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The association of proton movement with galactose transport into subcellular membrane vesicles of *Escherichia coli*

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1. Subcellular membrane vesicles were prepared from a strain of *Escherichia coli* constitutive for the GalP galactose-transport system. 2. The addition of substrates of the GalP transport system to vesicle suspensions promoted alkaline pH changes, which provided direct evidence for the coupling of sugar and proton transport. 3. Respiration-energized galactose transport was progressively inhibited at pH values above 6.0, and was abolished by agents that render the membrane permeable to protons. 4. The combined effects of valinomycin, the nigericin-like compound A217 and pH on galactose transport suggested that both ΔpH and $\Delta\psi$ components of the protonmotive force contributed to energization of galactose transport. 5. These results substantiate the conclusion that the GalP transport system operates by a chemiosmotic mechanism.

Although galactose is transported into *Escherichia coli* by at least seven different systems (for review, see Kornberg, 1976), only two of these are specifically induced by galactose (Buttin, 1968; Boos, 1974). They are the so-called 'methyl galactoside' and 'galactose specific' transport systems, designated MglP and GalP respectively (Buttin, 1963, 1968; Rotman *et al.*, 1968; Wilson, 1974*a*; Bachmann & Low, 1980). Both systems effect the active transport of galactose into the bacterial cells against a concentration gradient without chemical modification of the sugar, but they differ in gene location (Bachmann & Low, 1980), protein components and mechanism of energization. The MglP system contains a periplasmic binding protein (Boos, 1969, 1974; Boos & Sarvas, 1970; Silhavy *et al.*, 1978), is sensitive to arsenate (Vorisek & Kepes, 1972; Parnes & Boos, 1973; Boos, 1974; Wilson, 1974*b*; Daruwalla *et al.*, 1981) and does not translocate protons (Henderson *et al.*, 1977), whereas the GalP system has no binding protein, is arsenate-insensitive (Daruwalla *et al.*, 1981) and transports protons (Henderson *et al.*, 1977). The last observation indicates that the GalP system is energized by a chemiosmotic mechanism (Mitchell, 1961, 1963, 1970), i.e. it is a sugar/H^+ symport responding to a transmembrane proton gradient ($\Delta\bar{\mu}_{H^+}$), like the lactose carrier of *E. coli* (West, 1970; West & Mitchell, 1973).

All of the above studies used intact cells of *E. coli*. However, to confirm that a particular transport system operates by a chemiosmotic mechanism, there are several advantages in using the subcellular vesicles first described and elegantly exploited by Kaback (for review, see Kaback, 1972, 1974, 1976). Unlike intact cells, vesicles are susceptible to ionophore antibiotics that selectively modify either ΔpH or $\Delta\psi$ components of $\Delta\bar{\mu}_{H^+}$; also, the absence of soluble enzymes and endogenous substrates from vesicles prevents metabolism or internal binding of transported compounds, and ensures that alternative routes for galactose transport, such as the binding-protein-dependent systems MglP (Kerwar *et al.*, 1972) or AraF (Brown & Hogg, 1972; Kaback, 1972) and phosphotransferase systems (Kaback, 1971), are inactive.

Horne (1980) confirmed that vesicles derived from the GalP-constitutive $MglP^-$,$LacY^-$ *E. coli* strain S183-27T (Robbins *et al.*, 1976; Henderson & Giddens, 1977) retained a high level of GalP activity but no other galactose-transport systems. Such vesicles provided ideal material for the experiments described below, which demonstrate for the first time that alkaline pH changes can be observed on addition of appropriate substrates to anaerobic suspensions of vesicles. Together with the effects of protonophores and ionophores, these observations

Abbreviations used: $\Delta\psi$, transmembrane electrical potential; ΔpH, transmembrane pH gradient; $\Delta\bar{\mu}_{H^+}$, transmembrane electrochemical proton gradient or protonmotive force.

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confirm that the GalP system operates by a chemiosmotic mechanism.

Experimental procedures

Growth of cells and preparation of vesicles

Cells of *E. coli* S182-27T [*mglB,mglC,mglD,lac* (zya) deletion; $MglP^-$,GalP-constitutive], a gift from Dr. B. Rotman, Brown University, Providence, RI, U.S.A., were maintained and grown under the conditions described by Henderson *et al.* (1977), with the addition of L-methionine (80 μg/ml), L-threonine (80 μg/ml), L-leucine (80 μg/ml) and L-histidine (90 μg/ml). Where required, 50 mM-potassium nitrate was added. Cells were grown under aerobic (400 ml in a 2 litre flask) or relatively anaerobic (400 ml in a 500 ml flask) conditions and harvested at A_{680} 0.7–0.8.

Membrane vesicles were then prepared as described by Kaback (1971) from spheroplasts made by the method of Witholt *et al.* (1976). Vesicle preparations were essentially free of intact cells or spheroplasts as determined by phase-contrast light or electron microscopy and contained less than 2% of contaminating cytoplasmic enzymes (Horne, 1980). Vesicles were either used fresh or frozen in liquid N_2 immediately after preparation and stored at −20 °C before use.

Uptake of radioactively labelled compounds by vesicles

Membrane vesicles were diluted with water to give a known concentration between 1 and 2 mg of protein/ml in 50 mM-potassium phosphate/10 mM-$MgSO_4$, pH 6.6. The suspension was bubbled with air throughout the time course of the experiment and the assay mixture was pre-incubated at 25 °C for 3 min before the addition of radioactively-labelled compounds. Energization of transport was usually achieved by using phenazine methosulphate and potassium ascorbate (pH 6.5) added in sequence after 2.5 min and 2.8 min to final concentrations of 0.1 mM and 20 mM respectively. Other respiratory substrates were added after 2.5 min to a final concentration of 20 mM. Transport was initiated by the addition of D-[1-^3H]galactose at 3 min. Samples (0.2 ml) were withdrawn, filtered and washed on cellulose acetate filters (Oxoid; 0.45 μm pore size) by using 4 ml of 0.1 M-LiCl. Filters were transferred to vials and radioactivity was determined as described previously (Henderson *et al.*, 1977).

Estimates of the initial rate of transport were made by using the samples taken at 15 s and the amount of radioactive compound accumulated in 2 min was taken as a measure of the extent of uptake. The accumulation at either 15 s or 2 min was linearly related to the concentration of protein in the assay. Additions of uncouplers, ionophores etc. were made 3 min before the labelled compounds, unless otherwise indicated.

Different pH values were achieved by resuspending vesicles in 0.1 M-potassium phosphate at the required pH and incubating for 10 min at 25 °C. The vesicles were then washed once with 0.1 M-potassium phosphate at the appropriate pH and resuspended in the same buffer as for transport assays (Ramos *et al.*, 1976).

Protein assay

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard (Sigma).

Continuous measurement of sugar-promoted pH changes

Alkaline pH changes observed with vesicles were generally small compared with those obtained by using intact cells (see Henderson *et al.*, 1977). Therefore it was important to conduct these experiments in the absence of any buffer that could diminish the pH changes. Accordingly, membrane vesicles (20 mg of protein) were washed three times with 50 ml of 150 mM-KCl/10 mM-$MgSO_4$, pH 6.5, to remove phosphate. The vesicles were finally resuspended in 150 mM-KCl/10 mM-$MgSO_4$, pH 6.5, to a protein concentration of 8–10 mg/ml.

The glass cell and experimental apparatus used in these experiments were described by Henderson *et al.* (1977). The pH-meter was built according to the design of Professor P. B. Garland, Department of Biochemistry, University of Dundee, Dundee, Scotland, U.K. To observe alkaline pH changes elicited by sugar transport, the proton ejection caused by respiration must be prevented. All measurements were therefore performed under anaerobic conditions and the cell and electrodes were washed thoroughly several times with water and finally with anoxic 150 mM-KCl/10 mM-$MgSO_4$, pH 6.5. Air was flushed from the apparatus with argon. Vesicles were added to the cell to a final concentration of 1–2 mg/ml for pH measurements, and the cell filled with anoxic solution. Since the vesicle suspensions were unbuffered, drift rates were initially high, but slowed after about 10–15 min. At this point sugar additions (15 μmol) were made. The recorded pH changes were converted into nmol of H^+ by calibration with 3 μl of air-free standard 0.01 M-NaOH.

Materials

Valinomycin, carbonyl cyanide *m*-chlorophenylhydrazone, 2-deoxy-D-galactose, D-galactose (glucose-free grade) and D-fucose were obtained from Sigma, Kingston-upon-Thames, Surrey, U.K. D-Talose and methyl β-D-galactoside were from Koch–Light, Colnbrook, Bucks., U.K. D-Glucose and

2,4-dinitrophenol were from BDH Chemical, Poole, Dorset, U.K. Compound A217 was a gift from Dr. R. L. Hamill, Lilly Research Laboratories, Indianapolis, IN, U.S.A. Tetrachlorosalicylanilide was a gift from Dr. W. A. Hamilton, Department of Microbiology, University of Aberdeen, Aberdeen, Scotland, U.K. D-[1-^{3}H]Galactose was from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Sugar-promoted proton uptake into membrane vesicles

If a transport system operates by a chemiosmotic mechanism an alkaline pH change should be observed during proton-substrate carrier-mediated diffusion into de-energized cells (Mitchell, 1970, 1973). Such observations have been reported for the transport of lactose, galactose, arabinose and xylose into intact cells of *E. coli* (West, 1970; West & Mitchell, 1973; Henderson, 1974; Henderson *et al.*, 1977; Lam *et al.*, 1980). Similarly, if active galactose transport into membrane vesicles occurs by the same mechanism operating in intact cells, then addition of galactose to non-respiring vesicles should elicit on alkaline pH change in medium surrounding the vesicles.

Addition of various sugars to an anaerobic suspension of membrane vesicles of *E. coli* S183-27T resulted in an alkaline pH change corresponding to proton uptake into the vesicles (Fig. 1). Each of the successful sugars was a known substrate of the GalP system. Consistently, methyl β-galactoside, a specific substrate not for GalP but for the MglP system (Rotman *et al.*, 1968), failed to promote a pH change (Fig. 1*b*). In addition, it should be emphasized that talose and 2-deoxygalactose were shown to be substrates only for the GalP system in intact cells (Henderson & Giddens, 1977; Henderson & Horne, 1979), so their ability to elicit pH changes indicated that it is the GalP system operating in the vesicles.

The rate of 2-deoxygalactose-promoted H^+-diffusion appeared to be greater than that with the other sugars tested (Table 1). This result is similar to that of Henderson & Giddens (1977), who found that 2-deoxygalactose consistently gave a greater effective H^+ uptake when added to de-energized cell suspensions.

None of these H^+-diffusion rates (Table 1)

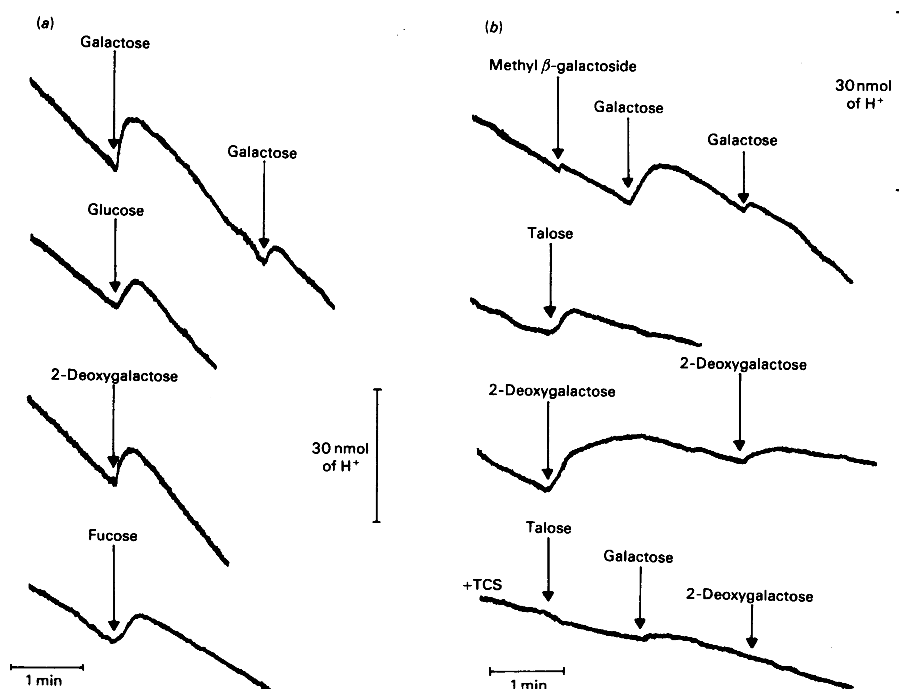

Fig. 1. *Alkaline pH changes elicited by addition of various sugars to membrane vesicles*
Vesicles were prepared from *E. coli* S183-27T grown on glycerol plus nitrate, and were resuspended in 150mM-KCl/10mM-$MgSO_4$ (see the Experimental procedures section). Results with two separate preparations are shown: (*a*) 1.6 mg/ml; (*b*) 1.0 mg/ml. Sugars (15 μmol) were added as indicated, generally in separate experiments, and tetrachlorosalicylanilide (TCS; 5 μM) was present in one experiment as shown.

Table 1. *Sugar-promoted pH changes in membrane vesicles*

The measurements were obtained from the experiments shown in Fig. 1. For experiment 1, pH changes elicited by the addition of sugars (15 μmol) to 150 mM-KCl/10 mM-$MgSO_4$, pH 6.5, were negligible in the absence of vesicles. For experiment 2, however, an alkaline pH change equivalent to 0.09 nmol of H^+ occurred in the absence of vesicles for galactose, 2-deoxygalactose and methyl β-D-galactoside, and talose gave an acid change of 0.26 nmol of H^+. The values below were corrected for these changes. Abbreviation: n.m., not measurable.

Sugar	Rate (nmol of H^+/min per mg)	Extent (nmol of H^+/mg)
Experiment 1		
Galactose	5.9	1.9
Second addition of galactose	n.m.	0.6
Glucose	3.1	1.7
2-Deoxygalactose	8.3	1.8
Fucose	3.2	1.5
Experiment 2		
Methyl β-galactoside	n.m.	0.03
Galactose	3.68	1.71
Talose	2.28	1.72
2-Deoxygalactose	5.37	2.66
Talose + tetrachlorosalicylanilide	n.m.	0.00

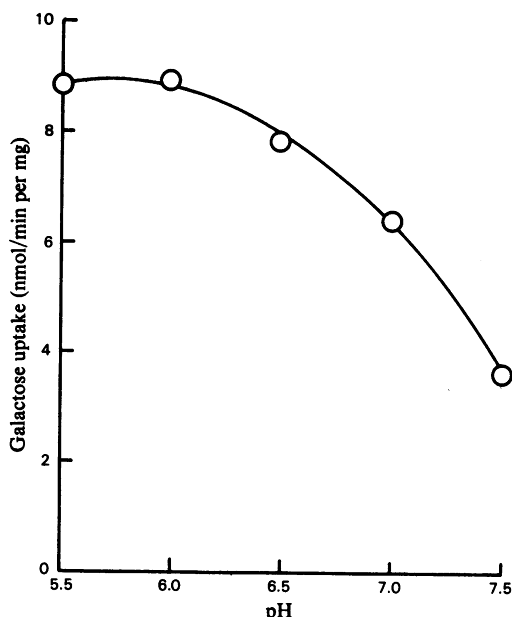

Fig. 2. *pH-dependence of galactose transport into membrane vesicles*

Membrane vesicles were prepared from *E. coli* S183-27T grown on glycerol plus nitrate and assayed for transport of [1-^{3}H]galactose (40 μM) energized by ascorbate plus phenazine methosulphate. Duplicate measurements of the initial rate at each pH were averaged.

correlated with the rates obtained for energized uptake of labelled sugars into vesicles (Horne & Henderson, 1979). There are two possible reasons for this discrepancy. First, the labelled sugar uptakes were measured under energized conditions (i.e. in the presence of reduced phenazine methosulphate) and it is likely that the affinity of the carrier for substrates increases on energization, as shown by studies with the lactose permease (Kaczorowski *et al.*, 1979; Wright *et al.*, 1979). Secondly, the concentration of substrate used in these experiments was about 5–6 mM final, over 100-fold higher than that used in energized labelled sugar uptakes (40 μM).

The uncoupler (tetrachlorosalicylanilide) eliminated the alkaline pH change due to the addition of talose and the subsequent addition of other sugars (Fig. 1*b*). In the presence of an uncoupler any protons entering the vesicles as a result of sugar transport will diffuse out again as a proton–uncoupler complex. Thus, no pH gradient will form across the vesicle membrane and no alkaline pH change will be detected.

These results were the first direct demonstration of sugar/H^+ symport in subcellular vesicles, and are consistent with the operation of a chemiosmotic mechanism for sugar transport by GalP.

Influence of pH on energized transport of galactose

The respiratory substrates ascorbate plus phenazine methosulphate, D-lactate, succinate, α-glycerophosphate and NADH plus ubiquinone were successful in energizing active galactose transport into membrane vesicles (Horne, 1980). As with other transport systems in vesicles (see, e.g., Kerwar *et al.*, 1972) the most efficient energy source for galactose transport was found to be ascorbate plus phenazine methosulphate, so these substrates have been used to examine the pH-dependence of energized galactose transport. As shown in Fig. 2 maximum activity occurred at pH 5.5–6.0, where $\Delta\bar{\mu}_{H^+}$ is known to be maximal (Ramos *et al.*, 1976). Indeed, the pH-dependence of energized galactose transport is remarkably similar to the reported pH-dependence of $\Delta\bar{\mu}_{H^+}$ (Ramos *et al.*, 1976), which suggests that galactose transport responds to the total $\Delta\bar{\mu}_{H^+}$ and not solely to either the ΔpH or the Δψ component. Energized lactose transport has a similar pH-dependence (Ramos & Kaback, 1977), consistent with a similarity in mechanism of the two sugar-transport systems.

Table 2. *Inhibition of galactose transport by uncouplers or ionophores at pH 6.6*

Membrane vesicles were prepared from *E. coli* S183-27T grown on glycerol in the presence of nitrate. Galactose transport was assayed as described in the Experimental procedures section with energization by ascorbate plus phenazine methosulphate, all inhibitors being added 3 min before the labelled galactose (40 μM). The inhibitors were all dissolved in ethanol except 2,4-dinitrophenol. The maximal ethanol concentration was 1%, and comparisons were made with the ethanol control where appropriate. Control values for energized initial rate and extent were 7.5 nmol/min per mg and 3.9 nmol/2 min per mg respectively. Abbreviation used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Inhibitor	Initial rate (% of control value)	Extent (% of control value)
Energized control	100.0	100.0
Unenergized control	5.3	10.3
5 μM-Tetrachloro-salicylanilide	2.5	1.7
1 mM-2,4-Dinitrophenol	25.6	32.9
5 μM-CCCP	2.9	3.6
5 μM-CCCP + 5 mM-mercaptoethanol	26.5	60.6
5 μM-Valinomycin	19.5	13.5
A217 (5 μg/ml)	51.8	49.2
1% Ethanol	133.3	121.6

Inhibition of energized galactose transport by protonophores

Despite the diversity of their chemical structures, the classical uncoupling agents, 2,4-dinitrophenol, carbonyl cyanide *m*-chlorophenylhydrazone and tetrachlorosalicylanilide all catalyse electrogenic proton movement through a variety of biological membranes (Mitchell & Moyle, 1967; Henderson, 1971). This would be expected to discharge $\Delta\bar{\mu}_{H^+}$ and prevent energization of proton-linked transport systems. All these compounds inhibited energized galactose transport into vesicles (Table 2), implying that $\Delta\bar{\mu}_{H^+}$ is the driving force. The potency of carbonyl cyanide *m*-chlorophenylhydrazone and tetrachlorosalicylanilide was greater than that of 2,4-dinitrophenol, consistent with their relative effects on other energized functions. However, these reagents may have more than one effect. Carbonyl cyanide *m*-chlorophenylhydrazone, for example, was reported to act as a thiol-specific reagent during long incubation periods (Kaback *et al.*, 1974), and in our experiments the inhibitory effect of carbonyl cyanide *m*-chlorophenylhydrazone was reduced by 2-mercaptoethanol (Table 2). The mercaptoethanol may actually be protecting susceptible thiol groups of proteins in the vesicle membrane, or it may be reacting directly with carbonyl cyanide *m*-chlorophenylhydrazone and decreasing the effective concentration of the uncoupler. Susceptible thiol groups are probably present in functional proteins of the respiratory chain as well as in the GalP transport protein itself (Kaethner & Horne, 1980), and impairment of either would reduce energized galactose transport. Nevertheless, the susceptibility to three different uncoupling agents is another indication that galactose transport is energized by $\Delta\bar{\mu}_{H^+}$.

Inhibition of energized galactose transport by ionophores

Valinomycin is an ionophore that specifically increases the potassium permeability of membranes and dissipates the electrical potential component, $\Delta\psi$, of $\Delta\bar{\mu}_{H^+}$ (Harold, 1970; Henderson, 1971). At pH 6.6 energized galactose transport was reduced to about 20% of control values by 5 μM-valinomycin (Table 2). Thus, it would appear that energized galactose transport was susceptible to changes in $\Delta\psi$.

Nigericin-like ionophores, such as A217, catalyse an electrically neutral exchange of K^+ for protons, and so reduce the transmembrane pH gradient without affecting $\Delta\psi$ (Henderson, 1971; Harold *et al.*, 1974). At pH 6.6, energized galactose transport was reduced to about 50% by 5 μg of A217/ml (Table 2), suggesting that galactose transport responds to changes in the pH gradient.

However, from these observations it is difficult to determine whether galactose is responding mainly to $\Delta\psi$, to ΔpH, or both, especially since ΔpH varies according to the external pH, whereas $\Delta\psi$ is essentially constant in membrane vesicles between pH 5.5 and 7.5 (Kaback, 1976, 1977). Also the effects of valinomycin and A217 depend on the concentration of each ionophore. Thus, a more thorough investigation was carried out by measuring the inhibition of galactose transport at various concentrations of the ionophores and different external pH values based on the strategy described by Kaback and co-workers (see, e.g., Ramos *et al.*, 1976; Ramos & Kaback, 1977; Kaback, 1977). To allow for the inherent dependence of galactose transport on pH (Fig. 2), the results are expressed as a percentage of control values measured in the absence of ionophore.

Increasing concentrations of valinomycin inhibited energized galactose transport much more at pH 7.5 than at pH 5.5 (Fig. 3*a*). This can be interpreted in terms of a chemiosmotic mechanism as follows. At pH 7.5 the ΔpH is effectively zero (Ramos *et al.*, 1976) and so valinomycin, which prevents formation of $\Delta\psi$ and not ΔpH, should have its greatest effect on galactose transport at pH 7.5. Whereas, at pH 5.5 approximately two-thirds of the total protonmotive force is ΔpH and one-third is $\Delta\psi$ (Ramos *et al.*, 1976), so that, even if valinomycin

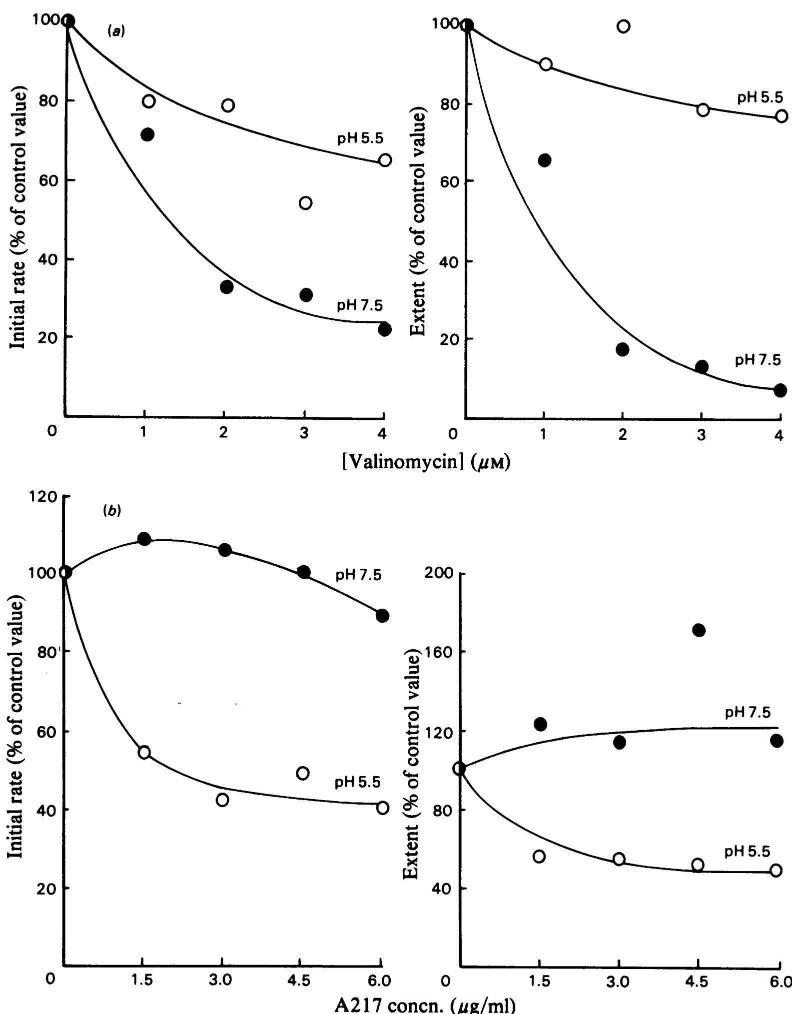

Fig. 3. *Effects of valinomycin or A217 on energized galactose transport at pH 5.5 and 7.5*
Galactose transport energized by ascorbate plus phenazine methosulphate was measured at pH 5.5 or pH 7.5 as described in the Experimental procedures section. Valinomycin (*a*) or A217 (*b*) was added at various concentrations 3 min before the galactose (40 μM). The results shown are means of duplicate measurements, with the control values for the initial rates being 10.5 and 4.4 nmol/min per mg at pH 5.5 and 7.5 respectively; for the extents, the control values were 6.1 and 3.4 nmol/2 min per mg at pH 5.5 and 7.5 respectively.

prevents formation of $\Delta\psi$, there is still a significant driving force for transport in the form of ΔpH.

By contrast, increasing concentrations of the nigericin-like compound, A217, inhibited energized galactose transport at pH 5.5, but had little effect at pH 7.5 (Fig. 3*b*). Again, these results correlate with what is known about the variation of the pH gradient and membrane potential with pH. Since no ΔpH exists at pH 7.5, A217 would not be expected to affect transport at this pH (Ramos *et al.*, 1976), whereas at pH 5.5 the protonmotive force consists mainly of ΔpH (Ramos *et al.*, 1976), so that A217 would be expected to have a maximal inhibitory effect on energization of transport.

Thus the differential effects of valinomycin, A217 and pH on energized galactose transport into membrane vesicles suggest that galactose is being translocated by a chemiosmotic mechanism. Two classes of transport system were defined by Ramos & Kaback (1977) according to their response to ionophores at pH 5.5; one class responded to changes in both ΔpH and $\Delta\psi$, whereas the other appeared to depend only on ΔpH. The results obtained above show that galactose responds to both ΔpH and $\Delta\psi$,

like the lactose permease of *E. coli* (Ramos & Kaback, 1977).

In conclusion, it has been demonstrated in the present paper that energized galactose transport into membrane vesicles could be driven by respiration, and was sensitive to uncouplers and ionophores in a manner that suggested the driving force was the total protonmotive force rather than any separate component. The coupling of sugar transport to proton movement could be demonstrated directly by showing that galactose and other substrates for the GalP system elicited an alkaline pH response when added to a suspension of de-energized membrane vesicles; in addition, this sugar-mediated movement of protons was found to be sensitive to uncouplers. All these results suggest that GalP is a membrane-bound transport system independent of periplasmic binding proteins, which operates by an electrogenic sugar/H^+ symport mechanism driven by the transmembrane electrochemical H^+ gradient ($\Delta\bar{\mu}_{H^+}$) generated by respiration.

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