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SELECTIVE PENETRATION OF AMMONIA AND ALKYLAMINES INTO *STREPTOCOCCUS FECALIS* AND THEIR EFFECT ON GLYCOLYSIS*

M. ZARLENGO AND A. ABRAMS

Department of Biochemistry, University of Colorado School of Medicine, Denver, Colo. (U.S.A.)

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SUMMARY

Evidence was presented for the selective penetration of free base into *Streptococcus fecalis* cells suspended in solutions of NH_4Cl, CH_3NH_3Cl, $(CH_3)_2NH_2Cl$ and $(CH_3)_3NHCl$ at pH 7.0 or below. The selective penetration was measured by automatic continuous titration of the appearance of H^+ and was found to be practically instantaneous. Selective penetration of NH_3 was freely reversible and led to passive accumulation of NH_4^+. A mathematical equation was developed which satisfactorily describes the observed relation between amine uptake and extracellular amine concentration at constant pH. This equation permits an estimation of the intracellular pH and the rise in intracellular pH following penetration of free amine. Tentatively the intracellular pH of *S. fecalis* cells obtained from stationary growth phase is estimated to be about 5.0. Glycolysis in "aged" cells is retarded but is restored immediately following selective penetration of NH_3 or alkylamines at constant extracellular pH. K^+ and Na^+ also restored glycolysis but their action was slower. K^+ and Na^+ were found to elicit an efflux of H^+ at constant extracellular pH from non-glycolyzing cells possibly by ion exchange. It is concluded from these findings and a number of others as well that glycolysis in "aged" cells is inhibited by their low intracellular pH and is restored when the intracellular pH is raised.

INTRODUCTION

It has been known for about a half century that NH_3 penetrates selectively into various plant and animal cells when they are placed in contact with NH_4^+ salt solutions[1,2]. In some instances the expected rise in intracellular pH has been directly observed in the living cell. In recent years evidence has been obtained for selective NH_3 penetration through membranes of isolated mammalian mitochondria[3], and in intact animals including man[4,5]. It is also well recognized that the uncharged form of many organic amines is more toxic to certain microorganisms than the corresponding undissociated charged form and this is also attributed to the selective penetration of the free base[6]. Apparently, however, the selective penetration of NH_3 or alkylamines into bacteria has not been previously investigated. Moreover, in no case has there been a determination of the metabolic effects, if any, associated primarily

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with the rise in intracellular pH which accompanies the selective penetration of a free base.

The present investigation stems from previous studies concerned with the effects of monovalent cations on glycolysis and permeability in the lactic acid bacterium, *Streptococcus fecalis*[7-9]. Experiments will be described showing that NH_3 and several alkylamines penetrate practically instantaneously as free bases into resting non-glycolyzing *S. fecalis* cells. A simple mathematical formulation of the relation between extracellular NH_3 concentration, NH_3 uptake and rise in intracellular pH at constant extracellular pH will be developed which is in accord with experimental measurements. In these experiments the means for observing selective penetration of NH_3 and alkylamines, quantitatively and kinetically, was afforded by automatic continuous titration (pH-stat) of the extracellular H^+ ions which arise by dissociation from NH_4^+ (or alkylammonium) ions concomitant with the entry of free base into the cells.

We have also studied, again by means of the pH-stat device, the effect on the glycolysis rate of cells following the entry of NH_3 or alkylamines. An immediate and striking acceleration of glycolysis was produced equally by NH_3 and various alkylamines, especially with aged cells. This finding suggested that a rise in intracellular pH was responsible for accelerating glycolysis and further investigations to be presented substantiate this view. Of most general interest are experiments which reveal that K^+ and Na^+ ions elicit an efflux of H^+ ions from non-glycolyzing cells, possibly by ion exchange, and following this action the glycolysis rate observed on addition of glucose is accelerated.

MATERIALS AND METHODS

S. fecalis cells (ATCC No. 9790) were grown in a medium containing tryptone, yeast extract, glucose and potassium phosphate and were harvested by centrifugation after reaching the stationary phase of growth[10]. The pH of the medium at the time of harvest was about 4.5. The cells were washed 3 times with cold distilled water by repeated centrifugation and finally suspended in water. The cells obtained from 300 ml of medium were suspended in 24 ml of water (stock suspension) and stored at 4°. The stock suspension contained 15–20 mg dry weight of cells/ml. Unless stated otherwise, the stock cells were used in an experiment after one or more days of storage (aged cells). Cells used on the same day that they were harvested will be referred to as fresh cells.

All experiments were conducted in an open 20-ml beaker at room temperature using 1 or 2 ml stock cells in a volume of 5–10 ml water. Measurements of the appearance of extracellular H^+ ions at constant pH whether from non-glycolyzing cells or during glycolysis, were made by continuous titration with automatic recording using a pH-stat device[11] (Radiometer-Copenhagen TTTIa Titrator with SBR2 Titrigraph). The titrant was 0.02 N NaOH except in one experiment in which HCl was used to titrate the release of alkali. An Agla 0.5-ml syringe was used for delivery of the titrant. The selected pH was 7.0 except when stated otherwise. At the start of each experiment the cell suspension in water was adjusted to the selected pH from its initial pH of about 5 by addition of about 1–2 μmoles of NaOH. The salt solutions to be tested were also adjusted to the selected pH, usually pH 7.0, with small amounts of NaOH before the start of an experiment. Only the chloride salts were used. No

buffer salts were used at any time, thus Cl^- ion was the only anion (other than OH^-) added to the cell suspension.

RESULTS

Equivalence of NH_3 penetration into cells and H^+ release from NH_4^+

The addition of NH_4Cl solutions to cell suspensions both previously adjusted to the same pH results in an instantaneous appearance of H^+ ions the magnitude of which is given by the amount of alkali required to maintain the initial pH. The quantity of H^+ ions released when 1.0 or 2.0 ml of stock cells were mixed with various

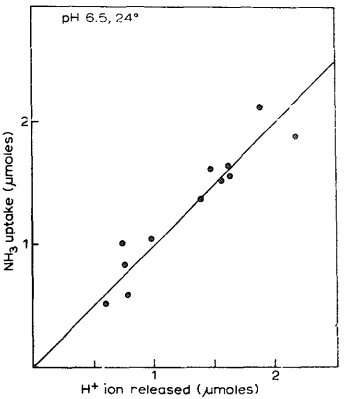

Fig. 1. Equivalence of H^+ ion release and NH_3 uptake by *S. fecalis* cells suspended in NH_4Cl solutions at pH 6.5. NH_3 uptake was measured with Nessler's reagent and H^+ ion release with pH-stat machine. NH_4Cl initial concentration ranged from 0.25 to 6.0 μmoles/ml and 0.5, 1.0, and 2.0 ml of stock cells were used.

concentrations of NH_4Cl at pH 6.5 was determined by titration. Following each titration the cells were centrifuged and the disappearance of NH_3 from the extracellular fluid was determined with Nesslers reagent. The results (Fig. 1) show that the appearance of extracellular H^+ ions is equivalent to NH_3 disappearance, mole for mole, and that this equivalence holds over a wide range of cell concentrations and NH_4Cl concentrations. Such an equivalence is expected for a system which permits the penetration only of free NH_3 at a pH (pH 6.5 in this instance) considerably lower than the pK of the NH_4^+ system (pK = 9.25). This is readily proved as follows: when a = moles of free NH_3 which have disappeared from the extracellular fluid, b = moles of H^+ ions released from NH_4^+ when free NH_3 penetrates at constant extracellular pH; NH_3 = moles of NH_3 present initially; NH_4^+ = moles of NH_4^+ present initially, then, at equilibrium after NH_3 penetration, where K is the dissociation constant of NH_4^+,

$$\frac{K}{[H^+]} = \frac{NH_3 - a + b}{NH_4^+ - b} \qquad (1)$$

($K = 10^{-9.25}$). But prior to NH_3 penetration

$$\frac{K}{[H^+]} = \frac{NH_3}{NH_4^+} \tag{2}$$

Then by substituting Eqn. 2 into Eqn. 1 and simplifying, the following relation is obtained.

$$a = b\left(1 + \frac{K}{[H^+]}\right) \tag{3}$$

Thus, it can be readily seen from Eqn. 3 that at pH 7 or below, the H^+ ions released from NH_4^+ differs from NH_3 uptake by less than 1%. It is also to be noted that titration of proton release affords a convenient and sensitive method of quantitative measurement of selective NH_3 penetration and can be extended to the selective penetration of amines in general.

In order to substantiate a process of non-ionic diffusion of NH_3 into the cells, it was of interest to demonstrate the reversibility of NH_3 penetration. For this purpose, NH_3 was first introduced into the cells by suspension in an NH_4Cl solution at pH 7 and the quantity taken up was measured by titration of H^+ ions as described above. The cells were then centrifuged and resuspended in water. Alkali appeared immediately in the extracellular medium as indicated by a rise in pH above that of the original suspension, and the amount of alkali appearing was then measured by titration with HCl to pH 7.0. The cells were centrifuged and resuspended in water three more times and the alkali appearing each time was titrated with HCl to pH 7.0.

TABLE I

THE REVERSIBILITY OF SELECTIVE NH_3 PENETRATION

Cells (1.0-ml stock) at pH 7.0 were loaded with intracellular NH_4^+ by suspension in 0.02 M NH_4Cl at pH 7.0 in a total volume of 5.0 ml. The quantity of H^+ ions appearing (total NH_3 uptake) was measured by titration to pH 7.0. The cells were then centrifuged and resuspended in 5.0 ml distilled water repeatedly. The release of base after each resuspension was measured by titration to pH 7.0 with HCl.

Total intracellular NH_4^+ (μmoles)	*Number of resuspensions*	*Amount of base released (cumulative) (μmoles)*
6.8	1	2.2
6.8	2	3.0
6.8	3	4.0

The results are shown in Table I. They indicate that free NH_3 is freely diffusible across the cell membrane from the inside, although complete recovery was not obtained under the conditions used.

The effect of extracellular pH and NH_4^+ concentration on NH_3 uptake

The relationship between NH_3 uptake and free extracellular NH_3 concentration was determined at pH 5.5 and pH 6.5. In order to provide the same range of extracellular free NH_3 concentration at the two pHs, the concentrations of extracellular NH_4Cl used at pH 5.5 were 10-fold greater than those at pH 6.5. The results are shown in Fig. 2 and it can be seen that the two curves are virtually identical. This means

that the extracellular pH together with NH_4^+ ions influence the NH_3 uptake only insofar as they determine the concentration of extracellular free NH_3. Thus, NH_3 is the penetrating species while NH_4^- and H^+ penetrate the membrane only at a relatively slow rate, if at all, under these conditions.

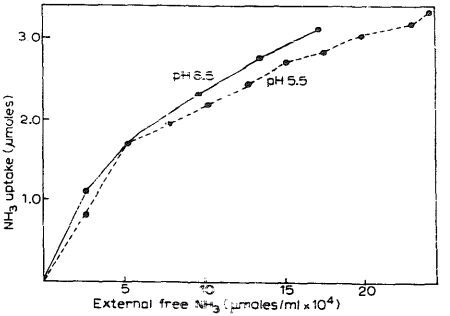

Fig. 2. The effect of extracellular pH and NH_4^- concentration on NH_3 uptake. A constant amount of cells (1.0-ml stock) at pH 6.5 was suspended in a series of NH_4Cl solution at pH 6.5 ranging in concentration from $1.6 \cdot 10^{-4}$ M to $1.4 \cdot 10^{-2}$ M. Similarly, cells were suspended in NH_4Cl solutions at pH 5.5 ranging in concentration from $1.6 \cdot 10^{-3}$ M to $1.4 \cdot 10^{-2}$ M. The H^+ ion release (total NH_3 uptake) was measured by titration and plotted against the extracellular free NH_3 concentration at equilibrium.

The passive accumulation of NH_4^- ions

Cells which have taken up free NH_3 from extracellular NH_4^+ as measured by titration of the H^+ ion release may contain a concentration of total NH_4^+ many fold greater than the extracellular NH_4^- concentration (Table II). The accumulation takes place very rapidly and in the absence of energy metabolism. This finding indicates that the intracellular pH initially was less than pH 7 (the extracellular pH) since the lower intracellular pH constitutes the driving force for accumulation against a concentration gradient as pointed out by JACOBS[1] in connection with studies on

TABLE II

THE PASSIVE ACCUMULATION OF NH_4^-

S. fecalis cells (1.0-ml stock) were suspended in solutions of NH_4Cl at pH 7.0 and the total NH_3 uptake was measured by titration of H^- appearance at pH 7.0. The intracellular concentration of NH_4^+ was calculated on the basis of an intracellular volume of NH_4^+ distribution estimated to be 0.7 of the wet cell weight. It is assumed that practically all NH_3 taken up is converted to NH_4^- by reaction with intracellular acid (see text).

Extracellular NH_4^- (μmoles/ml)	*Accumulation ratio* $\frac{(Intracellular\ NH_4^+)}{(Extracellular\ NH_4^+)}$
0.85	19
1.4	15
2.8	8

erythrocytes. In the case of bacteria, osmotic swelling is prevented by the rigid cell wall.

Theory of NH_3 uptake

A series of cell suspensions adjusted to pH 7.0 were mixed with various concentrations of NH_4Cl also previously adjusted to pH 7.0, and the NH_3 uptake was determined in each case by titration of H^+ ion release. Two different levels of cell concentrations were employed. The results are shown in Fig. 3 as a plot of NH_3 uptake against extracellular free NH_3 concentration at equilibrium. The latter values

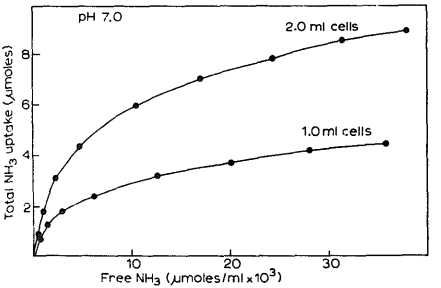

Fig. 3. NH_3-uptake curves for two different amounts of cells suspended in NH_4Cl solutions at pH 7.0. The abscissa is the free NH_3 concentration at equilibrium taking into account the NH_3 taken up. NH_3 uptake was measured by titration of H^+ ion release.

were calculated from the known extracellular NH_4Cl concentration, the pH (7.0) and pK (9.25), taking into account the NH_3 taken up. The non-linear relation between NH_3 uptake and extracellular NH_3 concentration shown in Fig. 3 can be accounted for by the following theoretical treatment. Let: $[NH_3]_e$, extracellular NH_3 concentration after equilibration of cells with NH_4Cl at constant pH; $[NH_3]_i$, intracellular NH_3 concentration; $[NH_4^+]_i$, intracellular NH_4^+ concentration; pH_i°, initial intracellular pH; $[H^+]_i^\circ$, initial intracellular H^+ concentration; pH_i, final intracellular pH; μ, total amount of NH_3 uptake; β, intracellular buffer capacity ($dpH/d\mu$) (assumed constant); I, intracellular volume of distribution of NH_3 (assumed constant); K, acid dissociation constant of NH_4^+ ($10^{-9.15}$).

The findings described in the previous sections indicate that free NH_3 rapidly equilibrates across the cell membrane by simple diffusion while H^+ and NH_4^+ are comparatively impermeable. Then at equilibrium,

$$[NH_3]_i = [NH_3]_e \tag{4}$$

The actual free intracellular NH_3 is only a small fraction of the total uptake of NH_3 (μ) since a major portion of the total NH_3 taken up may be assumed to react with intracellular acid to form intracellular NH_4^+ ions. This intracellular acid–base reaction results in a rise in intracellular pH according to the following equation:

$$pH_i = pH_i^\circ + \beta\mu \tag{5}$$

If we assume that pH_i° is two units or more below the pK (9.25), then 99 % or more of the NH_3 taken up is converted to intracellular NH_4^+ and therefore

$$[NH_4^+]_i = \frac{\mu}{v} \tag{6}$$

The Henderson-Hasselbach equation for the NH_4^+ system inside the cells then can be written as

$$pH_i = pK + \log \frac{[NH_3]_i \cdot v}{\mu} \tag{7}$$

Substituting Eqns. 4 and 5 into Eqn. 7 gives:

$$pH_i^\circ + \beta\mu = pK + \log \frac{[NH_3]_e \cdot v}{\mu} \tag{8}$$

Finally, rearrangement gives

$$\log \frac{[NH_3]_e}{\mu} = \log \frac{K}{v[H^+]_i^\circ} + \beta\mu \tag{9}$$

Eqn. 9 predicts a linear relation when $\log[NH_3]_e/\mu$ is plotted against μ. It also demands that β, the slope of this line, which is the total buffer capacity of the amine space should be inversely proportional to the amount of cells used. Moreover, the intercept $\log K/v[H^+]_i^\circ$ obtained by extrapolation of μ to zero contains v, the volume of distribution of total amine, and therefore its value should change by $+\log 2$ when the amount of cells is doubled. Fig. 4 shows a plot of $\log[NH_3]_e/\mu$ *versus* μ, using the data previously shown in Fig. 3, for 1.0 ml and 2.0 ml of cells. Satisfactory linear plots were obtained. Furthermore, the values of the slope and the intercept at the two different cell concentrations presented in Table III are also in satisfactory agreement with the prediction of Eqn. 9. It should be noted that Eqn. 9 applies generally to all weak bases which penetrate selectively by non-ionic diffusion.

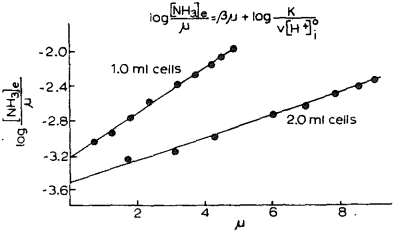

Fig. 4. Test of theory of NH_3 uptake. The experimental values of the NH_3 uptake in μmoles (μ) and free extracellular NH_3 concentration (μmoles/ml) are taken from the data of Fig. 3. The values of the slopes (β) and the intercepts are given in Table III.

Selective penetration of monomethylamine, dimethylamine and trimethylamine

These amines were found to penetrate solely in the form of the uncharged amine rather than as the quarternary ion. This was established by the finding that these

TABLE III

TEST OF NH_3 UPTAKE THEORY

Numerical values for the slopes (buffer capacities) and intercepts of Eqn. 9 for 2 different quantities of cells were determined by the method of least squares using the data presented in Fig. 3 and Fig. 4. See text for meaning of symbols.

	1.0-ml stock cells	*2.0-ml stock cells*	
Slope (β)	0.251	0.124	Predicted ratio: 2.00 Experimental ratio: 2.02
Intercept $\log K/r[H^+]_1^c$	−3.228	−3.493	Predicted difference: 0.301 Experimental difference: 0.265

amines produced an abrupt appearance of extracellular H^+ ions when added to the cells at pH 7 just as in the case of NH_3. Furthermore, the experimentally observed relation between H^+ ion release (amine uptake) and extracellular free base concentration was in excellent agreement with the theory formulated for ammonia uptake described in the previous section. This agreement with the theory is illustrated with methylamine in Fig. 5 which shows a linear relation in conformity with Eqn. 9.

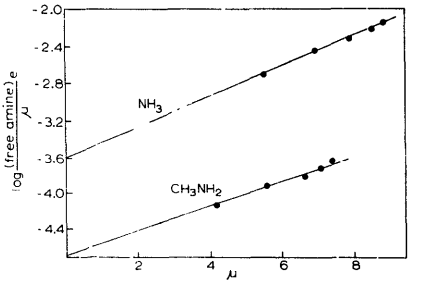

Fig. 5. Methylamine uptake (μ) in micromoles. Free extracellular methylamine concentration (μmoles/ml) plotted according to Eqn. 9. The measurements were carried out with 2.0 ml of stock cells at pH 7.0. The plot of the data for NH_3 is also shown for comparison of the intercepts.

It is to be noted particularly in Fig. 5 that the line for methylamine is parallel to the line for NH_3 as it should be since the value of β, the buffer capacity of the cells is the same. Furthermore, the difference in the values of the intercepts on the ordinate for NH_3 uptake and methylamine uptake is reasonably close to the difference in their pK values, 9.25 and 10.62 respectively, as Eqn. 9 predicts.

Kinetics of glycolysis in fresh and aged cells

The typical kinetics of glycolysis at a constant pH of 7.0 in freshly harvested cells and in aged cells (see METHODS) are shown in Fig. 6. As can be seen, in aged cells glycolysis is markedly retarded for no acid production from glucose can be detected for about 10 min after addition of glucose but then acid production from glucose commences and gradually accelerates until the glucose is completely consumed.

Glycolysis in the fresh cells on the other hand proceeds almost immediately (a lag less than 1 min) at a constant and rapid rate. In both cases the amount of acid produced after addition of glucose, when corrected for the acid appearing in the absence of glucose, is close to (sometimes slightly greater than) two moles per mole of glucose expected for the glycolytic breakdown of glucose to lactic acid[7].

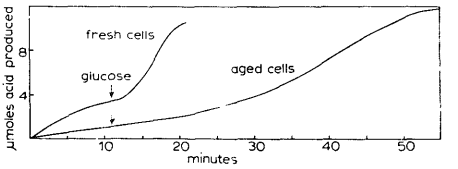

Fig. 6. Comparison of glycolysis in fresh and aged cells. The rate of H^+ ion production was measured at pH 7.0; 1.0-ml stock cells were used. Glucose (2 μmoles) was added to the aged cells and fresh cells 11 min after the cell suspension was adjusted to pH 7.0. Note the difference in H^+ ion released prior to the addition of glucose.

Inspection of Fig. 6 shows that prior to the addition of glucose, fresh cells and aged cells release H^+ ions into the medium when the pH is maintained at 7.0 by the pH-stat device. However, the release of H^+ from the fresh cells before glycolysis is initiated is quite rapid while the release from aged cells is relatively slow. This observation provides an explanation for the long lag in the glycolysis kinetics of aged cells compared to absence of lag in fresh cells, if it is assumed that excessive amounts of H^+ ions initially present within the cells are inhibitory to glycolysis and that this inhibition is relieved when excessive intracellular H^+ ions are removed from the cell. Evidence to support this view was obtained by an experiment shown in Fig. 7 in which aged cells were allowed to incubate before addition of glucose at a constant pH of 7 for a few hours during which time about 5 μmoles of H^+ ions were released from the cells. Glucose was then added, and glycolysis was now found to proceed

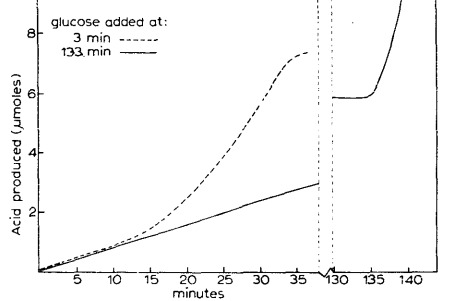

Fig. 7. Restoration of glycolysis in aged cells by pre-incubation at pH 7.0. 1.0 ml of stock cells were used. Glucose (2 μmoles) was added at the times indicated.

with no lag at a rapid rate exactly as in fresh cells. Further evidence for the inhibition of glycolysis by low intracellular pH comes from experiments described in the next sections showing restoration of glycolysis in aged cells by NH_4^+ alkylammonium, K^+ and Na^+ ions.

The restoration of glycolysis in aged cells after selective penetration of NH_3 and alkylamines

The effect of NH_4Cl and trimethylammonium chloride on glycolysis in aged cells at pH 7 is shown in Fig. 8 and Fig. 9. The cells were mixed with the test compounds, the H^+ ions appearing almost instantaneously due to selective penetration were titrated, and within a few minutes glucose was added. It is evident that glycolysis in the aged cells was completely restored immediately following selective penetration of NH_3 or trimethylamine. The same result was obtained with the other alkylamines

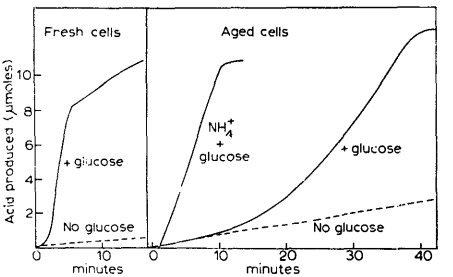

Fig. 8. Restoration of glycolysis in aged cells by NH_4Cl. Glucose (5.2 μmoles) was added at zero time to water suspensions of fresh cells, aged cells, and aged cells plus 0.01 M NH_4Cl, all at pH 7.0; 1.0-ml stock cells were used. Controls without glucose are shown by dashed lines. In the case of cells treated with NH_4Cl, the H^+ released due to NH_3 penetration were titrated a few minutes prior to the addition of glucose.

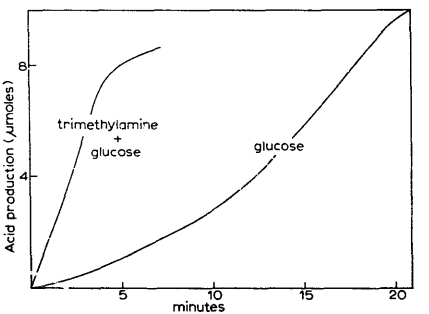

Fig. 9. Restoration of glycolysis in aged cells by trimethylamine. Glucose (4 μmoles) was added at zero time to a water suspension of aged cells, and aged cells plus 0.004 M trimethylamine hydrochloride, at pH 7.0; 1.0 ml of stock cells were used. The H^+ ions released due to selective penetration of trimethylamine was titrated a few minutes prior to the addition of glucose.

tested, $(CH_3)_2NH$ and $(CH_2)NH_2$. Since the selective penetration of free base raises the intracellular pH, these results are in accord with the idea that glycolysis in aged cells is inhibited by low intracellular pH and the restoration of glycolysis in aged cells is brought about by raising the intracellular pH. It is of interest to mention that Tris showed no selective penetration and did not stimulate glycolysis.

The restoration of glycolysis in aged cells by K^+ and Na^+ ions

The effect of K^+ and Na^+ ions on glycolysis in aged cells at pH 7 is shown in Fig. 10. It is clear that both K^+ and Na^+ ions restore glycolysis in aged cells. Fig. 10 also shows that K^+ and Na^+ ions elicited a gradual efflux of H^+ ions from the cells before glucose was added, with K^+ about twice as effective as Na^+. This is to be compared with the immediate appearance of H^+, also shown in Fig. 10, after selective penetration of NH_3. These findings, like those described in the previous two sections, indicate that glycolysis in aged cells is restored when excess intracellular H^+ ions are removed. If this view is correct, then the effect of K^+ and Na^+ on glycolysis

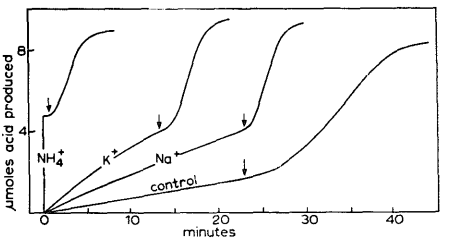

Fig. 10. Restoration of glycolysis in aged cells by K^+ and Na^+. Glucose (2 µmoles) was added at the time indicated by the arrows to aged cells suspensions containing 0.016 M KCl, NaCl, NH_4Cl and the pH was maintained at pH 7.0 by continuous titration with NaOH; the control contained no added salt; 1.0 ml of stock cells were used. Note the differences in rate of H^+ ion release produced by K^+, Na^+ and NH_4^+ prior to the addition of glucose.

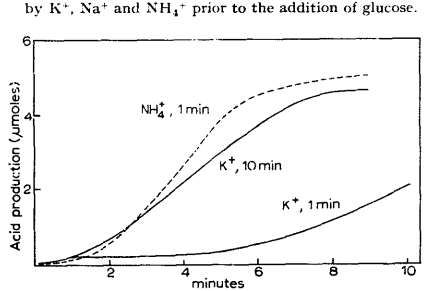

Fig. 11. The immediate effect of NH_4^+ and delayed effect of K^+ on glycolysis in aged cells. Glucose (2 µmoles) was added to aged cells at zero time, following previous preincubation with 0.0016 M NH_4Cl for 1 min and previous preincubation with 0.0016 M KCl for 1 min and 10 min; the pH was maintained at 7.0 throughout by continuous titration with NaOH; 1.0 ml of stock cells were used. The H^+ release in controls without glucose was subtracted.

should be strongly dependent on the time of preincubation with K^+ or Na^+ over that period of time during which they cause the gradual displacement of H^+ ions from cells. Therefore, an experiment was carried out in which glucose was added and the glycolysis rate measured after 1 min and after 10 min preincubation of the cells with K^+. For comparison, the effect of NH_4^+ after 1 min preincubation was also determined. The results, presented in Fig. 11, show that restoration of glycolysis was brought about after 10 min preincubation with K^+ but essentially no effect was observed after only 1 min preincubation with K^+. By contrast, NH_4^+, after only 1 min, completely restored glycolysis which is consistent with the practically instantaneous selective penetration of free NH_3 described earlier.

DISCUSSION

The extremely rapid, practically instantaneous, appearance of H^+ ions when non-glycolyzing *S. fecalis* cells come in contact with NH_4^+ or certain alkylammonium ions contrasts sharply with the relatively slow release of H^+ ions elicited by K^+ or Na^+ (Fig. 10) and reflects a fundamental difference in the mode of interaction of the two classes of monovalent cations with the cells. As the experimental findings indicate, the weak nitrogenous bases penetrate the cells by a process of non-ionic diffusion and extracellular H^+ ions arise directly by dissociation from the extracellular ammonium ions. On the other hand, in the case of the action of K^+ and Na^+ the H^+ ions obviously come from the cells. The process is likely to be one of K^+–H^+ or Na^+–H^+ exchange (at constant extracellular pH) without dependence on energy metabolism since the rate of H^+ ion release evoked by K^+ was about twice as fast as that with Na^+ and no source of energy was added (Fig. 10). K^+–H^+ and Na^+–H^+ exchanges have been observed to occur in yeast but only during glycolysis[12,13], and such a process was also suggested from studies of the K^+ requirement for the growth of *Bact. lactis aerogenes*[14].

A rise in intracellular pH is to be expected from either selective amine penetration or, H^+ "ion exchange" with K^+ or Na^+, and, on the assumption that a low intracellular pH inhibits glycolysis, accounts for the restoration of glycolysis in aged cells brought about, to the same extent, by ammonium, alkylammonium, K^+ and Na^+ ions. In support of the view that these cations stimulate glycolysis indirectly via a rise in intracellular pH is the abruptness with which the amines restored glycolysis in aged cells compared to the delayed restoration by K^+ (Fig. 11) since, as already pointed out, the intracellular pH is increased rapidly by the amines and much more slowly by K^+. It should be stressed that in these experiments the extracellular pH is maintained constant by means of the pH-stat machine. While it is known that NH_4^+ and K^+ ions specifically activate phosphoenolpyruvate transferase[15] and phosphofructokinase[16], Na^+ ions on the other hand are inhibitory to these enzyme activities. Thus, the observed restoration of glycolysis by Na^+ and various alkylammonium compounds as well as by K^+ and NH_4^+ under our particular conditions is not due to direct specific activation of the particular aforementioned glycolytic enzymes.

It was shown that fresh and aged cells both initially contain acid which leaks rapidly from the former and slowly from the latter when the cells are suspended in H_2O and maintained at pH 7 (Fig. 10). The reason for this difference between fresh and aged cells is not known at present. Nevertheless, it provides an explanation for

the rapid glycolysis in fresh cells and retarded glycolysis in aged cells again on the basis of an inhibition of glycolysis by the lower intracellular pH in aged cells. Furthermore, if a low intracellular pH is responsible for retarded glycolysis in aged cells, then clearly the gradual loss of excess endogenous H^+ from the aged cells while glycolysis is going on gradually relieves the inhibition and thus accounts for the S shaped kinetics of glycolysis characteristic of these cells. That excess acid initially present within the aged cells is responsible for retarding glycolysis was most convincingly demonstrated by an experiment showing complete restoration of glycolysis following a long period of incubation at pH 7 with loss of intracellular acid prior to the addition of glucose (Fig. 7).

From measurements of NH_3 uptake into bacterial cells, the theory of NH_3 uptake as expressed in Eqn. 9 permits, in principle, calculation of the intracellular pH (more precisely, the pH of the NH_3 space). The intracellular pH is contained in the value of the intercept at zero NH_3 uptake (Table III) and may be calculated provided the volume of the NH_3 space is known. Unfortunately, the volume of the NH_3 space is not known but if we assume as a reasonable first approximation that it is about 70% of the wet weight of the cells, then the data in Table III yield an intracellular pH of about 5.0.

The theory of NH_3 uptake also permits an evaluation of the intracellular (NH_3 space) buffer capacity (Table III) and thus it can be calculated that the intracellular pH of the NH_3 space increases by 0.25 pH unit for every micromole of free NH_3 (or free alkylamine) which penetrates into 1.0 ml of stock cells (approx. 15–20 mg dry wt.).

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