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Metabolism of D-Arabinose: Origin of a D-Ribulokinase Activity in *Escherichia coli*[1]

DONALD J. LEBLANC[2] AND ROBERT P. MORTLOCK
Department of Microbiology, University of Massachusetts, Amherst, Massachusetts, 01002

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The kinase responsible for the phosphorylation of D-ribulose was purified 45.5-fold from a strain of *Escherichia coli* K-12 capable of growth on D-arabinose with no separation of D-ribulo- or L-fuculokinase activities. Throughout the purification, the ratios of activities remained essentially constant. A nonadditive effect of combining both substrates in an assay mixture; identical K_m values for adenosine triphosphate with either L-fuculose or D-ribulose as substrate; and, the irreversible loss of activity on both substrates, after removal of magnesium ions from the enzyme preparation, suggest that the dual activity is due to the same enzyme. A fourfold greater affinity of the enzyme for L-fuculose than for D-ribulose, as well as a higher relative activity on L-fuculose, suggest that the natural substrate for this enzyme is L-fuculose. The product of the purified enzyme, with D-ribulose as substrate, was prepared. The ratio of total phosphorous to ribulose phosphate was 1.01:1, indicating that the product was ribulose monophosphate. The behavior of the kinase product in the cysteine-carbazole and orcinol reactions, as well as the results of periodate oxidation assays, provided evidence that it was not D-ribulose-5-phosphate. Reaction of this compound with a cell-free extract of *E. coli* possessing L-fuculose-1-phosphate aldolase activity resulted in the production of dihydroxyacetone phosphate and glycolaldehyde. The kinase product failed to reduce 2,3,5-triphenyltetrazolium and possessed a half-life of approximately 1.5 min in the presence of 1 N HCl at 100 C. These properties suggested that the phosphate group was attached to carbon atom 1 of D-ribulose.

Mutants of *Aerobacter aerogenes* PRL-R3 can be selected which possess the ability to utilize the aldopentose, D-arabinose, as a sole source of carbon and energy. These mutants degrade D-arabinose by isomerization to D-ribulose followed by phosphorylation to D-ribulose-5-phosphate. Camyre and Mortlock (5) showed that *A. aerogenes* acquires the ability to grow on D-arabinose by loss of control over the synthesis of L-fucose isomerase (EC 5.3.1.3). Oliver and Mortlock (Bacteriol. Proc., p. 123, 1968) purified the isomerase activity responsible for the conversion of D-arabinose to D-ribulose and showed it to be identical to L-fucose isomerase.

The D-ribulose produced by the action of L-fucose isomerase is also an intermediate in the metabolism of ribitol by *A. aerogenes*. Bisson et al. (3) showed that D-ribulose is the inducer of the two enzymes unique to the metabolism of ribitol, ribitol dehydrogenase (EC 1.1.1.56) and D-ribulokinase (EC 2.7.1.47). This D-ribulokinase from the ribitol pathway phosphorylates D-ribulose at carbon 5. Thus, mutants of *A. aerogenes*, during growth on D-arabinose, use the isomerase of the L-fucose pathway to catalyze the isomerization of D-arabinose to D-ribulose and the kinase of the ribitol pathway to phosphorylate the D-ribulose to D-ribulose-5-phosphate.

Mutants of *Escherichia coli* can be selected which have acquired the ability to grow on D-arabinose. Green and Cohen (13) provided evidence that L-fucose isomerase and D-arabinose isomerase activities in *E. coli* are due to the same enzyme. Whereas *A. aerogenes* utilizes the inducible D-ribulokinase of the ribitol pathway for further degradation of D-arabinose, no strains of *E. coli* were observed which utilize ribitol as a growth substrate. The lack of a functional ribitol pathway in *E. coli* presented a problem concerning the origin of a kinase for the phosphorylation of D-ribulose during the growth of this organism on D-arabinose. Heath and Ghalambor

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[2] Present address: Department of Microbiology, Georgetown University Medical School, Washington, D.C. 20007.

(15) reported activity of L-fuculokinase (EC 2.7.1.51) on D-ribulose, and similar results were obtained by Lim and Cohen (19). Although these latter authors reported the phosphorylation of D-ribulose at carbon 5 by L-fuculokinase, an enzyme known to phosphorylate L-fuculose at carbon 1 (15), the identity of the enzyme(s) responsible for the phosphorylation of L-fuculose and D-ribulose was not demonstrated. Up to 10% of the ribulose phosphorylated by the enzyme preparation of these authors was found to be D-ribulose-1-phosphate. Heath and Ghalambor (15) did not determine the nature of the product formed by the action of L-fuculokinase on D-ribulose.

The present investigation was undertaken to determine the origin of the kinase activity responsible for the phosphorylation of D-ribulose in *E. coli* during growth on D-arabinose, as well as to determine the nature of the phosphorylated product of this reaction.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. The parent strain used in this investigation was *E. coli* strain 1000, an HfrH derivative of K-12, obtained from C. B. Thorne (Univ. of Massachusetts, Amherst). This strain was incapable of growth on D-xylose (*xyl*$^-$). Mutants of strain 1000, capable of utilizing D-arabinose as sole source of carbon and energy, were selected by incubation in minimal salts (7) plus 0.3% D-arabinose. One of these D-arabinose-positive isolates, strain 1102, was selected for studies on the origin of D-ribulokinase in *E. coli* K-12.

Cells were grown aerobically at 37 C on a minimal medium (7) supplemented with 0.3% carbohydrate or 1.0% casein hydrolysate, or both. $MgSO_4$ and carbon and energy sources were autoclaved separately and added after cooling. For the preparation of cell-free extracts in the purification of D-ribulokinase activity, 5 liters of exponentially growing cells were obtained on minimal salts + 0.3% D-arabinose on a Microferm Laboratory Fermentor (New Brunswick Scientific Co., Inc., New Brunswick, N.J.).

Kinase assay and analytical procedures. Kinase assays, for monitoring the purification of D-ribulokinase activity, were based on the continuous spectrophotometric measurement of adenosine diphosphate (ADP) formation with the pyruvate kinase-lactic acid dehydrogenase system (2). Absorbancy changes at 340 nm, due to the oxidation of reduced nicotinamide adenine dinucleotide (NADH), were measured at room temperature in a DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) and monitored with a model 2000 recording attachment (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). For the determination of a K_m value for Mg^{2+} with the purified kinase, enzymatic activity was determined by the rate of disappearance of pentulose (19).

A unit of activity was defined as that amount of enzyme necessary to oxidize 1 nmole of NADH, or to result in the disappearance of 1 nmole of pentulose, in 1 min. The protein content was measured by determining the ratio of absorbancies at 280 and 260 nm (29).

Ketose sugars were determined by the cysteine-carbazole method (9). Absorption spectra with the orcinol test for pentose (21) were obtained on a ratio recording spectrophotometer (model DK-1A; Beckman Instruments, Inc., Fullerton, Calif.). Estimation of inorganic phosphate, either total or acid hydrolyzed, was by the method of Fiske and SubbaRow (11). Periodate consumption was determined by the colorimetric method, at 265 nm (16), and also by iodine titration (24).

Reducing sugar was determined by reaction with 2,3,5-triphenyltetrazolium (10). Because of the lability of some of the ketoses and ketose phosphates under the alkaline conditions employed, the 3-min heat step was replaced by a 10-min incubation at room temperature. Standard curves with D-ribulose and D-fructose were constructed for the quantitative determination of reducing sugar.

Glycoaldehyde was measured colorimetrically after formation of an osazone (27). Dihydroxyacetone phosphate was measured spectrophotometrically with α-glycerophosphate dehydrogenase and NADH (12).

Chemicals. D-Ribulose was synthesized enzymatically by the oxidation of ribitol (25), and isolated as the *o*-nitrophenylhydrazone derivative (6). For enzymatic studies, D-ribulose was regenerated by the procedure of Muller et al. (23).

L-Fuculose was prepared by the enzymatic isomerization of L-fucose (13). The L-fuculose was separated from residual L-fucose by paper chromatography in a solvent system consisting of pyridine-butanol-water (2:2:1). After drying the paper, the locations of L-fucose and L-fuculose were detected by the silver nitrate method (28). The L-fuculose was eluted from the paper with distilled water.

The alumina c gamma, for the purification of D-ribulokinase, was prepared by the method of Colowick (8). D-Xylulose, L-xylulose, and L-ribulose were prepared chemically by refluxing the corresponding aldopentose with dry pyridine (22). L-Ribulose-5-phosphate was synthesized enzymatically by using a cell-free extract of *A. aerogenes* constitutive for L-ribulokinase and deficient in L-arabinose isomerase and L-ribulose-5-phosphate 4-epimerase activities, by the procedure of Anderson (1).

Adenosine-5′-triphosphate (ATP), D-arabinose, L-fucose, and D-ribulose-5-phosphate (barium salt) were purchased from Sigma Chemical Co., St. Louis, Mo. Fructose-1-phosphate and fructose-6-phosphate were purchased from Calbiochem, Los Angeles, Calif.

Purification of D-ribulokinase: preparation of cell-free extract. An exponentially growing culture of strain 1102, in 5 liters of minimal salts plus 0.3% D-arabinose plus 0.1% casein hydrolysate, constituting 3.0 grams (dry weight) of cells, was harvested by centrifugation, washed twice in minimal salts solution, and suspended in 50 ml of buffer [0.01 M tris(hydroxymethyl)aminomethane-hydrochloride (*p*H 7.8), 0.004 M ethylenediaminetetraacetic acid (EDTA), 0.004 M dithiothreitol (DDT), and 0.008 M magnesium acetate]. This cell suspension was stored overnight at −20 C. The frozen cells were thawed and broken by sonic treatment. After removal of cellular debris by centrifugation, the supernatant fluid was stored in an ice bath. All subsequent

steps in the purification procedure were carried out in an ice bath, or under refrigeration at 4 C.

Protamine sulfate fractionation. The crude extract was diluted to 10 mg of protein per ml by the addition of buffer (80 ml, total volume). Solid ammonium sulfate was added slowly to 0.1 M, followed by the addition of a 2% solution of protamine sulfate to a final concentration of 15 mg of protamine sulfate per ml. The precipitate was removed by centrifugation.

Ammonium sulfate fractionation. Buffer was added to the supernatant fluid to dilute the protein to 2.2 mg per ml (total volume, 600 ml). Solid ammonium sulfate was added slowly to raise the ammonium sulfate concentration to 1.47 M. The precipitate was removed by centrifugation, and the supernatant fluid was treated with solid ammonium sulfate to raise the concentration to 2.28 M. This fraction, containing the majority of D-ribulokinase activity, was collected by centrifugation and resuspended in 21 ml of buffer.

Dialysis. The resuspended precipitate was dialyzed for 5 hr against 12.5 volumes of buffer, followed by transfer to 12.5 volumes of fresh buffer for an additional 12 hr.

Elution from DEAE cellulose. The dialyzed enzyme fraction was applied to a diethylaminoethyl (DEAE) cellulose column (17 by 115 mm) previously equilibrated with buffer. Elution was effected by collecting four 25-ml fractions at each of the following NaCl concentrations in buffer: 0.06, 0.10, 0.15, and 0.20 M. All fractions were collected at a rate of 1 ml per min. Most of the D-ribulokinase activity was found in the second, third, and fourth 0.15 M fractions which were pooled.

Adsorption on alumina c gamma. Pooled enzyme fractions were adsorbed to alumina c gamma by mixing the two components at a ratio of 6.75 mg of alumina c gamma per mg of protein. Elution of the protein from the gel was effected by washing with increasing concentrations of potassium phosphate in the buffer solution described previously. Most of the D-ribulokinase activity was eluted at 0.02 M phosphate. The enzyme at this point constituted a 45.5-fold purification, calculated from the activity observed after the protamine sulfate treatment, and was devoid of any detectable adenosine triphosphatase activity. The purification procedure is summarized in Table 1.

Preparation of ribulose phosphate. The product of the phosphorylation reaction was prepared by mixing 1.29 mmoles of D-ribulose, 2.57 mmoles of ATP, 40 μmoles of EDTA, 80 μmoles of magnesium acetate, and 40 μmoles of DDT. After adjustment of the *p*H to 7.5 with NaOH, 35,000 units of D-ribulokinase (nanomoles per minute) were added, raising the total reaction volume to 25 ml. The *p*H was maintained at 7.5 to 7.8 by periodic addition of 0.1 M NaOH. The reaction was complete in 3 hr. Glacial acetic acid, 0.7 ml, was added, and the precipitate was removed by centrifugation. The *p*H of the supernatant fluid was then adjusted to 7.0 with NaOH.

The phosphorylated sugar was separated from the reaction mixture by ion-exchange chromatography as follows. The reaction mixture was diluted with 2 volumes of water and applied to a Dowex 1-X4-formate column (17 by 330 mm). The column was washed with water and eluted by gradient procedures. A Varigrad gradient maker (Buchler Instrument, Inc., Fort Lee, N.J.) was used to establish a gradient from 0 to 0.03 N sodium formate plus 0 to 0.2 N formic acid. Fractions of 20-ml size were collected. The elution of nucleotide material was monitored by measuring the 255 nm absorbing material with a Canalco Widetrack 85 Ultraviolet Flow Analyzer (Canal Industrial Corp., Rockville, Md.). Sugar phosphate was monitored by assay for cysteine-carbazole-reactive material (9). Fractions 90 through 135 were pooled.

The solution was concentrated in vacuo, at 35 C, to a volume of 40 ml. The *p*H was adjusted to 6.7 with NaOH, and 4 mmoles of barium acetate were added. The *p*H was again adjusted to 6.7 by the addition of dilute acetic acid. The resultant precipitate was removed by centrifugation, and 4 volumes of 95% ethanol were added to the supernatant fluid. After refrigeration at 4 C for 3 hr, the white flocculent precipitate was collected by centrifugation, washed with 20 ml of absolute ethanol, and allowed to dry overnight at room temperature. The dried barium salt of D-ribulose phosphate weighed 161.6 mg.

RESULTS

Properties of D-ribulokinase. Cell-free extracts of *E. coli* strain 1102, previously grown on D-arabinose, were shown to possess kinase activity on L-fuculose, as well as on D-ribulose. Several properties of the purified enzyme preparation were studied, primarily to determine whether the dual activity observed in crude extracts was due to the same enzyme. Throughout the purification, the kinase catalyzed the phosphorylation of L-fuculose with twice the relative activity as that found for D-ribulose. When these two ketoses were combined in an assay mixture, the activity was the same as on L-fuculose alone. D-Xylulose, L-ribulose, L-xylulose, D-fructose, D-arabinose, and L-fucose were inactive.

The biological activities of D-ribulose and L-fuculose were determined by comparing the per cent of cysteine-carbazole-reactive material which was capable of serving as substrate for the purified D-ribulokinase. With the D-ribulose preparation used in these studies, 60% of the cysteine-carbazole-reactive material was capable of serving as substrate for the kinase. The biological activity of the L-fuculose was found to be 23%. In all of the kinetic studies, the biological activity was used for calculation of substrate concentration.

K_m values, calculated from double reciprocal plots, indicated that the affinity for L-fuculose (K_m of 1.9×10^{-4} M), was approximately four times greater than the affinity for D-ribulose (K_m of 7.3×10^{-4} M). The K_m values for ATP were 5.3×10^{-4} M with L-fuculose as sugar substrate, and 5.5×10^{-4} M with D-ribulose as sugar substrate.

When magnesium ions were removed from the enzyme preparation by addition of excess EDTA,

TABLE 1. *Purification of D-ribulokinase activity from Escherichia coli*

Step	Total activity (units)[a]	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Cell-free extract	358,000	—[b]	—[b]	—[b]
Protamine sulfate	540,000	385	1.00	100
Ammonium sulfate	126,000	600	1.56	23
Dialysis	116,000	580	1.50	21.5
DEAE cellulose	122,000	10,800	28.50	22.6
Alumina c gamma	70,000	17,500	45.50	13

[a] One unit equals 1 nmole of D-ribulose phosphorylated per minute.

[b] Because of the presence of interfering enzymes, particularly adenosine triphosphatase activities, it was difficult to determine accurately the total amount of D-ribulokinase in the extract before the protamine sulfate step.

TABLE 2. *Properties of D-ribulokinase from Escherichia coli*

Substrate	Result
Relative velocity[a]	
L-Fuculose	100
D-Ribulose	55
L-Fuculose + D-ribulose	100
D-Xylulose, L-ribulose, L-xylulose, D-fructose, D-arabinose, and L-fucose	0[b]
K_m value (10^{-4} M)	
L-Fuculose	1.9
D-Ribulose	7.3
ATP (L-fuculose)[c]	5.3
ATP (D-ribulose)[c]	5.5
Mg^{2+} (D-ribulose)[c]	7.3

[a] Substrate concentrations for measurement of relative activity were 10^{-2} M.

[b] Zero activity, not detectable by continuous spectrophotometric assay (2).

[c] Sugar used, at concentration normally permitting the reaction to proceed at V_{max}.

all activity was irreversibly lost with both L-fuculose and D-ribulose. To determine the K_m value for magnesium, the pentulose disappearance assay (19) was required, since the continuous spectrophotometric assay (2) was dependent on the presence of magnesium ions. Because of the greater amounts of enzyme required for the pentulose disappearance assay, kinetic studies with magnesium were carried out only with D-ribulose as sugar substrate. The K_m for magnesium was found to be 7.3×10^{-4} M.

The higher K_m for D-ribulose and the greater relative activity on L-fuculose suggested that D-ribulose was probably a secondary substrate, whereas L-fuculose was the natural substrate for this enzyme. The nonadditive effect of combining both substrates, the identical K_m values for ATP with either pentulose as sugar substrate, and the irreversible loss of activity on either substrate upon removal of magnesium ions suggested that the dual activity was due to the same enzyme. The properties discussed above are summarized in Table 2.

Identification of the kinase product. L-Fuculose-1-phosphate was isolated as the product of L-fuculokinase with L-fuculose in *E. coli* (15). Since the D-ribulokinase activity isolated from *E. coli* strain 1102 appeared to be due to L-fuculokinase, it was important to determine at which position D-ribulose was phosphorylated by this enzyme.

The product of the phosphorylation reaction was prepared and separated from the rest of the reaction mixture, particularly nucleotides, by ion-exchange chromatography as previously described. The peak representing cysteine-carbazole-reactive material occurred from fractions 90 through 135, and these tubes were pooled. These pooled fractions exhibited negligible absorption at 260 and 280 nm, suggesting that the sample was relatively free of nucleotide and protein material. Treatment of a portion of this solution with $Ba(OH)_2$ and $ZnSO_4$ resulted in the loss of all cysteine-carbazole-reactive material, indicating that all of the ketose sugar was phosphorylated. After concentration of the solution, the ribulose phosphate was isolated as the barium salt.

The 1,290 μmoles of D-ribulose initially added to the reaction mixture was measured as cysteine-carbazole-reactive material. As mentioned above, only 60% (775 μmoles) of this material was capable of serving as substrate for the purified kinase. Thus, the 161.6 mg (443 μmoles) of product represented nearly a 60% yield. This preparation was used in all subsequent studies on the nature of the phosphorylated product of D-ribulokinase.

The cysteine-carbazole reaction provided the first evidence that the product of D-ribulokinase from *E. coli* was not D-ribulose-5-phosphate. The time required for maximum color development with D-ribulose-5-phosphate was 135 min, whereas the unknown product required only 45 min and D-ribulose reached full color intensity within 15 min. With all three of the above compounds, maximal absorption was obtained at 540 nm. After hydrolysis of the phosphate group of both D-ribulose-5-phosphate and the unknown, the time required to reach maximal development of color in the cysteine-carbazole reaction was diminished to 15 min.

D-Ribulose-5-phosphate reduced 2 moles of periodic acid per mole of sugar phosphate. The unknown kinase product was found to reduce approximately 1 mole of periodic acid per mole of sugar phosphate. The spectrophotometric assay (16) produced a ratio of 1.04:1, whereas

the iodine titration procedure (24) gave a ratio of 1.3:1, which did not increase after 1 week of incubation at room temperature.

Further evidence that the unknown product was not D-ribulose-5-phosphate was provided by the orcinol reaction. After heating for 40 min with the orcinol reagents, the 540:670 nm ratios were 0.34 for D-ribulose-5-phosphate, 0.71 for D-ribulose, and 1.44 for the kinase product. These properties are summarized in Table 3. The absorption spectra of the orcinol reaction for the above compounds, as well as for L-ribulose-5-phosphate, D-arabinose, and ATP can be seen in Fig. 1.

Estimation of total phosphate (18) confirmed the assumption that the kinase product was ribulose monophosphate. From 0.725 μmole of ketose phosphate, 0.73 μmole of inorganic phosphate was produced.

Ketoses, under alkaline conditions, undergo a Lobry de Bruyn-van Ekenstein transformation. Reagents used to assay reducing sugars are alkaline reagents, and thus give a positive test with ketoses. Ribulose and fructose, as well as ribulose-5-phosphate and fructose-6-phosphate, should, therefore, reduce 2,3,5-triphenyltetra-

TABLE 3. *Cysteine-carbazole, orcinol, and periodate reactions of ribulose phosphates*

Reaction	Kinase product	D-Ribulose-5-phosphate	D-Ribulose
Cysteine-carbazole			
Maximum absorption (nm)	540	540	540
Development time (min)	45	135	15
Development time after acid hydrolysis (min)[a]	15	15	15
Orcinol: 540:670 nm	1.44	0.34	0.71
Periodate reduced per μmole			
Spectrophotometric assay	1.04	1.93	
Iodine titration assay	1.30	2.40	

[a] Each compound was heated for 10 min in 1 N HCl at 100 C. Any unhydrolyzed sugar phosphate was precipitated with $Ba(OH)_2$ and $ZnSO_4$, and the development time of the hydrolyzed product in the supernatant fluid was determined.

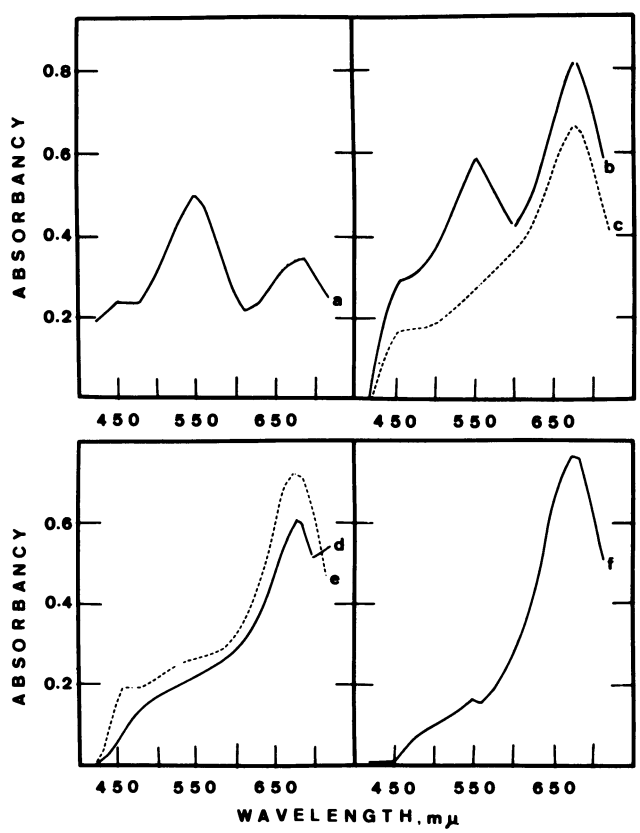

FIG. 1. *Absorption spectra of sugars and sugar phosphates with the orcinol reaction. Curve a, kinase product; curve b, D-ribulose; curve c, L-ribulose-5-phosphate; curve d, D-ribulose-5-phosphate; curve e, D-arabinose; curve f, adenosine triphosphate.*

zolium. However, since the Lobry de Bruyn-van Ekenstein transformation initially occurs between the 1 and 2 carbon atoms, phosphorylation of fructose or ribulose at carbon atom 1 should block this reaction, thus abolishing the reducing ability of these two ketoses. Standard curves, using D-ribulose and D-fructose, were prepared to test the reducing ability of the above ketose phosphates. One micromole each of fructose-1-phosphate, fructose-6-phosphate, D-ribulose-5-phosphate, and the kinase product were assayed for ability to reduce 2,3,5-triphenyltetrazolium, as previously described. The results of this experiment can be seen in Table 4. One micromole of fructose-6-phosphate and D-ribulose-5-phosphate were equivalent to 1 μmole and 0.97 μmole of reducing sugar, respectively. However, fructose-1-phosphate and the kinase product were equivalent to 0.04 and 0 μmoles of reducing sugar.

If the phosphate ester of a ketose-1-phosphate was hydrolyzed, reducing ability should be recovered. One micromole of each of the above compounds was heated at 100 C in 1 N HCl for 10 min, followed by cooling and neutralization. These solutions were then assayed for reducing ability. As can be seen in Table 4, there is a direct correlation between the amount of inorganic phosphate released by acid hydrolysis and the amount of reducing sugar present with fructose-1-phosphate and the kinase product. With fructose-6-phosphate and ribulose-5-phosphate, the amount of reducing sugar remained essentially constant before and after hydrolysis. The data presented in Table 4 strongly suggest, therefore, that the product of D-ribulokinase in *E. coli* K-12 is D-ribulose-1-phosphate.

Information on the identity of phosphoric esters can be obtained by measurement of the rate of hydrolysis. The time required for 50% hydrolysis of fructose-6-phosphate in 1 N acid at 100 C is approximately 25 times longer than for 50% hydrolysis of fructose-1-phosphate under the same conditions (18). Horecker, et al. (16) showed that D-ribulose-1,5-diphosphate was considerably more acid labile than D-ribulose-5-phosphate. These authors suggested that the presence of the 1-phosphate also renders the 5-position linkage more labile to acid. One would predict, therefore, that D-ribulose-1-phosphate would be considerably more acid labile than D-ribulose-5-phosphate. To determine the acid lability of the kinase product, eight test tubes were prepared containing 1 μmole of the unknown product and 1 N HCl in a volume of 1 ml. The tubes were heated in a boiling-water bath; at the end of 0, 2, 5, 7, 10, 30, 60, and 90 min, a tube was removed, cooled, and neutralized. The inorganic phosphate present in each tube was estimated (11). The same experiment was also performed with fructose-1-phosphate, fructose-6-phosphate, and ribulose-5-phosphate. From plots of micromoles of inorganic phosphate versus time of hydrolysis, the time for 50% hydrolysis ($t_{½}$) was determined. The results (Table 5) demonstrated that the kinase product was 50% hydrolyzed approximately 50 times faster than D-ribulose-5-phosphate.

Ghalambor and Heath (12) showed that the condensation product of L-fuculose-1-phosphate aldolase (EC 4.1.2.17) with dihydroxyacetone phosphate and glycolaldehyde was D-ribulose-1-phosphate. If the unknown kinase product were D-ribulose-1-phosphate, then the cleavage products with this aldolase should be the above-mentioned 3- and 2-carbon units.

To test the ability of the kinase product to serve as substrate for L-fuculose-1-phosphate aldolase, the two-step assay procedure of Ghalambor and Heath (12) was used. The kinase product was converted to the sodium salt, and "crude extracts" were prepared for aldolase assays as described in the accompanying paper (17). Specific activities were determined by measuring the production of dihydroxyacetone phosphate, as well as glycoladehyde, as previously described. When the assay was performed with a crude extract prepared from a culture of *E. coli* strain 1000 previously grown in the presence of L-fu-

TABLE 4. *Reducing ability of ketose phosphates*[a]

Compound	Reducing sugar (μmoles)		Inorganic phosphate (μmoles)	
	Before hydrolysis	After hydrolysis	Before hydrolysis	After hydrolysis
Fructose-6-phosphate ..	1.00	0.98	0	0.125
Ribulose-5-phosphate ..	0.97	0.86	0	0.215
Fructose-1-phosphate ..	0.04	0.86	0	0.830
Kinase product	0	0.83	0	0.800

[a] In all cases, 1.0 μmole of sugar phosphate was assayed. Hydrolysis was for 10 min in 1 N HCl at 100 C.

TABLE 5. *Acid lability of ketose phosphates*

Compound	$t_{1/2}$ (min)	
	Found[a]	Expected[b]
Fructose-1-phosphate	3.8	2.8
Fructose-6-phosphate	60.0	70.0
Ribulose-5-phosphate	70.0	60.0
Kinase product	1.4	

[a] Time required for 50% hydrolysis at 100 C in 1 N HCl.

[b] Values for $t_{1/2}$ previously reported (18).

cose, the kinase product served as substrate and exhibited specific activities of 38 nmoles per min per mg of protein when dihydroxyacetone phosphate was determined, and 21 nmoles per min per mg of protein when glycolaldehyde was determined. No activity could be detected for the production of either compound when a crude extract was prepared from a culture of strain 1000 grown on casein hydrolysate without L-fucose. The sodium salt of D-ribulose-5-phosphate failed to produce activity in the aldolase assay when any of the above conditions were used.

DISCUSSION

The enzyme responsible for the phosphorylation of D-ribulose, during growth of *E. coli* on D-arabinose, was partially purified and identified as L-fuculokinase. The product of this enzyme, with D-ribulose as substrate, was identified as D-ribulose-1-phosphate on the basis of its biological and chemical properties.

The results of periodate oxidation studies were inconclusive regarding the structure of the kinase product. D-Ribulose-5-phosphate exists in the open chain form (19) and should consume 2 moles of periodate per mole of sugar phosphate (4). This 2:1 ratio for D-ribulose-5-phosphate was confirmed in this work and by others (19, 26). One mole of the kinase product was shown to consume 1.04 to 1.3 moles of periodate. An open chain form of D-ribulose-1-phosphate should consume 3 moles of periodate per mole of ketose phosphate (4). However, D-ribulose-1-phosphate should exist in the furanose form (19). The initial reaction with periodate, in this case, would result in cleavage of the furanose ring between carbons 3 and 4, thus producing a dialdehyde. The production of a dialdehyde, which is common in periodate oxidations of cyclic carbohydrates, may then be followed by the formation of an internal hemiacetal or hemialdal (14). These phosphorylated intermediates may be stable under the conditions employed, and thus resist further oxidation by periodate. The failure to obtain the theoretical 3 moles of periodate consumption by D-ribose-5-phosphate was attributed to the stability of a formic acid ester intermediate in acid solution (20). Because of the uncertainties introduced by the above possibilities, further identification of the kinase product by periodate oxidation studies was not attempted.

Lim and Cohen (19) reported the purification of a D-ribulokinase from *E. coli* strain B_{a15} capable of phosphorylating D-ribulose at carbon 5 and L-fuculose at carbon 1. One explanation offered by these authors proposed that their preparation contained two separate enzymes, one phosphorylating D-ribulose at position 5 and another which phosphorylated L-fuculose at position 1. This was suggested by the fact that the reactivity of the enzyme system toward the two substrates differed as a function of the extent of purification. Lim and Cohen also suggested that the small amount of ribulose-1-phosphate detected might be due to cross reactivity of the L-fuculokinase on D-ribulose. In light of the present work, this would appear to be the most logical explanation for the results of these authors.

Further evidence substantiating L-fuculokinase as the enzyme responsible for the phosphorylation of D-ribulose was provided by a mutant, strain 1407, selected from strain 1102 for its inability to grow on L-fucose. As shown in the accompanying paper (17), this strain, deficient in the synthesis of L-fuculokinase activity, also lost the ability to grow on D-arabinose.

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