Biochem. J. (1987) 243, 345–350 (Printed in Great Britain)

The stimulation by salts of hexose phosphate uptake by *Escherichia coli*

Richard C. ESSENBERG
Department of Biochemistry, Oklahoma State University, Stillwater, OK 74078, U.S.A.

Hexose phosphate uptake in *Escherichia coli* is stimulated by salts. KCl and $MgCl_2$ stimulate to about the same extent, but Mg^{2+} is effective at a tenth the concentration of K^+. At higher concentrations, both salts inhibit. The stimulation by a series of salts correlates strongly with the hydrated radius of the cation, with small ions more effective than large. There are effects by the anion, but they do not correlate with any simple property. Cells accumulate glucose 6-phosphate to a higher concentration in the presence of KCl than in its absence. The maximum velocity of glucose 6-phosphate uptake is stimulated by KCl, as is the ratio V/K_m.

INTRODUCTION

Many transport systems in bacteria are stimulated by salts (Lanyi, 1979). The hexose phosphate transport system of *Escherichia coli*, an inducible energy-dependent system, was shown to be stimulated by NaCl and KCl to extents not explainable by the effect of these salts on osmolarity (Essenberg & Kornberg, 1975). For many of the Na^+-stimulated systems, the effect is due to an Na^+–substrate co-transport. This possibility seems unlikely for the hexose phosphate system for two reasons: Na^+ symport systems are usually quite specific for cation, with K^+ providing no stimulation, and good evidence exists that the hexose phosphate system is H^+ symport (Essenberg & Kornberg, 1975; Ramos & Kaback, 1977). I therefore undertook the set of studies reported in the present paper to investigate the nature of the stimulation more fully in the hope that it would shed some light on its mechanism.

EXPERIMENTAL

Growth of cells

Escherichia coli K12 strain RE48, blocked for utilization of glucose 6-phosphate and constitutive for hexose phosphate uptake (Essenberg & Kornberg, 1975), was streaked on plates containing fructose 1-phosphate as sole carbon source to maintain constitutive hexose phosphate uptake. Single colonies from this plate were inoculated into medium A (Miller, 1972) with 20 mM-glycerol as carbon source and 0.1% casamino acids (Difco). After overnight growth at 37 °C, the cells were sedimented in a clinical centrifuge and resuspended in a volume of medium A without carbon source, equal to the original volume of the culture, to make a starter culture, which was stored at 4 °C. This inoculum maintained its viability for several weeks. Cultures for experiments were grown from a 1% inoculum in medium A with 20 mM-glycerol as carbon source at 37 °C overnight. The cells were diluted to one-half their original density with fresh medium A with 20 mM-glycerol and grown for a further 2 h before harvest to get them into late-exponential phase. They were harvested by centrifugation at 5000 *g* for 5 min and washed twice in an appropriate medium for the experiment, usually 5 mM-Pipes neutralized to pH 6.6 with Tris (Pipes/Tris buffer). They were resuspended in 0.1 vol. of the same medium. Resuspension in this low-osmolarity buffer was not expected to have a large effect on transport of glucose 6-phosphate or its stimulation by salts on the basis of earlier results in which sucrose was used to maintain osmolarity (Essenberg & Kornberg, 1975). An additional experiment was done to be certain of this point. In this experiment, cells were resuspended in buffer alone, as described above, or in the same buffer containing 80 mM-LiCl to avoid osmotic downshock. A concentration-dependence curve was done on both samples with KCl. The rates of transport were slightly higher in the cells resuspended in LiCl, with the maximum difference being 15%. Kinetic parameters calculated for the stimulation were not significantly different for the two resuspension media.

Transport assay

Assays were typically done in a total volume of 0.5 ml in 15 ml scintillation vials at room temperature. A 50 μl aliquot of the concentrated cell suspension was added to the desired medium and the assay started by addition of [^{14}C]glucose 6-phosphate (specific radioactivity 1 mCi/mmol; ICN Radiochemicals, Irvine, CA, U.S.A.) to 0.1 mM. Samples (0.2 ml) were taken at 10 and 70 s and filtered through 0.45 μm-pore-size membrane filters (Enzo Biochem, New York, NY, U.S.A.). The filters were immediately washed with the same medium used for the assay. They were dried, and radioactivity determined by liquid-scintillation counting in toluene containing 0.4% 2,5-diphenyloxazole and 0.02% *p*-bis-(*O*-methylstyryl)benzene (Sigma Chemical Co., St. Louis, MO, U.S.A.). A sample of the radioactive glucose 6-phosphate was counted for radioactivity in the same way after drying on a filter to determine the counting efficiency (typically about 80%). Variations in this general scheme are noted in the legends.

Analysis of results

The results of experiments varied, especially the absolute rate of uptake. Also, the response of cultures to various treatments was not always quantitative. Since the uptake of substrate varied between experiments, results were usually normalized to give an average of one for each experiment. The combined experiments were analysed by the random-effects model, and homogeneity of variance was tested with Levene's test (Snedecor & Cochran, 1980). Where appropriate, a two-factor

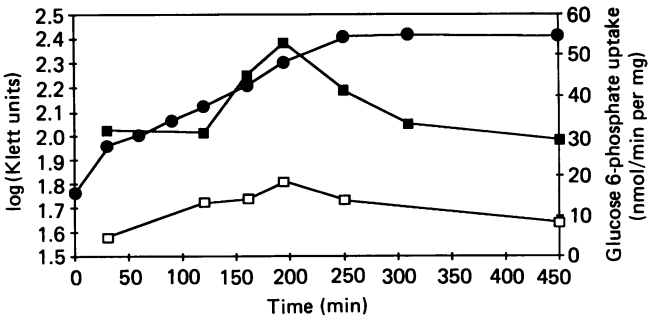

Fig. 1. Specific activity of hexose phosphate transport system as a function of growth stage

Cells of strain RE48 were grown on glycerol minimal medium with shaking at 37 °C. Growth (●) was monitored turbidimetrically on a Klett–Summerson colorimeter, a no. 42 filter being used. At intervals, 5 ml samples were removed and washed, half in 80 mM-KCl in Pipes/Tris buffer, the other half in Pipes/Tris buffer alone. Glucose 6-phosphate uptake was measured as described in the Experimental section. □, Rate of uptake in the absence of KCl; ■, that in the presence of KCl.

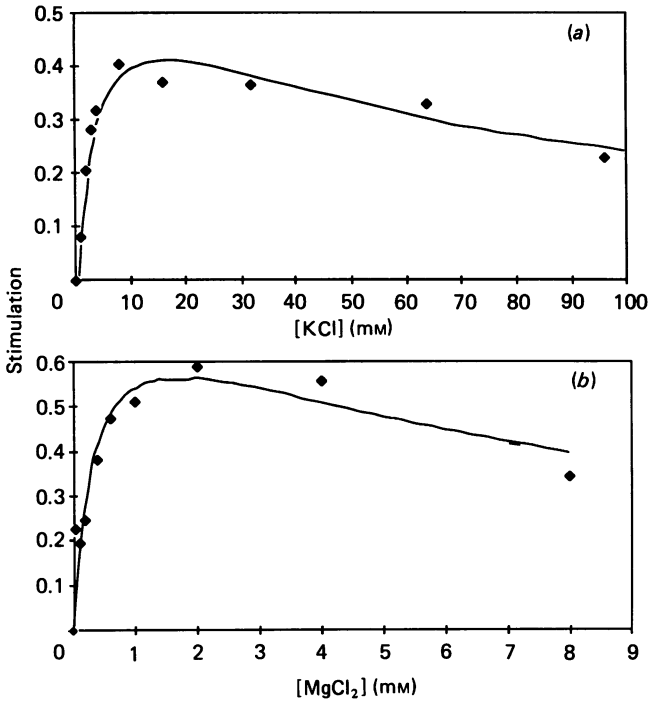

Fig. 2. Stimulation of the rate of transport of glucose 6-phosphate by KCl and $MgCl_2$

Glucose 6-phosphate uptake was measured as described in the Experimental section in the presence of various concentrations of KCl (*a*) or $MgCl_2$ (*b*) in 5 mM-Pipes/Tris buffer. The stimulation is calculated as the rate increase in the presence of salt divided by that in its absence. These values summarize the results of three experiments. Treatment ($F = 5.548$ for 19 and 40 degrees of freedom) and experiment ($F = 2.379$ for 40 and 120 degrees of freedom) effects were significant with $P < 1\%$. The standard error was 0.083 with 40 degrees of freedom. The lines drawn are the theoretical lines calculated from the best-fit parameters for the substrate-inhibition model.

Table 1. Stimulation by chloride salts of different univalent cations

Rates of transport (relative to that in the presence of K^+) were determined in the presence of 20 mM-concentrations of the indicated chloride salt. Three independent experiments were done, with the total number of replicates for each salt indicated in the Table. The standard error for the combined experiments is 0.10 with 20 degrees of freedom. Rates marked with a superscript A were significantly different from the rest, with $P < 5\%$. [Newman & Keul's test (Snedecor & Cochran, 1980)]. Treatment ($F = 10.455$ for 9 and 20 degrees of freedom) and experiment ($F = 2.453$ for 20 and 103 degrees of freedom) effects were significant with $P < 1\%$.

Cation	Relative rate	No. of determinations
Rb^+	1.18^A	13
Cs^+	1.11^A	13
K^+	1.00^A	17
$CH_3NH_3^+$	0.68	13
Na^+	0.57	11
$(CH_3)_4N^+$	0.48	11
NH_4^+	0.47	12
$Choline^+$	0.46	11
Li^+	0.36	12
None	0.32	20

analysis of variance was also done. Average values for the treatments were fitted to models by using the computer programs of Cleland (1967), which calculate the standard deviations in the parameters. The standard error for the series of experiments was taken as the constant error for weighting unless the variance was not homogeneous, in which case a smoothed error calculated from the standard deviations for each treatment average was used. Quality of fit was tested by using an analysis of variance, and different possible models were compared in the same way. The statistical methods were taken from Snedecor & Cochran (1980).

RESULTS

Transport activity at different stages of growth

The variability in the absolute rate of hexose phosphate transport suggested that the activity may depend on some factor in the growth of the cells that was not adequately controlled. As a test of one possibility, the rate of transport as a function of cell growth through the exponential phase into the stationary phase was measured, the results being shown in Fig. 1. The specific activity of hexose phosphate transport increased essentially linearly with cell density up to 200 Klett units, then declined above 250 units as the cells stopped growing. In other experiments (not shown), the transport activity declined almost to zero as the cells were held in stationary phase. Except for the first sample, the rate in the presence of KCl was a constant multiple of the unstimulated rate (2.7 ± 0.4). It was found that cells grown from 1:100 inoculum overnight, diluted 1:2 and grown for 2 h had rates of transport as good as, or better than, those of cells harvested in late-exponential growth, so this method of growth was used for all experiments described here.

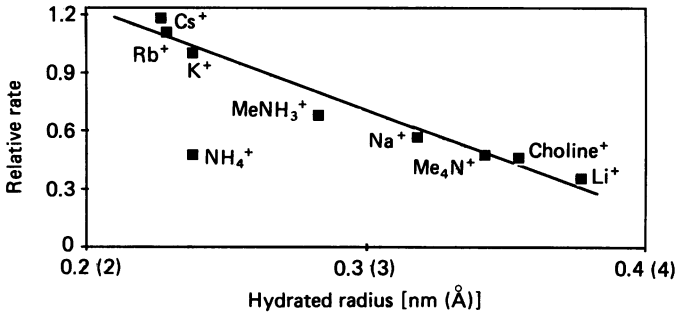

Fig. 3. Rate of uptake as a function of the hydrated radius of the cation

The rates from Table 1 are plotted against the hydrated radius of the cation. The hydrated radii were calculated from the mobilities of the ions (Robinson & Stokes, 1955). The mobility for choline$^+$ is from Spivey & Snell (1964). The straight line is the least-squares best-fit line through all the points except NH_4^+.

Stimulation of transport by KCl and $MgCl_2$

The data shown in Fig. 2 show the effect of KCl and $MgCl_2$ at various concentrations on glucose 6-phosphate uptake. Both salts stimulate transport to a similar extent [maximum stimulation ('$V_{max.}$') = 0.60 ± 0.09 for KCl, 0.79 ± 0.16 for $MgCl_2$], but $MgCl_2$ is effective at a much lower concentration than is KCl ($K_m = 3.7 \pm 1.2$ mM for KCl, $= 0.35 \pm 0.16$ mM for $MgCl_2$). At higher concentrations, both salts inhibit, with the effect fitted adequately by a simple substrate-inhibition model.

Table 2. Stimulation by anions of different potassium salts

Rates of transport (relative to that in the presence of Cl^-) were measured in 20 mM concentrations of the indicated potassium salts. Phosphate was a mixture of uni- and bi-basic salts to give pH 6.6. Four independent experiments were done, but not all salts were used in all experiments. The number of experiments and total number of replicates for each salt are indicated. The standard error for the total was 0.099 with 39 degrees of freedom. Rates marked with a superscript A were significantly different from that for F^-, those marked with a superscript B were different from that for no salt, and those marked with superscript C were different from that for HCO_3^-, all with $P < 5\%$ [Newman & Keul's test]. Both treatment ($F = 6.518$ for 14 and 39 degrees of freedom) and experiment ($F = 5.342$ for 39 and 199 degrees of freedom) effects were significant with $P < 1\%$.

Anion	Relative rate	No. of experiments	No. of determinations
Acetate$^-$	1.224^{ABC}	3	13
Citrate^{3-}	1.219^{ABC}	4	17
Tartrate^{2-}	1.135^{BC}	3	13
PO_4^{3-}	1.107^{BC}	4	17
Br^-	1.089^{BC}	4	17
NO_3^-	1.080^{BC}	4	16
SCN^-	1.053^{BC}	4	17
Cl^-	1.000^{BC}	4	28
Formate$^-$	0.971^{BC}	2	9
SO_4^{2-}	0.843^{C}	4	16
I^-	0.805^{C}	4	16
ClO_4^-	0.748^{C}	2	9
F^-	0.683^{C}	4	16
None	0.462	4	33
HCO_3^-	0.325	4	16

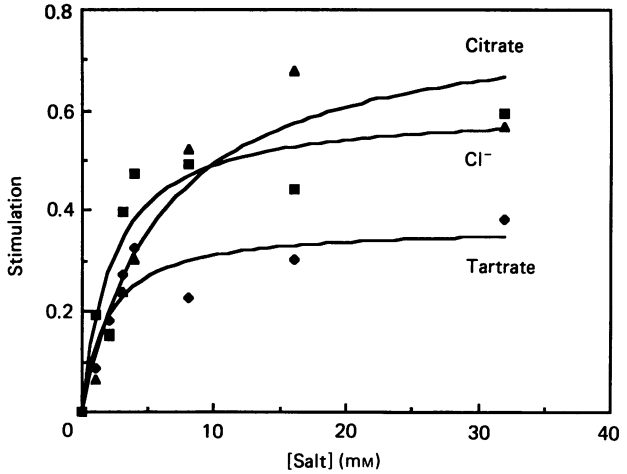

Fig. 4. Stimulation of uptake by KCl, potassium tartrate and potassium citrate

Glucose 6-phosphate uptake was measured as described in the Experimental section the presence of various concentrations of KCl (■), potassium tartrate (◆) or potassium citrate (▲). Stimulation is as defined in Fig. 2. This Figure summarizes the results of four experiments. Treatment ($F = 3.285$ for 23 and 72 degrees of freedom) and experiment ($F = 4.276$ for 72 and 191 degrees of freedom) effects were significant with $P < 0.1\%$. The standard error was 0.126 with 72 degrees of freedom. The lines are calculated from the best-fit parameters given in Table 3.

Stimulation by different salts

Stimulation of transport was measured at a fixed concentration of 20 mM in a series of chloride salts of different univalent metals. As shown in Table 1, there were significant differences between the various cations. Large alkali cations (Rb^+, Cs^+ and K^+) were more effective than small ions (Li^+) or the various ammonium ions [$CH_3NH_3^+$, $(CH_3)_4N^+$, NH_4^+]. If the relative transport rate is plotted against the hydrated radii of the ions (Fig. 3), a straight line results. NH_4^+, with the same solvated radius as K^+, is clearly anomalous, having a lower stimulation than expected. If NH_4^+ is excluded, the straight line accounts for 96% of the variance due to the cation.

A similar experiment with various anions of potassium salts is shown in Table 2. In this case, the differences between the different salts are not so pronounced, with the exception of $KHCO_3$, which is clearly inhibitory. The differences that exist are not clearly correlated with any property of the ion. Organic ions seem to be more effective than inorganic ions, but size or charge make little difference. If the polyvalent ions are excluded, there is still nearly as much variation, and citrate and acetate appear equally good.

The concentration-dependence of stimulation for a series of salts of potassium with anions of different valence was determined in an effort to see what property of the salt was best correlated with stimulation (Fig. 4). The curves for all three salts were fitted well by Michaelis–Menten saturation curves. The parameters

Table 3. Kinetic parameters for Michaelis–Menten fits to stimulation curves for chloride, tartrate and citrate salts

Salt	Maximum stimulation ('V max.')	K_m (mM)	'$V_{max.}$'/K_m
KCl	0.60±0.08	2.3±1.0	0.26±0.10
K_2 tartrate	0.37±0.05	1.8±0.9	0.20±0.08
K_3 citrate	0.79±0.11	5.9±2.2	0.13±0.03

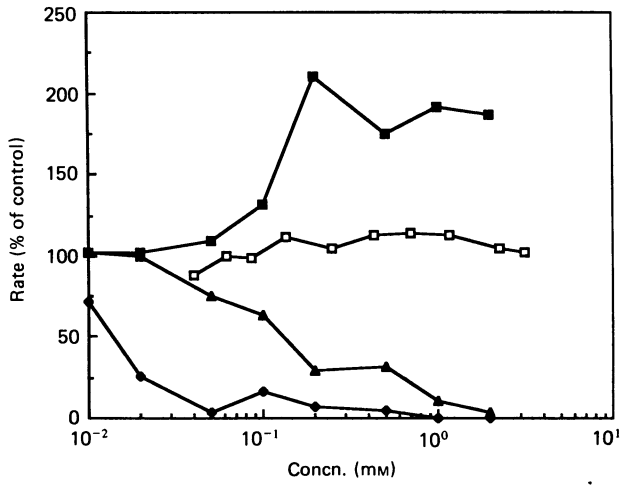

Fig. 5. Effect of polyvalent-metal salts on glucose 6-phosphate uptake in the presence of KCl

Glucose 6-phosphate uptakes were measured as described in the Experimental section in 140 mM-KCl/1 mM-Pipes, pH 6.6 in the presence of the indicated amount of $LaCl_3$ (■), $CuCl_2$ (▲), $HgCl_2$ (◆) or $CaCl_2$ (□). The control contained only 140 mM-KCl.

for these three fits are shown in Table 3. Of particular interest are the values of $V_{max.}/K_m$, which are the initial slopes of the response curves. These are significantly different ($t' = 2.57$ for 30 degrees of freedom for Cl^- versus tartrate, $P < 5\%$; other t' values are larger) and are in the proportion 4:3:2 for Cl^-/tartrate^{2-}/citrate^{3-} (t' is an analogue of Student's t, used when the variances in the samples being compared are not the same).

Response to bivalent cations added to KCl solutions

Essenberg & Kornberg (1975) reported that Ca^{2+} and Mg^{2+} added to solutions of 140 mM-KCl stimulated hexose phosphate uptake. These ions were tested more extensively along with a series of other multivalent cations: Be^{2+}, Sr^{2+}, Ba^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Sn^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+} and La^{3+}. Of these, Hg^{2+} and Cu^{2+} inhibited and La^{3+} activated, with the others having no effect (Fig. 5).

Influence of endogenous K^+ stores on stimulation by exogenous K^+

Two methods were used to deplete endogenous K^+ stores. The first was to wash in cold $MgSO_4$ (Thompson & MacLeod, 1971) and the second was to leave the cells to 4 °C overnight in buffer lacking K^+ (Saier *et al.*, 1975). Both treatments decreased the level of uptake in the absence of KCl, but both also decreased the level in the presence of KCl, so the stimulation by KCl was similar in all cases (Table 4).

Table 4. Effect of depletion of endogenous K^+ on transport

One portion of cells was washed three times in 50 mM-$MgSO_4$ at 4 °C, then equal portions were washed and resuspended in 5 mM-Pipes/Tris buffer, pH 6.6, with or without 140 mM-KCl. Another portion was washed three times in Pipes/Tris buffer, pH 6.6, then left overnight at 4 °C in the Pipes/Tris buffer. They were then washed and resuspended as for the $MgSO_4$ treatment. The control was directly washed and resuspended in Pipes/Tris buffer with or without 140 mM-KCl.

Treatment	Rate of transport (nmol/min per mg dry wt.) No KCl	+KCl	Increase
None	6.6±1.0	14.4±1.0	7.7±1.4
$MgSO_4$	2.6±0.2	9.0±0.2	6.4±0.2
Buffer	2.0±0.6	5.7±0.4	3.7±0.7

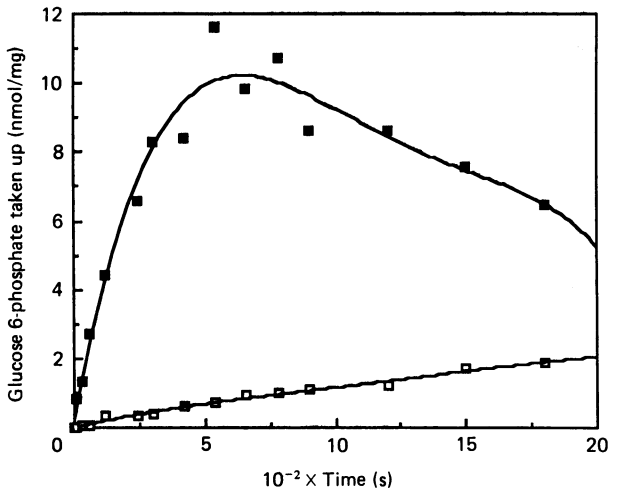

Fig. 6. Accumulation of glucose 6-phosphate in the presence and absence of KCl

Cells of strain RE48 were grown, harvested and washed in Pipes/Tris buffer with (■) or without (□) 80 mM-KCl as described in the Experimental section. [^{14}C]Glucose 6-phosphate was added to 2 ml samples of cells to a final concentration of 0.1 mM, and samples of 0.1 ml were withdrawn at intervals and filtered and washed as described in the Experimental section. Another uptake experiment was done in which samples were added to 5% (w/v) trichloroacetic acid and filtered through glass-fibre filters. These were washed three times with 5% trichloroacetic acid, then twice with propan-2-ol, then dried before counting radioactivity. The radioactivity in the trichloroacetic acid precipitates was deducted from those measured in the normal uptake experiment to correct for incorporation into other cellular components.

Effect of KCl on energetics and kinetics of hexose phosphate transport

Two experiments were done to assess the effect of KCl on the energetics and mechanism of the transport system. In the first, transport was monitored until the internal

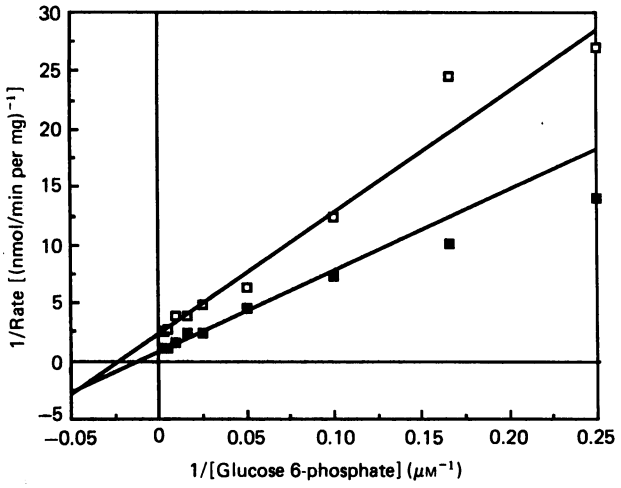

Fig. 7. Kinetics of glucose 6-phosphate uptake with and without KCl

Glucose 6-phosphate uptake was measured as described in the Experimental section at various concentrations of glucose 6-phosphate in 80 mM-KCl in Pipes/Tris buffer (■) or in Pipes/Tris buffer alone (□). This Figure summarizes five experiments. The lines are drawn from the parameters in Table 5.

Table 5. Kinetic parameters for glucose 6-phosphate uptake with and without KCl

Errors were not homogeneous, so a smooth curve was fitted to the observed errors for each concentration of glucose 6-phosphate, and the calculated values for this error were used for the weights in the fitting program.

	$V_{max.}$ (relative to no KCl)	K_m (μM)	$V_{max.}/K_m$
No KCl	1.00±0.07	45±6	0.022±0.002
80 mM-KCl	2.84±0.09	86±9	0.033±0.003

glucose 6-phosphate concentration reached a steady state in order to determine whether the rate and extent of uptake were both affected by KCl. As shown in Fig. 6, in the absence of KCl the extent of uptake was less than in its presence, suggesting a lesser capacity to maintain a gradient of hexose phosphate.

The second experiment was a determination of the kinetic parameters of the transport system in the presence or absence of KCl. Fig. 7 shows that, under both conditions, the response to glucose 6-phosphate was represented by a Michaelis–Menten saturation curve. The parameters of these curves are shown in Table 5. Both $V_{max.}/K_m$ and $V_{max.}$ values are significantly altered in the presence of KCl.

DISCUSSION

Many salts stimulate the rate of uptake of glucose 6-phosphate by cells of *E. coli*. With the exception of the inhibition by HCO_3^-, there is little difference between the anions tested, and what difference exists does not correlate with any simple property of the anion, such as radius, or the salt, such as osmolarity. Among the univalent cations, however, there is a clear and strong correlation with the hydrated radius of the ions, with ions having the smallest hydrated radius being most effective. Thus most of the stimulation appears to be a property of the cation. The results in Table 3, showing the kinetic parameters for stimulation by potassium salts of a uni-, a bi- and a ter-valent anion, are not entirely consistent with this idea. One would expect only the concentration of K^+ to determine the stimulation and the initial slopes to fall in the ratio 1:2:3 for chloride/tartrate/citrate. The observed ratio is not consistent with any simple model, since the order of effectiveness is chloride > tartrate > citrate. Given the difference in maximal stimulation with these three anions, the nature of the anion *does* play a role, but it is not possible to say what this role is. Neither tartrate nor citrate is transported in *E. coli*. Bivalent cations also stimulate, as shown by the results in Fig. 2. The hydrated radius of Mg^{2+} is quite different from that of K^+, so it appears that the bivalent cations fall on a different curve. Also, Mg^{2+} is effective at much lower concentrations than K^+. That the mechanisms of stimulation by the two ions are similar is suggested by the fact that no additional stimulation was seen when bivalent ions were added to a high concentration of KCl.

The stimulation by both KCl and $MgCl_2$ follows a simple kinetic model. It appears that the stimulation is saturable, which would imply a fixed number of sites on the cell where the salt can act. At higher salt concentrations, however, there is inhibition, which could arise for a variety of reasons. Both salts cause about the same stimulation, but the concentration of $MgCl_2$ required is only a tenth that for KCl, which suggests that the binding is much tighter for Mg^{2+}.

There are two major pieces of evidence that salts exert their effect by way of energy coupling. The first is the kinetics. Both basic parameters of the glucose 6-phosphate concentration curve are affected. If the effect of ions was simply to perturb the membrane in some way, one might expect the maximum velocity to change, but not the affinity for glucose 6-phosphate. More obviously, the effect of KCl on the accumulation of glucose 6-phosphate indicates that the salt affects the ability of the cell to maintain a concentration gradient. This could be a result of K^+ being directly involved in the coupling of energy to hexose phosphate uptake, or it could be that K^+ is required to maintain the proton gradient thought to energize the glucose 6-phosphate uptake.

The effects seen in the present work are very similar to those seen by Roomans *et al.* (1979) and Roomans & Borst-Pauwels (1979) for sulphate and phosphate uptake by *Saccharomyces cerevisiae* (baker's yeast). Both these processes appear to be carried out by symport with protons, and, in both cases, cations stimulate the rate of uptake. Bivalent cations stimulate at lower concentrations than univalent ones, and tervalent cations are effective at even lower concentrations than are bivalent ones. These effects are attributed to the effects of the ions on the surface potential of the cell, which tends to repel the anionic substrate. The cations screen the potential, and polyvalent ions are more effective because they bind more tightly to the anionic surface charges. The possible effects are quite complex and depend on the various kinetic parameters of the system. Also, a thorough test of this model would require a knowledge of the surface potential of *E. coli*.

Also relevant is the observation of Russell & Rosenberg (1980) that K^+ is co-transported with hexose phosphates when phosphate is not present. These authors (Russell & Rosenberg, 1979) found that K^+ is required for phosphate uptake, and that there is a co-transport of the two ions. This co-transport appears not to be a property of the phosphate transport system alone, since it requires a functional K^+ transport system. They also found that the requirement for potassium for phosphate uptake was absolute, but that, under normal conditions, cells leaked enough K^+ into the medium to allow some phosphate transport. If the cells were first depleted of K^+ then the dependence became absolute. In the case of hexose phosphates, they did not explore whether K^+ was required, as is the case with the phosphate. The studies reported here do not indicate an absolute requirement for K^+, even after depletion of endogenous stores. Russell & Rosenberg (1979) interpret their data to show that phosphate uptake is primarily a co-transport with protons, but that the resulting acidification of the cell interior leads to a $H^+ \rightleftharpoons K^+$ exchange. It is not clear why the coupling is so tight that the initial rates of K^+ uptake show no lag. At any rate, the coupling between K^+ and hexose phosphate uptake does not appear to be this tight.

We thank Miss Bobbye Hill for competent technical assistance. This work was supported in part by grant BMS 74-17347 of the National Science Foundation and by the Oklahoma Agricultural Experiment Station, of which this is paper no. J-4985.

Received 7 April 1986/1 October 1986; accepted 9 December 1986

REFERENCES

Cleland, W. W. (1967) Adv. Enzymol. Relat. Areas Mol. Biol. **29**, 1–32

Essenberg, R. C. & Kornberg, H. L. (1975) J. Biol. Chem. **250**, 939–945

Lanyi, J. K. (1979) Biochim. Biophys. Acta **559**, 377–397

Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor

Ramos, S. & Kaback, H. R. (1977) Biochemistry **16**, 854–859

Robinson, R. A. & Stokes, R. H. (1955) Electrolyte Solutions, pp. 266–272, Butterworths, London

Roomans, G. M. & Borst-Pauwels, G. W. F. (1979) Biochem. J. **178**, 521–527

Roomans, G. M., Kuypers, G. A. J., Theuvenet, A. P. R. & Borst-Pauwels, G. W. F. H. (1979) Biochim. Biophys. Acta **551**, 197–206

Russell, L. M. & Rosenberg, H. (1979) Biochem. J. **184**, 13–21

Russell, L. M. & Rosenberg, H. (1980) Biochem. J. **188**, 715–723

Saier, M. H., Jr., Wentzel, D. L., Feucht, B. U. & Judice, J. J. (1975) J. Biol. Chem. **250**, 5089–5096

Snedecor, G. W. & Cochran, W. G. (1980) Statistical Methods, 7th edn., Iowa State University Press, Ames

Spivey, H. O. & Snell, F. M. (1964) J. Phys. Chem. **68**, 2126–2130

Thompson, J. & MacLeod, R. A. (1971) J. Biol. Chem. **246**, 4066–4074