Proton-linked L-rhamnose transport, and its comparison with L-fucose transport in Enterobacteriaceae

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1. An alkaline pH change occurred when L-rhamnose, L-mannose or L-lyxose was added to L-rhamnose-grown energy-depleted suspensions of strains of *Escherichia coli*. This is diagnostic of sugar–H^+ symport activity. 2. L-Rhamnose, L-mannose and L-lyxose were inducers of the sugar–H^+ symport and of L-[^{14}C]rhamnose transport activity. L-Rhamnose also induced the biochemically and genetically distinct L-fucose–H^+ symport activity in strains competent for L-rhamnose metabolism. 3. Steady-state kinetic measurements showed that L-mannose and L-lyxose were competitive inhibitors (alternative substrates) for the L-rhamnose transport system, and that L-galactose and D-arabinose were competitive inhibitors (alternative substrates) for the L-fucose transport system. Additional measurements with other sugars of related structure defined the different substrate specificities of the two transport systems. 4. The relative rates of H^+ symport and of sugar metabolism, and the relative values of their kinetic parameters, suggested that the physiological role of the transport activity was primarily for utilization of L-rhamnose, not for L-mannose or L-lyxose. 5. L-Rhamnose transport into subcellular vesicles of *E. coli* was dependent on respiration, was optimal at pH 7, and was inhibited by protonophores and ionophores. It was insensitive to *N*-ethylmaleimide or cytochalasin B. 6. L-Rhamnose, L-mannose and L-lyxose each elicited an alkaline pH change when added to energy-depleted suspensions of L-rhamnose-grown *Salmonella typhimurium* LT2, *Klebsiella pneumoniae*, *Klebsiella aerogenes*, *Erwinia carotovora carotovora* and *Erwinia carotovora atroseptica*. The relative rates of subsequent acidification varied, depending on both the organism and the sugar. L-Fucose promoted an alkaline pH change in all the L-rhamnose-induced organisms except the *Erwinia* species. No L-rhamnose–H^+ symport occurred in any organism grown on L-fucose. 7. All these results showed that L-rhamnose transport into the micro-organisms occurred by a system different from that for L-fucose transport. Both systems are energized by the trans-membrane electrochemical gradient of protons. 8. Neither steady-state kinetic measurements nor binding-protein assays revealed the existence of a second L-rhamnose transport system in *E. coli*.

INTRODUCTION

Escherichia coli and other Enterobacteriaceae grow on L-rhamnose (6-deoxy-L-mannose; Figure 1) as sole carbon source (Cooper, 1986; Lin, 1987). Utilization of L-rhamnose involves a relatively uncharacterized transport process across the cytoplasmic membrane (Buttin, 1968; Power, 1967). This is followed by metabolism via the cytoplasmic enzymes L-rhamnose isomerase, L-rhamnulose kinase and L-rhamnulose-1-phosphate aldolase (Wilson and Ajl, 1957a,b; Takagi and Sawada, 1964a,b,c). These transport and metabolic enzymes are regulated co-ordinately, and are induced during growth on L-rhamnose (Power, 1967; Buttin, 1968; Al-Zarban et al., 1984; Chen and Lin, 1984; Tobin and Schlief, 1987; Badia et al., 1989). We now present evidence that the initial transport of L-rhamnose is coupled to the trans-membrane electrochemical gradient of protons, $\Delta\mu_{H^+}$ (Mitchell, 1961, 1963, 1973; Nicholls, 1982). The L-rhamnose–H^+ symport system appears also to catalyse L-mannose–H^+ and L-lyxose–H^+ symport, which may be of physiological significance in those Enterobacteriaceae that grow on L-mannose or L-lyxose (Cooper, 1986). There are now at least six biochemically and genetically distinguishable bacterial transport systems with the common property of coupling inward sugar transport to uptake of protons, sugar–H^+ symport [or the experimentally indistinguishable antiport of hydroxyl ions (Mitchell, 1973)]; their substrates are lactose, D-galactose, L-arabinose, D-xylose, L-fucose or L-rhamnose (reviewed by Kaback, 1986; Henderson, 1986, 1990).

MATERIALS AND METHODS

L-Rhamnose, L-mannose, L-fucose, L-galactose, D-arabinose, D-fucose, D-galactose (glucose-free grade), D-glucose, D-talose, D-lyxose, D-altrose, D-xylose, D-ribose, D-gulose, D-mannose, L-glucose, L-arabinose, L-allose, L-gulose, L-talose, L-lyxose, L-xylose, xylitol and maltose were obtained from Sigma Chemical Co. D-Glucose was also obtained from Fisons, and D-talose from Koch–Light. Lactose was obtained from Hopkin and Williams, and melibiose from BDH. L-[1-^{3}H]Fucose and L-[1-^{14}C]fucose were from Amersham, and L-[1-^{14}C]rhamnose was from Centre Energetique Atomique (Lyon, France); each was diluted with unlabelled L-sugar to give stock 2 mM solutions of specific radioactivity 1–5 Ci/mol.

The strains of *E. coli* used in this work were JM2418 [Δ*hisgnd*, Δ*lac*, *araD*, *str*, *ptsF*, *ptsM*], JAR2 [Δ*hisgnd*, Δ*lac*, *araD*, *str*, *ptsF*, *ptsM*, *rha*::Mu d(Ap^r*lac*)I (Pl clr 100Cm)], JAR3 [Δ*hisgnd*, Δ*lac*,

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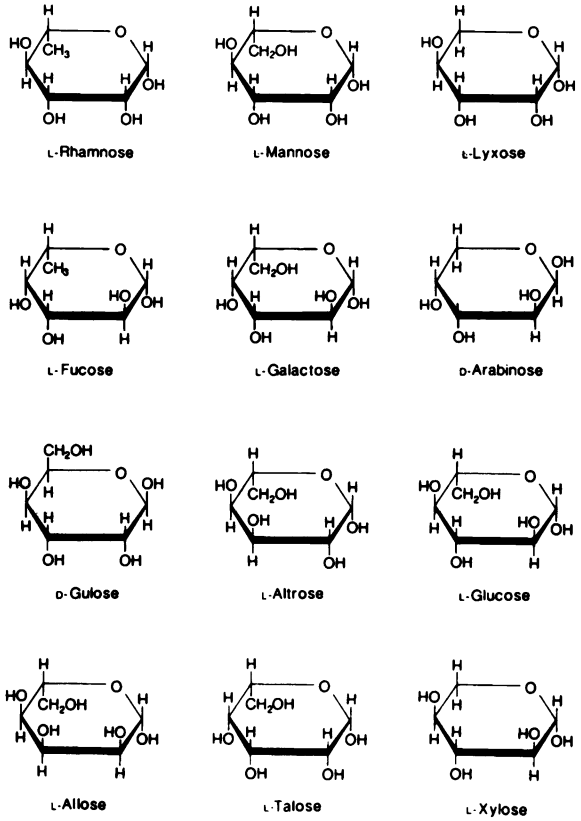

Figure 1 Structures of sugars related to L-rhamnose

The β-form of each sugar is shown.

araD, *str*, *ptsF*, *ptsM*, *rha*::*plac*MuI (Pl clr 100Cm)] and JM2513 [Δ*hisgnd*, Δ*lac*, *araD*, *str*, *ptsF*, *ptsM*, *rha*::*plac*MuI (Pl clr 100Cm)]. *E. coli* strains JM2418 and JAR3 were wild-type for L-rhamnose transport and metabolism; strain JAR2 was L-rhamnose-transport-negative by virtue of fusion between the *rhaT* and *lacZ* genes (Tate et al., 1992); strain JM2513 was L-rhamnose-metabolism-negative by virtue of fusion between an unidentified *rha* gene and *lacZ*. They were grown on basal salts medium (Henderson et al., 1977) supplemented with 10 mM L-rhamnose (1 mM for strain JM2513), 20 mM glycerol and 80 μg/ml L-histidine with shaking at 30 °C. Culture volumes were 1–3 ml in a 12 ml test tube for experiments investigating induction, 200 ml in a 250 ml conical flask for assays of energized transport into intact cells, 400 ml in a 500 ml conical flask for assays of sugar–H^+ symport, or 1250 ml in a 2-litre flask for vesicle preparations. Right-side-out subcellular vesicles were made from spheroplasts (Witholt et al., 1976; Witholt and Boekhout, 1978) of *E. coli* JM2513 by the method of Kaback (1972).

Salmonella typhimurium strains LT2 (laboratory stock) and C5 (kindly donated by Dr. C. E. Hormaeche) were grown on basal salts plus 10 mM L-rhamnose supplemented with 20 mM glycerol, or with 20 mM succinate in some experiments. *Klebsiella pneumoniae* 8017 and *Klebsiella aerogenes* 418 were kindly donated by Dr. M. Macpherson, of the Biotechnology Unit, University of Leeds, and *Erwinia carotovora carotovora* and *Erwinia carotovora atroseptica* by Dr. G. P. C. Salmond, of the School of Biological Sciences, University of Warwick; these organisms were grown on media identical with that for *S. typhimurium*, except that 0.06% nutrient broth (Oxoid) was also added to cultures of *Erwinia*.

Unless indicated otherwise, cultures of each organism were harvested, depleted of energy reserves, and washed as described by Henderson et al. (1977). For measurements of radioactive L-rhamnose and L-fucose transport into intact cells, 150 mM KCl/5 mM Mes, pH 6.5, was the supporting medium, the assays being carried out with 10 mM glycerol plus aeration as described by Henderson et al. (1977). Experiments were performed to confirm that the sample taken 15 s after addition of the labelled substrate would give an accurate value for the initial rate. The method referred to above was followed, but samples were taken at many timed points (5 s to 10 min). The amount of sugar transported was plotted against the sample time for labelled substrate at 2 μM or 400 μM final concentration. The 15 s time point fell within the linear phase of the measurement, confirming that it would give an accurate value for the initial rate (Muiry, 1989). Potential inhibitors of sugar transport were added at a final concentration of 5 mM for 3 min before addition of labelled substrate. Each measurement was carried out in duplicate, except that measurements were made in triplicate for sugar concentrations below 8 μM. Each sample was counted for radioactivity for 10000 counts or 20 min in a Packard Tri-Carb liquid scintillation spectrophotometer model 3385 or a Beckman LS2800 instrument.

Values for the kinetic constants K_m, K_i, $V_{max.}$ and their standard deviations were calculated by using the computerized least-squares fit to a hyperbola, or to the full rate equation for competitive inhibition, by the method of Cleland (1967). The values were not significantly different from those determined by the method of Eisenthal and Cornish-Bowden (1974).

Transport of radioactive L-rhamnose (80 μM) into subcellular vesicles in 50 mM potassium phosphate (pH 6.5)/10 mM $MgSO_4$ was energized by 20 mM ascorbate, 0.1 mM phenazine methosulphate plus oxygen and was measured as described by Horne and Henderson (1983). Possible inhibitors were added to the suspensions 3 min before the labelled sugar.

Sugar-promoted pH changes (sugar–H^+ symport) were measured with energy-depleted anaerobic suspensions of intact cells in 150 mM KCl/2 mM glycylglycine, pH 5.8–6.7, as detailed by Henderson and Macpherson (1986).

RESULTS

Sugar-promoted pH changes in *E. coli* cells grown on L-rhamnose

E. coli strain JAR3 was grown on glycerol plus L-rhamnose, harvested, depleted and resuspended (Henderson and Macpherson, 1986). Addition of L-rhamnose, L-mannose or L-lyxose to an anaerobic suspension elicited an initial alkaline pH change, followed by rapid acidification for L-rhamnose and slower acidification for the other two sugars (Figure 2). The alkaline pH change was diagnostic of sugar–H^+ symport activity (West, 1970; West and Mitchell, 1972), while the acidification was probably due to metabolism of the sugars to acidic end-products (Hacking and Lin, 1976, 1977; Cooper, 1986; Henderson and Macpherson, 1986). The extent of the proton influx with L-mannose or L-lyxose (Table 1, Figure 2a) was comparable with that described for other sugar–H^+ symport systems in *E. coli* (West and Mitchell, 1972; Henderson, 1974; Henderson et al., 1977; Lam et al., 1980; Daruwalla et al., 1981; Bradley et al., 1987). The pH changes were absent from cells grown on glycerol alone (Figure 2b). Similar results were obtained with *E. coli* JM2418 (Figure 3a), the strain previously used to characterize the L-fucose–H^+ symport activity (Bradley et al., 1987).

E. coli strains grown on L-rhamnose exhibited an alkaline pH

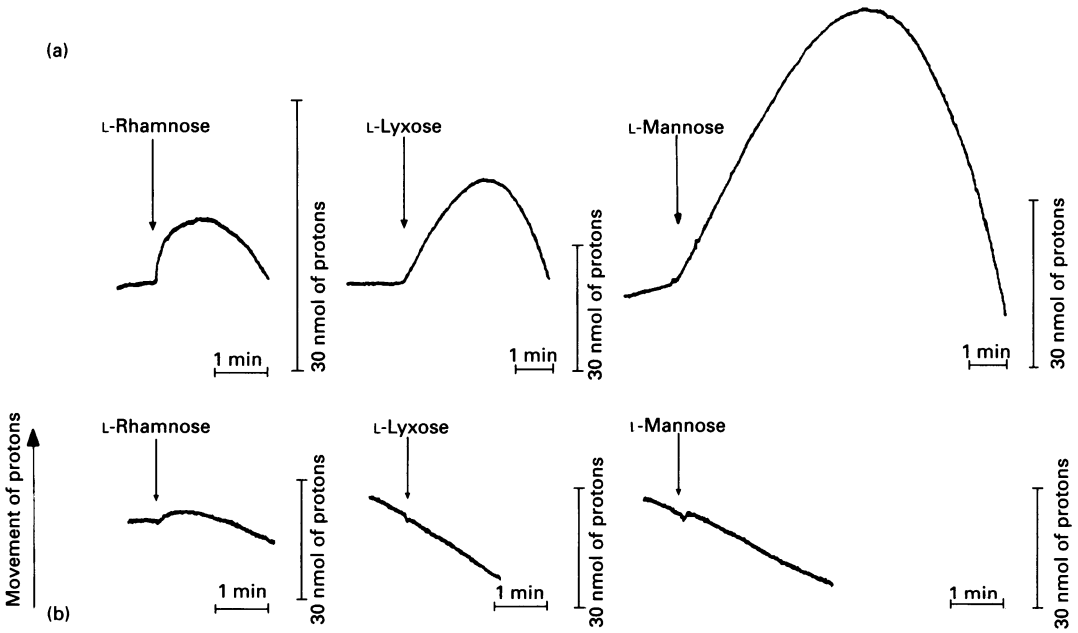

Figure 2 Induction by L-rhamnose of alkaline pH changes promoted by L-rhamnose, L-mannose and L-lyxose in *E. coli* JAR3

E. coli strain JAR3 was grown on 20 mM glycerol with (**a**) or without (**b**) 10 mM L-rhamnose and harvested as described in the Materials and methods section and by Henderson and Macpherson (1986). In the separate experiments shown, 10 μmol of the indicated sugar was added to an anaerobic suspension of cells (17.3 mg dry mass) in 3.7 ml of 150 mM KCl/2 mM glycylglycine, pH 6.24–6.34. The pH was measured as described by Henderson and Macpherson (1986). An upward deflection represents an alkaline pH change. In separate experiments (results not shown) 4 μmol of 2,4-dinitrophenol or 15 nmol of tetrachlorosalicylanilide was added 35 min before the sugar; they both prevented appearance of the sugar-promoted alkaline pH changes in L-rhamnose-grown cells. Note that the calibration marks and time scales have been varied to facilitate illustration.

Table 1 Substrate specificity of sugar–H+ symport in *E. coli* strain JM2418

The alkaline pH changes caused by the indicated sugars were measured as described in the Materials and methods section and illustrated in Figure 2(a). The pH values at the point of sugar addition varied between 5.6 and 6.6.

	Proton uptake		
Sugar	Rate (nmol of H^+/min per mg)	Extent (nmol of H^+ per mg)	No. of measurements
L-Rhamnose	1.28*	0.40*	4
L-Mannose	1.34	3.01	3
L-Lyxose	0.91	3.83	5
L-Glucose	0.00	0.00	3
L-Xylose	0.02	0.13	3
D-Xylose	0.00	0.00	3
L-Arabinose	0.00	0.00	1
D-Arabinose	2.60	0.39	4
L-Fucose	1.22	1.13	3

* Probably under-estimated owing to the rapidity of acidification.

change and acidification when L-fucose was added (see e.g. Figure 3a), and an alkaline pH change without the acidification when L-galactose or D-arabinose was used (Table 1; J. A. R. Muiry and T. C. Gunn, unpublished work). This is consistent with the known substrate specificity of the L-fucose-inducible L-fucose–H^+ symport system (Bradley et al., 1987). The conclusion that L-rhamnose induces both L-rhamnose–H^+ and L-fucose–H^+ transport activities in wild-type strains was confirmed by measuring uptake of the labelled sugars into differently induced strains (Table 2), and was supported by the inability of L-fucose, L-galactose or D-arabinose to inhibit L-rhamnose transport activity (Table 3). These data also showed that L-fucose induced only the L-fucose–H^+ transport system (Table 2). Interestingly, L-rhamnose did not induce L-fucose transport in an *E. coli* strain unable to metabolize L-rhamnose (Table 2), consistent with the conclusion that it is not L-rhamnose itself, but a metabolic derivative, that induces L-fucose transport (Chen et al., 1987b).

The appearance of the alkaline pH changes (Figures 2 and 3) was abolished by reagents that render the membrane permeable to protons (Mitchell, 1961, 1970), 4 μM tetrachlorosalicylanilide or 1 mM 2,4-dinitrophenol (results not shown). This indicated that the external pH change reflected a difference of pH across the membrane rather than a net change of pH inside and outside the cells.

The substrate specificity of this rhamnose-inducible sugar–H^+ symport was investigated in *E. coli* JM2418 (Table 1). Only L-rhamnose, L-mannose and L-lyxose were effective substrates of the L-rhamnose induced sugar–H^+ symport, given that L-fucose, L-galactose and D-arabinose entered by the L-fucose–H^+ symport in this strain of *E. coli* (Bradley et al., 1987). The average initial rates of H^+ uptake into *E. coli* strain JM2418 (in nmol/min per mg dry mass) were 1.28 (4) for L-rhamnose, 1.34 (3) for L-mannose and 1.12 (4) for L-lyxose, and in strain JAR3 they were 2.58 (3) for L-rhamnose, 0.97 (3) for L-mannose and 0.88 (3) for L-lyxose. These are very similar to the values obtained with L-fucose, 1.22 (3) in the present work and 1.05 (14) previously (Bradley et al., 1987). The numbers of measurements are given in parentheses.

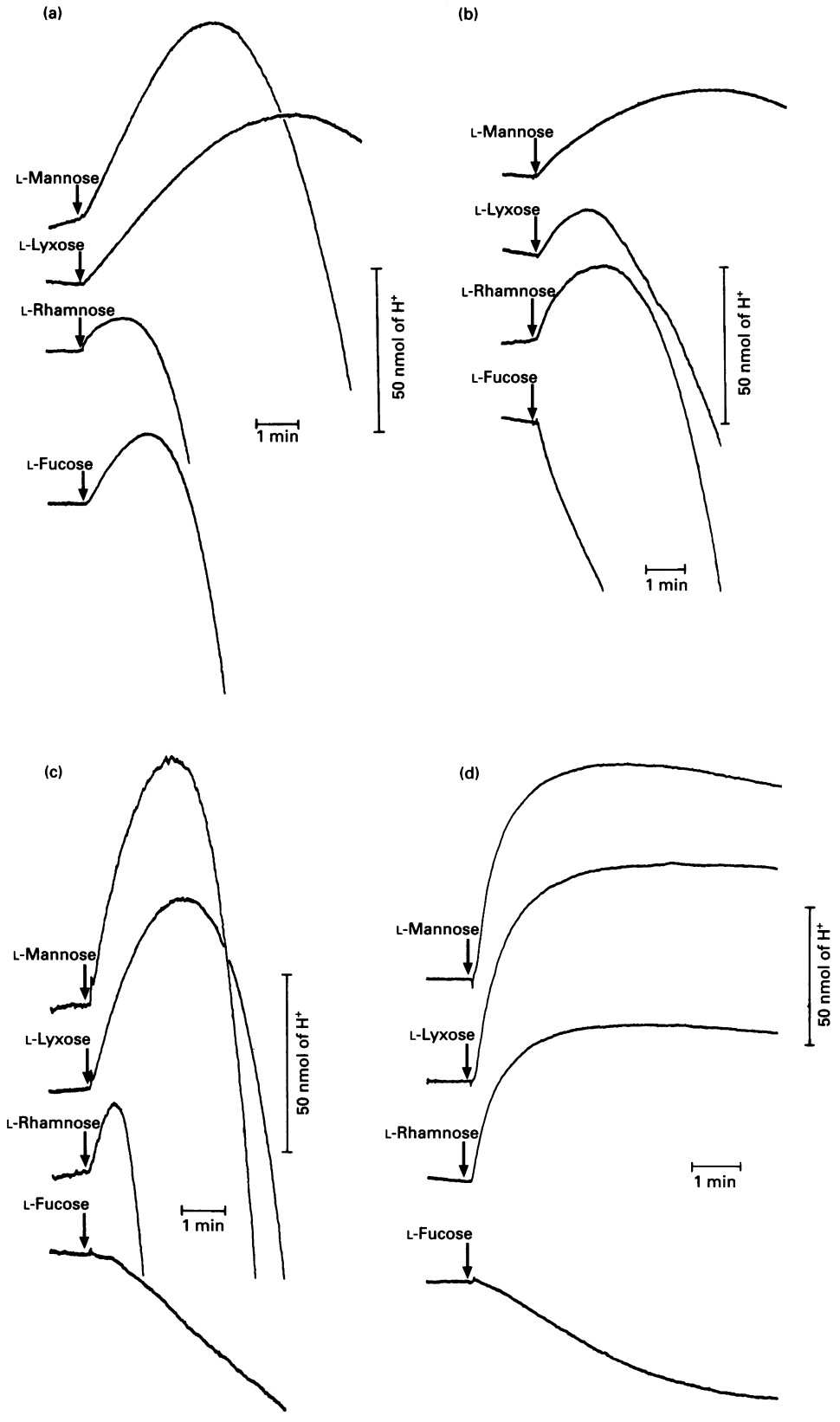

Figure 3 Alkaline pH changes promoted by L-sugars in L-rhamnose-grown species of Enterobacteriaceae

Sugar-promoted pH changes were measured as described for Figure 2 and Table 1. The pH at the point of sugar addition was in the range 5.8–6.7. The calibration marks all represent 50 nmol of H^+. (**a**) *E. coli* strain JM2418; (**b**) *Klebsiella aerogenes*; (**c**) *Erwinia carotovora carotovora*; (**d**) *Salmonella typhimurium* strain C5 *rhaT* gene (Tate et al., 1992) expressed in *E. coli* strain AR120 [wild-type *S. typhimurium* strain C5 did not yield alkaline pH changes, whereas wild-type *S. typhimurium* strain LT2 gave changes very similar to those seen with the other organisms (results not shown)].

Table 2 Induction of L-rhamnose and L-fucose transport into strains of *E. coli*

The indicated *E. coli* strain was grown at 30 °C in 3 ml of minimal salts medium containing 20 mM glycerol, 80 μg/ml L-histidine and 5 mM added sugar as indicated. The cells were harvested at A_{680} values of 1.8–1.9, sedimented, and washed with 4 × 5 ml of 150 mM KCl/5 mM Mes, pH 6.5. Samples of the final suspensions (A_{680} = 1.8–2.1) in the same buffer were aerated with 10 mM glycerol before measurement of the uptake of 50 μM L-[^{14}C]rhamnose or L-[^{3}H]fucose in duplicate as described elsewhere (Henderson, 1986).

E. coli strain	Addition	L-Rhamnose uptake (nmol/min per mg)	L-Fucose uptake (nmol/min per mg)	β-Galactosidase (Miller units)
JM2418	None	0.08	0.34	–
	L-Rhamnose	2.64	2.80	–
	L-Fucose	0.00	5.74	–
	L-Mannose	4.40	0.64	–
	L-Lyxose	7.00	0.72	–
	L-Xylose	0.48	0.32	–
	D-Xylose	0.62	0.42	–
JAR3	None	2.4	0.5	0.0
	L-Rhamnose	14.6	25.3	0.1
	L-Fucose	0.4	33.5	0.1
JM2513	None	2.0	0.5	0.3
	L-Rhamnose	6.4	0.1	680.6
	L-Fucose	0.8	6.8	4.7
JAR2	None	0.4	1.0	1.6
	L-Rhamnose	0.4	2.0	672.7
	L-Fucose	0.2	13.0	10.8

Sugar-promoted pH changes in species of Enterobacteria grown on L-rhamnose

When sugar–H+ symport measurements were made on L-rhamnose-grown cells of *Klebsiella aerogenes* similar results to those with *E. coli* were obtained (Figures 3a and 3b). However, in *K. aerogenes* the metabolism of L-mannose was relatively slow and the metabolism of L-fucose was very fast, as indicated by the rates of acidification (Figure 3b). The substantial alkaline pH changes observed when non-metabolizable analogues of L-fucose (L-galactose or D-arabinose) were used (results not shown) confirmed that this organism contained the L-fucose–H+ symport system in addition to the L-rhamnose–H+ symport system.

When L-rhamnose-grown cells of *Erwinia carotovora carotovora* were used, L-rhamnose, L-mannose or L-lyxose all gave initial alkaline pH changes (Figure 3c). They differed significantly from those with *E. coli* in the rates of acidification, and in their non-appearance when L-fucose, L-galactose or D-arabinose was used (Figure 3c; J. A. R. Muiry and T. P. McDonald, unpublished work). Additional measurements of sugar transport confirmed that the L-fucose–H+ symport system appeared to be absent from this organism (results not shown).

Rhamnose-grown cells of wild-type *Salmonella typhimurium* LT2 gave results very similar to those with *E. coli* strains (results not shown). When the *rhaT* gene from *S. typhimurium* C5 was expressed in an *E. coli* host strain under control of the lambda promoter (Tate et al., 1992) extensive alkalinization occurred with L-rhamnose, L-mannose or L-lyxose, but there was no acidification. Furthermore, there was no alkalinization with L-fucose (Figure 3d), nor with L-galactose or D-arabinose. These results were consistent with the absence of expression of the enzymes for L-rhamnose metabolism, preventing the acid secretion and the induction, by a derivative of L-rhamnose, of L-fucose–H+ symport.

Inducer specificities of L-rhamnose and L-fucose transport

Transport of L-rhamnose into intact cells of *E. coli* is conveniently measured by using the radioisotope-labelled sugar. When strain JM2418 was grown in the presence of different sugars, only cells grown with L-rhamnose, L-mannose or L-lyxose displayed subsequent accumulation of L-rhamnose (Table 2), whereas L-fucose, L-xylose and D-xylose were not effective inducers (Table 2). In strains JM2513 and JAR2, which have gene fusions in the *rha* operon (Muiry, 1989), L-rhamnose, but not L-fucose, induced β-galactosidase activity (Table 2). The unexpected induction of the L-fucose–H+ transport system by L-rhamnose (Tables 1 and 2) has been discussed above (cf. Bradley et al., 1987), and occurs because L-rhamnose may be converted into the normal inducer, L-fuculose 1-phosphate, of the *fuc* operon via L-lactaldehyde (Chen et al., 1987a,b). Consistent with this, L-rhamnose failed to induce L-fucose transport in strain JM2513, which cannot metabolize L-rhamnose.

Steady-state kinetics of L-rhamnose and L-fucose transport

The initial rates of L-rhamnose transport were measured over a wide range of substrate concentrations at pH 6.5 and 25 °C in *E. coli* strain JAR3. Provided that the external rhamnose concentration was not depleted by more than 15%, the relationship between v and s was a rectangular hyperbola, and the linearity of the reciprocal plots (Figure 4) was an important indication that only one L-rhamnose transport system was present. The apparent K_m values determined for L-rhamnose varied between 16 and 43 μM and the apparent $V_{max.}$ values between 11 and 17 nmol/min per mg dry mass; the corresponding values for L-fucose transport were 18–36 μM and 9–36 nmol/min per mg dry mass (Muiry, 1989; see the Discussion section).

Inhibition of L-rhamnose and L-fucose transport by potential alternative substrates

L-Mannose or L-lyxose inhibited L-rhamnose transport with a good fit to a competitive mechanism (Figure 4), enabling the calculation of apparent K_i values of 899 ± 125 μM and 915 ± 127 μM respectively. These can probably be equated with their apparent K_m values as alternative substrates for the L-rhamnose transporter (Segel, 1975). In similar experiments L-

Table 3 Inhibition of L-rhamnose and L-fucose transport by substrate analogues

Uptake of ^{14}C-labelled substrate was measured in induced cells of *E. coli* strain JAR3 for L-rhamnose and strain JM2418 for L-fucose as described in Table 2. Cells were preincubated with the test sugar at 5 mM final concentration for 3 min before addition of the ^{14}C-labelled substrate. Samples were taken at 15 s to calculate the rate of accumulation of radiolabelled sugar. With experiments of this type there were often small fluctuations in transport from day to day, as well as over the time-span of the experiment. For this reason, control measurements, with buffer added in place of potential inhibitor, were performed periodically throughout the experiment to identify and compensate for the inherent variations in the transport activity. For each potential inhibitor tested, the average value was used to express the initial rate of uptake as a percentage of the values for L-rhamnose or L-fucose alone. Numbers of samples are given in parentheses.

Potential inhibitor	Sugar transport in the presence of inhibitor (%)	
	L-Rhamnose	L-Fucose
None	100 (18)	100 (18)
D-Allose	95 (2)	103 (2)
L-Allose	96 (2)	100 (2)
D-Altrose	91 (6)	77 (4)
D-Arabinose	77 (6)	19 (4)
L-Arabinose	98 (4)	95 (4)
D-Fucose	96 (4)	94 (4)
L-Fucose	94 (6)	2 (6)
D-Galactose	98 (4)	101 (2)
L-Galactose	95 (2)	11 (4)
D-Glucose	126 (2)	96 (2)
L-Glucose	107 (2)	83 (2)
D-Gulose	109 (2)	107 (2)
L-Gulose	105 (2)	107 (2)
Lactose	98 (2)	97 (2)
D-Lyxose	95 (4)	93 (4)
L-Lyxose	42 (6)	92 (4)
Maltose	99 (4)	100 (2)
D-Mannose	88 (2)	94 (4)
L-Mannose	37 (6)	95 (4)
Melibiose	97 (6)	90 (2)
L-Rhamnose	3 (6)	99 (4)
Ribose	99 (2)	106 (2)
D-Talose	96 (4)	97 (2)
L-Talose	92 (4)	90 (2)
Xylitol	90 (4)	101 (2)
D-Xylose	93 (2)	95 (4)
L-Xylose	91 (2)	97 (2)

galactose and D-arabinose inhibited L-fucose transport competitively, with apparent K_i (K_m) values of 109 ± 6 μM and 310 ± 14 μM respectively (Figure 5). In all cases replots of the slopes against inhibitor concentration were linear, and there was no significant increase in intercept, although only one example is shown (Figure 4b); this is diagnostic of a simple competitive interaction (Segel, 1975).

In order to confirm and extend the substrate recognition properties of both transport systems, the ability of a wide range of D- and L-sugars (5 mM) to inhibit uptake of radioisotope-labelled L-rhamnose or L-fucose (0.05 mM) into *E. coli* strain JAR3 was measured (Table 3). Significant inhibition (greater than 10%) of L-rhamnose uptake occurred with L-mannose, L-lyxose, and, to a much lesser extent, D-arabinose, whereas significant inhibition of L-fucose uptake occurred with L-galactose, D-arabinose, D-altrose and perhaps L-glucose (see Figure 1 for structures). Sugars differing in configuration from L-mannose at only a single -OH residue (L-glucose at C-2, L-talose at C-4; Figure 1) did not affect L-rhamnose transport (Table 3). Similarly, sugars differing in configuration from L-galactose at a single -OH residue (L-talose at C-2, L-gulose at C-3, L-glucose at C-4) did not affect L-fucose transport. D-Gulose, the sugar that differs from L-mannose only in the configuration of -CH_2OH around C-5 (Figure 1), did not affect L-rhamnose transport, whereas D-altrose, the equivalent differing from L-galactose only in the configuration of -CH_2OH at C-5 (Figure 1), was a weak inhibitor of L-fucose transport.

The failure of L-xylose to inhibit L-rhamnose or L-fucose transport confirmed how critical is the configuration of the -OH at C-2 in substrates of the former (compare with L-lyxose; Figure 1), and of the -OH at C-4 in substrates of the latter (compare with D-arabinose, Figure 1).

The configuration of -OH in L-allose is different from L-rhamnose at both C-2 and C-3 and from L-fucose at both C-3 and C-4 (Figure 1). In view of the detrimental effect of only one of these differences on ability to inhibit uptake activity (above), it is not surprising that L-allose fails to affect either L-rhamnose or L-fucose transport (Table 3). In fact, any sugar that differed from L-rhamnose or L-fucose in configuration of -OH at more than one position failed to interact with either transport system (Table 3). These results are discussed further below.

L-Rhamnose and L-fucose transport into subcellular vesicles of *E. coli*

Right-side-out subcellular vesicles made by the method of Kaback (1972) have been invaluable for studying the energization of substrate transport into micro-organisms (see, e.g., Kaback, 1972, 1986; Ramos et al., 1976; Ramos and Kaback, 1977).

Radioisotope-labelled L-rhamnose was accumulated by such vesicles made from cells of *E. coli* JM2513 grown on L-rhamnose plus glycerol (Figure 6, Table 4). The transport was critically dependent on the presence of a respiratory substrate, ascorbate + phenazine methosulphate (Figure 6); respiration was necessary to generate $\Delta\mu_{H^+}$ (Ramos et al., 1976; Ramos and Kaback, 1977; Ingledew and Poole, 1984). Even when respiratory substrate was present, transport of L-rhamnose was inhibited by protonophores (Henderson, 1974) and ionophores that diminish $\Delta\mu_{H^+}$ (Figure 6, Table 3). Maximum activity of the L-rhamnose transport system occurred at pH 7 (Figure 7), different from the lower pH optimum found for proton-linked lactose, D-galactose or L-fucose transport into subcellular vesicles (Ramos et al., 1976; Ramos and Kaback, 1977; Horne and Henderson, 1983; Bradley et al., 1987). These results all showed that L-rhamnose transport was linked to $\Delta\mu_{H^+}$.

At pH 6.5 the apparent K_m for transport of L-rhamnose into vesicles was in the range of 40–55 μM, with an average apparent $V_{max.}$ of about 12 nmol/min per mg of protein (results from three batches of vesicles). This apparent K_m was similar to that for L-fucose in vesicles from *E. coli* strain JM2418 (Bradley et al., 1987), but the apparent $V_{max.}$ for L-rhamnose was substantially higher than that for L-fucose, probably because the absence of L-rhamnose metabolism in *E. coli* strain JM2513 caused hyper-induction of transport activity.

Insensitivity of L-rhamnose and L-fucose transport to inhibitors of sugar transport proteins

N-Ethylmaleimide is a thiol reagent that inhibits the activities of the separate proton-linked transport systems for lactose, L-arabinose, D-galactose and D-xylose in *E. coli* (Fox and Kennedy, 1965; Macpherson et al., 1981, 1983; Horne and Henderson, 1983; Kaback, 1986; Henderson and Macpherson, 1986; Davis, 1986; Menick et al., 1987). When vesicles from the L-rhamnose-grown *E. coli* strain JM2513 were treated with 1 mM *N*-ethylmaleimide for 15 min at 25 °C, L-rhamnose transport was relatively unaffected (Table 4), although this was sufficient to

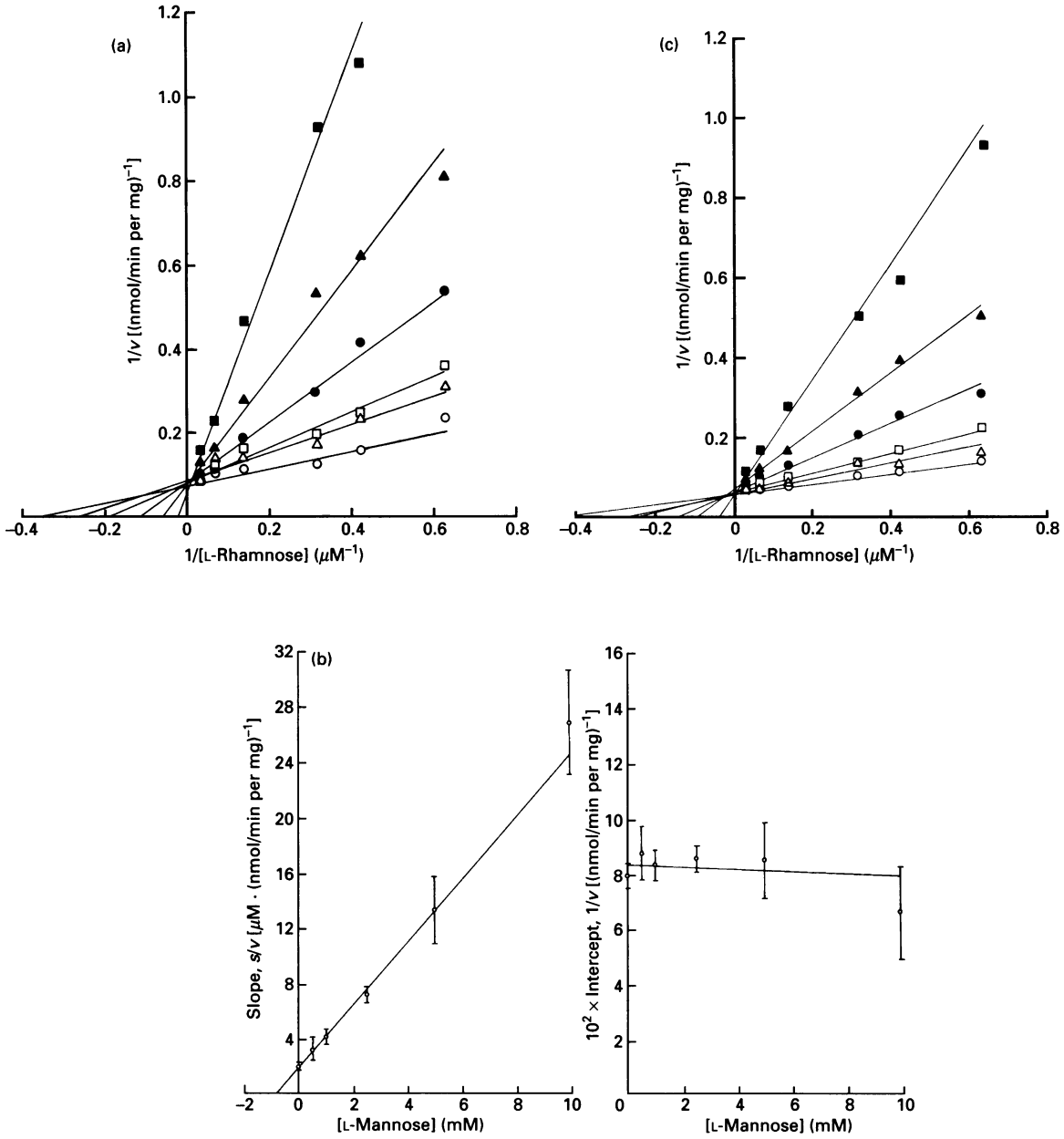

Figure 4 Kinetics of inhibition of L-rhamnose transport

E. coli strain JAR3 was grown on 20 mM glycerol + 10 mM L-rhamnose, harvested, and resuspended in 150 mM KCl/5 mM Mes, pH 6.5, plus 1 mM mercaptoethanol for 1 h at 30 °C. The resedimented cells were resuspended in the same medium but without mercaptoethanol, sedimented again and finally resuspended to a density of 0.68 mg dry cell mass/ml. Aerated samples (0.2 ml) were incubated at 25 °C for 3 min with 25 mM glycerol before addition of 50 μl of radiolabelled substrate solution to give the indicated concentration range. After 15 s, 0.2 ml was filtered rapidly and washed twice with approx. 2 ml of the above medium. The radioactivity trapped on the filter was measured and converted into nmol/min per mg dry mass as described elsewhere (Henderson et al., 1977). Each point shown is the mean of two measurements. (**a**) Reciprocal Lineweaver–Burk plots of initial rates of L-rhamnose transport measured in the presence of six different concentrations of L-mannose: ○, 0.0 mM; △, 0.5 mM; □, 1.0 mM; ●, 2.5 mM; ▲, 5.0 mM; ■, 10.0 mM. (**b**) Replots of slope, apparent K_m/apparent V_{max}, versus concentration of L-mannose, and of intercept, 1/apparent V_{max}, versus concentration of L-mannose. (**c**) Reciprocal Lineweaver–Burk plots of initial rates of L-rhamnose transport measured in the presence of six different concentrations of L-lyxose: ○, 0.0 mM; △, 0.5 mM; □, 1.0 mM; ●, 2.5 mM; ▲, 5.0 mM; ■, 10.0 mM.

abolish activity of LacY in the same vesicles (results not shown). Even concentrations of *N*-ethylmaleimide up to 10 mM failed to abolish either L-rhamnose transport or L-fucose transport into vesicles from L-fucose-induced *E. coli* strain JM2418.

Cytochalasin B inhibits the activity of the proton-linked transport systems for L-arabinose or D-galactose in *E. coli* and the passive glucose transporter of mammalian cells (G. Smith, K. R. Petro and T. P. McDonald, unpublished work; Cairns et al., 1991; Charalambous et al., 1989; Baldwin and Henderson, 1989; Shanahan, 1982; Carter-Su et al., 1982), though the *E. coli* systems for lactose and D-xylose transport are insensitive (G. Smith, K. R. Petro and T. P. McDonald, unpublished work). Concentrations of cytochalasin B (20–80 μM) sufficient to inhibit L-arabinose or D-galactose transport by 55–95% had only a mild effect on L-rhamnose transport (Table 4). L-Fucose transport was even less sensitive to cytochalasin B (results not shown).

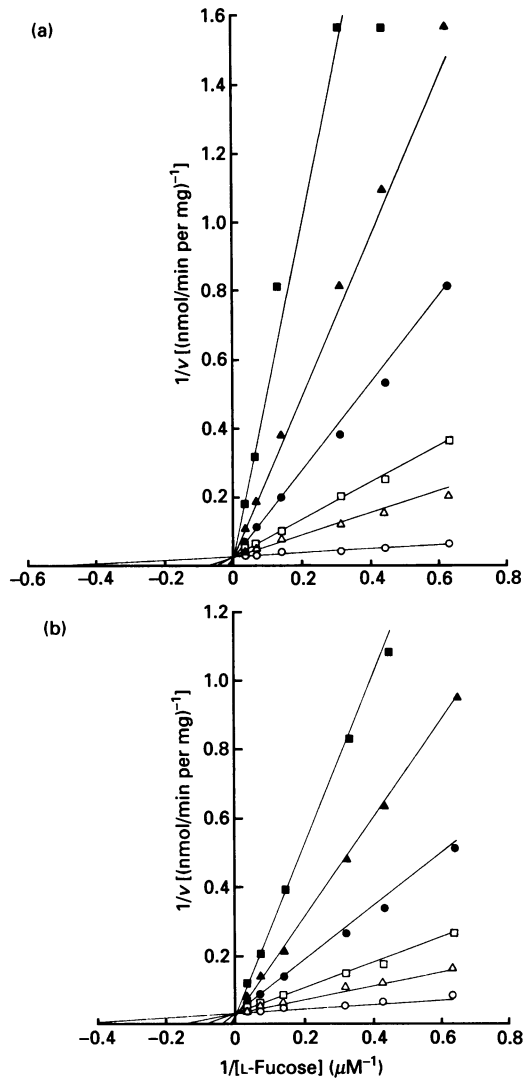

Figure 5 Kinetics of inhibition of L-fucose transport

E. coli strain JM2418 was grown on 20 mM glycerol + 10 mM L-fucose, harvested, and transport was measured as described for Figure 4. (**a**) Reciprocal Lineweaver–Burk plots of initial rates of L-fucose transport measured in the presence of six different concentrations of L-galactose: ○, 0.0 mM; △, 0.5 mM; □, 1.0 mM; ●, 2.5 mM; ▲, 5.0 mM; ■, 10.0 mM. (**b**) Reciprocal Lineweaver–Burk plots of initial rates of L-fucose transport measured in the presence of six different concentrations of D-arabinose: ○, 0.0 mM; △, 0.5 mM; □, 1.0 mM; ●, 2.5 mM; ▲, 5.0 mM; ■, 10.0 mM.

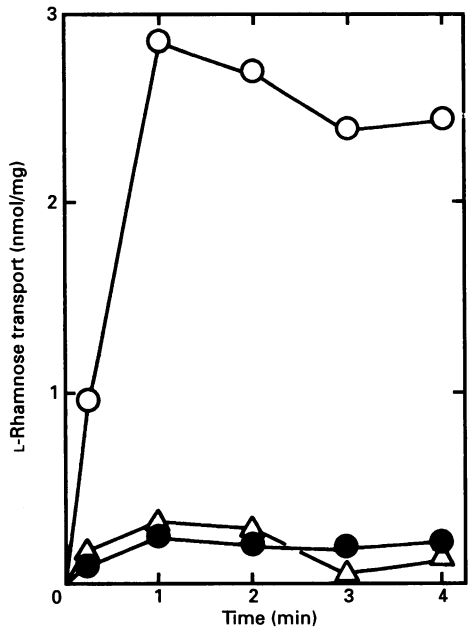

Figure 6 Respiration-dependence and uncoupler-sensitivity of L-rhamnose transport into subcellular vesicles of *E. coli*

Subcellular vesicles were prepared from glycerol + L-rhamnose-grown cells of strain JM2513, and transport of 80 μM radioisotope-labelled L-rhamnose was measured as described by Horne and Henderson (1983): ○, + ascorbate/phenazine methosulphate as respiratory substrate; △, + ascorbate/phenazine methosulphate + 1 mM 2,4-dinitrophenol; ●, no additions. Each point is the mean of four measurements on two batches of vesicles.

Table 4 Effects of ionophores and inhibitors on L-rhamnose transport into subcellular vesicles of *E. coli* JM2513

The conditions were as for Figure 6, except that the concentration of L-rhamnose was lowered from 80 μM to 40 μM in the experiment with cytochalasin B to minimize any competition by substrate. Each value is the mean of 4–10 measurements of the initial rate of transport, the average control value being 3.72 nmol/min per mg.

Addition	Activity (% of control)
None	100.0
1 mM Dinitrophenol	20.6
20 μM Tetrachlorosalicylanilide	20.8
4 μg/ml Nigericin	81.6
4 μM Valinomycin	36.2
20 μM Cytochalasin B	92.9
1 mM *N*-ethylmaleimide	88.6
No ascorbate or phenazine methosulphate	13.6

DISCUSSION

The observation of an L-rhamnose-inducible alkaline pH change promoted by L-mannose, L-lyxose and, to a lesser extent, L-rhamnose in intact cells, the dependence of L-rhamnose transport into vesicles on respiration, and its sensitivity to uncoupling agents confirmed the existence of an L-rhamnose–H^+ symport system in *E. coli*. The possibility of L-rhamnose uptake by one of the other sugar–H^+ symporters (for lactose, D-galactose, L-arabinose, D-xylose or L-fucose) was eliminated by its occurrence in *lac* deletion mutants, and by its different substrate and inducer specificities compared with D-galactose–H^+ symport (Henderson et al., 1977; Henderson and Giddens, 1977), D-xylose–H^+ symport (Lam et al., 1980), L-arabinose–H^+ symport (Daruwalla et al., 1981) or L-fucose–H^+ symport (Bradley et al., 1987). A similar L-rhamnose–H^+ symport was present in *Salmonella typhimurium* LT2, in strains of *Erwinia carotovora* or *Erwinia atroseptica* and in strains of *Klebsiella pneumoniae* and *Klebsiella aerogenes* (Figure 3). The common occurrence of a system for transport and metabolism of L-rhamnose may reflect the relative abundance of this sugar in the environment (Cheshire, 1979).

For each of the sugars D-galactose, L-arabinose and D-xylose, there exists in *E. coli* a second transport system containing a binding protein (see, e.g., Boos, 1969; Schleif, 1969; Brown and Hogg, 1972; Ahlem et al., 1982). The linearity of all the reciprocal plots (Figure 4; and J. A. R. Muiry, unpublished work) and the failure to detect significant binding of L-[^{14}C]rhamnose to peri-

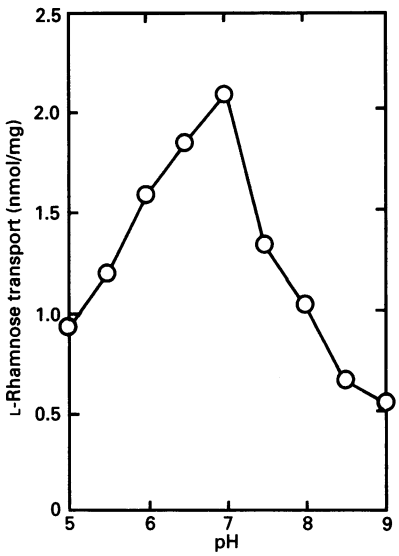

Figure 7 Dependence of L-rhamnose transport on pH

Vesicles made from L-rhamnose-induced *E. coli* strain JM2513 were washed twice in 50 mM potassium phosphate of the indicated pH before measuring transport of radioisotope-labelled L-rhamnose in the presence of ascorbate/phenazine methosulphate. Mean values from six measurements of initial rate are shown.

plasmic proteins (T. C. Gunn and S. A. Bradley, unpublished work) indicate that the existence of a second transport system for L-rhamnose is unlikely.

L-Mannose and L-lyxose will be useful alternative substrates for studying the L-rhamnose–H^+ symport. The relatively slow rates of acid extrusion (Figures 2 and 3) showed that they were metabolized much more slowly than L-rhamnose by the strains of Enterobacteriaceae investigated. In *E. coli* at least, L-mannose and L-lyxose were also inducers of the L-rhamnose operon; such gratuitous inducers are often valuable tools for investigating regulation of gene expression. In most types of experiment, L-mannose was a more effective substrate/inhibitor than L-lyxose (Tables 1 and 3), but their apparent K_i values were quite similar (Figure 4).

It is informative to compare the transport systems for L-rhamnose and L-fucose, first with each other and then with other proton-linked transport systems in *E. coli*. Their substrate specificities were different, L-rhamnose, L-mannose and L-lyxose for one and L-fucose, L-galactose and D-arabinose for the other. By comparing the structures of these sugars with those of L-glucose, L-talose and L-xylose (Figure 1), which were not substrates or inhibitors (Tables 2 and 4), it is clear that each transport system simultaneously recognizes the configuration of -OH groups at both C-2 and C-4 of the pyranose ring; the L-rhamnose transporter 'accepts' the L-mannose configuration at C-2 and the L-glucose configuration at C-4 and 'rejects' the L-glucose configuration at C-2 and the L-galactose configuration at C-4. The L-fucose transporter does the reverse. However, both the 6-deoxy-L-sugar transporters are tolerant to a similar degree of changes at C-6; either introduction of an -OH group in L-mannose (L-rhamnose transport) or L-galactose (L-fucose transport) or removal of $-CH_3$ in L-lyxose (rhamnose transport) or D-arabinose (L-fucose transport) decreases, but does not abolish, transport activity. Since D-gulose does not interact with the L-rhamnose transporter, although different from the natural substrate only in the configuration at C-5, this transporter does 'reject' the configuration of $-CH_2OH$ at C-5 characteristic of the D-series of hexoses. However, D-altrose, which bears the corresponding relationship to L-fucose at the C-5 position, weakly inhibited L-fucose transport, indicating some tolerance of the 'wrong' configuration of the $-CH_2OH$ group at C-5. The L-fucose transporter must also recognize the configuration of the -OH at C-3, since L-gulose failed to inhibit L-fucose uptake, though it differs from L-galactose only at the C-3 position. L-Altrose, the sugar that differs from L-mannose only in the C-3 position, was not available to test.

The overall conclusions are as follows. The configuration of the -OH residue at each of the C-2, C-3 and C-4 positions in a sugar is critical for recognition by the L-fucose transport system. The presence or absence of a $-CH_3$ or $-CH_2OH$ group in the L-configuration at the C-5 position is not so important, though a $-CH_2OH$ in the D-configuration decreases interaction. Essentially the same is true for the L-rhamnose transport system, except that the effect of changes at the C-3 position could not be investigated. This characterization of substrate recognition by the two transport systems should be invaluable in the future elucidation of the structure–activity relationships of the proteins involved.

The average apparent K_m value for L-rhamnose was 27.9 μM (5 determinations; range 16–43 μM), not significantly different from that found for L-fucose, 24.7 μM (5 determinations, range 18–36 μM, cf. Bradley et al., 1987). These values were at the low end of the range determined for the proton-linked D-galactose (Henderson et al., 1977), L-arabinose (Daruwalla et al., 1981), D-xylose (Davis et al., 1984; Davis, 1986) and lactose (Wright et al., 1981) transporters in intact cells of appropriately induced strains of *E. coli* (Henderson, 1986). Binding-protein transport systems (probably energized by ATP; Ames, 1986) exhibit higher affinities for substrate, K_m 0.2–6 μM (Henderson, 1986). The average apparent $V_{max.}$ for L-rhamnose transport was 14.0 nmol/min per mg dry mass (5 determinations; range 11–17 nmol/min per mg dry mass), very similar to the values for other transport systems (Henderson, 1986; Ames, 1986), but the apparent $V_{max.}$ for L-fucose was double this, 27.9 nmol/min per mg (5 determinations; range 9–36 nmol/min per mg). All these values were determined at 25 °C. Perhaps these differences may account for variations in the efficiency of utilization of these sugars under physiological conditions.

This investigation characterized the properties of a proton-linked transport system for L-rhamnose and compared it with systems for transport of L-fucose and other sugars. Their similarities and differences must reside in the three-dimensional architecture of each individual protein as determined by the sequence of its constituent amino acids. In fact, the primary sequences of the L-rhamnose–H^+ transporter (Tate et al., 1992) and of the L-fucose–H^+ transporter (Lu and Lin, 1989) are very different from each other and from other families of proton-linked sugar transporters (Henderson, 1990). It will be necessary to determine the three-dimensional structures of several such transporters to elucidate the molecular mechanism(s) of substrate recognition and sugar–H^+ symport.

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