.wt. 35000); *K. aerogenes* ribitol dehyrogenase (9) (mol.wt. 27000).

coupled assay system (Fig. 5). The deduced kinetic constants, designated $K_{app.}$ and $V_{app.}$, are presented in Table 3.

Experiments on the pentitol operons of *E. coli* C have prompted speculation about the substrate specificities of the pentulokinases from this organism (Scangos & Reiner, 1978*b*, 1979). Unlike *E. coli* K12, *E. coli* C can utilize both ribitol and D-arabitol as sole source of carbon and energy because, for each of these pentitol substrates, the organism possesses an operon encoding a pentitol dehydrogenase and a pentulokinase. The two operons are clustered on the bacterial chromosome with a gene order identical with that of the *K. aerogenes* operons (Scangos & Reiner, 1978*a*), and the ribitol dehydrogenases of *E. coli* C and *K. aerogenes* show about 95% homology in their amino acid sequences (Altosaar & Hartley, 1976). Scangos & Reiner (1979) have shown that D-arabitol is toxic to mutants of *E. coli* C that are constitutive for D-arabitol operon expression but that lack D-arabitol dehydrogenase activity. D-Arabitol-resistant mutants derived from such strains have lost the constitutive D-xylulokinase activity. These authors have therefore proposed that the D-xylulokinase activity encoded in the *E. coli* C D-arabitol operon can phosphorylate D-arabitol, and that this action is lethal to the organism as the D-arabitol phosphate formed cannot be further metabolized. Similar experiments on the *E. coli* C ribitol operon led Scangos & Reiner (1978*b*) to propose that D-ribulokinase can phosphorylate galactitol, L-arabitol and D-arabitol.

We therefore tested the substrate specificities of the purified *K. aerogenes* pentulokinases. No activity of purified D-xylulokinase towards D-arabitol was detected (i.e. an activity of less than 0.3 μmol/min per mg on 200 mM-substrate). However, D-ribulokinase showed activity towards both ribitol and D-arabitol (Fig. 5), but no activity towards D-xylulose, xylitol, L-arabitol, galactitol, D-mannitol, D-sorbitol, erythritol, glycerol, D-ribose, D-arabinose, D-fructose or D-glucose (an activity of less than 0.1 μmol/min per mg on 100 mM-substrate). Thus D-ribulokinase will phosphorylate D-ketopentoses and D-pentitols that contain vicinal hydroxy groups

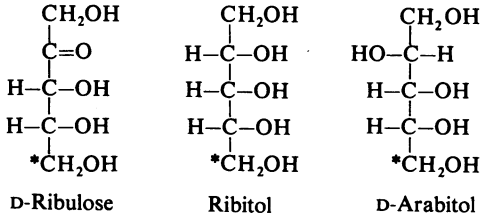
Table 2. *Amino acid analysis of* D-*ribulokinase and* D-*xylulokinase*
Experimental details are given in the text.

Amino acid	Amino acid composition (residues/subunit): D-Ribulokinase (subunit mol.wt. 60000)	D-Xylulokinase (subunit mol.wt. 54000)
Lysine	13.3	18.4
Histidine	17.9	18.8
Arginine	29.2	23.6
Aspartic acid and asparagine	45.1	37.3
Threonine	32.1	24.1
Serine	32.3	32.6
Glutamic acid and glutamine	53.5	51.0
Proline	30.0	27.4
Glycine	57.4	44.4
Alanine	78.5	68.3
Valine	46.9	28.6
Methionine	11.2	14.6
Isoleucine	31.2	22.1
Leucine	46.8	61.6
Tyrosine	14.4	8.7
Phenylalanine	13.8	8.7
Tryptophan	8.6	8.6
Cysteic acid	5.4	7.4
Total	567.6	506.2

Table 3. *Substrate specificities of the* D-*pentulokinases*
Experimental details are given in the text.

Enzyme	Substrate	$K_{app.}$ (mM)	$V_{app.}$ (μmol/min per mg)
D-Xylulokinase	D-Xylulose	0.8	150
D-Ribulokinase	D-Ribulose	0.4	71
	Ribitol	220	12
	D-Arabitol	140	6.6

on the carbon positions α and β to the position of phosphorylation. This is illustrated below, with the carbon position at which phosphorylation takes place being indicated by an asterisk:



Aldo-sugars and hexitols will not act as substrates.

The kinetic constants of D-ribulokinase for ribitol

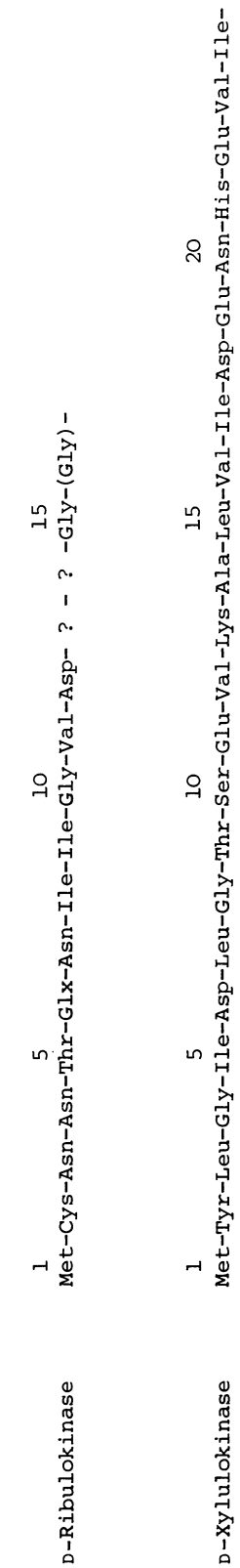


Fig. 4. *N-Terminal sequences of the pentulokinases*
The residue in parenthesis indicates that the assignment is tentative, and '?' indicates that no assignment could be made for this residue.

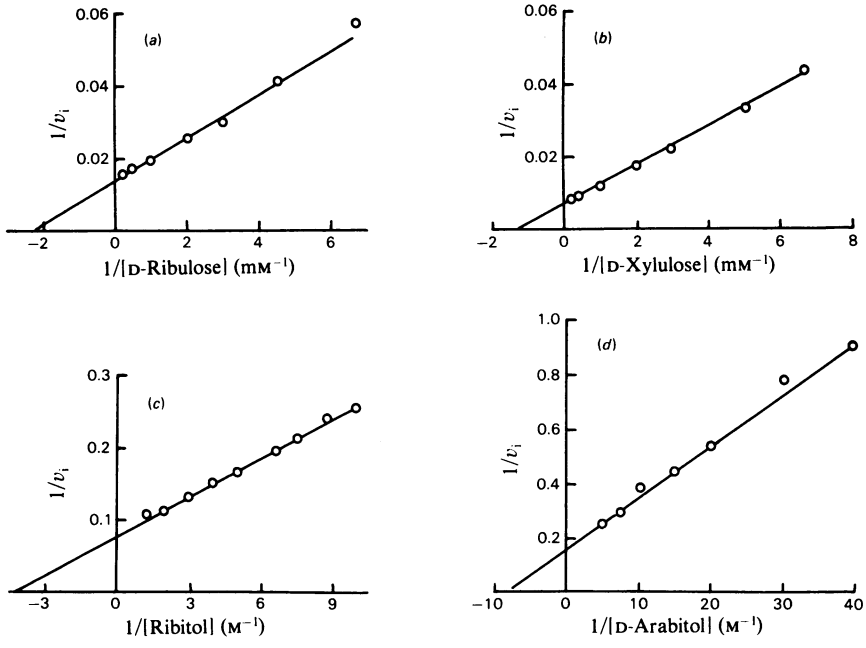


Fig. 5. *Plots of the activities of the pentulokinases on various substrates*

Initial rates of reaction (v_i), which are given in μmol/min per mg, were determined by using the pyruvate kinase/lactate dehydrogenase coupled assay at a single concentration (0.5 mM) of ATP. (*a*) Activity of D-ribulokinase on D-ribulose; (*b*) activity of D-xylulokinase on D-xylulose; (*c*) activity of D-ribulokinase on ribitol; (*d*) activity of D-ribulokinase on D-arabitol.

and D-arabitol are presented in Table 3. However, because of the nature of the assay, it had only, in fact, been demonstrated that D-ribulokinase can catalyse a ribitol- or D-arabitol-dependent generation of ADP from ATP. To demonstrate that the pentitol phosphates were indeed products of the reactions, samples of the reaction mixtures were subjected to high-voltage paper electrophoresis. After staining with $AgNO_3$, the reaction mixtures were observed to give rise to spots of the same electrophoretic mobility as the glucose 1-phosphate marker. These spots, putatively due to the pentitol phosphates, were eluted and analysed by field-desorption mass spectrometry. In each case a major peak at an *m/e* value of 232 was observed; this corresponds to the free acid form of the pentitol phosphates. However, there is apparent disagreement between the prediction by Scangos & Reiner (1978*b*) that the *E. coli* C D-ribulokinase can phosphorylate L-arabitol and galactitol and our inability to detect such activities with the purified *K. aerogenes* enzyme. Clearly the data could be reconciled by a difference in the substrate specificities of the D-ribulokinases of the two organisms. We therefore tested whether L-arabitol and galactitol were toxic to an *E. coli* K12 strain harbouring a constitutive *K. aerogenes rbt* operon. *E. coli* K12 strains NC621 (a λ*prbt-101* lysogen of strain CSH62) and NC63 (a λ627 lysogen of strain CSH62, to serve as a control) were streaked on CH plates and on CH plates supplemented with 10 mM-galactitol, -L-arabitol or -D-arabitol and incubated at 32°C. It was apparent that all three polyols were toxic to strain NC621 but not to strain NC63. Thus a difference between the substrate specificities of the D-ribulokinases from the two organisms cannot account for the discrepancy between the predictions made from the toxicity of galactitol and L-arabitol and our inability to detect their phosphorylation by the purified *K. aerogenes* enzyme. Two types of explanation can be proposed to reconcile the data. The D-ribulokinases might phosphorylate galactitol and L-ribitol, but so inefficiently that the activity was not detected with the purified enzyme. Alternatively, it could be proposed that it is not galactitol and L-arabitol, but one of their catabolic intermediates, that serve as substrates for a toxic phosphorylation. For example, from the data of Scangos & Reiner (1978*b*), it cannot be excluded that L-arabitol is oxidized to L-xylulose by the action of ribitol dehydrogenase (Burleigh *et al.*, 1974), and that it is the phosphorylation of L-xylulose that is toxic to the cell. However, at present, it is not possible to decide which type of explanation is correct.

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