Quantitative Analysis of Proton-Linked Transport Systems

GLUTAMATE TRANSPORT IN *STAPHYLOCOCCUS AUREUS*

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1. The magnitude of the protonmotive force in respiring *Staphylococcus aureus* was measured over the range of extracellular pH from 5.6 to 7.8. 2. The membrane potential remains constant at 150 mV, inside-negative, but the pH gradient decreases from 2.1 units, inside-alkaline, at pH 5.6 to zero at pH 7.5 and above. 3. The accumulation of glutamate in the soluble cell pool is pH-independent at a value equivalent to 100 mV. 4. The results of experiments studying co-transport of protons are consistent with a proton/glutamate stoichiometry of 2 and electrogenic transport across the pH range examined. 5. The amount of glutamate uptake is the result of a kinetic steady state between influx and efflux pathways. 6. Evidence is presented for the regulation of this kinetic steady state by the response of the initial rate of uptake to changes in the protonmotive force.

In previous studies of amino acid uptake by *Staphylococcus aureus* in this laboratory (Hamilton & Niven, 1974; Niven & Hamilton, 1974), it was concluded that transport of an anion such as glutamate is an electroneutral process catalysed by a proton symport with a stoichiometry of one, and that the amount of accumulation is equal to the transmembrane pH gradient. The experimental system used in that study consisted of resting cell suspensions in which gradients of potential and of pH were generated by treatment of the cells with uncouplers and ionophores, and manipulation of the ionic content of the medium.

More recently, however, Lagarde (1977) has claimed that 3-deoxy-2-oxo-D-gluconate is taken up by *Escherichia coli* on a symport with a stoichiometry of two protons per acid anion translocated, and that the transport is electrogenic at all pH values tested. Also, Booth *et al.* (1979) have demonstrated that, in the transport and accumulation of non-metabolized β -galactosides by *E. coli*, thermodynamic equilibrium is not attained between the driving force and the amount of solute uptake into the cell pool. The driving force is greater than the accumulation ratio achieved, and the amount of uptake represents rather a kinetic steady state ultimately determined by the rate of carrier-independent efflux.

In the light of this greater insight we have therefore re-examined the transport of glutamate in actively metabolizing *Staph. aureus* over a range of pH values. Collins & Hamilton (1976) have already measured the protonmotive force in *Staph. aureus* at an extracellular pH (pH_o) of 6.5. The protonmotive force was estimated at 211 mV, consisting of a membrane potential ($\Delta\psi$) of 134 mV, inside-negative, and a pH gradient (ΔpH) of 1.3 units, inside-alkaline. $\Delta\psi$ and ΔpH have, however, been found to be pH-dependent variables in whole cells and vesicles of *E. coli* (Padan *et al.*, 1976; Ramos & Kaback, 1977a; Booth *et al.*, 1979), and Rottenberg (1976) has proposed that the proton/solute stoichiometry might also be a pH-dependent variable. It was therefore considered important to examine these parameters also in *Staph. aureus*.

We were able to demonstrate, across the pH range studied, that glutamate transport is an electrogenic process with a proton stoichiometry of greater than one, and that the amount of glutamate in the amino acid pool results from the balance between the rates of entry through transport, and of exit through efflux, metabolism and incorporation into cellular macromolecules.

Materials and Methods

Media and growth conditions

Staphylococcus aureus (Colworth House Culture Collection no. 273) was grown at 30°C, with shaking, in 100 ml volumes of a medium containing: tryptone, 10.0 g/litre; Lab Lemco, 5.0 g/litre; yeast extract,

Abbreviations used: DMO, 5,5-dimethyl-2,4-oxazolidinedione; TCS, 3,3',4',5-tetrachlorosalicylanilide.

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1.0g/litre; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 5.0g/litre; HCl to pH 6.5. Overnight cultures were diluted to approx. 0.2mg bacterial dry wt./ml in fresh medium and reincubated until the cell density had doubled. Cells in the late-exponential phase of growth were then harvested by centrifugation at 4°C, (13 200g, 10min), washed once in 150mM-KCl and twice in medium as detailed for each individual experiment, before final resuspension in that medium to a density of approx. 25mg bacterial dry wt./ml. This suspension was stored on ice until required, up to a maximum period of 4h.

For starvation of cells, harvesting was carried out as described above. Cells were washed once in 150mM-KCl and once in the buffer appropriate to each individual experiment before resuspension in that buffer to a density of 0.5–1.0mg bacterial dry wt./ml. Portions (50ml) of these suspensions were incubated at 37°C, with shaking, for 2.5h. The cells were then harvested by centrifugation at room temperature (3100g, 5min), washed once, and resuspended in buffer to a density of approx. 15mg bacterial dry wt./ml.

Experimental conditions

All incubations were carried out at 25°C in a water-jacketed glass vessel of capacity 10ml, the contents of which were stirred magnetically. All additions and sampling were performed by using Hamilton micro-syringes or larger-volumed glass-barrelled syringes. Samples were filtered (Oxoid MF25, 0.45 μm pore size, 25mm diameter) by using a Millipore 1225 sampling manifold, or, in the case of ΔpH determinations, with individual ground-glass-sinter assemblies. Each filter for radioactivity counting was dried in a hot-air oven and assayed for radioactivity by liquid-scintillation counting with 6.0ml of scintillation fluid containing PPO (2,5-diphenyloxazole; 6.0g) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene; 75mg] in toluene (1 litre). Radioactivity was determined in a Packard Tri-Carb model 3255 liquid-scintillation spectrometer at 8°C. Counting efficiency, obtained by comparison with suitably quenched standards, was estimated as 75% for ^{14}C , and 50% for ^3H .

Protonmotive force and glutamate accumulation

Cells washed and resuspended in 250mM-sucrose/30mM-choline chloride were diluted to a density of approx. 1mg bacterial dry wt./ml in buffer* composed of solutions of 0.1M-citric acid and 0.2M- Na_2HPO_4 , at a number of pH values.

pH gradient. The pH gradient was estimated from the distribution of the lipid-soluble weak acid DMO (Waddell & Butler, 1959). The rationale of the double-isotope method used has been discussed

previously (Harold *et al.*, 1970). A portion (5ml) of cell suspension was incubated with [^{14}C]DMO (0.78mCi/mmol. final concn. 54 μM) and [^3H]inulin (610mCi/mmol; final concn. 0.12 μM). After 15min, triplicate 1ml samples were removed and filtered, but not washed; at the same time duplicate 50 μl samples of the suspension were removed for direct assay of radioactivity.

For each sample, the counts in the ^3H channel due to [^{14}C]DMO were estimated from the counts in the ^{14}C channel and the percentage overlap measured with ^{14}C -labelled standards. From the total counts in the ^3H channel, the [^3H]inulin content of each sample was obtained by difference. The [^3H]inulin content of each filter was taken as a measure of extracellular water, and the amount of extracellular [^{14}C]DMO was calculated from this amount of [^3H]inulin and the ratio of [^{14}C]DMO to [^3H]inulin in the whole suspension; this was subtracted from the total [^{14}C]DMO to yield intracellular [^{14}C]DMO.

The pH gradient was calculated from the relationship:

$$\Delta\text{pH} = \text{pH}_i + \text{pH}_o$$

$$= \log \left[\frac{[\text{DMO}]_{\text{in}}}{[\text{DMO}]_{\text{out}}} (1 + 10^{\text{pK} - \text{pH}_o}) - 10^{\text{pK} - \text{pH}_o} \right]$$

where pK refers to the pK of DMO, which was taken to be 6.32 (Addanki *et al.*, 1968), and pH_i and pH_o are the intracellular and extracellular pH values respectively.

Membrane potential. The membrane potential was estimated from the distribution of K^+ in valinomycin-treated cells (Maloney *et al.*, 1975). To the 5ml suspension were added KCl to 0.1mM and [^3H]inulin to 0.2 μM . After 5min incubation, valinomycin was added to a final concentration of 2 $\mu\text{g}/\text{ml}$ of suspension. After a further 10min, triplicate 1ml samples were removed and filtered, but washed. Each filter was added to a test tube with two drops of butanol and placed in a boiling-water bath for 15min. After cooling, 2.5ml water was added, and each tube was agitated vigorously to remove all material from the filters.

The concentration of K^+ in each extract and the filtrates, diluted where required, was assayed by using an EEL model 150 clinical flame photometer. A 50 μl portion of each extract or filtrate was also assayed for radioactivity. The amount of [^3H]inulin in the extract gives a measure of the volume of extracellular water trapped on the filter, and knowing the K^+ concentration of the extracellular medium, the appropriate correction to the intracellular K^+ concentration can be made. The membrane potential was calculated by using the Nernst Equation:

$$\Delta\psi = Z \log \left(\frac{[\text{K}^+]_{\text{in}}}{[\text{K}^+]_{\text{out}}} \right)$$

where Z is 2.303 RT/F and equals 59 at 25°C.

Glutamate accumulation. L-[^{14}C]Glutamic acid (10mCi/mmol; final concn. 2 μM) was added to 3 ml of cell suspension and the accumulation of the amino acid allowed to proceed until a plateau was reached after approx. 15 min. A small volume of a solution of unlabelled sodium glutamate (final concn. 1 mM) was then added, and efflux of accumulated label monitored by assay of filtered samples (0.1 ml) for radioactivity: samples were washed three times with 1 ml volumes of experiment buffer. The steady-state accumulation of glutamate was estimated from the extent of efflux of label under these conditions. The calculated accumulation of glutamate was converted into mV by using the relationship:

$$\Delta p \text{ Glutamate} = Z \log \left(\frac{[\text{glutamate}]_{\text{in}}}{[\text{glutamate}]_{\text{out}}} \right)$$

Initial rates of glutamate uptake were determined by measuring accumulation over the first 2 min of an experiment, during which time uptake of the amino acid proceeded linearly.

Calculation of all transmembrane concentration ratios were performed assuming an intracellular volume of 1.55 $\mu\text{l}/\text{mg}$ bacterial dry wt. (Niven & Hamilton, 1972).

Proton movement and proton/glutamate stoichiometry

Cells were harvested, and washed once in 150 mM-KCl and twice in 150 mM-KCl containing 0.3 mM-Tes (2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-ethanesulphonic acid). Cell suspensions (4 ml) in 150 mM-KCl at approx. 2 mg bacterial dry wt./ml contained carbonic anhydrase (20 $\mu\text{g}/\text{ml}$) to catalyse carbonic acid/ CO_2 equilibration (Scholes & Mitchell, 1970). Incubation was carried out in a stoppered vessel, the contents being kept anaerobically bubbling with water-saturated O_2 -free N_2 introduced through a port in the stopper. The pH of the cell suspension was monitored with a fully screened glass micro-electrode (part no. 103) supplied by Probion Ltd., Leslie, Fife, Scotland, U.K., and a Beckman Research pH-meter (model 101901). A calomel reference electrode was connected through a remote liquid junction in a three-way ground-glass tap. The output from the meter was recorded on a Rikadenki multichannel pen recorder (model B341).

Throughout the equilibration period, small additions of anaerobic HCl or KOH were made as required to bring the system to pH 6.5. The pH-recording system was adjusted to give a full-scale deflection on the chart of 0.1 pH unit. Anaerobic potassium glutamate, which had previously been adjusted to pH 6.5, was added to a concentration of 1 mM, and the ensuing pH changes recorded. Proton movement in the suspension was calibrated by using small portions (10 nmol) of anaerobic HCl.

The stoichiometry of proton/glutamate absorbed was estimated by comparing the initial rates of uptake of the two species by a modification of the method of West & Mitchell (1973) as used by Lagarde (1977). During the first 2 min after addition of glutamate, tangents were drawn to the proton-uptake curve at several points. The slope of these tangents was plotted as a logarithmic function against time to yield a straight line, which was extrapolated back to zero time to give an estimate of the initial rate of proton uptake. Since introduction of a syringe needle for sample withdrawal disturbed the recorder trace, the initial rate of glutamate uptake had to be estimated in separate parallel experiments. Potassium [^{14}C]glutamate (0.499 mCi/mmol; final concn. 1 mM) was added, and, during the first 5 min, 0.2 ml samples were filtered and washed three times with 1 ml portions of 150 mM-KCl. In calculation of the initial rate, uptake was assumed to be linear with respect to time for 5 min.

Glutamate uptake in response to gradients of pH, potential and sodium concentration in starved cells

(a) In one series of experiments (see Fig. 4a below), cells were starved in 100 mM- KH_2PO_4 , pH 8.0, and resuspended in the same buffer. Small portions of the suspension, at approx. 15 mg bacterial dry wt./ml, were incubated with 5 mM-KCN and 4 μg of valinomycin/ml, and the transport assay initiated by dilution of cells 33-fold into buffer solutions containing KCN and valinomycin to the same concentrations, and 40 μM -[^{14}C]glutamate (5 mCi/mmol). Gradients imposed across the cell membrane depend on the composition of the dilution buffer, which was either 100 mM- KH_2PO_4 at pH 6.0 or 8.0, or 100 mM- NaH_2PO_4 at pH 6.0 or 8.0. The pH of potassium- and sodium-containing buffers was adjusted with KOH and NaOH respectively. Samples (0.2 ml) for assay of radioactivity were withdrawn, filtered, and washed three times with 1 ml portions of the dilution buffer.

(b) A second series of experiments was carried out as described by Tsuchiya *et al.* (1977) (Fig. 4b). Cells were starved and resuspended in one of the four buffers used for dilution in the experiments described above. Portions of each cell type were incubated with 5 mM-KCN and 3.6 μg of the uncoupler TCS/ml, and transport assays initiated by dilution of cells 33-fold into 100 mM- NaH_2PO_4 , pH 8.0, containing KCN and TCS to the same concentrations, plus 40 μM -[^{14}C]glutamate (5 mCi/mmol). Samples were taken and treated as described in (a) above.

Chemicals

Analytical-grade reagents were used whenever possible. Tes was obtained from BDH, Poole, Dorset, U.K. Carbonic anhydrase and valinomycin were

obtained from Sigma, Kingston upon Thames, Surrey, U.K. Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and DMO for dilution of the isotope solution from Koch–Light Laboratories, Bucks., U.K. PPO was also purchased from Koch–Light, and POPOP was from Nuclear Enterprises, Edinburgh, Scotland, U.K. Bacteriological media were obtained from Oxoid, London, E.C.4, U.K. The uncoupler TCS was generously given by Unilever Research Laboratories, Sharnbrook, Beds., U.K. Stock solutions of both TCS and valinomycin were made up to 2 mg/ml in ethanol, and stored at -20°C .

Results

The protonmotive force

The results of determinations of the protonmotive force in respiring cells are shown in Fig. 1. Throughout a range of pH_o from 5.6 to 7.8, the membrane potential was found to be constant at a value of

Fig. 1. *The protonmotive force in respiring cells*
Respiring cells were suspended in citric acid/sodium phosphate buffers at a density of 1 mg bacterial dry wt./ml. Experimental conditions are described in the Materials and Methods section. Membrane potential (□) was measured in the presence of 0.1 mM-KCl, 2 μg of valinomycin/ml and 0.2 μM - ^3H inulin. The results presented are means of nine determinations. The pH gradient (■) was measured in the presence of 54 μM - ^{14}C DMO and 0.12 μM - ^3H inulin. Each point represents a separate experimental determination. The protonmotive force (●) was obtained from the addition of the membrane potential and pH gradient.

150 mV. This value is rather higher than the 134 mV measured previously by an ion-flux method (Collins & Hamilton, 1976). Although this may represent a genuine difference in the magnitude of the membrane potential under the conditions used here, it is more likely that the present values are slight overestimates arising from the method of assay. Indeed, similar high values for the membrane potential were found by the method of measuring the steady-state distribution of K^+ when experimental conditions were identical with those used by the previous workers.

Any error in estimation of the membrane potential may be due firstly to a difference in activity of K^+ inside and outside the cell; however, since it is difficult to measure intracellular ion activities, concentration terms must be substituted in the Nernst Equation. Secondly, if ^3H inulin is excluded by the cell wall rather than by the cell membrane, the amount of extramembranal K^+ will be underestimated, giving rise to an equivalent overestimation of the intracellular K^+ concentration, and hence of $\Delta\psi$. However, since the K^+ concentration in the medium is low, any error introduced in this manner will be almost negligible. Thirdly, and more significantly, owing to the ion-exchange properties of the cell wall, it is almost certain that the concentration of K^+ close to the membrane, in the intramural space, is higher than in the bulk medium; thus, since it is the bulk medium concentration that is measured, once again the intracellular K^+ concentration and $\Delta\psi$ will be liable to overestimation.

As the pH of the medium is raised, the magnitude of the transmembrane pH gradient, inside-alkaline, decreases from a value of about 2.1 units at pH_o 5.6 to zero at pH_o 7.5 and above. The measurement of the pH gradient by distribution of an acid such as DMO is dependent on the assumptions that only the undissociated form of the acid is capable of permeating the cell membrane, and that accumulation is a passive process; additionally, the indicator should

Table 1. *Effect of DMO concentration on the measured value of the pH gradient*

In a total volume of 6.0 ml, respiring cell suspensions (1.1–1.2 mg dry bacterial dry wt./ml) in 50 mM-sodium phosphate buffer, pH 6.7, contained ^{14}C DMO at the concentrations shown, 0.2 μM - ^3H inulin, 0.1 mM-KCl and 2 μg of valinomycin/ml. The increase in DMO concentration was achieved by using unlabelled acid. The DMO accumulation ratio and ΔpH were calculated as described in the Materials and Methods section.

[DMO] (μM)	Accumulation ratio	ΔpH
4.5	4.86	0.798
52.7	5.15	0.827
108.9	4.84	0.772

not be bound to or metabolized within the cell. The results shown in Table 1 strongly suggest that these conditions are satisfied by DMO in the *Staph. aureus* system; over a 25-fold concentration range, the accumulation ratio of DMO is not significantly altered. These data also show that, at the concentration used in the main experiments (54 μM), DMO itself does not affect the magnitude of the pH gradient. The value for ΔpH at pH 6.5 is close to that obtained previously by an ion-flux method (Collins & Hamilton, 1976).

The total protonmotive force, obtained by adding the potential and pH gradient components, therefore decreases in parallel with ΔpH as the extracellular pH increases. Almost identical results were obtained for cells suspended in 50 mM-sodium phosphate buffer (results not shown).

Glutamate accumulation

The steady-state glutamate accumulation was measured under the conditions in which protonmotive force determinations were made. As shown in Fig. 2, throughout the pH range the accumulation of glutamate remains constant. When compared with the magnitude of ΔpH , it becomes clear that, at pH_o 6 and above, there is insufficient energy in ΔpH alone to drive this accumulation on the basis of an electro-neutral proton-symport mechanism as originally proposed (Hamilton & Niven, 1974; Niven &

Hamilton, 1974). Under these metabolizing conditions, therefore, another explanation is necessary. The most likely possibilities are that (1) the proton/glutamate stoichiometry is greater than 1; (2) glutamate is accumulated in symport with Na^+ ; or (3) glutamate accumulation is driven by an energy source other than the protonmotive force, e.g. by ATP. In view of the fact that glutamate uptake is almost completely inhibited by the uncoupler TCS (Hamilton, 1968), and is insensitive to arsenate (results not shown), this last possibility has been discounted.

Glutamate-associated influx of protons and the proton/glutamate stoichiometry

When glutamate, as the potassium salt, was added to an anaerobic suspension of *Staph. aureus* cells, the pH of the extracellular medium became more alkaline (Fig. 3). Although control experiments using strains lacking the glutamate-transport system (or in which glutamate transport was inhibited) were not possible, the pH change was not seen in the absence of cells, and it is therefore believed that it represents influx of protons coupled to the influx of glutamate, as previously demonstrated by Gale & Llewellyn (1972). A comparison of the initial rates of uptake of protons and of glutamate at pH_o 6.5 yielded the results shown

Fig. 2. The protonmotive force and steady state of accumulation of glutamate in respiring cells

The protonmotive force was measured as described in Fig. 1. Glutamate uptake (○) was measured in the presence of 2 μM -[^{14}C]glutamate and recorded as described in the Materials and Methods section. Each point represents a separate experimental determination.

Fig. 3. Glutamate-associated proton movements in resting cells

Anaerobic cell suspension (0.95 mg bacterial dry wt./ml) in 150 mM-KCl contained, in a total volume of 4.0 ml, 1 mM-potassium iodoacetate, 20 μg of carbonic anhydrase/ml and 0.3 mM-Tes. After equilibration at about pH 6.5, 0.33 M-potassium glutamate, pH 6.5, was added anaerobically to a final concentration of 3.3 mM.

Table 2. Initial rate of proton and glutamate uptake in anaerobic cell suspensions

Anaerobic cell suspensions (1.5–2.0 mg bacterial dry wt./ml) in 150 mM-KCl contained 20 μ g of carbonic anhydrase/ml in a total volume of 4.0 ml. After equilibration at around pH 6.5, anaerobic potassium glutamate, pH 6.5, was added to a final concentration of 1 mM; initial rates of uptake of protons and glutamate were calculated as described in the Materials and Methods section. Results are means $\pm$ s.d. for the number of determinations given in parentheses.

	Initial rate of uptake of:	
	H ⁺ (ng-ion/min per mg dry wt)	Glutamate (nmol/min per mg dry wt)
(a)	1.64 $\pm$ 0.40 (36)	1.01 $\pm$ 0.43 (7)
(b)	2.05 $\pm$ 0.56 (3)	0.91 $\pm$ 0.31 (4)

in Table 2. Table 2(a) shows the rates estimated from all experiments performed. As is evident from the s.d., there is a considerable amount of variation in both parameters. The initial rate of proton uptake, although relatively straightforward to measure, is subject to variability due, for example, to the condition of cells with regard to proton permeability, which tends to vary from day to day. The initial rate of glutamate uptake, on the other hand, was difficult to measure accurately, owing to the low rate and the necessity of using glutamate of low specific activity.

It was not possible to measure both rates simultaneously in a single cell suspension, and when only experiments done on the same day and with the same batch of cells were compared, the average values for the uptake rates obtained were as shown in Table 2(b). s.d. values are included with these data to indicate the large variation that still remains. Although these values are consistent with a H⁺/glutamate stoichiometry of greater than 1, owing to the limitations of the method, they cannot be regarded as unequivocal proof that the stoichiometry is equal to 2.

Glutamate uptake driven by artificial gradients of pH, potential and sodium concentration

The results of experiments with starved cells to examine glutamate uptake driven by artificial gradient of pH, potential and sodium concentration are presented in Fig. 4. The first series of experiments (Fig. 4a) were designed to examine the effects of a transmembrane pH gradient, inside-alkaline, and an electrical potential, inside-negative, on the uptake of glutamate. However, owing to the way in which the experiments were done, a potential was always accompanied by a transmembrane sodium gradient. Dilution of valinomycin-treated cells from a K⁺-containing medium to an Na⁺-containing medium will cause K⁺ to leave the cells, and an electrical im-

balance, in the form of a transient potassium-diffusion potential, will be established across the cell membrane. Dilution from pH 8 to 6 will result in the imposition of a large pH gradient. As shown in Fig. 4(a), glutamate is taken up by the cells in response to this pH gradient, and the extent of uptake is increased by the simultaneous imposition of a K⁺-diffusion potential. A potential alone, however, results in little or no accumulation above the control value.

The second series of experiments (Fig. 4b) discriminated between the effects of a membrane potential and a sodium concentration gradient. Dilution of TCS-treated cells from a K⁺-containing medium to an Na⁺-containing medium at the same pH will impose only a gradient sodium concentration across the membrane. Dilution of such cells from pH 6 to 8 will establish a membrane potential in the form of a transient proton-diffusion potential. Neither of these gradients, alone or in concert, supported any accumulation of glutamate.

Fig. 4. Glutamate accumulation driven by artificial gradients in starved cells

(a) Suspensions of energy-starved cells in 100 mM-KH₂PO₄, pH 8.0, contained 5 mM-KCN and 4 μ g of valinomycin/ml. At zero time, a portion was diluted 33.3-fold into 100 mM-NaH₂PO₄, pH 6.0 (○), 100 mM-KH₂PO₄, pH 6.0 (●), 100 mM-NaH₂PO₄, pH 8.0 (■), or 100 mM-KH₂PO₄, pH 8.0 (□); the final cell suspension had a density of 0.5 mg of bacterial dry wt./ml and contained 5 mM-KCN, 4 μ g of valinomycin/ml and 40 μ M-[¹⁴C]-L-glutamic acid in a total volume of 2.5 ml. (b) Suspensions of energy-starved cells in 100 mM-NaH₂PO₄, pH 8.0 (●), 100 mM-NaH₂PO₄, pH 6.0 (○), 100 mM-KH₂PO₄, pH 8.0 (■) or 100 mM-KH₂PO₄, pH 6.0 (□) contained 5 mM-KCN and 3.6 μ g of TCS/ml. At zero time a portion was diluted 33.3-fold into 100 mM-NaH₂PO₄, pH 8.0; the final cell suspension had a density of 0.5 mg of bacterial dry wt./ml and contained 5 mM-KCN, 3.6 μ g of TCS/ml, and 40 μ M-[¹⁴C]-L-glutamic acid in a total volume of 2.5 ml.

In interpreting these results it must be realized that, in experiments in which a proton-diffusion potential is being created (Fig. 4*b*), there is initially a large pH gradient, inside-acid, that will oppose glutamate accumulation. It is possible that a small pH gradient, inside-acid, also exists in the experiment to test the action of a K⁺-diffusion potential (Fig. 4*a*): certainly, accumulation of glutamate in symport with protons will tend to establish such a gradient. It is therefore possible that, in order to see glutamate uptake in response to a membrane potential, there must also be a pH gradient of favourable orientation for uptake of glutamate. Under conditions where this holds, potential-dependent accumulation was in fact convincingly demonstrated (Fig. 4*a*).

It does appear, however, that glutamate uptake cannot be driven by a gradient of Na⁺ (Fig. 4*b*). This conclusion was supported by further experiments that showed that the rate and extent of accumulation of glutamate by respiring cells was independent of the Na⁺ concentration of the incubation medium (results not shown).

Effect of decreasing the membrane potential on glutamate uptake

The results presented in Table 3 show that, as the K⁺ concentration of the extracellular medium is increased, the membrane potential of metabolizing valinomycin-treated cells is dissipated; the intracellular K⁺ concentration is high, but the cells no longer maintain a large concentration gradient of the ion. The membrane potential is therefore collapsed by adding valinomycin to cells prepared and suspended in 50mM-sodium phosphate buffer containing 52.5mM-KCl, and the effect of this on the initial rate of glutamate uptake can be investigated. It should be noted, however, that the magnitude of the pH gradient was not affected by the presence of valinomycin in these experiments. As shown in Fig. 5, at all four pH values tested, decrease of the membrane potential causes an inhibition of the initial rate of glutamate transport. At p*H*_o 7 and above, where the pH gradient itself is small, this inhibition is almost complete; further, it is not reversed by addition of Na⁺. Even at

Fig. 5. Effect of valinomycin on accumulation of glutamate by cells in medium supplemented with KCl
In a total volume of 2.0ml, respiring cell suspensions (0.5mg of bacterial dry wt./ml) in 50mM-sodium phosphate buffers supplemented with 52.5mM-KCl contained 1μM-L-[¹⁴C]glutamic acid and 2μg of valinomycin/ml. Control suspensions contained 1μl of ethanol/ml. Experimental points refer to uptake rates compared with control rates at each pH value.

p*H*_o 5.8, where there is a large pH gradient, the rate of uptake is inhibited by over 20%. The membrane potential is thus implicated in the driving force for the accumulation of glutamate by respiring *Staph. aureus*, and modulation of this potential by valinomycin treatment in K⁺ medium is shown to affect accumulation through an effect of the initial rate of uptake.

Discussion

In parallel with the findings from *E. coli* whole cells and vesicles (Padan *et al.*, 1976; Ramos & Kaback, 1977*a*; Booth *et al.*, 1979), we have demonstrated that Δ*pH* and the total protonmotive force are p*H*_o-dependent variables in *Staph. aureus* (Fig. 1). Δ*ψ* remains constant over the range of p*H*_o values studied, and the cells maintain a steady value of intracellular pH between 7.6 and 7.8.

As pointed out above, the value of 150mV for the membrane potential, assayed by the steady-state distribution of K⁺ in valinomycin-treated cells, may be a slight overestimate as compared with the previously calculated value of 134mV, determined from the ion fluxes resulting on energization of the cell suspension (Collins & Hamilton, 1976). On the other hand the depolarizing effect of valinomycin treatment in K⁺ medium (Table 3; Booth *et al.*, 1979) is likely to lead to an underestimate of

Table 3. Variations of transmembrane K⁺ concentration gradient with external K⁺ concentration

In a total volume of 6.0ml, respiring cell suspensions (1.2mg bacterial dry wt./ml) in 50mM-phosphate buffers, p*H*6.7, containing K⁺ at the concentrations shown, were supplemented with 0.2μM-[³H]inulin.

[K ⁺] _{out} (mM)	[K ⁺] _{in} (mM)	[K ⁺] _{in} /[K ⁺] _{out}
0.64	293	458
1.24	438	353
4.75	570	120
19.37	794	41
51.96	624	12

$\Delta\psi$. At the experimental concentration of KCl of 0.1 mM, however, this latter effect is likely to be minimal in these studies. Also, the lack of any requirement to use EDTA treatment to render these Gram-positive cells valinomycin-sensitive decreases the risk of depolarization due to non-specific increases in ion permeability.

Preliminary kinetic analysis and studies of substrate and inhibitor specificity indicate that glutamate is transported by a single system with apparent K_m of 89.5 μM and V_{max} of 9.90 nmol/min per mg bacterial dry wt. In the absence of suitable non-metabolized analogues, or inhibitors or mutations affecting intracellular metabolism of glutamate, it was necessary to measure accumulation into the amino acid pool by the indirect method of determining the amount of labelled material exchanging with an excess of unlabelled glutamate added to the suspension after a steady-state condition had been established. Further addition of unlabelled α -oxoglutarate led to a second efflux of approximately the same amount of radioactive material from the cells, and the same result was obtained when the additions were carried out in the reverse order. Between them the [^{14}C] glutamate and α -oxo[^{14}C]glutarate fractions constituted 80–90% of the radioactivity in the cold-trichloroacetic acid-soluble pool material from the cells. Incorporation of radioactivity into trichloroacetic acid-insoluble cell polymers is slight under the conditions of the assay (<5% of pool after 50 min) and is not measurable until after 10 min incubation.

The value of approx. 100 mV obtained for the accumulation of glutamate in the cell pool remains constant over the pH_o range 5.6–7.8, and clearly bears no simple direct relationship to the protonmotive force, nor either of its component parts, $\Delta\psi$ or ΔpH . In particular, above pH_o 6 the accumulation observed is in excess of ΔpH , its putative driving force according to the previously derived model of an electroneutral proton symport with a stoichiometry of 1 (Hamilton & Niven, 1974; Niven & Hamilton, 1974). There exist three possible explanations of this result.

Harold & Spitz (1975) demonstrated that, in *Streptococcus faecalis*, glutamate uptake is not driven by the protonmotive force, but does require ATP. In *E. coli*, on the other hand, one of the glutamate-transport systems is Na^+ -linked, and uptake and accumulation of the amino acid is driven by the electrochemical gradient of that ion (Schellenberg & Furlong, 1977). Each of these mechanisms, either in isolation or in combination with the proton gradient as manifest by the protonmotive force, could give rise to the results given in Fig. 2. Experiments to test these possibilities in *Staph. aureus* were, however, entirely negative.

From their studies of transport of glutamate and other solutes by *E. coli* vesicles, Ramos & Kaback

(1977b) concluded that the proton/anion stoichiometry must increase from 1 to 2 as pH_o increases, with the uptake consequently becoming electrogenic and thus responsive to the membrane potential at alkaline pH_o values. This conclusion is, however, arrived at indirectly, and is dependent on the false assumption (Booth *et al.*, 1979) that solute accumulation is in thermodynamic equilibrium with the driving force. Lagarde (1977) has measured directly the proton/anion stoichiometry for the uptake of 3-deoxy-2-oxoglucuronate by whole cells of *E. coli* at pH_o 7; his findings are consistent with a stoichiometry of 2. Our own study of glutamate transport in *Staph. aureus* also indicates a proton stoichiometry of 2 at pH_o 6.5 (Table 2), and gives evidence of electrogenicity at pH_o 6.0 (Fig. 4a).

We consider that these data are consistent with proton/anion stoichiometries of 2 throughout the range pH_o 6.0–7.0, and that there is no requirement to hypothesize a pH_o -dependent variable stoichiometry. Such a mechanism for energy coupling would give a maximum driving force for accumulation of $\Delta\psi - 2Z\Delta\text{pH}$. This driving force is in excess of the accumulation actually attained across the range of pH_o studied (Fig. 2), and it therefore represents another example of intracellular accumulation of solute as the resultant of a kinetic steady state (Booth *et al.*, 1979). For glutamate and other metabolizable solutes, the 'efflux' pathways must include metabolism and incorporation into cell polymers as well as carrier-mediated and diffusion pathways of efflux from the cell pool.

At first sight this conclusion is quite at variance with our previous claim of a proton stoichiometry of 1 and electroneutral transport in response solely to ΔpH (Hamilton & Niven, 1974; Niven & Hamilton, 1974). The limitations of the experimental protocol used in those studies, however, can now be appreciated. Resting cell suspensions were used, uncoupled by treatment with TCS and valinomycin so that the protonmotive force was zero, i.e. $\Delta\psi = Z\Delta\text{pH}$. Under these highly artificial conditions, therefore, a driving force of $\Delta\psi - 2Z\Delta\text{pH}$ for the electrogenic uptake of an anion by metabolizing cells would simplify to $-Z\Delta\text{pH}$. That is to say, whatever the true value of the proton/anion stoichiometry, the uncoupled-resting-cell-suspension technique used in the original work will always give an apparent value of 1 for the stoichiometry.

These and other more complete discussions elsewhere of kinetic steady states in transport (Booth *et al.*, 1979; Seaston *et al.*, 1976), leave open the question of the mechanism of the response of the transport system to the protonmotive force. Most reasonably one might expect energy coupling to increase the availability and/or affinity of the carrier at the outer face of the membrane (and/or decrease these parameters at the inner face), and that these

effects might evidence themselves in increased rates of influx capable of characterization in terms of altered values of $V_{\max}$ and K_m . The results given in Fig. 5 show both the electrogenic character of glutamate transport throughout the pH_o range studied, and the decreased initial rate of uptake in response to diminished membrane potential.

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References

- Addanki, S., Cahill, F. D. & Sotos, J. F. (1968) *J. Biol. Chem.* **243**, 2337–2348
- Booth, I. R., Mitchell, W. J. & Hamilton, W. A. (1979) *Biochem. J.* **182**, 687–696
- Collins, S. H. & Hamilton, W. A. (1976) *J. Bacteriol.* **126**, 1224–1231
- Gale, E. F. & Llewellyn, J. M. (1972) *Biochim. Biophys. Acta* **266**, 182–205
- Hamilton, W. A. (1968) *J. Gen. Microbiol.* **50**, 441–458
- Hamilton, W. A. & Niven, D. F. (1974) *Biochem. Soc. Trans.* **2**, 797–800
- Harold, F. M. & Spitz, E. (1975) *J. Bacteriol.* **122**, 266–277
- Harold, F. M., Pavlasova, E. & Baarda, J. R. (1970) *Biochim. Biophys. Acta* **196**, 235–244
- Lagarde, A. (1977) *Biochem. J.* **168**, 211–221
- Maloney, P. C., Kashket, E. R. & Wilson, T. H. (1975) *Methods Membr. Biol.* **5**, 1–49
- Niven, D. F. & Hamilton, W. A. (1972) *Biochem. J.* **127**, 58p
- Niven, D. F. & Hamilton, W. A. (1974) *Eur. J. Biochem.* **44**, 517–522
- Padan, E., Zilberstein, D. & Rottenberg, H. (1976) *Eur. J. Biochem.* **63**, 533–541
- Ramos, S. & Kaback, H. R. (1977a) *Biochemistry* **16**, 848–854
- Ramos, S. & Kaback, H. R. (1977b) *Biochemistry* **16**, 855–859
- Rottenberg, H. (1976) *FEBS Lett.* **66**, 159–163
- Schellenberg, G. D. & Furlong, C. E. (1977) *J. Biol. Chem.* **252**, 9055–9064
- Scholes, P. & Mitchell, P. (1970) *J. Bioenerg.* **1**, 61–72
- Seaston, A., Carr, G. & Eddy, A. A. (1976) *Biochem. J.* **154**, 669–676
- Tsuchiya, T., Raven, J. & Wilson T. H. (1977) *Biochem. Biophys. Res. Commun.* **76**, 26–31
- Waddell, W. J. & Butler, T. C. (1959) *J. Clin. Invest.* **38**, 720–729
- West, I. C. & Mitchell, P. (1973) *Biochem. J.* **132**, 587–592