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THE ROLE OF HYDROGEN AND POTASSIUM IONS IN THE TRANSPORT OF ACIDIC AMINO ACIDS IN *STAPHYLOCOCCUS AUREUS*

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SUMMARY

1. The uptake of acidic amino acids (aspartate and glutamate) by cell suspensions of *Staphylococcus aureus* is not sensitive to valinomycin unless potassium ions K^+ at a concentration of 50 mM or higher are added to the incubation medium, when the antibiotic becomes strongly inhibitory. For a given concentration of K^+ the sensitivity to valinomycin increases over the pH range 5.5–8.5. The rate of aspartate uptake is not significantly affected by the presence of K^+ when cells are incubated in the presence of glucose and phosphate buffer at pH value 7 or less.

2. The rate aspartate uptake and the ratio of the internal to external concentration attained increase as the pH falls whether glucose is present or not. At pH 5.5–6.0 in the presence of valinomycin, K^+ and iodoacetamide 0.62 equiv of H^+ are taken up per molecule of aspartate, or 0.91 equiv of H^+ per molecule of glutamate, accumulated within the cell.

3. The uptake of aspartate or glutamate is accompanied by an increased uptake of K^+ but the relationship between the amount of K^+ taken up and amino acid accumulated is variable; in the absence of an external source of energy such as glucose, aspartate uptake can take place without any increase in the K^+ uptake. Aspartate uptake at pH 8 in the absence of glucose is markedly greater in potassium than in sodium phosphate buffers; the effect of K^+ is smaller in the presence of glucose and KCl is less effective than potassium phosphate. The possible nature of the relationship between aspartate transport and the translocation of H^+ and K^+ is discussed.

4. A survey of the uptake of other amino acids shows that, in general, the rate of uptake increases with decreasing pH and, when K^+ is present, the sensitivity to inhibition by valinomycin decreases with decreasing pH. Comparable experiments with *Streptococcus faecalis* show that amino acid transport in that organism is not affected by pH in the same way and is highly sensitive to valinomycin in the absence of added K^+.

INTRODUCTION

Staphylococcus aureus accumulates aspartate and glutamate by an energy-dependent process and the concentration within the cell may reach a value 2–3 orders of magnitude higher than that in the external medium[2–3]. The rate of accumulation

and the concentration gradient attained across the cell membrane are decreased by osmotic shock and can be restored by the addition of staphylococcal lipid; fractionation of the lipid reveals that the activity lies in the unsaturated fatty acid fraction[3,4]. A wide range of unsaturated fatty acids stimulate the uptake of aspartate and glutamate but not lysine or other amino acids[5]. The uptake of glutamate is accompanied by uptake of K^+ (ref. 6).

The transport of amino acids in yeast or mammalian cells is frequently accompanied by, and coupled to, translocation of H^+, Na^+, or K^+ (ref. 7); thus Eddy and Nowacki[8] have shown that the uptake of glycine by *Saccharomyces carlsbergensis* is accompanied by an uptake of H^+ and an efflux of K^+. Relatively little information is available on the movement of ions during amino acid uptake in bacteria. Harold and Baarda[9,10] have shown that valinomycin uncouples the transport of glutamate in *Streptococcus faecalis* and that the antibacterial effect of this antibiotic is due to depletion of K^+ within the cells. Valinomycin forms a lipid-soluble complex with K^+ and, by acting as a K^+ conductor, renders membranes permeable to K^+ and brings about the equilibration of any K^+ gradient across a membrane[11–13]. The present paper shows that the transport of acidic amino acids in *Staphylococcus aureus* is coupled to proton translocation but becomes sensitive to valinomycin when a high concentration of K^+ is added to the external medium, the sensitivity increasing as the pH rises.

METHODS

Organism and conditions of growth

Staphylococcus aureus Duncan was used for these studies and was grown for 18 h as previously described[14]. The growth medium contained salts at the following concentrations: 30 mM $(NH_4)_2HPO_4$, 7.3 mM KH_2PO_4, 17 mM NaCl, 0.3 mM $MgSO_4$, 0.04 mM $FeSO_4$. For some experiments, described below, NaH_2PO_4 was substituted for KH_2PO_4 to give a "K^+-deficient medium". For routine culture of organisms, the salt mixture was supplemented with 1 % (w/v) glucose, 0.1 % (w/v) arginine and 0.1 % commercial yeast extract or marmite.

Cells were centrifuged down from the growth medium, resuspended and washed once in whatever salt solution or buffer was required for the experimental test, suspended in the same salt or buffer solution at a cell density of 2.0 or 20.0 mg dry wt./ml, and eventually diluted as described below. Dry weights of cell suspensions were determined turbidimetrically on a Hilger absorptiometer calibrated for the organism used.

In some of the early experiments *Streptococcus faecalis* Trowbridge (N.C.T.C. 6782 B) was grown in the same medium and used under the same conditions as for *Staphylococcus aureus*.

Salt solutions

(A) The solution previously described as "buffered saline"[15] was used: this is prepared from 3.0 g NaCl, 3.0 g KH_2PO_4, 10.0 g Na_2HPO_4, 2.1 g $MgSO_4 \cdot 7H_2O$, water to 1 l and is described below as "100 mM phosphate–saline". The pH value was adjusted to required values between 5.5 and 8.5 by addition of either 1 M NaOH or 2 M HCl.

(B) Sodium phosphate–saline: prepared as (A) with NaH_2PO_4 substituted for KH_2PO_4 and pH adjusted with 1 M NaOH.

(C) Potassium phosphate–saline: prepared as (A) with K_2HPO_4 substituted for Na_2HPO_4 and pH adjusted with 1 M KOH.

(D) Tris buffers: 100 mM Tris with pH adjusted with 2 M HCl.

(E) Phosphate buffers: 100 mM Na_2HPO_4 or K_2HPO_4 with pH adjusted with 2 M HCl.

Determination of amino acid uptake and concentration within cells

The uptake and concentration of ^{14}C-labelled amino acids were determined as described previously[3] except that incubations were carried out at 15 or 20°, samples routinely taken at 15, 30 and 45 min and the reaction stopped by cooling in an ice-bath together with the addition to each tube (containing 1.5 ml reaction mixture) of 0.1 ml 30 mM 2,4-dinitrophenol. Concentration ratios are given empirically by the ratio P/S where P = total radioactivity in the hot water extract of the cell pellet and S = total radioactivity in the supernatant medium; a value of P/S = 1 corresponds approximately to an internal concentration 1000 times that in the external medium. The P/S value increases during the first 30 min and reaches a steady value after 45–60 min at 15°; rates of uptake are compared on the basis of values at 15 or 30 min while values at 45 or 60 min are taken as a measure of the concentration gradient attained.

Determination of proton concentration

Changes in H^+ concentration were measured with a micro glass electrode (Type G2222C, Radiometer of Copenhagen) assembled in the titration unit TTA31, measuring unit C33B-2 and Vibron electrometer 33B-2 (Electronic Instruments) recording on a potentiometric recorder (Servoscribe RE511.20). Full scale deflection could be obtained for intervals of 0.1, 0.3 or 1.0 pH unit; the middle range was found satisfactory for the experiments described below. The titration unit was set up with 6.0 ml 2 mM sodium phosphate–saline, 0.03 ml M NaCl, 0.6 ml chloramphenicol (1.0 mg/ml), 0.5 ml cell suspension (20 mg dry wt./ml), water and other additions, as below, to 10.0 ml. The pH was adjusted to the required value in the unit and measurements calibrated by the addition of known amounts (10–50 nequiv.) of H^+ at the end of each experiment.

Determination of K^+ concentration

K^+ concentrations were measured both by the use of a K^+-sensitive electrode and ^{42}K. The electrode was Type BH115 (Electronic Instruments) and was connected to the pH measuring unit used above; the readings were calibrated against standard KCl solutions and experiments performed in a manner similar to that used for proton studies. Experiments with $^{42}K^+$ were carried out under conditions similar to those used for the investigation of amino acid uptake; samples were removed and the cells filtered off and washed on membrane filters (Oxoid membrane filters Grade 0.45). The filter discs were transferred to metal planchets and radioactivity determined with an end-window Geiger–Müller tube connected to a conventional counter. All experiments with $^{42}K^+$ were completed within 3 days of receipt of the isotope and estimations corrected for isotopic decay.

Isotopes

Isotopes were obtained from the Radiochemical Centre, Amersham, Bucks. Amino acids were uniformly labelled with ^{14}C and used at the following specific activities: aspartate 10, glutamate 12.7, lysine 12 mCi/mmole. $^{42}K^+$ was supplied in isotonic saline at an initial specific activity 20 mCi/g K^+ and used at a final concentration of 10mM KCl. In all cases the counting methods were calibrated against known amounts of amino acid or K^+ distributed either on lens tissue or membrane filter discs as used in the relevant experimental procedures.

Inhibitors

Valinomycin, gramicidin and oligomycin were prepared as 1 or 0.1 mM solutions in ethanol; equivalent amounts of ethanol were added to control mixtures whenever these inhibitors were used.

RESULTS

The uptake of aspartate by Staphylococcus aureus

Competition with glutamate. The uptake of aspartate is affected in the same way as that of glutamate by the presence of a source of energy, inhibitors, lipids and fatty acids[3–5]. Gale and Van Halteren[16] found that the uptake of glutamate was inhibited

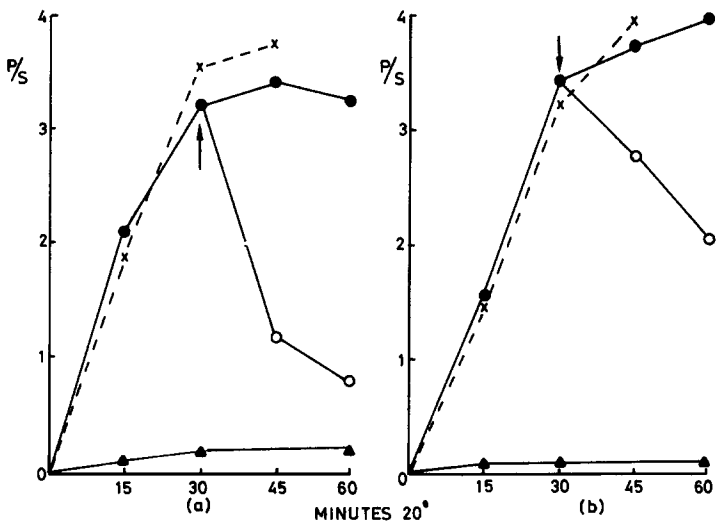

Fig. 1. Transport of (a) aspartate and (b) glutamate by *Staphylococcus aureus*. (a) *Staphylococcus aureus* cells suspended at 0.4 mg dry wt./ml in 1.5 ml 100 mM phosphate–saline (pH 7.0) containing 1% (w/v) glucose, 60 μg chloramphenicol/ml, and 20 μM [U-^{14}C]aspartate (spec. act. 10 mC/mmole) with additions (final concns.): ●, none; ▲, 1 mM glutamate at time 0; ○, 1 mM glutamate at time 30 min (at arrow); ×, 1 mM lysine at time 0. Samples taken at times indicated at 15°; reaction stopped by rapid cooling and addition of 0.1 ml 30 mM 2,4-dinitrophenol; cells centrifuged down (supernatant fluid sampled for radioactivity determination = S) and resuspended in 1.0 ml water at 100° for 10 min; centrifuged and extracted pool-radioactivity estimated = P. P/S = radioactivity in pool extracted from 0.6 mg cells/radioactivity in supernatant fluid from which these cells were removed. (b) As for (a) with aspartate and glutamate interchanged; additions: ●, 20 μM [U-^{14}C]glutamate (spec. act. 12.7 mC/mmole), no aspartate; ▲, 1 mM aspartate added at time 0; ○, 1 mM aspartate added at time 30 min (at arrow); ×, 1 mM lysine added at time 0.

by the presence of aspartate and *vice versa*. Fig. 1 confirms this mutual antagonism and shows that the addition of excess unlabelled glutamate resulted in a "chase" of labelled aspartate previously accumulated within the cells; likewise excess unlabelled aspartate "chased" labelled glutamate. Neither uptake nor retention of either acidic amino acid was affected by the presence of excess lysine. It therefore appears that the transport of aspartate is mediated by the same system as that of glutamate.

Inhibitors. Fig. 2 shows the effect of number of inhibitors on the uptake of aspartate by staphylococcal cells suspended in 100 mM phosphate–saline at pH 7.0 in the presence of glucose. At sufficient concentrations either gramicidin or 2,4-dinitrophenol gave complete inhibition whereas the inhibition obtained with valinomycin, oligomycin or antimycin reached a plateau at a value considerably less than 100%. Greater than 90% inhibition was obtained with valinomycin and oligomycin added together whereas the value obtained with antimycin and valinomycin fell between the values obtained with these antibiotics added separately (Fig. 3). The sensitivity to oligomycin or dinitrophenol was not affected by the presence of absence of K^+ in the incubation medium.

The action of valinomycin. Harold and Baarda[9] found that valinomycin inhibited the uptake of glutamate by *Streptococcus faecalis* and that the inhibition could be antagonised by high concentrations of K^+. A similar result was obtained for the uptake of aspartate by *Streptococcus faecalis* but the uptake by *Staphyloccus aureus* was inhibited by valinomycin only when a high concentration of K^+ was added to the incubation medium as shown in Fig. 4. Staphylococci were grown under the usual conditions in medium containing $^{42}K^+$ and the K^+ content of the harvested cells determined in the usual manner. Assuming that K^+ was distributed throughout the volume of the whole cell, the K^+ concentration in the cells was of the order 5–20 mM; it can therefore be seen from Fig. 4 that the uptake of aspartate by *Staphy-*

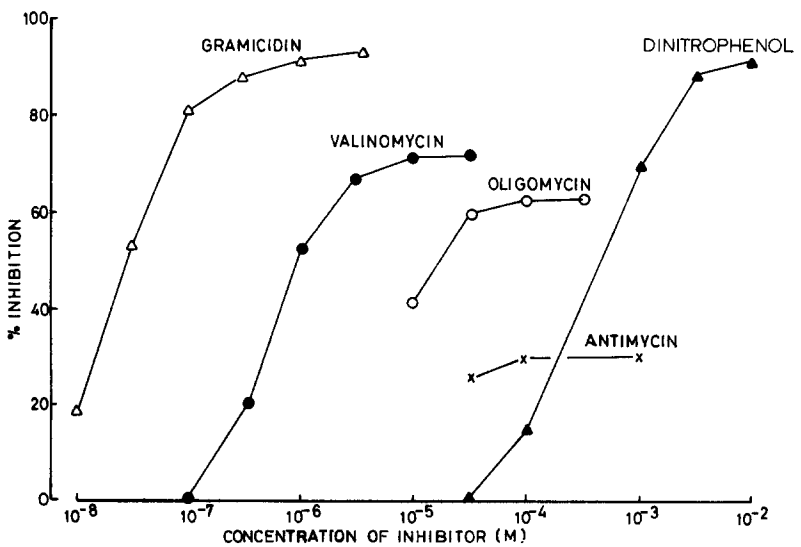

Fig. 2. Inhibitors of aspartate transport in *Staphylococcus aureus* at pH 7.0. Conditions as for Fig. 1a; results expressed as percent inhibition of rate of increase in P/S value in control without inhibitor.

lococcus aureus did not become significantly sensitive to valinomycin until the K^+ concentration was greater outside than inside the cell. Cells grown in unlabelled medium were then suspended in 100 mM sodium phosphate–saline at pH 7.0 containing glucose and aspartate with KCl added to final concentrations ranging from 1 to 300 mM; in one series the aspartate was labelled with ^{14}C and K^+ was unlabelled, while in a parallel series $^{42}K^+$ was added and aspartate was unlabelled. Fig. 5 shows the close parallelism that was found between the degree of inhibition of aspartate uptake by valinomycin on the one hand, and the rate of uptake of K^+ on the other.

The uptake of aspartate (or glutamate) and its inhibition by valinomycin, were then studied with cells suspended at pH 7.0 in (1) 100 mM phosphate–saline (containing both Na^+ and K^+), (2) 100 mM sodium phosphate–saline, (3) 100 mM potassium phosphate–saline and (4) 100 mM sodium phosphate–saline *plus* 100 mM KCl. The rate and extent of aspartate (or glutamate) uptake were the same, within experimental error, in all four buffer systems; 3 μM valinomycin was without effect in (2) but gave 70–80 % inhibition in all the K^+-containing media. These results confirm that valinomycin acts as an inhibitor of acidic amino acid transport in the presence of K^+ but show also that the uptake of aspartate or glutamate is not affected by the addition of K^+ to the medium. Fig. 6 shows the effect of adding K^+ to the incubation medium in the course of aspartate uptake: the uptake was insensitive to valinomycin until K^+ was added when inhibition occurred immediately. The results suggest that some factor other than K^+ concentration exerts an overriding control of aspartate uptake.

Effect of pH and energy source. Cells were incubated in sodium phosphate–saline containing 100 mM KCl and the pH adjusted to values at intervals of 0.5 pH unit

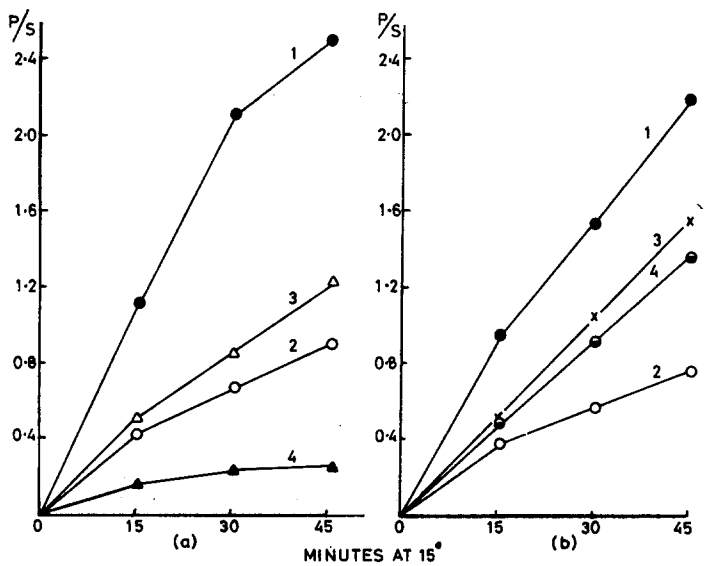

Fig. 3. Effect of valinomycin and (a) oligomycin or (b) antimycin on aspartate uptake by *Staphylococcus aureus* at pH 7.0. Conditions as for Fig. 1a with additions: (a) Curve 1, none; Curve 2, 3 μM valinomycin; Curve 3, 30 μM oligomycin; Curve 4, 3 μM valinomycin + 30 μM oligomycin. (b) Curve 1, none; Curve 2, 3 μM valinomycin; Curve 3, 0.1 mM antimycin; Curve 4, 3 μM valinomycin + 0.1 mM antimycin.

between 5.5 and 8.5. The inhibition of uptake by 1 μM valinomycin was determined at each pH value. Fig. 7 shows that (a) the rate of aspartate uptake and (b) the concentration ratio (judged by the P/S value) attained across the cell surface after 60 min

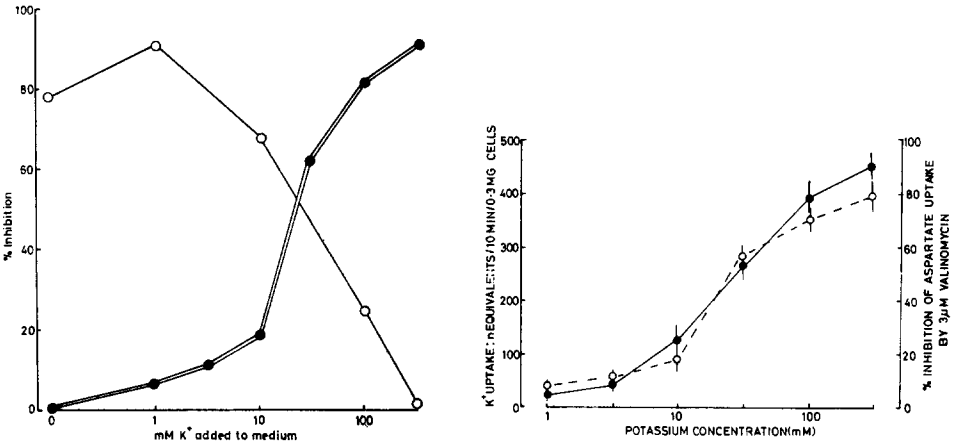

Fig. 4. Effect of K^+ on the inhibition of aspartate uptake by valinomycin in *Staph. aureus* and *S. faecalis*. Conditions as for Fig. 1a with KCl added to final concns. shown ○, inhibition by 1 μM valinomycin *Streptococcus faecalis*; ●, inhibition by 3 μM valinomycin *Staphylococcus aureus*.

Fig. 5. Effect of K^+ concentration on (●) uptake of K^+ and (○) inhibition of aspartate uptake by 3 μM valinomycin in *Staphylococcus aureus* at pH 7.0. Conditions for aspartate uptake as for Fig. 1a using sodium phosphate–saline with KCl added to final concns. shown; K^+ uptake measured in parallel samples containing unlabelled aspartate and ^{42}KCl.

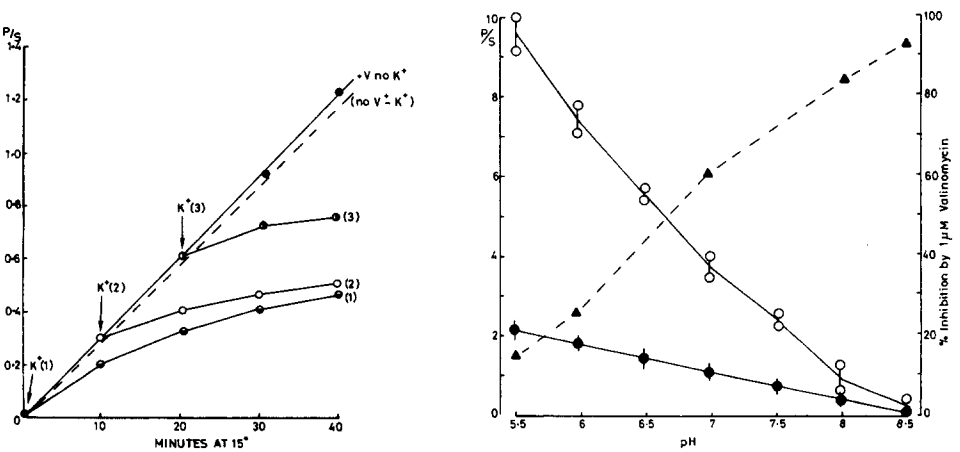

Fig. 6. Effect of K^+ on the accumulation of aspartate by *Staphylococcus aureus* at pH 7.0 in the presence of valinomycin. Cells suspended in 100 mM phosphate–saline (pH 7.0) with glucose, chloramphenicol and [U-^{14}C]aspartate as for Fig. 1a; KCl added to final concn. 100 mM at times indicated by arrows. ------, no additions; ———, 3 μM valinomycin (V).

Fig. 7. Effect of pH on uptake of aspartate and its inhibition by valinomycin in *Staphylococcus aureus*. Cells suspended in 100 mM sodium phosphate–saline adjusted to pH values shown and containing 100 mM KCl, glucose, chloramphenicol and [U-^{14}C]aspartate as for Fig. 1a. ●, rate of accumulation of aspartate: P/S value after 15 min at 15°; ○, concentration ratio attained (P/S) after 60 min at 15°; △, percent inhibition of rate of aspartate uptake by 1 μM valinomycin.

at 15° decreased as the pH rose while (c) the valinomycin inhibition increased from approx. 15 % at pH 5.5 to over 90 % at pH 8.5. Again it was found that the addition of K^+ did not significantly affect the rate or amount of aspartate taken up in the absence of valinomycin and it appeared that the controlling factor was the external proton concentration. If the aspartate uptake is coupled to proton translocation and controlled by the proton gradient across the cell surface, then it should be possible

TABLE I

EFFECT OF GLUCOSE ON ASPARTATE UPTAKE BY *Staphylococcus aureus*

0.6 mg dry wt. *Staphylococcus aureus* suspended in 1.5 ml 100 mM phosphate–saline (pH adjusted to values below) containing 60 μg chloramphenicol/ml and 20 μM [U-^{14}C]aspartate (spec. act. 10 mC/mmole) with and without 1 % (w/v) glucose; incubated at 20° for 15 min; reaction stopped by cooling and addition 0.1 ml 30 mM 2,4-dinitrophenol; cells centrifuged down, resuspended in 1.0 ml water for 10 min at 100°, cells centrifuged down and hot water extract sampled for radioactivity estimation (1000 counts/min = 0.55 nmole aspartate).

pH	*nmoles aspartate/mg dry wt. of cells*		
1 % glucose:	+	—	—
2 mM iodoacetate:	—	—	+
5.5	6.16	3.15	2.43
6.0	5.66	2.99	2.26
7.0	4.81	2.20	1.42
8.0	3.00	1.22	0.76

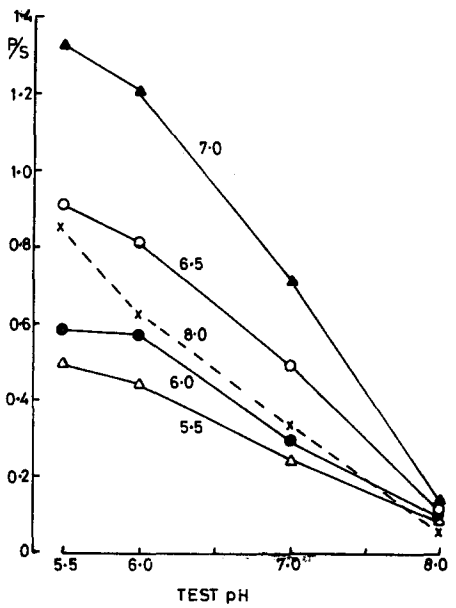

Fig. 8. Effect of pre-incubating cells at various pH values on their subsequent uptake of aspartate. Cells suspended for 20 min at 37° at 2.0 mg dry wt./ml in 30 mM sodium phosphate–saline adjusted to pH value shown on curve; each batch of cells then centrifuged down, resuspended at 0.4 mg/ml in 100 mM sodium phosphate–saline adjusted to test pH values as shown on abscissa, containing chloramphenicol, [U-^{14}C]aspartate but no glucose; P/S value determined after 30 min at 20° in each case.

to replace glucose by a proton gradient and alter the uptake by changing that proton gradient. Table I shows the amount of aspartate accumulated in the pool of the cells when the latter were incubated in 100 mM phosphate–saline, adjusted to various pH values, in the presence and absence of glucose or iodoacetate. The effect of pH was qualitatively the same in all cases; the rate of aspartate uptake was approximately halved by the omission of glucose and further decreased but not abolished by the addition of 2 mM iodoacetate. Fig. 8 shows the effect of holding the cells suspended in 30 mM sodium phosphate–saline adjusted to various pH values between 5.5 and 8.0 for 20 min at 37° before determining, in each case, the rate of aspartate uptake on resuspending the cells in the usual 100 mM sodium phosphate–saline over the pH range 5.5–8.0 in the absence of glucose: the rate of uptake increased with the external proton concentration as before but the rates at all test pH values increased as the "holding" pH rose from 5.5 to 7.0. This relationship broke down if the holding pH was greater than 7 when subsequent rates of aspartate uptake were slow. Assuming that some equilibration with the external medium took place during the holding period, then the lower the external pH during this period, the higher the internal H^+ concentration on transfer to the aspartate medium and the smaller the H^+ gradient from the external to the internal medium; it can be seen that holding the cells at pH 6 markedly reduced the subsequent aspartate uptake at pH 7 compared with that obtained for cells held at pH 7.

The uptake of protons

Preliminary experiments. Cells were incubated at room temperature in the assembly described under Methods. Early tests were carried out in 2 mM glycylglycine buffers at pH 5.5–6.5 but proton changes were widely variable and of a nature that suggested metabolism of the buffer under certain conditions. Further experiments were carried out with 2 mM sodium phosphate buffers; the electrode was found to generate considerable "noise" under these conditions but the fluctuations were largely abolished by addition to the buffer of 30 mM NaCl. When cells were suspended at 1.0 mg dry wt/ml in this medium at pH 5.5 or 6.0 a steady uptake of H^+ at 2–4 nequiv/min per mg cells was observed. On the addition of aspartate to a final concentration of 0.3 mM the rate of H^+ uptake increased 2–3 times but this increased rate declined rapidly and returned to the control rate after 8–12 min; the extra H^+ uptake after aspartate addition varied between 5 and 10 nequiv per mg cells. As in the case of aspartate uptake the increase was greatest when the pH was between 5.0 and 6.0 and decreased at higher pH values, while holding the cells at pH 7.0 before test gave higher uptakes than holding at pH 5.5, 6.0 or 8.0. Increasing the cell density from 1.0 to 2.0 mg/ml approximately halved the extra uptake; decreasing the cell density resulted in differences too small for accurate measurement. The extra uptake increased about 2.5 times when the final concentration of aspartate was increased from 0.3 to 1 mM but further increases in aspartate concentration gave no enhancement of H^+ uptake. Conditions adopted as a result of these preliminary experiments were: cells were first suspended at 10 mg/ml in 2 mM sodium phosphate buffer (pH 7.0) and held at room temperature for 30–40 min; 1.0 ml of this suspension was then added to 9 ml 2 mM sodium phosphate buffer (pH 5.5) containing 30 mM NaCl and 60 μg chloramphenicol/ml in the titration assembly, the pH was adjusted if necessary, and the rate of $[H^+]$ change recorded. 0.3 ml 30 mM sodium aspartate

was then injected into the assembly and the record continued until the new rate of H^+ uptake had returned to that obtained before aspartate addition. It was evident that a variable H^+ excretion by the cells was masking the change taking place on amino acid addition; occasionally there was an output of H^+ with the cells alone and this was reduced on aspartate addition. With *Staphylococcus aureus* an increased H^+ uptake was obtained on addition of aspartate or glutamate but not lysine; similar experiments with suspensions of *Streptococcus faecalis* showed no increased H^+ uptake on addition of any of these amino acids.

Quantitative relationship between uptake of H^+ and of aspartate. In order to reduce variations in H^+ uptake in the control, conditions were first sought which would reduce endogenous H^+ formation. The addition of iodoacetate or iodoacetamide reduced not only the control H^+ production but also the uptake occurring after aspartate addition. Addition of valinomycin to the cell suspension was followed by a rapid H^+ uptake (not illustrated); if a final concentration of 100 mM KCl was added first this gave an increased H^+ output and the further addition of valinomycin then resulted in a burst of H^+ output lasting 2–4 min (see Fig. 9). Parallel experiments with cells incubated with $^{42}K^+$ or preloaded with $^{42}K^+$, showed that K^+ was lost from cells incubated with valinomycin alone, and gained by cells incubated with K^+ and valinomycin. The transport of K^+ across the cell membrane, in the presence or absence of valinomycin, is thus coupled to counter transport of H^+. In the absence of a proton

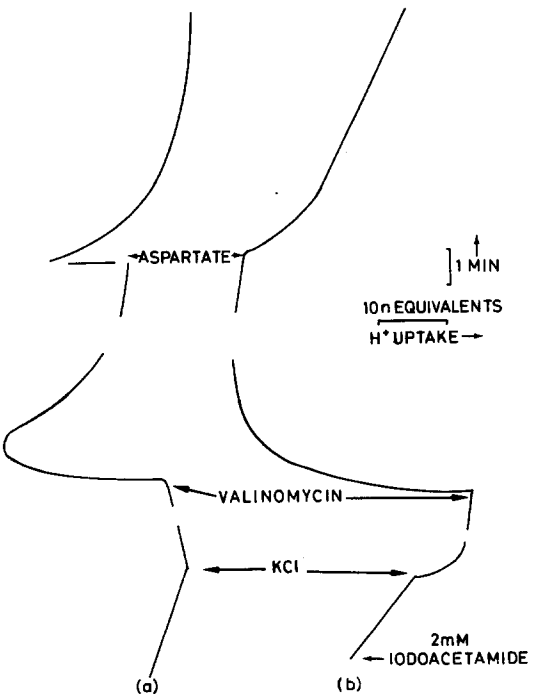

Fig. 9. Recorder tracing of H^+ changes in *Staphylococcus aureus* suspensions. Cells suspended at 1.0 mg dry wt./ml in 2 mM sodium phosphate–saline (pH 5.55) containing 30 mM NaCl and 60 μg chloramphenicol/ml. Iodoacetamide added to final concentration 2 mM in suspension (b) before recording began. Additions made at intervals as shown (final concns.): 100 mM KCl; 1 μM valinomycin; 1 mM sodium aspartate. H^+ uptake left to right; time reads upwards.

conductor the movement of K^+ will be limited[17]; the addition of gramicidin, which is a conductor of both K^+ and H^+ (ref. 17), is followed by extensive movement of both K^+ and H^+. The addition of KCl followed by valinomycin reduces the H^+ content of the cells and has little effect on aspartate uptake at pH 5.5 (see Fig. 7); it seemed probable therefore that the aim of reducing H^+ production and excretion in the absence of aspartate could be obtained by suitable combinations of K^+, valinomycin and an inhibitor such as iodoacetamide. Table II sets out some of the mixtures tested: valinomycin added alone had no effect on the increase in H^+ uptake during the first 10 min after aspartate addition; KCl followed by valinomycin doubled the increase, while iodoacetamide followed by KCl and valinomycin gave an increase more than 4 times that obtained in the control, and it can be seen from Fig. 9 that the increased rate was then maintained for the period recorded. Iodoacetate did not replace iodoacetamide and the order in which the substances were added proved to be important; the procedure eventually adopted was to add the iodoacetamide to the incubation mixture, containing the cells, in the titration assembly, begin recording, add 100 mM KCl (final concn.) 3–5 min later, and 1 μM valinomycin (final conc.) 2–3 min after that; the mixture was then left until a steady H^+ uptake was recorded (5–10 min) and the aspartate or glutamate then injected. Two methods of determining H^+ and aspartate uptake were adopted: (a) [U-^{14}C]aspartate was added to a final concentration of 0.1–0.3 mM and incubation continued for a known period at the end of which samples were removed from the assembly and the cells removed on a membrane filter for counting, while $[H^+]$ was estimated from the pH recording, or (b) [U-^{14}C]-aspartate was added at 0.03–0.05 mM final concn., (thus corresponding to the concentration used in the aspartate uptake experiments described in Section A), and the experiment continued until the rate of H^+ uptake had returned to that occurring before aspartate addition (see Fig. 10); samples were then removed and the cells filtered off for radioactivity assay. Considerable error was possible in the estimation of H^+ uptake by either method: in (a) since the assumption had to be made that H^+ uptake in the control remained constant throughout the experimental period; in (b)

TABLE II

EFFECT OF KCl, VALINOMYCIN AND IODOACETAMIDE ON H^+ UPTAKE DURING ASPARTATE ACCUMULATION BY *Staphylococcus aureus*

Cells suspended at 1.0 mg dry wt./ml in 10 ml 2 mM sodium phosphate–saline, containing 30 mM NaCl, and adjusted to pH 5.5; H^+ uptake recorded before and after the addition of 1 mM sodium aspartate. The following additions were made before the aspartate, at intervals of 3–5 min in order left to right. a, b, c = different experiments.

Iodoacetate (2 mM)	*Iodoacetamide (2 mM)*	*KCl (100 mM)*	*Valinomycin (1 μM)*	*Increased H^+ uptake during 10 min after aspartate addition (nequiv. H^+ per mg cells)*		
				a	*b*	*c*
–	–	–	–	5	6	6
–	–	–	+		6	6
–	–	+	+	11	9	15
–	+	–	–		2	
–	+	+	+	18	25	
+	–	+	+	3		

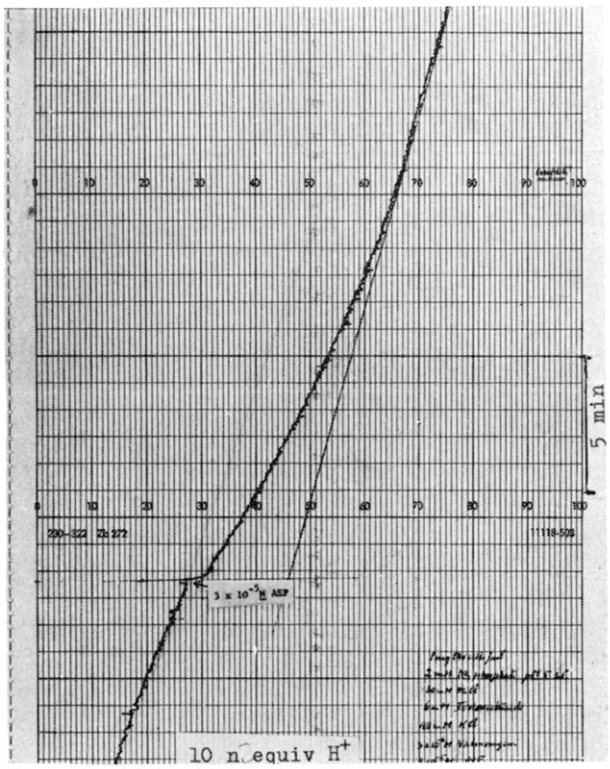

Fig. 10. Record of H^+ uptake during aspartate uptake by *Staphylococcus aureus*. Suspension prepared and treated as Fig. 9b before addition of aspartate to final concn. 30 μM.

when, in some experiments, it was found that the steady rate obtained, after ^{14}C estimations indicated that aspartate uptake had finished, was not the same as that obtained before the addition of the aspartate, and allowance consequently had to be made for this change in the control rate. EDDY AND NOWACKI[8] encountered similar difficulties in computing the H^+ translocation accompanying glycine uptake in yeast cells.

Table III gives a series of results obtained by the two methods and also shows the effect of iodoacetamide concentration on the net H^+ uptake. It can be seen that a ratio of 0.41 H^+ per molecule aspartate taken up was obtained in the absence of iodoacetamide and this increased to 0.66 when 6 mM iodoacetamide was present; increasing the iodoacetamide concentration to 10 mM did not raise the ratio further and the mean value of 9 estimations (with aspartate concentration 0.03–0.3 mM) was 0.62. The mean of three estimations of the ratio for glutamate uptake was 0.91.

The uptake of potassium

Effect of glucose and aspartate. Fig. 11a shows the uptake of $^{42}K^+$ obtained for suspensions of *Staphylococcus aureus* suspended in 100 mM sodium phosphate–saline (pH 7.0); there was little uptake in the absence of glucose (Curve 4). K^+ uptake obtained in the absence of glucose has varied between 10 and 35 % of that occurring

TABLE III

PROTON UPTAKE IN THE PRESENCE OF ASPARTATE OR GLUTAMATE

Assembly set up as described under Methods; cells at 1.0 mg dry wt./ml in 10 ml 2 mM sodium phosphate–saline (pH 5.5) containing 30 mM NaCl, iodoacetamide as below and 100 mM KCl, 1 μM valinomycin and aspartate or glutamate added as described in text.

Amino acid added (mM)	*Iodoacetamide (mM)*	*Time (min)*	*nequiv taken up per mg cells*		*H⁺/amino acid*	*Mean*
			Amino acid	*H⁺*		
Aspartate						
0.3	0	10	32.4 ± 3	11 ± 2	0.34 ± 0.09	
		10	19.2 ± 1.5	6 ± 1	0.31 ± 0.08	0.41 ± 0.10
		10	22.5 ± 2	11 ± 2	0.49 ± 0.13	
0.3	2	20	33.7 ± 3	18 ± 2	0.54 ± 0.11	0.46 ± 0.10
	4	20	33.5 ± 3	12.5 ± 2	0.38 ± 0.10	
0.3	6	25	41.6 ± 3.5	24.5 ± 2	0.59 ± 0.09	[0.66 ± 0.10]
	6	45	56.2 ± 5	41.3 ± 3	0.73 ± 0.12	
0.1	6	25	38.5 ± 3.5	24.5 ± 2	0.64 ± 0.10	
	10	25	37.0 ± 3	19.0 ± 2	0.52 ± 0.10	0.62 ± 0.11
0.05	6	30	22.0 ± 2	15.5 ± 1	0.70 ± 0.11	S.E. = 0.03
	6	30	28.2 ± 2.5	16.0 ± 2	0.57 ± 0.12	
0.03	6	25	21.4 ± 1.5	16.3 ± 2	0.76 ± 0.14	
	6	20	17.3 ± 1.5	8.5 ± 1	0.49 ± 0.10	
	6	25	20.3 ± 2	14.5 ± 1.5	0.71 ± 0.15	
Glutamate						
0.06	6	30	24.2 ± 2	23.0 ± 2	0.95 ± 0.15	
0.03	6	25	18.0 ± 1.5	18.6 ± 1.5	1.03 ± 0.18	0.91 ± 0.15
0.03	6	25	16.2 ± 0.5	12.0 ± 1	0.75 ± 0.05	

in its presence. In the presence of glucose, K^+ uptake was influenced by the further addition of aspartate, 20 μM increasing (Curve 2), 1 mM decreasing the uptake (Curve 3). Fig. 11b shows that the K^+ uptake was increased by aspartate concentrations between 10 and about 300 μM while concentrations higher than 300 μM were inhibitory; the largest increase was given by 60–70 μM aspartate. The effect of K^+ concentration on $^{42}K^+$ uptake was studied in the presence of glucose; half-maximal rate of K^+ uptake was obtained at 24 mM K^+ and the system was saturated at approx. 150 mM K^+. Varying the K^+ concentration between 10 and 100 mM did not affect the amount of extra K^+ taken up in the presence of 60 mM aspartate; further experiments were carried out in 10 mM K^+. Essentially similar results were obtained when Tris buffer (pH 7.0) was substituted for the sodium phosphate–saline (pH 7.0).

Relation between K^+ and aspartate uptake. It is evident from Fig. 11 that, assuming that the K^+ uptake coupled to glucose metabolism is not itself altered by the presence of aspartate, there is no direct relationship between aspartate uptake and the K^+ uptake over and above that due to glucose alone. Two series of determinations were carried out to confirm this: one series contained [U-^{14}C]aspartate and $^{39}K^+$, the other contained unlabelled aspartate and $^{42}K^+$. Table IV shows that the ratio of the extra K^+ taken up to aspartate taken up varied not only with the aspartate concentration but also with incubation time.

It would appear from the inhibitory actions of valinomycin in Fig. 7 that K^+

plays a more important role in aspartate transport at high than at low pH values. The amount of extra K^+ taken up in the presence of aspartate was therefore determined, in the presence and absence of glucose, at pH 6.0, 7.0 and 8.0 and the results are set out in Table V. When glucose was omitted from the incubation medium, the addition of aspartate gave no increased K^+ uptake in 14 out of 24 tests and the positive results showed a wide quantitative scatter. In the presence of glucose, no increased K^+ uptake in the presence of aspartate was recorded in 6 out of 51 tests and, again, the positive results were widely variable. It was not convenient to carry out experiments with a K^+ concentration less than 10 mM owing to the form in which the $^{42}K^+$ was received and a few experiments were carried out with a K^+-sensitive electrode in order to work at low concentrations. The electrode was calibrated against standard KCl solutions but was found to be sufficiently sensitive only when the K^+ concentration was kept below 0.5 mM. Staphylococcal cells suspended at 2 mg/ml in Tris buffer were found to leak K^+ on standing at room temperature (see below) and the most convenient way of performing these experiments was to suspend the cells in Tris buffer and allow K^+ to leak until the electrode indicated that the concentration in the medium had risen to 0.1–0.3 mM. The addition of aspartate had no effect on the rate of leakage over the range pH 6–8. Addition of glucose (final concn. 10 mM) was followed by uptake of K^+ which continued for 30–40 min before the initial rate of leakage was re-established. If aspartate was added before or at the same time as the glucose, the K^+ uptake increased and Table V includes a few results obtained by this method.

It appears that the uptake of aspartate in the presence of glucose is accom-

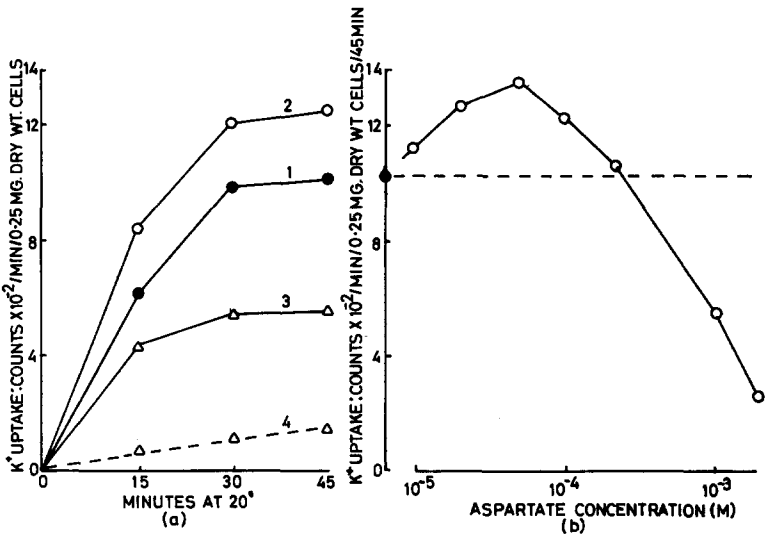

Fig. 11. Effect of aspartate and glucose on K^+ uptake by *Staphylococcus aureus* at pH 7.0. (a) Cells suspended at 0.5 mg dry wt./ml in 100 mM sodium phosphate–saline (pH 7.0) containing 60 μg chloramphenicol/ml, 10 mM ^{42}KCl (spec. act., 0.4 mC/g) and additions as below. Samples taken at 15-min intervals at 20°; filtered and washed twice with water on membrane filters before counting. Curve 1, 1% (w/v) glucose; Curve 2, as (1) + 20 μM aspartate; Curve 3, as (1) + 1 mM aspartate; Curve 4, no glucose. (b) Conditions as for (a) with glucose, and aspartate concentrations as shown.

TABLE IV

UPTAKE OF ASPARTATE AND K^+ BY *Staphylococcus aureus*

1.0 mg dry wt. of staphylococcal cells suspended in 2.0 ml 100 mM sodium phosphate–saline (pH 7.0) containing 1% (w/v) glucose, 120 μg chloramphenicol, 10 mM KCl and aspartate as below; series (a) with [U-^{14}C]aspartate (spec. act. as below) and unlabelled KCl; series (b) unlabelled aspartate and ^{42}KCl (spec. act. 0.68 mC/g). 0.5-ml samples taken at 15, 30 and 45 min at 20°, cells removed, washed and radioactivity determined on membrane filters. Values for K^+ uptake = increase over value (in parentheses) for glucose control without aspartate.

Aspartate		*15 min*			*30 min*			*45 min*		
μM	*mC/mmole*	*nequiv taken up*		*K⁺/aspartate*	*nequiv taken up*		*K⁺/aspartate*	*nequiv taken up*		*K⁺/aspartate*
		(*a*) *Aspartate*	(*b*) *K⁺*		(*a*) *Aspartate*	(*b*) *K⁺*		(*a*) *Aspartate*	(*b*) *K⁺*	
0	—		(155)			(233)			(285)	
60	5.0	22.5	49	2.18	37	51	1.38	37	44	1.20
120	3.33	33	37	1.15	50	50	1.00	54	40	0.74
180	2.5	36	26	0.72	51	44	0.86	60.5	54	0.89

TABLE V

RELATIONSHIP BETWEEN ASPARTATE UPTAKE AND ASSOCIATED EXTRA K^+ UPTAKE IN *Staphylococcus aureus*

Determinations as for Table IV except for final column marked * where K^+-sensitive electrode was used as described in text.

Glucose:	*Absent*					*50 mM*					*50 mM*				*10 mM**		
Sodium phosphate–saline:	*1 mM*					*1 mM*					*100 mM*				(*100 mM Tris*)		
KCl:	*10 mM*					*10 mM*					*10 mM*				*0.2 mM*		
pH	*No.*	*No. of zero results*	K^+/*aspartate* *Range*	*Mean*	*S.E.*	*No.*	*No. of zero results*	K^+/*aspartate* *Range*	*Mean*	*S.E.*	*No.*	K^+/*aspartate* *Range*	*Mean*	*S.E.*	*No.*	K^+/*aspartate* *Values*	*Mean*
6.0	9	7	0.0 1.90	0.41	0.26	9	2	0.0 1.23	0.69	0.17	6	0.85 3.80	1.70	0.42	2	1.27 2.76	2.01
7.0	9	4	0.0 1.47	0.26	0.15	9	1	0.0 5.08	2.07	0.75	6	0.42 2.10	1.47	0.23	2	1.30 1.40	1.35
8.0	6	3	0.0 2.6	1.04	0.30	9	2	0.0 2.05	0.90	0.30	6	0.0 0.85	0.46	0.35	2	4.05 5.90	4.97

panied by an increased uptake of K^+ but it has not been possible to establish a quantitative relationship between the two quantities.

Release of K^+ from staphylococcal cells. The decrease in K^+ uptake when high concentrations of aspartate are added to cell suspensions in the presence of glucose (Figs. 11a and 11b) could be explained by an efflux of K^+ from the cells under these conditions. Cells were grown in the usual medium with the addition of $^{42}K^+$, harvested and suspended in either 100 mM sodium phosphate–saline (pH 7.0) or Tris buffer (pH 7.0). The suspensions were incubated at 20° in the presence of glucose, and aspartate added at final concentrations from 0.03 to 1.0 mM; samples were taken at 10-min intervals from 0 to 40 min and the $^{42}K^+$ content of the cells determined. Some loss of K^+ occurred in all cases but in no case did the presence of aspartate increase this loss by an amount greater than the experimental error. The experiment was repeated with 10 mM KCl added to the suspension medium and, again, there was no greater loss of $^{42}K^+$ from cells in the presence of aspartate than from those in its absence. The leak of K^+ from cells suspended in 100 mM Tris buffer at pH 6, 7 or 8 was also followed with the K^+-sensitive electrode as described above; addition of aspartate to a final concentration of 20–100 μM gave no change in the rate of leakage. It can be concluded that the uptake of aspartate is not coupled to an increased efflux or turnover of K^+ in the cells.

Fig. 12 shows the effect of adding valinomycin to staphylococci pre-loaded

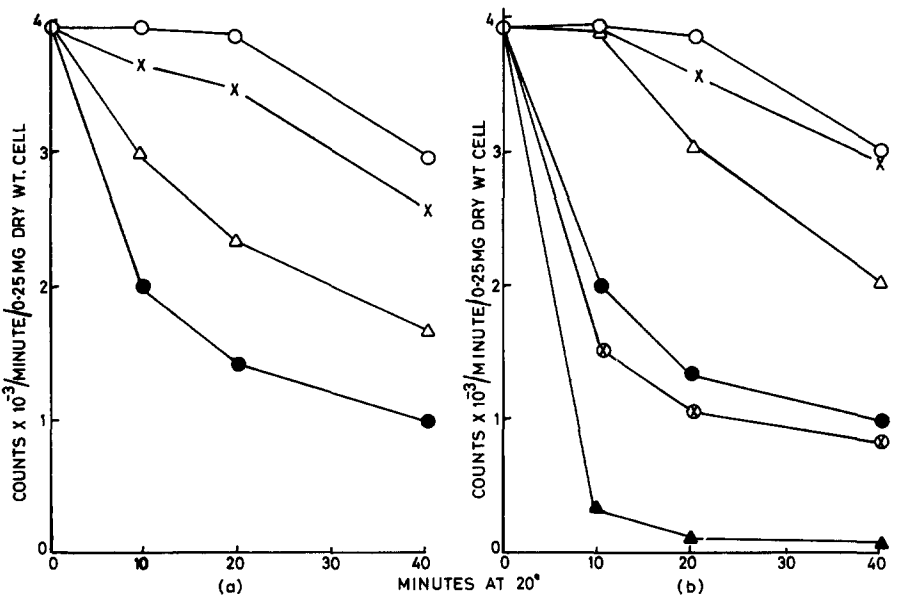

Fig. 12. Release of K^+ from *Staphylococcus aureus* in the presence of valinomycin. *Staphylococcus aureus* grown as in Methods in medium containing ^{42}KCl (0.5 mC at initial specific activity 5 mC/g added to 150 ml medium); cells harvested after 18 h growth at 25°; washed in 30 mM sodium phosphate–saline (pH 7.0) and suspended at 0.5 mg dry wt./ml in the presence of 1% (w/v) glucose in: (a) 100 mM sodium phosphate–saline (pH 7.0) + (○) 0; (●) 1.0; (△) 0.3; (×) 0.1 μM valinomycin. (b) 100 mM sodium phosphate–saline (pH 7.0) with (●) and without (○) 1 μM valinomycin. 100 mM Tris–HCl buffer (pH 7.0) with (⊗) and without (×) 1 μM valinomycin; 100 mM potassium phosphate–saline with (▲) and without (△) 1 μM valinomycin. 0.5-ml samples taken at times shown, cells removed, washed and radioactivity determined on membrane filters.

with $^{42}K^+$ and suspended in different buffers in the presence of glucose. In 100 mM Tris buffer or sodium phosphate-saline (pH 7.0), cells lost K^+ to an extent that amounted to 20–30 % of their initial content in 40 min at 20°; in 100 mM potassium phosphate-saline the loss of $^{42}K^+$ was approximately twice that in the sodium or Tris system (Fig. 12b). If glucose was omitted the rate of loss increased by 10–15 %. In all cases the rate of loss was greatly increased by the presence of 1 μM valinomycin; exchange was almost complete within 10 min in potassium phosphate–saline. Reducing the valinomycin concentration to 0.3 μM (Fig. 12a) approximately halved the rate of $^{42}K^+$ efflux. Valinomycin did not cause the cells to leak aspartate.

Sodium. Cells were grown in media labelled with $^{22}Na^+$ and also grown in unlabelled media and then incubated in $^{22}Na^+$ phosphate–saline with and without glucose and aspartate. Samples were taken and the cells removed on a membrane filter, washed twice with water and radioactivity determined; no count significantly above background was obtained in any experiment.

Effect of the ionic composition of the incubation medium

In Section A it was shown that aspartate uptake was inhibited by valinomycin when high concentrations of K^+ were added to the incubation medium and that the sensitivity increased as the pH rose. However, it was found that the addition of K^+ had no effect on the rate or extent of aspartate uptake under the conditions then used (100 mM sodium phosphate–saline (pH 7.0), 100 mM KCl, 1 % glucose). If K^+ has an indirect effect on aspartate transport (such as increasing proton translocation) then it seemed that positive effects should be sought under conditions of low buffering power and high pH. Table VI shows some typical results.

Effect of NaCl. Following the above line of reasoning, experiments were first carried out in 1 mM sodium phosphate–saline (pH 8.0), with and without glucose, with the addition of 100 mequiv NaCl, KCl, sodium phosphate (adjusted to pH 8.0) or potassium (adjusted to pH 8.0). Table VI, Expt. (a), shows that, in the absence of glucose, aspartate uptake was stimulated in order of decreasing effect by the salts NaCl, KCl or potassium phosphate, and decreased by sodium phosphate. There was little buffering power in the basal sodium phosphate–saline so the effect of 100 mequivalent sodium phosphate could reflect the greater buffering power so provided. In Expt. b the strength of the basal buffer solution was increased to 10 mM with little effect on the relative actions of the various salts. The ionic strength of the growth medium was considerably greater than that of the basal incubation medium in either Expt. a or b and the action of NaCl could have been due to osmotic stabilisation. Since KCl was less effective than NaCl in the absence, but more effective in the presence, of glucose, it would seem that K^+ and Na^+ have different actions. Expts. c and d show that the effect of increasing the NaCl concentration in the absence of glucose was to increase the rates of aspartate uptake in the presence of sodium phosphate, potassium phosphate of KCl while the rate in the presence of 100 mM KCl became greater than that in 100 mM NaCl.

Effect of sodium and potassium phosphates. In all the experiments shown in Table VI, the rate of aspartate uptake in 100 mequiv potassium phosphate was 2–6 times greater than in 100 mequiv sodium phosphate. The addition of NaCl increased both rates but the ratio of the rate in potassium phosphate to that in sodium phosphate remained approximately constant (Expts. c and d). The presence of glucose

TABLE VI

EFFECT OF SALTS ON ASPARTATE UPTAKE BY *Staphylococcus aureus*

Cells incubated at 0.4 mg dry wt./ml in salt mixtures at pH 8.0 as below with 60 μg chloramphenicol/ml, 20 μM [U-^{14}C]aspartate (10 mC/mmole) and glucose where indicated; samples taken at 15, 30 and 45 min at 20° and P/S value determined as for Fig. 1a; P/S value at 30 min given below.

Basal solution:	*Expt. a*		*Expt. b*		*Expt. c*			*Expt. d*			
Sodium phosphate–saline, pH 8.0:	*1 mM*	*1 mM*	*10 mM*	*10 mM*	*10 mM*	*10 mM*	*10 mM*	*10 mM*	*10 mM*	*10 mM*	*10 mM*
5 mM glucose:	—	+	—	+	—	—	—	—	—	—	—
NaCl:	—	—	—	—	*0 mM*	*60 mM*	*100 mM*	*0 mM*	*20 mM*	*60 mM*	*100 mM*
Additions (*100 mequiv*)											
None	0.3	0.97	0.42	1.66	0.26	0.35	0.74	—	—	—	—
NaCl	1.36	3.65	1.26	2.31	0.74	—	—	—	—	—	—
KCl	0.82	4.60	0.47	2.48	0.29	0.52	0.80	—	—	0.67	—
Sodium phosphate	0.10	0.80	0.24	1.59	0.19	0.33	0.53	0.13	0.17	0.23	0.27
Potassium phosphate	0.60	2.91	0.77	2.35	0.52	0.85	1.64	0.48	0.67	0.81	1.03
Potassium phosphate / Sodium phosphate	(6.0)	(3.6)	(3.2)	(1.48)	(2.73)	(2.57)	(3.2)	(3.7)	(3.8)	(3.5)	(3.8)
Sodium phosphate + potassium phosphate	—	—	—	—	0.28	0.59	—	—	—	0.66	—
Sodium phosphate + KCl	—	—	—	—	0.19	0.40	—	—	—	0.45	—

decreased this ratio and Table VII shows that the ratio increased with rising pH whether glucose was present or not. A mixture of sodium and potassium phosphates had an effect intermediate between those of the phosphates tested separately (Table VI, Expt. c) but the addition of KCl to sodium phosphate was less effective than the addition of an equivalent concentration of potassium phosphate. The uptake of aspartate by cells suspended in 100 mM Tris buffer (pH 8.0) was very slow in the absence of glucose and was not stimulated by the addition of KCl.

Action of valinomycin. Fig. 13 shows the action of 1 μM valinomycin on the uptake of aspartate in 100 mequivalent sodium or potassium phosphate buffers at

TABLE VII

EFFECT OF pH ON ASPARTATE UPTAKE IN SODIUM OR POTASSIUM PHOSPHATE

Conditions as for Table VI; P/S values after 30 min at 20° quoted.

Addition (*100 mequiv*)	*P/S*					
pH:	*6.0*	*7.0*	*8.0*	*6.0*	*7.0*	*8.0*
5 mM glucose:	–	–	–	+	+	+
Sodium phosphate	1.46	1.04	0.30	4.24	2.75	0.84
Potassium phosphate	1.61	1.56	0.95	4.30	3.30	1.28
Potassium phosphate/ Sodium phosphate	(1.10)	(1.50)	(2.62)	(1.02)	(1.20)	(1.52)

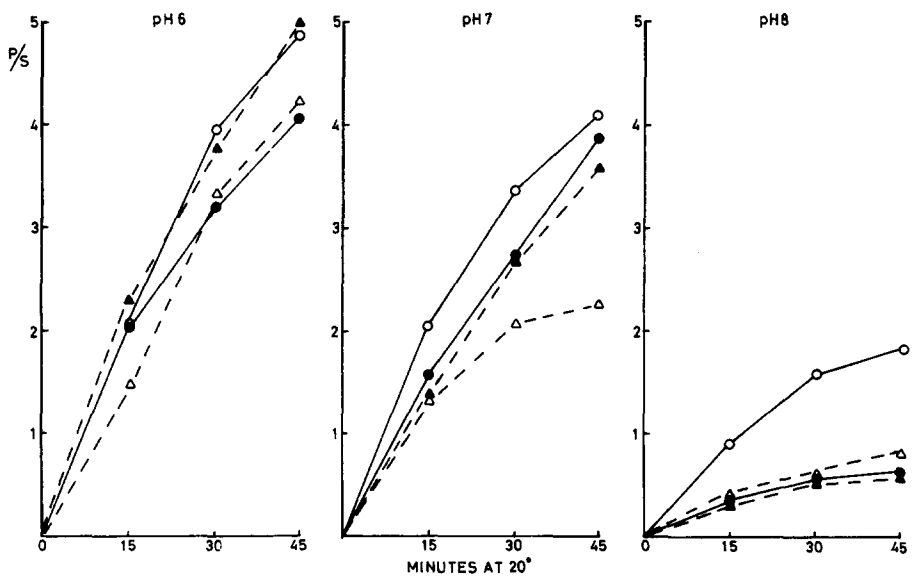

Fig. 13. Effect of valinomycin on aspartate uptake in potassium or sodium phosphate buffers at pH values 6.0, 7.0 and 8.0. Cells suspended at 0.4 mg dry wt./ml in 1.5 ml. 100 mM sodium phosphate (●, ▲) or potassium phosphate (○, △) with (▲, △) and without (●, ○) 1 μM valinomycin; incubation media also contained 60 μg chloramphenicol/ml and 20 μM [U-^{14}C]aspartate and 15 μl ethanol (equivalent to valinomycin solution added). Temp. 15°; pH as shown; estimations as for Fig. 1a.

pH 6, 7 or 8 in the absence of glucose. The increasing effect of potassium phosphate as opposed to sodium phosphate with rising pH can again be seen. At pH 6 valinomycin gave a small (20 %) increase in the rate of aspartate uptake in potassium phosphate (an effect frequently but not consistently obtained at acid pH values) and a similar decrease in the rate in potassium phosphate: valinomycin thus decreased the rate in the presence of K^+ to that obtained in the sodium phosphate control. Although the values are quantitatively different, the same overall effect of valinomycin can be observed at pH 8. However, at pH 7 valinomycin decreased the rate in the presence of K^+ to a value markedly below that obtained in the presence of Na^+ with or without valinomycin; this effect can also be seen in Fig. 6 and was consistently obtained in the earlier experiments using 100 mM sodium phosphate-saline, 100 mM KCl and 1 % glucose as the suspending medium.

Transport of other amino acids

Table VIII shows some of the effects of pH and inhibitors on the uptake of amino acids by *Staphylococcus aureus* and *Streptococcus faecalis*. In *Staphylococcus aureus* aspartate, glutamate and lysine are concentrated to a greater extent than the other amino acids tested and it has been shown (Section A), that aspartate and glutamate probably have a common transport mechanism. With the exception of proline the transport of all the amino acids tested took place more rapidly at pH 6 than at pH 8. All the transport systems showed a similar sensitivity to valinomycin and 2,4-dinitrophenol; the former was effective only when K^+ was added to the incubation medium and the sensitivity increased as the pH value increased. In contrast, the uptake of aspartate and glutamate by *Streptococcus faecalis* was independent of pH within the range 6–8; the sensitivity to valinomycin decreased with rising pH and was reduced by the addition of K^+ to the incubation medium as shown in Fig. 4.

DISCUSSION

The uptake of aspartate and glutamate by *Staphylococcus aureus* is accompanied by proton uptake and continues until the concentration inside the cell reaches a value controlled by the proton gradient across the cell surface. The transport is completely inhibited by dinitrophenol or gramicidin as would be expected if these substances act as proton conductors[9,18]. Under the artificial conditions necessary to inhibit endogenous H^+ excretion, it has been possible to demonstrate the uptake of between 0.6 and 1.0 H^+ equiv per molecule of aspartate or glutamate accumulated within the cell. Acidic amino acids are probably transported as anions and their translocation would be accompanied by movement either of cations in the same direction or of anions in the opposite direction. The effect of pH and the uptake of protons could thus arise from H^+ acting as the charge-balancing cation. This role could also be filled by K^+ but the sensitivity of the transport to dinitrophenol is not affected by the presence of K^+. Moreover the rates of uptake of glycine, alanine or lysine are also promoted by low external pH values and display the same sensitivity to dinitrophenol and valinomycin as the acidic amino acids. In *Streptococcus faecalis*, where the pH effect is not demonstrable, it is only the acidic amino acids whose transport is sensitive to dinitrophenol. It seems probably that protons play more than

TABLE VIII

THE EFFECT OF pH, VALINOMYCIN AND DINITROPHENOL ON THE UPTAKE OF AMINO ACIDS BY *Staphylococcus aureus* AND *Streptococcus faecalis*

Cells suspended at 0.4 mg dry wt./ml in 1.5 ml 100 mM sodium phosphate–saline, 1% (w/v) glucose, 20 μM [U-^{14}C]amino acid (spec. act. 3–14.7 mC/mmole), 60 μg chloramphenicol/ml; for *Staphylococcus aureus* 100 mM KCl is added in all experiments except those in final column (*); for *Streptococcus faecalis* 100 mM KCl is added in experiments in last column only. Samples taken at 15, 30 and 45 min at 15° and P/S values determined as in Fig. 1.

Amino acid	*P/S value pH 7; 45 min*	*Rate of uptake at pH 6 / Rate of uptake at pH 8*	*Inhibition (%)*				
			1 mM dinitrophenol, pH 7	*3 µM valinomycin*			*3 µM valinomycin, pH 7 (no K+)**
				pH 6	*pH 7*	*pH 8*	
Staphylococcus aureus							
Asp	3.8	4.0	70	37	70	95	0
Glu	5.75	3.4	70	24	70	92	0
Lys	1.01	1.3	75	62	70	90	0
Pro	0.30	1.0	53	66	70	90	7
Ala	0.21	2.5	72	32	56	66	0
Gly	0.09	3.7	55	17	34	52	0
Leu	0.06	1.4		45		69	
Streptococcus faecalis							*(100 mM K+)**
Asp	2.8	1.1	50	84	80	20	25
Glu	8.7	0.9	63	81	80	50	0
Lys	0.45	0.9	0	5	0	0	0
Ala	0.12	0.4	0	39	40	0	0

a simple charge-balancing role in the transport of acidic amino acids in *Staphylococcus aureus*.

The inhibitory effects of valinomycin are unusual. It is evident from Fig. 4 that the action of valinomycin on aspartate uptake in *Staphylococcus aureus* is different from that in *Streptococcus faecalis*, while the effects of pH on valinomycin inhibition shown in Figs. 7 and 13 suggest that K^+ is of more importance in aspartate transport in *Staphylococcus aureus* at high than low pH values. If the transport of aspartate is dependent on a proton gradient across the cell membrane, then such transport would be minimal at high pH values and some alternative or secondary mechanism might come into play. Three simple hypotheses could be proposed to explain the experimental findings:

(A) That two separate and independent mechanisms are involved, (i) coupled to H^+ translocation and (ii) coupled to K^+ translocation.

(B) That an aspartate carrier is involved and requires (i) protonation and (ii) the presence of either H^+ or K^+ as co-substrate.

(C) That aspartate transport is coupled to H^+ uptake and this can, under appropriate conditions, be driven by a K^+/H^+ pump acting as one of the mechanisms for the ejection of H^+ from the cell.

The three hypotheses require a stimulation of aspartate uptake by K^+ and B and C predict that this stimulation would be proportionately greater at high than low pH values; as set out in Section D, it has been possible to demonstrate this situation under certain conditions although results are complicated by osmotic and specific ion effects. The fact that it has not been possible to establish a quantitative relationship between aspartate and K^+ uptake argues in favour of Hypothesis C and against A. Also difficult to reconcile with Hypothesis A are (1) the complete inhibition of aspartate uptake by dinitrophenol whether K^+ is present or not, and (2) the findings set out in Figs. 6, 7 and Table VII where, particularly at pH 7, valinomycin has a large inhibitory effect yet the addition of K^+ has relatively little effect on aspartate uptake. An increased uptake of K^+ on addition of aspartate has only been demonstrated consistently in the presence of glucose when the cells are excreting H^+; on Hypothesis B, H^+ would activate the carrier and lead to an increased uptake of both aspartate and K^+; on Hypothesis C, glucose would activate the K^+/H^+ pump and the coupling of H^+ translocation to aspartate uptake result in a secondary stimulation of K^+ uptake.

Valinomycin will dispel any K^+ concentration gradient across a membrane and consequently inactivate any transport process coupled to K^+ translocation down such a gradient. On any of the hypotheses, valinomycin would therefore be expected to inactivate that part of aspartate transport dependent on K^+ translocation. At pH 6 and 8 valinomycin cuts down aspartate uptake in the presence of K^+ to that obtained in the presence of Na^+ but at pH 7, whether in the presence (Fig. 6) or absence (Fig. 13) of glucose, the rate of uptake in the presence of K^+ *plus* valinomycin is markedly smaller than that in the presence of Na^+ with or without valinomycin. Neither Hypothesis A or C can provide a ready explanation but on Hypothesis B this could be explained if the aspartate carrier binds K^+ and is then immobilised on removal of the K^+ gradient — at pH 6 the carrier is essentially proton activated, whereas at pH 8 the uptake in the absence of K^+ is too small for these changes to be evident.

Prof. A. A. Eddy has pointed out that the proton gradient presumably contains two components, one the difference in H^+ concentration on the two sides of the membrane, and the other the potential difference across the membrane. One effect of valinomycin in the presence of high concentrations of K^+ would be to decrease or even abolish the membrane potential and this could be a major factor in the action of valinomycin at high pH values.

In the absence of an artificially maintained H^+ gradient, aspartate uptake will be dependent on a metabolically generated gradient such as that obtained in the presence of glucose or alcohol. It can be seen from Fig. 3 that aspartate uptake in media containing both K^+ and glucose at pH 7 is completely inhibited by valinomycin and oligomycin together although neither antibiotic is fully inhibitory when tested alone. This suggests that the H^+ production upon which aspartate transport is dependent under these conditions has two components, one sensitive to valinomycin and the other oligomycin. The possible roles of valinomycin have been discussed above; oligomycin is believed to act as an inhibitor of membrane-bound ATPase in mitochondria[19].

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