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The active transport of 2-keto-D-gluconate in vesicles prepared from *Pseudomonas purida*

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The transport of 2-keto-D-gluconate (α-D-*arabino*-2-hexulopyranosonic acid; 2KGA) in vesicles prepared from glucose-grown *Pseudomonas putida* occurs by a saturable process with a K_m of $110.0 \pm 2.9\ \mu M$ and a $V_{max.}$ of 0.55 ± 0.04 nmol·min^{-1}·(mg of protein)$^{-1}$. The provision of phenazine methosulphate/ascorbate or L-malate leads to an accumulation of intravescular 2KGA, a decrease in the K_m value to $50 \pm 2.1\ \mu M$ and $35 \pm 2.9\ \mu M$ respectively and no change in the $V_{max.}$. In the presence of electron donors the transport of 2KGA is inhibited by the respiratory poisons antimycin A, rotenone and the uncoupler 2,4-dinitrophenol. 2KGA transport is also competitively inhibited by 4-deoxy-4-fluoro-2-keto- or 3-deoxy-3-fluoro-2-keto-D-gluconate with K_i values of $50\ \mu M$ and $160\ \mu M$ respectively. The carrier system for 2KGA is repressed in vesicles from cells grown on succinate. Such vesicles transport 2KGA by non-specific physical diffusion with a K_m value of ∞ in the absence or presence of electron donors. Vesicles from glucose or succinate grown cells, in the presence of phenazine methosulphate/ascorbate at pH 6.6, generate a proton-motive force (Δp) of approx. 140 mV. The Δp, composed of proton gradient (ΔpH) and a membrane potential ($\Delta\psi$), is collapsed in the presence of dinitrophenol. Based on the results obtained with valinomycin, nigericin and carbonyl cyanide *m*-chlorophenylhydrazone, the active transport of 2KGA at pH 6.6 is coupled predominately to the ΔpH component of Δp.

Because of the presence of cytoplasmic membrane-bound glucose dehydrogenase (EC 1.1.99.10) and gluconate dehydrogenase (EC 1.1.99.3) in aerobic fluorescent pseudomonads (Midgley & Dawes, 1973; Roberts *et al.*, 1973), a model for the separate transport of glucose, gluconate and 2KGA has been proposed (Roberts *et al.*, 1973; Guyman & Eagon, 1974; Eisenberg *et al.*, 1974). Briefly, the model proposes the presence of three separate transport carriers in the cytoplasmic membrane for glucose, gluconate and 2KGA.

Abbreviations used: 2KGA, 2-keto-D-gluconic acid (α-D-*arabino*-2-hexulopyranosonic acid); 4F2KGA, 4-deoxy-4-fluoro-2-keto-D-gluconic acid; 3F2KGA, 3-deoxy-3-fluoro-2-keto-D-gluconic acid; 3FG, 3-deoxy-3-fluoro-D-glucose; 4FG, 4-deoxy-4-fluoro-D-glucose; PMS, phenazine methosulphate; $TPMP^+$, triphenylmethylphosphonium (iodide salt); DNP, 2,4-dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Δp, proton-motive force; $\Delta\psi$ and ΔpH, differences in electrical potential and pH respectively between the outside and inside of the membrane.

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The active transport of these solutes is considered to be coupled to the respiration of L-malate, glucose or gluconate via the appropriate enzymes and the electron transport chain. In support of this model vesicles prepared from *Pseudomonas aeruginosa* have a separate carrier for the active transport of gluconate which may be coupled to the respiration of electron donors (e.g. L-malate) (Stinnet *et al.*, 1973). Subsequently it was shown in vesicles prepared from *P. putida* that separate active transport systems exist for both glucose and gluconate (Al-Jobore *et al.*, 1980).

In none of these studies were measurements of Δp generated by electron donors undertaken. Although Roberts *et al.* (1973) provided evidence for the active uptake of 2KGA in whole cells of *P. aeruginosa*, no detailed characterization of this transport system in the cytoplasmic membrane was undertaken.

In the present paper we report, in vesicles prepared from *P. putida*, (*a*) the presence of an inducible/repressible active transport system for 2KGA, (*b*) the kinetics and specificity of this transport system, and (*c*) that the provision of

ascorbate/PMS or L-malate/FAD generates a Δp composed of $\Delta\psi$ and ΔpH of sufficient magnitude to account for the accumulation of 2KGA as well as that of glucose and gluconate previously reported (Al-Jobore *et al.*, 1980). A preliminary account of some of these results has appeared elsewhere (Agbanyo & Taylor, 1983).

Materials and methods

Chemicals

3FG and 4FG were synthesized by the methods of Foster *et al.* (1967) and Lopes & Taylor (1979) respectively. D-[U-^{14}C]Glucose (283mCi/mmol), L-[^{14}C]glucose (58mCi/mmol), sodium [1-^{14}C]acetate (54.2mCi/mmol) and [U-^{14}C]TPMP$^+$ (9.0mCi/mmol) were from The Radiochemical Centre (Amersham, Bucks., U.K.). PMS, ascorbic acid, *o*-phenylenediamine, lysozyme, ribonuclease A, deoxyribonuclease I, bovine serum albumin, valinomycin, nigericin and CCCP were from Sigma (St. Louis, MO, U.S.A.). All other chemicals and reagents were from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Culture conditions, organism and vesicle preparation

Pseudomonas putida ATCC 12633 was routinely maintained on a mineral salts agar medium containing glucose or succinate (Davis & Mingoli, 1950). Whole cells were harvested from the appropriate carbon source and vesicles were isolated at a final concentration of 5–10mg of protein·ml^{-1}, suspended in 100mM-potassium phosphate buffer, pH6.6, and characterized by electron microscopy as previously reported (Al-Jobore *et al.*, 1980). The orientation of vesicles was estimated to be 75% right-side-out. This was based on the accessibility of NADH–cytochrome *c* reductase in native and Triton X-100-treated vesicles. The intravesicular volume, as determined by the method of Rottenberg (1979) using L-[1-^{14}C]glucose, was found to be 3.6μl·(mg of protein)$^{-1}$. Protein determinations were by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Preparation of D-[U-^{14}C]2KGA, 3F2KGA and 4F2KGA

A stock solution of D-[U-^{14}C]2KGA (2.07mM, 3.2mCi/mmol) was prepared from 0.565μmol of D-[U-^{14}C]glucose diluted with 50μmol of glucose before treatment with 2mg of partially purified glucose dehydrogenase/gluconate dehydrogenase from *P. putida* (Taylor *et al.*, 1975). D-[U-^{14}C]2KGA was eluted from a Dowex-1 formate anion-exchange column (1cm×15cm) with a formic acid gradient (0.0–1.0M) at a flow rate of 1.0ml·min^{-1} as described elsewhere (Kay & Gronlund, 1969). The concentration of 2KGA was determined by the *o*-phenylenediamine method (Lanning & Cohen, 1951). Radioactive measurements were made on 0.1ml fractions in 10ml of Beckman Ready-Solv with a LS-7500 liquid-scintillation counter (Beckman Inc., Irvine, CA, U.S.A). Stock solutions of 4.7mM-4F2KGA and 3.6mM-3F2KGA were prepared in a similar manner from 40μmol of 4FG and 41.2μmol of 3FG respectively. The concentrations of the fluoro-α-ketoaldonic acids was determined by the same method as for 2KGA.

Transport and kinetic studies with vesicles

Transport, in the absence or presence of electron donors, was initiated by the addition of appropriate concentrations of D-[U-^{14}C]2KGA (3.2mCi/mmol) to vesicles as previously reported (Al-Jobore *et al.*, 1980). Kinetic data were obtained by Lineweaver–Burk (1934) or Dixon (1953) plots. For inhibition studies (Dixon plot), vesicles (1mg of protein), 50mM-potassium phosphate buffer, pH6.6, 12mM-$MgSO_4$ and a series of known 3F2KGA or 4F2KGA concentrations were incubated for 10min. 2KGA transport was initiated by the addition of a known concentration of D-[U-^{14}C]2KGA. All materials were in a total volume of 1.0ml. The Lineweaver–Burk and Dixon plots were estimated by the method of least squares. The K_m and $V_{max.}$ values are the average of duplicate determinations.

Measurement of Δp in vesicles

The Δp was calculated from $\Delta p = \Delta\psi - (2.303RT/F)\cdot\Delta$pH (Mitchell & Moyle, 1968). $\Delta\psi$ and ΔpH were measured by using the distribution of [^{14}C]TPMP$^+$ and [1-^{14}C]acetate respectively. The flow analysis cell and method of calculating ΔpH was as reported by Ramos *et al.* (1979). $\Delta\psi$ was calculated from the Nernst equation [where $\Delta\psi = 58.8\log_{10}(\text{TPMP}^+_{in}/\text{TPMP}^+_{out})$ at room temperature]. Membrane vesicles (3.01mg of protein) suspended in 0.05M-potassium phosphate buffer, pH 6.6, containing 0.01M-$MgSO_4$ were added (total volume 0.8ml) to the upper chamber. At appropriate times, 20μl of TPMP$^+$ (125μM, 9mCi/mmol) or 40μl of [1-^{14}C]acetate (44.3μM, 54.2mCi/mmol) was added through the aperture in the top well. The uncoupler DNP (20μl, 2μM) was added in a similar manner. Potassium phosphate (0.05M, pH6.6) was pumped through the lower chamber at a rate of 3.4ml·min^{-1} by using a Perspex pump (LKB). Fractions (1.7ml) were collected. Samples (1.0ml) of these fractions in 10ml of Ready-Solv were assayed for radioactivity with a Beckman LS-7500 scintillation counter.

Effects of valinomycin, nigericin and CCCP on 2KGA transport

Vesicles from glucose-grown *P. putida* (1.2mg of protein) were suspended in 50mM-potassium phosphate buffer, pH6.6, containing 12mM-$MgSO_4$ and 10mM-L-malate/50μM-FAD or 0.1mM-PMS/20mM-ascorbate. The mixture was incubated at 30°C for 10min in the presence or absence of known concentrations of ionophores or CCCP (Fig. 4). Transport was initiated by the addition of 82.78μM-D-[U-^{14}C]2KGA in a final volume of 1.0ml. At time intervals 0.1ml aliquots were withdrawn, diluted into 2.0ml of 0.1M-LiCl at 25°C, filtered through a membrane (HRMP02400; Millipore Corp., Bedford, MA, U.S.A.) and immediately washed with another 2.0ml of LiCl. The filters were then dried and the radioactivity determined with a Beckman LS-7500 liquid-scintillation spectrometer as previously reported (Al-Jobore *et al.*, 1980). The results are the average of duplicate determinations.

Results and discussion

In vesicles obtained from glucose-grown *P. putida* the provision of L-malate/FAD or PMS/ascorbate increases the rate of transport and accumulation of 2KGA (Table 1). The result with L-malate is consistent with the presence of L-malate dehydrogenase (Stinnett *et al.*, 1973) and the transfer of electrons through the respiratory chain to oxygen. A similar respiratory couple is observed with glucose, gluconate, 2-deoxyglucose and 3FG, all of which are substrates for glucose dehydrogenase or gluconate dehydrogenase (Al-Jobore *et al.*, 1980). In the absence of electron donors, these vesicles transport 2KGA by saturable kinetics with K_m value 111.0$\pm$2.9μM and $V_{max.}$ 0.55$\pm$0.04nmol·min^{-1}·(mg of protein^{-1}) (Fig. 1). In the presence of PMS/ascorbate or L-malate/FAD the K_m values for 2KGA decreases to 50$\pm$2.1μM and 35$\pm$2.9μM respectively although the $V_{max.}$ remains the same (Fig. 1). Evidence for the coupling of 2KGA transport to respiration is provided by the effect of the inhibitors antimycin A, rotenone and DNP. The inhibition of 2KGA transport is only observed (Table 2) in the presence of the electron donors (e.g. L-malate/FAD). The non-inhibitory effect of cyanide (Table 2) may be due to a cyanide-insensitive terminal *o*-type cytochrome. This type of terminal oxidase has been identified in *P. aeruginosa* (Matsushita *et al.*, 1980). Moreover, a purified oxygenated cytochrome *o* obtained from a strain of *Vitreoscilla* sp. has been shown to not react with cyanide (Liu & Webster, 1974).

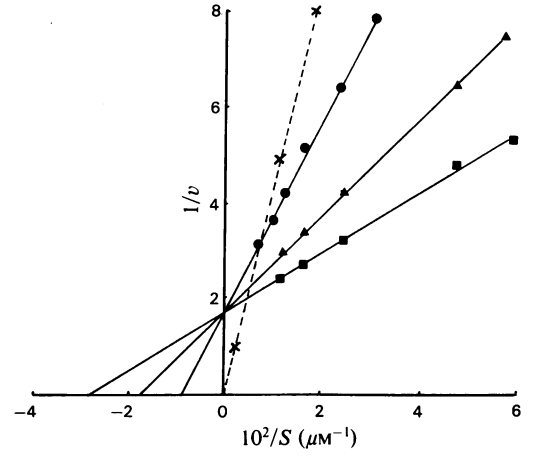

Fig. 1. *Lineweaver–Burk plots of 2KGA transport in vesicles from glucose- or succinate-grown P. putida in the absence or presence of electron donors*

v, initial rate of 2KGA transport [nmol·min^{-1}·(mg of protein)$^{-1}$]; *S*, [2KGA] in μM. With glucose-grown cells: ●, 2KGA only; ▲, 2KGA plus PMS (0.1mM) and ascorbate (20mM); ■, 2KGA plus L-malate (10mM) and FAD (50μM). With succinate-grown cells: ×, 2KGA in the absence or presence of electron donors (see the Materials and methods section).

Table 1. *Effect of various electron donors on the initial rate of transport and accumulation of 2KGA in vesicles prepared from glucose-grown P. putida*

The initial rates and accumulation of 2KGA are based on initial external [2KGA] of 144.9μM. The intravesicular volume of 3.6μl·(mg of protein)$^{-1}$ was determined by the method of Rottenberg (1979) and the intravesicular [2KGA] measured after 10min.

Electron donors added (final concn.)	Initial rate of transport [nmol·min^{-1}·(mg of protein)$^{-1}$]	Intravesicular [2KGA] (μM)	$\frac{[KGA]_{int.}}{[KGA]_{ext.}}$
None	0.30	143	0.99
L-Malate (10mM)+FAD (50μM)	0.45	207	1.43
PMS (0.1mM)+ascorbate (20mM)	0.50	297	2.05
Glucose (100μM)	0.85	369	2.55
Gluconate (100μM)	1.10	256	1.56
3FG (260μM)	0.60	269	1.86
2-Deoxyglucose (10mM)	1.70	536	3.70

Table 2. *Effect of respiratory inhibitors and DNP on the rate of 2KGA transport in vesicles prepared from glucose-grown P. putida*

The reaction mixtures (total volume 1.0ml) contained 50 μmol of potassium phosphate buffer, pH6.6, 12 μmol of $MgSO_4,7H_2O$, vesicles (1.0mg of protein), the concentration of inhibitor indicated and, when present, the electron donor L-malate (10mM)+FAD (50 μM). Preincubation of this mixture with shaking and oxygen gasing at 30°C for 10min was followed by the addition of known concentrations of 2KGA. The initial rates of transport were measured as previously reported (Al-Jobore *et al.*, 1980).

Compound added (final concn.)	Initial rate of 2KGA transport [nmol·min^{-1}·(mg of protein)$^{-1}$]	
	In the absence of electron donor +124.2 μM-2KGA	In the presence of electron donor +82.8 μM-2KGA
None	0.44	0.35
Antimycin A (0.24mM)	0.45	0.24
Rotenone (0.24mM)	0.42	0.24
DNP (30.0mM)	0.40	0.22
KCN (20mM)	0.44	0.44

Table 3. *Determination of $\Delta\psi$ and ΔpH in vesicles from glucose- or succinate-grown P. putida*

Data were obtained from Fig. 3(*a*) and Fig. 3(*b*), $\Delta\psi$ and ΔpH values are in mV (see the Materials and methods section).

Electron donors added (final concn.)	Vesicles from glucose-grown cells			Vesicles from succinate-grown cells		
	$\Delta\psi$	ΔpH	Δp	$\Delta\psi$	ΔpH	Δp
L-Malate (10mM)+FAD (50 μM)	60	69	129	0	0	0
PMS (0.1mM)+ascorbate (20mM)	71	74	145	70	70	140

Further evidence for the presence of a 2KGA transport protein is provided by the competitive inhibition of 2KGA transport by fluorinated sugar analogues of 2KGA. Thus 4F2KGA and 3F2KGA both display competitive inhibition, with K_i values of 50 μM and 160 μM respectively (Figs. 2*a* and 2*b*). Similar values were obtained by Lineweaver–Burk plots (results not shown). With vesicles from succinate-grown cells the carrier protein for 2KGA is repressed, as shown by a K_m value of ∞ in the presence or absence of electron donors (Fig. 1). Based on similar kinetic results (Al-Jobore *et al.*, 1980) we have shown that the glucose carrier, in vesicles prepared from succinate-grown *P. putida*, is also repressed (K_m ∞) whereas the gluconate carrier is not (K_m 47.0 μM). It is apparent, therefore, that whereas both glucose and 2KGA carriers are inducible/repressible the gluconate carrier is not.

According to the chemiosmotic theory (Mitchell, 1961) accumulation of 2KGA subsequent to the oxidation of electron donors (Table 1) would be a consequence of electrogenic proton extrusion from the vesicles and the generation of a transmembrane potential ($\Delta\psi$, interior negative). This is supported by the provision of PMS/ascorbate or L-malate/FAD (results not shown) whereupon $TPMP^+$ in vesicles from glucose-grown cells accumulates (Fig. 3*a*). Moreover the provision of DNP collapses the proton gradient and hence the accumulation of $TPMP^+$ (Fig. 3*a*). A ΔpH is also evident from the accumulation of acetate in the presence of the electron donor, which likewise is collapsed in the presence of DNP (Fig. 3*b*). From these data the $\Delta\psi$ and ΔpH (and hence Δp) may be calculated. The values obtained for vesicles obtained from glucose- or succinate-grown *P. putida* are shown in Table 3. Thus vesicles from glucose-grown cells, in the presence of PMS/ascorbate or L-malate/FAD generate a Δp of −145mV (composed of $\Delta\psi$, −71mV and ΔpH, −74mV) and −129mV (composed of $\Delta\psi$, −60mV and ΔpH, −69mV) respectively. In vesicles from succinate-grown cells, however, PMS/ascorbate generates a Δp of −140mV (composed of $\Delta\psi$, −70mV and ΔpH, −70mV) whereas the electron donor L-malate/FAD generates no detectable Δp, (i.e., acetate and $TPMP^+$ merely equilibrate). We have preliminary evidence that this is due to the induction of the L-malate transport system. Thus in vesicles from glucose-grown cells the uptake of L-malate is by non-specific physical diffusion with a K_m of ∞. In vesicles from succinate-grown cells, however, L-malate transport has a K_m value of 14.3mM and $V_{max.}$ 313nmol·min^{-1}·(mg of protein)$^{-1}$ (N. F. Taylor, unpublished work). The activity of L-malate dehydrogenase in either glucose- or succinate grown vesicles remains the same. The fact that neither $\Delta\psi$ or ΔpH can be detected in the case of succinate grown vesicles,

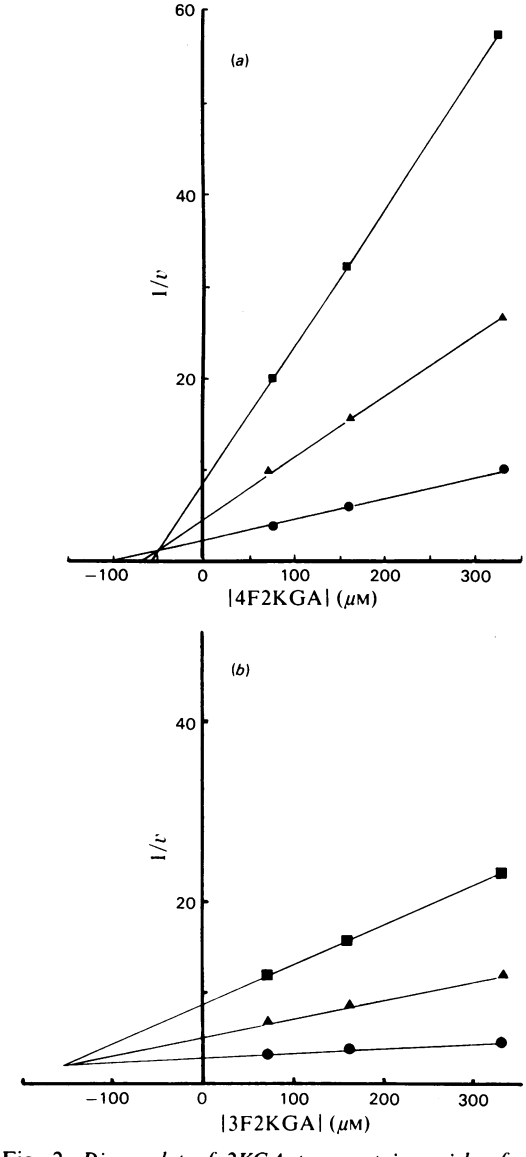

Fig. 2. *Dixon plot of 2KGA transport in vesicles from glucose-grown P. putida in the presence of known (a) 4F2KGA or (b) 3F2KGA concentrations*

v, initial rate of 2KGA transport [nmol·min^{-1}·(mg of protein)$^{-1}$]. 2KGA concentrations: ●, 154 μM; ▲, 95 μM; ■, 47.5 μM. All kinetic measurements were made in the absence of electron donors (see the Materials and methods section).

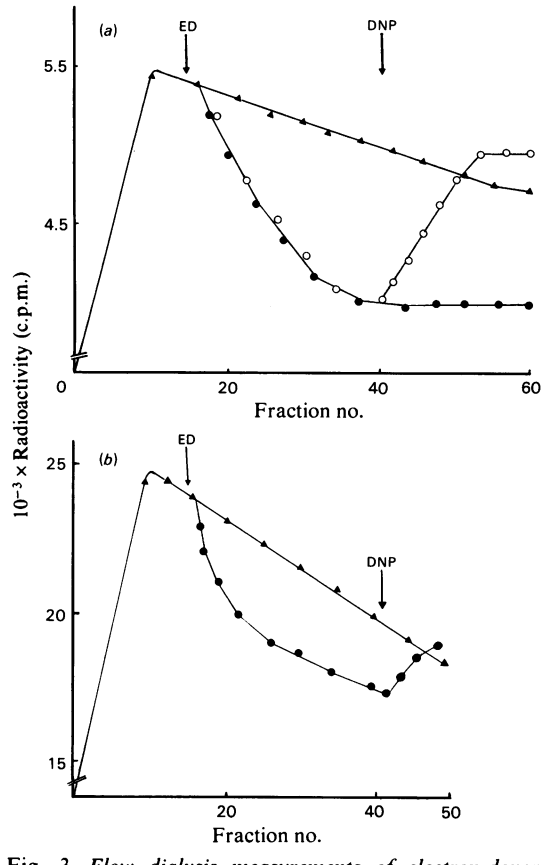

Fig. 3. *Flow dialysis measurements of electron-donor-dependent (a) TPMP$^+$ or (b) acetate uptake in vesicles from glucose-grown P. putida*

▲, Equilibration of TPMP$^+$ or acetate; ●, uptake of TPMP$^+$ or acetate after addition of electron donor (ED) which was a mixture of 9.0 μl of PMS (0.1 mM) and 37.0 μl of ascorbate (20 mM); ○, uptake of TPMP$^+$ or (●) acetate after addition of 20.0 μl of DNP (2 μM). Arrows indicate points of additions (see the Materials and methods section).

therefore, may be due to the preferential active facilitated transport of malate by electrogenic proton symport; hence the Δp generated by malate would not be detected. In the case of glucose-grown vesicles the malate carrier is absent and hence proton-symport cannot occur. Thus ΔpH and $\Delta\psi$ generated by the respiration of L-malate/FAD, detected by acetate and TPMP$^+$ accumulation, is evident (Table 3). Further details of the L-malate transport system will be reported elsewhere.

The active transport of organic anions (e.g. glucose-6-phosphate) is considered to be driven by ΔpH at pH 5.5 (Ramos *et al.*, 1976). It was of interest, therefore, to establish which component of Δp drives the transport and accumulation of 2KGA at pH 6.6. The effects of ionophores or CCCP on the transport and accumulation of 2KGA after 5 min in the presence of L-malate/FAD at pH 6.6 are shown (Fig. 4). In the presence of valinomycin, which will collapse $\Delta\psi$, only a 38% inhibition of accumulated 2KGA is observed. In the presence of nigericin, however, which exchanges H^+ for K^+ and hence collapses ΔpH but not $\Delta\psi$, a 73% inhibition of 2KGA accumulation occurs. A larger inhibition is observed in the presence of CCCP which translocates protons

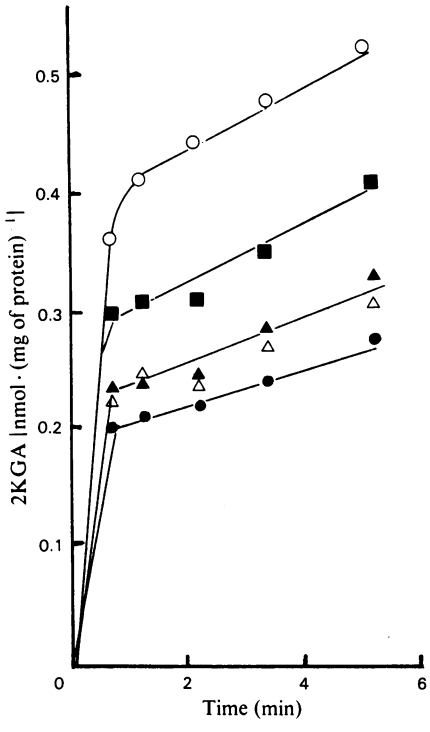

Fig. 4. *Effect of ionophores and CCCP on 2KGA transport in vesicles from glucose-grown P. putida at pH6.6*
The transport of 82.78 μM 2KGA was studied in the absence of an electron donor (●) or in the presence of an electron donor alone (○) or in the presence of an electron donor plus 5.0 μM-valinomycin (■) or 1.0 μM-nigericin (▲) or 1.0 μM-CCCP (△). The electron donor consisted of 10 mM-L-malate/50 μM-FAD (see the Materials and methods section).

only. Thus it is apparent that, at pH6.6, the major component of Δp that drives 2KGA transport is derived from ΔpH. Similar results were obtained when PMS/ascorbate was used as the electron donor (results not shown).

These results, while providing further evidence for the chemiosmotic theory, demonstrates the presence of a repressible/inducible carrier system for 2KGA. The active transport of this substrate is capable of being coupled to the respiration of L-malate/FAD as well as other electron donors. The resulting proton extrusion generates a Δp (with ΔpH as a major contribution) of sufficient magnitude to account for the observed intra-vesicular accumulation of 2KGA. The fairly low but significant intra-vesicular accumulations of 2KGA (Table 1) are similar to those previously reported for glucose and gluconate (Al-Jobore *et al.*, 1980). The reasons for these low accumulations are not known but similar observations have been made for the transport of D- and L-lactate in vesicles prepared from a *Pseudomonas* sp. (Martin & Konings, 1973). The question as to whether 2KGA transport occurs with or without proton-symport remains to be established. Finally, our transport studies with vesicles indicate that the 2KGA and glucose carrier systems are repressible whereas the gluconate carrier system is not (Al-Jobore *et al.*, 1980). These results are in agreement with those reported for whole cells in *P. aeruginosa* for the regulation of glucose, gluconate and 2KGA transport (Whiting *et al.*, 1975).

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