VALINOMYCIN-INDUCED AMINO ACID UPTAKE BY *STAPHYLOCOCCUS AUREUS*

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Received 31 August 1973

1. Introduction

Following the demonstration of a valinomycin-induced protonmotive force in mitochondria [1, 2], several investigators have recently employed similar techniques in their studies of bacterial substrate translocation. By inducing potassium efflux on the addition of valinomycin, the uptake of β-galactosides and/or several amino acids by whole cells of *Streptococcus lactis* [3] and *Strep. faecalis* [4], and also by *Escherichia coli* membrane vesicles [5], has been demonstrated.

In an attempt to extend our understanding of the mechanisms of energy coupling to the transport of amino acids by *Staphylococcus aureus*, the effect of such valinomycin-induced transmembrane electrical potentials on the uptake of basic, acidic and neutral amino acids has been investigated. Valinomycin treatment was found to stimulate the rate of lysine uptake until equilibrium had been achieved; whereas the characteristics of glutamate uptake were unaffected, a transient stimulation of glycine and isoleucine uptake was found to occur. These results are in keeping with the driving forces suggested previously for amino acid accumulation by *Staph. aureus* [6], the basic, acidic and neutral amino acids behaving as cationic, anionic and neutral substrates respectively, and responding to the membrane potential, pH gradient and total protonmotive force.

2. Materials and methods

2.2. *Preparation of cell suspension*

Staph. aureus was grown and harvested as described previously [6]. Cells from a 100 ml culture were washed once in 33 mM potassium phosphate buffer, pH 7.0, containing 10 mM $MgSO_4$ (70 ml) and resuspended in that buffer system (50 ml). To reduce both the amino acid pool and endogenous metabolism to negligible values, this suspension was incubated, with shaking, for 3 hr at 37°C, after which the cells were harvested by centrifugation (3 100 *g*, 7 min), washed with 10 mM $MgSO_4$ (35 ml) and resuspended to approx. 2 ml in 0.25 mM *N*-Tris (hydroxymethyl)-methyl-2-amino-ethanesulphonic acid (TES), 0.1 mM Tris, pH 7.0, containing 10 mM $MgSO_4$. Such suspensions (approx. 42 mg dry bacterial weight/ml) were stored at 0–4°C until required (up to a maximum period of 3 hr).

2.2. *Uptake studies*

42 ml reaction volumes were contained in a glass vessel, surrounded by a water jacket, which allowed pH and oxygen content to be monitored while the suspension was both stirred and bubbled with nitrogen. In all experiments, endogenous-reduced cells were used at a final concentration of approx. 1 mg dry bacterial weight/ml, 1.0 ml of the prepared cell suspension being added to 40.9 ml 0.25 mM TES, 0.1 mM Tris, 10 mM $MgSO_4$, pH ~7 at 30°C. Once anaerobiosis had been achieved by nitrogen bubbling, the incubations were started by the addition of 100 μl aliquots of the appropriate L-[^{14}C] amino acid (2.6 mM, 1.923 mCi/mmole) (Radiochemical Centre, Amersham, Bucks.). The ionophorous antibiotics were added in acetone (20 μl/42 ml suspension volume), 3, 5, 3′, 4′-tetrachlorosalicylanilide (TCS) and

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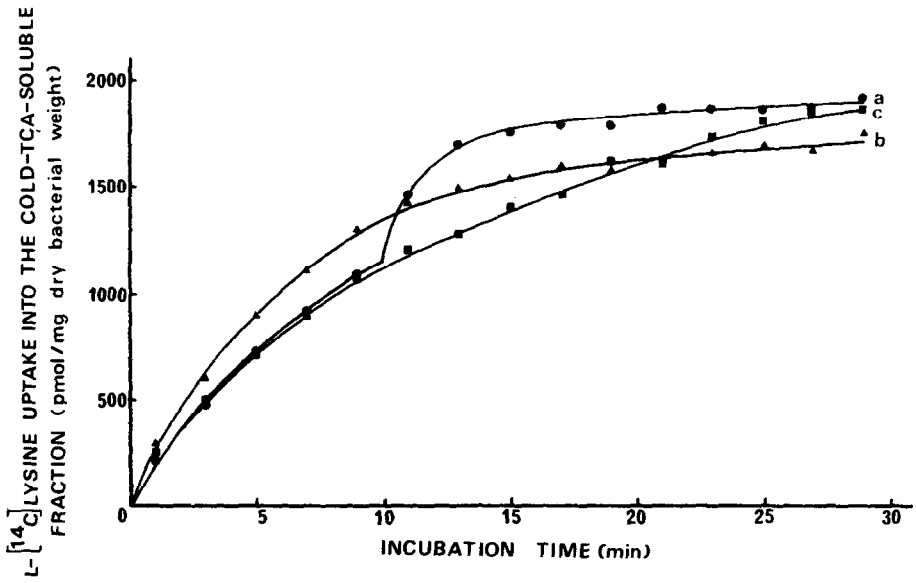

Fig. 1. [^{14}C]lysine uptake by anaerobic *Staph. aureus:* a) valinomycin and TCS added at 10 min and 20 min respectively (•); b) TCS and valinomycin added at 10 min and 20 min respectively (▲); c) no antibiotic additions (■).

valinomycin being added to 1.4×10^{-6} M and 1.8×10^{6} M, respectively. Samples (2 X 1.0 ml) were removed and the radioactivity uptake into the amino acid pool determined as described previously [6].

In all but the lysine experiments, low levels of uptake into both the whole cell and cold-trichloroacetic acid (TCA)-insoluble fractions were encountered. As the cold-TCA-insoluble uptake was found to vary

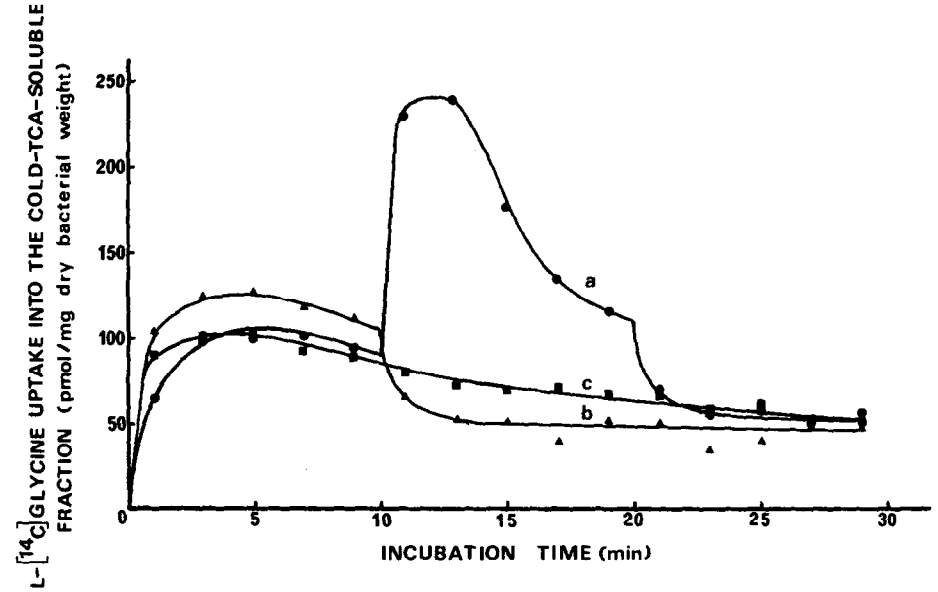

Fig. 2. [^{14}C]glycine uptake by anaerobic *Staph. aureus*: a) valinomycin and TCS added at 10 min and 20 min respectively (•); b) TCS and valinomycin added at 10 min and 20 min respectively (▲); c) no antibiotic additions (■).

about a mean value, the cold-TCA-insoluble count used for the calculation of the cold-TCA-soluble uptake in any such experiment, was the average of the figures obtained during that particular experiment.

2.3. *Flame photometry*

Intracellular potassium contents were obtained by removing 1.0 ml aliquots during the experiments. The samples were centrifuged (9 000 *g*, 5 min) and the pellets resuspended to 1.0 ml in distilled water. These samples were assayed for potassium using an EEL Model 150 Clinical Flame Photometer and the intracellular potassium concentrations subsequently estimated, the intracellular pool volume being taken as 1.55 ml/g dry bacterial weight [7].

3. Results

By preincubating the cells used in these studies in the presence of both potassium and magnesium, it was possible to obtain cells which retained high intracellular potassium concentrations, were relatively impermeable to proton and potassium, and therefore, at the beginning of the incubation period, had not attained a state of Donnan equilibrium when the protonmotive force would be zero. The intracellular potassium concentration of freshly prepared cell suspensions was approx. 270 mM. Although this decreased with time, the addition of TCS and/or valinomycin during an uptake assay was found to increase the rate of cellular potassium efflux. During the first experiment (a) of any set of three, the average values for the intracellular potassium concentrations at 8 min, 18 min and 28 min were 224 mM, 77 mM and 26 mM, respectively, valinomycin and TCS being added at 10 min and 20 min, respectively. In the second experiment (b) of any set, where the order of the antibiotic additions was reversed, the average values at the times stated above, were 165 mM, 57 mM and 26 mM, respectively. In the absence of antibiotics (the last experiment (c) of any set) the respective average values were 154 mM, 117 mM and 91 mM. These results and the pH shifts observed on the addition of TCS and valinomycin, reflect the ion-translocating properties of these antibiotics.

Fig. 1 shows the uptake of [^{14}C]lysine into the cold-TCA-soluble pool of *Staph. aureus*, and the effect of adding valinomycin and TCS during an uptake assay. When valinomycin was added at 10 min (fig. 1a), the rate of lysine uptake was found to be stimulated; the extent of uptake, however, was identical to that found in the control experiment (fig. 1c). The addition of TCS at 20 min had no effect on the process. When the antibiotic addition sequence was reversed, as in fig. 1b, TCS and valinomycin had no detectable effect on the characteristics of lysine uptake.

Fig. 2 shows the characteristics of [^{14}C]glycine uptake by *Staph. aureus*. Prior to the addition of antibiotics, glycine uptake achieved a maximum after approximately 5 min, followed by a slow efflux phase as observed in the control experiment (fig. 2c). The addition of valinomycin at 10 min (fig. 2a) induced transient glycine uptake, while the further addition of TCS at 20 min increased the rate of glycine efflux. As seen in fig. 2b, the addition of TCS at 10 min induced rapid glycine efflux and the attainment of a plateau level of uptake which was unaffected by further addition of valinomycin. Similar results were obtained in comparable experiments with [^{14}C]isoleucine.

The addition of TCS and valinomycin during experiments designed to investigate the effect of these antibiotics on glutamate uptake (fig. 3) had no detectable effect on the characteristics of this process.

4. Discussion

According to the chemiosmotic interpretation of transport phenomena [8], proton-coupled symporters, displaying a stoichiometry of 1:1 with respect to proton and amino acid, may be involved in the translocation of neutral and acidic amino acids by bacteria. Such symporters may recognise respectively the uncharged and monovalent anionic forms of these amino acids, the resultant permeant species bearing a net positive and no charge, respectively. Whereas the intracellular accumulation of neutral amino acids would then occur in response to the total protonmotive force, that of acidic amino acids would be dependent only on the transmembrane pH gradient (inside alkaline). Basic amino acids on the other hand, may be transported as the monovalent cations by means of uniport mechanisms, in response to the membrane potential (inside negative).

With respect to *Staph. aureus*, considerable evidence has been presented in favour of this latter suggestion [6]. In this present investigation, we have extended these studies and examined the effect of valinomycin-induced potassium efflux on the uptake not only of basic, but also of neutral and acidic amino acids.

In the absence of antibiotics (fig. 1c) lysine accumulation was found to occur in response to the membrane potential [6]. The addition of valinomycin at 10 min (fig. 1a) induced a marked stimulation of the rate of lysine influx. Valinomycin would induce a rapid potassium efflux, the extent of which would be dependent on the presence of a permeant counterion; provided the permeant lysine species is in fact positively charged, it would be expected to act as such a counterion, resulting in the observed stimulation of lysine uptake. The futher addition of TCS had no effect as the cells had already attained Donnan equilibrium.

Although the addition of TCS at 10 min (fig. 1b) would induce a rapid influx of protons (and consequently perhaps a slightly increased rate of cation efflux), lysine uptake was not affected. In the presence of TCS, proton, rather than lysine cation, would respond to any change in the electrical potential. The further addition of valinomycin had no effect on the lysine uptake process, as again protons would respond more efficiently.

In the absence of antibiotics, the cells were found to accumulate glycine in response to the residual protonmotive force (fig. 2c); the extent of glycine uptake, however, was found to decrease with time, ion permeation allowing the cells to tend towards a Donnan equilibrium state when the protonmotive force would have zero magnitude. The addition of TCS (fig. 2b) accelerated this latter process resulting in marked glycine efflux; the further addition of valinomycin had no effect on the extent of glycine uptake as these cells would already be at Donnan equilibrium. When, however, valinomycin was added prior to TCS (fig. 2a), a transient stimulation of glycine uptake was observed. This result would be expected if the permeant glycine species is positively charged; this cationic entity acting as a counterion for potassium efflux. Due to the fast potassium efflux, and proton influx associated with glycine translocation or other leak pathways, the cells would quickly approach a Donnan equilibrium state. The protonmotive force would therefore tend towards zero and glycine efflux would be expected, as was observed. The further addition of TCS would allow the final attainment of Donnan equilibrium and glycine efflux to the point of transmembrane equilibration. The finding that the final extent of glycine (and of isoleucine), uptake, even in the presence of both TCS and valinomycin, was representative of a concentrative transport rather than transmembrane equilibration, suggested that a portion of this material might be bound by some unknown mechanism.

The uptake of glutamate (fig. 3) was found to be unaffected by the addition of TCS or valinomycin during an uptake assay. This result suggested that the

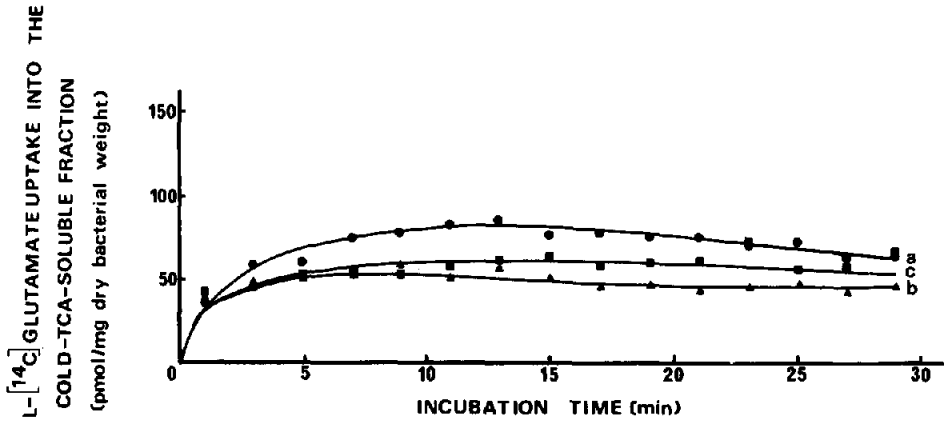

Fig. 3. [^{14}C]glutamate uptake by anaerobic *Staph. aureus*: a) valinomycin and TCS added at 10 min and 20 min respectively (●); b) TCS and valinomycin added at 10 min and 20 min respectively (▲); c) no antibiotic additions (■).

translocation of glutamate did not involve the net movement of positive charge and that the accumulation of glutamate in *Staph. aureus* may occur rather in response to the transmembrane pH gradient (inside alkaline). Although any pH gradient in these experiments would have been inside acid, glutamate exclusion was not however observed. As the extent of glutamate uptake was not significantly altered by antibiotic treatment, it was concluded that this uptake might also be due to binding effects.

These results therefore demonstrated that although the characteristics of uptake are significantly different, the translocation of lysine and that of glycine (and isoleucine), involves the net movement of positive charge, an increased electrochemical potential stimulating the uptake of these amino acids. In contrast, the uptake of glutamate did not respond to the increased electrical potential, suggesting that the translocation of this amino acid involves the permeation of an effectively uncharged entity.

It has therefore been possible to verify the predictions as to how bacterial amino acid transport will respond to changes in applied driving force. The results obtained are in agreement with the hypothesis that the basic, acidic and neutral amino acids are accumulated by *Staph. aureus* in response to the membrane potential (inside negative), pH gradient (inside alkaline) and the total protonmotive force, respectively; while the basic amino acids are translocated by uniport mechanisms, the acidic and neutral amino acids cross the membrane by means of proton-coupled symport mechanisms. These conclusions are further strengthened by experimental results to be reported shortly in which the uptake of amino acids in response to applied pH gradients is examined.

Acknowledgements

This work has been supported by a grant from the Science Research Council. We would also like to thank Robin E. Jeacocke for his help in the development of the ideas presented.

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