JOURNAL OF BACTERIOLOGY, Jan. 1974, p. 189-195
Vol. 117, No. 1

Printed in U.S.A.

Induction of Morphogenesis in *Geodermatophilus* by Inorganic Cations and by Organic Nitrogenous Cations

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Received for publication 4 June 1973

Morphogenesis of *Geodermatophilus* strain 22-68 involves two stages, a motile rod (R form) and an irregularly shaped cluster of coccoid cells (C form). A variety of mono- and divalent cations have been found to induce R-form to C-form morphogenesis and to maintain the organism in the C form. Concentration optima for all cations exceeded 100 mM. Results indicated that uptake of cations was accompanied by extrusion of intracellular protons, causing an increase in intracellular pH. A variety of organic amines also induced morphogenesis. Organic amines were taken up in the dissociated free base form, causing the intracellular pH to rise. None of these compounds was utilized as a carbon or nitrogen source.

The actinomycete *Geodermatophilus* exhibits a complex morphogenetic growth cycle involving two major forms, a motile budding-rod (R form) and a nonmotile, irregularly shaped aggregate of coccoid cells (C form). Recently, we demonstrated synchronous morphogenesis of this organism in undefined media and characterized the growth cycle by electron microscopy (5). We reported that cellular differentiation was controlled by an unidentified factor present in one medium constituent, tryptose (Difco). This factor was required for morphogenesis of the R form to the C form. Furthermore, in the presence of the factor, growth was exclusively in the C form, suggesting that it also was required for maintaining the organism in the C form. This was supported by the observation that removal of tryptose resulted in C-form to R-form differentiation. During attempts to identify the tryptose factor, we found that a variety of inorganic cations and organic nitrogenous cations will control morphogenesis of *Geodermatophilus*.

MATERIALS AND METHODS

Geodermatophilus sp. strain 22-68 was used exclusively in this study. Cultural conditions and compositions of media CB and TYB have been described previously (5). Medium CB was modified by increasing the K^+ (medium CBK) or the Na^+ (CBNa) concentrations to values designated in the text. Medium A was developed in the present study and is a chemically defined medium lacking the factor which is required for maintenance of the C form and for R-form-to-C-form morphogenesis. Morphological characteristics of strain 22-68 in medium A were therefore identical to those reported previously for medium CB (5). Stock solutions for medium A were prepared in glass-distilled water as follows. Amino acid stock solution consisted of (per 200 ml of water): L-methionine, 0.4 g; L-leucine, 1.0 g; and monosodium L-glutamate, 4.0 g. Vitamin stock solution consisted of (per liter of water): biotin, 2 mg; thiamine-hydrochloride, 5 mg; and cyanocobalamin, 0.1 mg. Stock solution A-2X contained 0.4% (wt/vol) L-asparagine, 0.1% (wt/vol) $MgSO_4$, 1.0% (vol/vol) trace mineral solution (9), 1.0% (vol/vol) vitamin stock solution, 0.5% (vol/vol) amino acid stock solution, and 40 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.0). All solutions were stored frozen. Medium A was prepared by diluting solution A-2X with an equal volume of glass-distilled water followed by steam sterilization. Glucose (20% wt/vol) was sterilized separately and added aseptically to the medium at a final concentration of 0.2%. Medium AK and medium ANa were modifications of A in which K^+ and Na^+ concentrations, respectively, had been increased as designated in the text.

Cell dry weights were estimated turbidimetrically at 660 nm from standard curves prepared with washed exponential phase R-form and C-form cells. These plots were identical for both forms and were linear up to an optical density reading at 660 nm (OD_{660}) of 0.700 (an OD_{660} of 0.700 representing 0.85 mg of dry cells per ml).

Two systems, designated I and II, were devised to assay compounds for ability to induce morphogenesis in *Geodermatophilus*. The medium in both cases was prepared as follows. The amount of each compound to be tested was contained in less than 0.2 ml. The appropriate volume was transferred to a 5-ml flask. Medium CB was added to give a final volume of 2 ml,

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and the pH was adjusted to 7.0 with NaOH. Two additional flasks (one contained 2 ml of medium CB and the other contained 2 ml of medium TYB) served as controls. Medium A was used in place of medium CB when a defined medium was desirable. Media were inoculated as described below.

System I was a qualitative assay for compounds which could maintain strain 22-68 in the C form. C-form cells cultured in 25 ml of medium TYB for 3 to 5 days were used as inocula. Cells were harvested aseptically, washed three times with sterile distilled water, and suspended in water to an OD_{660} of 0.2 to 0.3. A sample of 0.01 ml was used to inoculate each portion of assay media. After 24 h of incubation at 30 C in a water bath shaker, a sample was examined with a microscope with a Petroff-Hausser counting chamber, a minimum of 300 cells being counted in each sample. When compounds reported in the text were used at optimal concentrations, better than 95% of the cells were in the easily distinguishable C form at this time.

System II was designed to assay for compounds which induced R-form to C-form morphogenesis. R-form inocula cultured in medium CB for 2 to 5 days were harvested by centrifugation and resuspended in distilled water to an OD_{660} of 0.2 to 0.3. Samples of 0.01 ml were inoculated into the assay medium. The percentage of cells in the C form was determined with a Petroff-Hausser counting chamber after 48 h of incubation. Since the inducers which were tested prevented significant increase in colony-forming units over this period (see below), system II provides a quantitative estimation of differentiation.

The abbreviation [Me]i, where Me represents an inorganic cation, stands for the intracellular concentration of that cation. Extracellular concentration of a cation is designated by [Me]e. $[Na^+]i$ and $[K^+]i$ were determined on 5% trichloroacetic acid extracts of cells prepared by the method of Harold et al. (4). Appropriate dilutions of extracts were analyzed for Na^+ and K^+ by atomic absorption spectrophotometry (Perkin-Elmer model 303). Extracellular cation concentrations were determined on appropriate dilutions of media.

Starved cells used in a few experiments were prepared by aseptically harvesting exponential-phase R-form cells grown in medium A. Cells were washed three times with distilled water, resuspended in medium A without carbon or nitrogen sources, and incubated at 30 C for 24 to 48 h. After this period, cells were harvested, washed three times, and resuspended in 0.01 M Tris-hydrochloride (pH 7.0). No loss of viability occurred during the starvation period. (We found that such starved cells apparently had sufficient endogenous reserves to support energy-dependent transport in the absence of added energy sources.) Therefore, washed exponential-phase cells were used without the starvation treatment in most experiments.

Specific conditions for certain uptake experiments are described in the text.

All work was performed at 24 C. Cells from samples taken at designated intervals were collected by rapid membrane filtration (Millipore filter type HAWP 025). Cells on filters were washed three times with ice-cold distilled water and were treated and analyzed by atomic absorption spectrophotometry as described above.

Acidification of the external medium upon uptake of inorganic cations or organic amines was followed by continuous titration with a pH meter (model E-300, Metrohm Ltd., Herisan, Switzerland) combined with a recording pH-stat (Impulsomat model E-373; Dosigraph model E-364, Metrohm Ltd.). Experiments were performed by the method of Zarlengo and Abrams (10), except that an energy source for the cells was not added to the reaction mixture; as noted above, *Geodermatophilus* apparently has sufficient endogenous reserves to support uptake of compounds. $[H^+]i$ was estimated by the method of Harold et al. (3).

All inorganic compounds were reagent grade salts obtained from commercial sources. Organic nitrogenous cations were purchased from Eastman Organic Chemicals, with the exception of Tris (Sigma Chemical Co.).

RESULTS

Inorganic cations were tested for ability to induce R-form-to-C-form morphogenesis. Results for chloride salts are presented in Table 1. Effects of sulfate and acetate salts were identical. Typical examples of the assay are shown in Fig. 1. The optimal concentrations are clearly represented by micrographs e, h, k, and n. The apparent nonspecificity of inorganic cations was striking, and there was no relationship between valence of the cationic species and ability to induce morphogenesis. Of those tested, Fe^{3+}, Co^{2+} and Mn^{2+} inhibited growth at concentrations higher than 5 mM and failed to function as inducers. A small but significant amount (18 to 25%) of induction was observed with 25 mM

TABLE 1. *Induction of R-form to C-form morphogenesis by inorganic cation*

Cation[a]	Optimal concn (mM)	Morphogenesis[b] (%)
NH_4^+	100	100
Li^+	125	82–95
Na^+	100	97–98
Rb^+	150	73–86
K^+	150	88–92
Cs^+	25	18–25
Ca^{2+}	200	62–93
Mg^{2+}	125	83–90
Sr^{2+}	125	97–98

[a] Chloride salts were used.

[b] Inducing activity was determined by assay system II. Percentage of differentiated cells was determined after 48 h of incubation, and the range obtained in three experiments is presented.

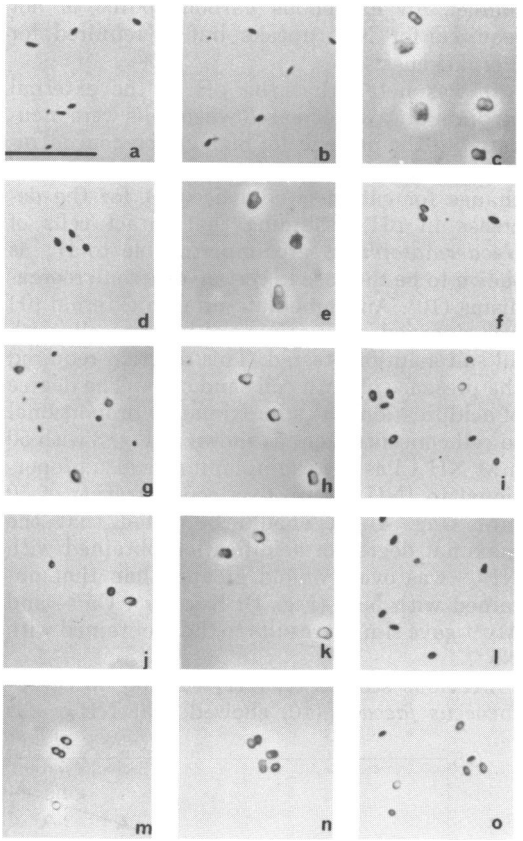

FIG. 1. *Typical example of assay by system II. (a) Inoculum cells at zero time. All other micrographs represent typical microscope fields after 48 h of incubation. (b) Control in medium CB; (c) control in medium TYB; (d, e, f) NH_4Cl at 25, 100, and 150 mM; (g, h, i) NaCl at 40, 100, and 125 mM; (j, k, l) KCl at 75, 150, and 175 mM; (m, n, o) $MgCl_2$ at 75, 125, and 150 mM. Bar indicates 10 μm.*

Cs^+, but complete inhibition of growth occurred at higher concentrations. Although growth was restored at lower concentrations, no morphogenesis resulted. Inefficiency of Fe^{3+}, Co^{2+}, Mn^{2+}, and Cs^+ as inducers was apparently due to their toxicity, since inhibition of growth also resulted in abnormal, rounded cells resembling spheroplasts. With these exceptions, it should be noted that concentration optima of other cations were high (100 mM or greater). Although they also caused retardation of growth, they were highly efficient inducers. The resulting C-form cells were ultrastructurally identical to those induced with tryptose.

Na^+, NH_4^+, and K^+ were tested for ability to maintain *Geodermatophilus* in the C form by assay system I. All three were active at a concentration of 100 mM.

The effects of increased extracellular cation concentrations on the cation contents of differentiating cells were studied by comparing the Na^+ and K^+ contents of exponential-phase cells cultured in different media (Table 2). In experiment 1, $[Na^+]e$ and $[K^+]e$ were varied in the defined medium, A, and the effects on morphogenesis and intracellular cation concentrations were determined. R-form cells growing exponentially in medium A had $[Na^+]i$ and $[K^+]i$ of approximately 29 and 567 μmol/g dry cell weight, respectively. Morphogenesis to the C form resulted when $[Na^+]e$ was increased to 100 mM, and a 10-fold increase in $[Na^+]i$ was observed. However, the changes were less dramatic when the inducer was 100 mM K^+. $[K^+]i$ increased by less than 1.1-fold, whereas $[Na^+]i$ decreased 1.7-fold. In experiment 2, chemically undefined media were used. $[Na^+]i$ and $[K^+]i$ of R-form cells growing exponentially in CB were approximately 339 and 350 μmol/g dry cell weight, respectively. These cells were therefore enriched for Na^+. Their $[Na^+]i$ was over 10-fold greater than that of R-form cells cultured in medium A and was approximately equal to $[Na^+]i$ of C-form cells grown in medium ANa. These results indicated that high $[Na^+]i$ alone is insufficient to cause induction of morphogenesis, and that $[Na^+]i$ is regulated by $[Na^+]e$, since $[Na^+]e$ of medium CB is greater than that of medium A. Increasing the $[Na^+]e$ of CB to 100 mM resulted in morphogenesis to the C form and slight increases in both $[Na^+]i$ (1.2-fold) and $[K^+]i$ (1.3-fold). With 100 mM K^+ as the inducer, a 2.4-fold increase in $[K^+]i$ and a 1.9-fold decrease in $[Na^+]i$ resulted.

TABLE 2. *Effect of varying extracellular cation concentrations on intracellular Na^+ and K^+ content*

Expt no.	Cell type	Medium	Concn in medium (mM)		$[Na^+]i$ (μmol/g dry cell wt)	$[K^+]i$ (μmol/g dry cell wt)
			Na^+	K^+		
1[a]	R	A	7	4	28.8	566.6
	C	ANa	100	4	281.8	602.5
	C	AK	7	100	16.5	617.9
2[b]	R	CB	27	33	338.7	349.9
	C	CBNa	100	33	418.4	467.2
	C	CBK	27	100	182.5	825.0

[a] R-form cells cultured in medium A were inoculated into the various media. Samples were removed after 4 days of incubation for determination of $[Na^+]i$ and $[K^+]i$ by atomic absorption spectrophotometry.

[b] R-form cells cultured in CB were inoculated into the various media. Determinations of $[Na^+]i$ and $[K^+]i$ were made after 4 days of incubation.

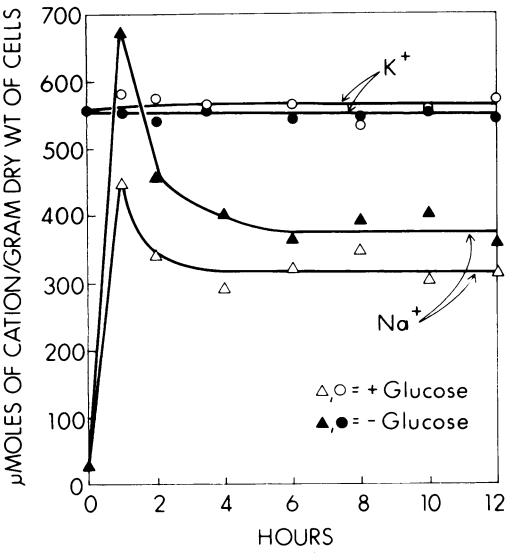

FIG. 2. *Uptake of Na^+ in presence and absence of exogenous carbon source. Starved R-form cells were suspended in 0.01 M Tris-hydrochloride (pH 7.1) (closed symbols) or in medium A (open symbols) at initial density of 0.1 mg of dry cell weight per ml. NaCl (75 mM) was added to both flasks at zero time.*

Thus, a significant change in K^+ content was demonstrated for differentiating cells in medium CBK. By comparison, $[Na^+]i$ of cells differentiating in medium TYB increased less than 1.1-fold, whereas $[K^+]i$ increased 1.6-fold. Results from these experiments suggested that inducing cations were taken up by differentiating cells but that induction was not solely dependent on high intracellular cation concentrations.

The effects of high $[Na^+]e$ on cellular Na^+ content were studied further with starved R-form cell suspensions. A cell suspension (0.1 mg dry cell weight per ml) was divided equally, one-half being suspended in 0.01 M Tris (pH 7.1); the other half was diluted similarly in medium A. NaCl was added, to a final concentration of 75 mM to both preparations, and changes in $[Na^+]i$ and $[K^+]i$ were followed. K^+ and Na^+ movements were identical for both sets of cells (Fig. 2). $[K^+]i$ did not change during the course of the experiment, and a value of approximately 550 μmol/g of dry cells was maintained over a 12-h period. $[Na^+]i$ increased rapidly, reaching a maximum within 1 h, then decreased over a period of 3 h before finally reaching a steady-state level. The increase in probably due to counterflow. The initial stage of morphogenesis appeared after 6 h in medium A. No differentiation occurred in the absence of a carbon source. An exogenous carbon source is not required for Na^+ uptake but is required for morphogenesis.

It was noted that the pH of the external medium always decreased when cells were incubated in the presence of high cation concentrations. Accumulation of extruded H^+ in exchange for cations would account for the decrease in pH, assuming that intact cells of *Geodermatophilus* are impermeable to H^+ as shown to be the case in certain other microorganisms (10). An immediate drop in external pH was recorded upon mixing of R-form cells with all salt solutions tested. This reaction required the presence of both cells and salts. The degree of acidification was approximately proportional to cell concentration, as shown in Fig. 3 with 50 mM NH_4Cl as the example; it was also proportional to $[NH_4^+]e$ up to a concentration of 50 mM (Fig. 4). It should be noted that the maximal degree of acidification obtained with NH_4^+ was over twofold greater than that obtained with Na^+ (Fig. 4). K^+, Rb^+, Ca^{2+}, and Mg^{2+} gave similar results to those obtained with Na^+.

Previous studies on NH_4^+ uptake in *Streptococcus faecalis* (10) showed that NH_4^+ was

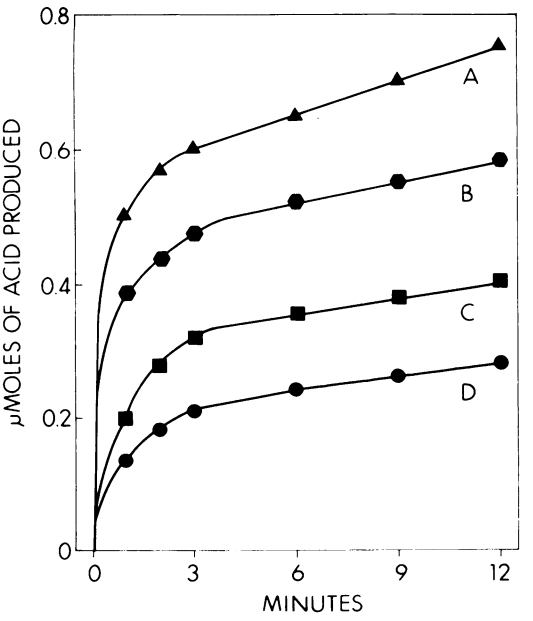

FIG. 3. *Effect of varying cell concentrations on acidification of external medium. At 0 min, R-form cells at 4 mg/ml (A), 3 mg/ml (B), 2 mg/ml (C), and 1 mg/ml (D) were mixed with 50 mM NH_4Cl in a total volume of 2 ml. The pH of the mixture was maintained at 7.0 by continuous titration with a recording pH-stat.*

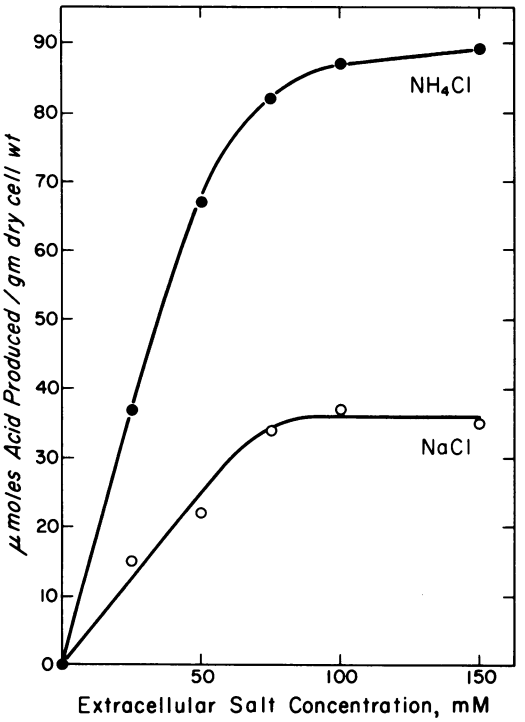

FIG. 4. *Effects of varying* $[NH_4^+]e$ *and* $[Na^+]e$ *on acidification of external medium. R-form cells (3 mg/ml) were mixed with varying concentrations of* NH_4Cl *and NaCl. The pH of the mixture was followed by continuous titration with a pH-stat until no further changes occurred (15 min in all cases). The final amount of acid produced is plotted against the initial extracellular salt concentration.*

passively transported as the free base NH_3; the dissociated H^+ remained in the external medium, causing a decrease in the pH. If this were the case in *Geodermatophilus*, organic amines with strong base properties, capable of penetrating the cell membrane, also should be taken up passively in the form of the dissociated base. Accumulation of the impermeable dissociated H^+ should, consequently, lower the pH of the culture. All organic amines tested were potent inducers of R-form-to-C-form morphogeneis (Table 3). It should be noted that medium A is normally buffered with 20 mM Tris. This consideration is insufficient to induce morphogenesis and a minimum of 50 mM is required. We have shown that NH_4^+ and organic amines were not metabolized as nitrogen or carbon sources (Ishiguro, Ph.D. thesis, University of Illinois, Urbana, 1971).

Results of continuous titration experiments with a pH-stat showed an immediate decrease in external pH upon mixing of cells with solutions of all organic amines except Tris, diethanolamine, and triethanolamine. This was expected because of the buffering properties of these compounds. To test the effect of external pH, a series of flasks which contained medium CB or medium A was prepared without added inducers. The media were adjusted to initial pH values ranging from 5.0 to 6.5, and were inoculated with R-form cells. In all cases no growth or morphogenesis occurred.

Proton extrusion or uptake of the free-base forms of NH_4^+ and organic amines should also result in an increase in intracellular pH. The effect of inducers on $[H^+]i$ was determined. Incubation of cells with inducers resulted in a decrease in $[H^+]i$ in all cases (Table 4). The results obtained for NH_4^+ and the organic nitrogenous cations may have been distorted by the washing procedure and may thus represent minimal values. Zarlengo and Abrams (10) showed that NH_3 penetration in *S. faecalis* was freely reversible and that intracellular NH_3 could easily be removed by washing.

DISCUSSION

In attempts to find a common mode of action for the wide variety of inorganic cations capable of inducing morphogenesis in *Geodermatophilus*, we found that uptake of these compounds was accompanied by extrusion of H^+ in all cases. This phenomenon is well established in other microorganisms (2). The efflux of H^+ did not appear to be stoichiometric. Although we have not studied this in detail, incoming cations probably also exchange for other intra-

TABLE 3. *Induction of R-form to C-form morphogenesis by organic amines*[a]

Compound	Optimal concn (mM)	Morphogenesis (%)
Methylamine-hydrochloride	200	98–100
Dimethylamine-hydrochloride	150	76–88
Trimethylamine-hydrochloride	100	81–84
Diethylamine-hydrochloride	25	99–100
Triethylamine-hydrochloride	17.5	96–100
Butylamine-hydrochloride	10	89–96
Ethanolamine-hydrochloride	40	93–96
Diethanolamine-hydrochloride	25	72–93
Triethanolamine-hydrochloride	25	88–100
Tris-hydrochloride	75	97–100

[a] Compounds were assayed by system II in CB. The percentage of differentiated cells was determined after 48 h, and the results presented represent the ranges obtained in four experiments.

TABLE 4. *Effect of inorganic cations and organic amines on intracellular H^+ content*[a]

Compound added	Concn (mM)	$[H^+]i$ (μmol of H^+/g dry cell wt)
None		383
NaCl	100	212
KCl	150	235
RbCl	150	247
$CaCl_2$	200	206
$MgCl_2$	125	217
NH_4Cl	100	282
Methylamine-hydrochloride	200	263
Triethylamine-hydrochloride	20	290
Ethanolamine-hydrochloride	40	254
Tris-hydrochloride	75	292

[a] Each inducer was added to a separate portion of medium A, and the $[H^+]i$ of R-form cells was determined after 30 min of incubation in the mixture. Averages of duplicate determinations are presented. Duplicates varied by less than 8%.

cellular cationic species. In any case, cation uptake resulted in a detectable increase in intracellular pH, and we believe that this is the primary cause of morphogenetic induction. This proposal is supported strongly by the observation that organic nitrogenous cations also induced morphogenesis. *Geodermatophilus*, like *S. faecalis* (10), appeared to passively take up organic amines (and NH_4^+) in the form of the dissociated base. The dissociated H^+ remained in the medium and could be detected by a decrease in extracellular pH. The phenomena of passive uptake of dissociated base and impermeability of the dissociated H^+ make it possible to explain why the degree of acidification caused by uptake of NH_4^+ and organic amines had a more stoichiometric relationship than that caused by cation uptake (Fig. 4). Like the inorganic cations, uptake of organic amines caused an increase in intracellular pH.

Reichenbach and Dworkin (6) reported a similar nonspecific cation-induced morphogenetic system in *Stigmatella aurantiaca*. Myxospore formation in this organism was induced by a variety of monovalent cations. A mode of action was not suggested. In another example, Roberts (7) reported that sodium and potassium salts induced differentiation of *Dermatophilus* to the hyphal stage and inhibited the appearance of the motile stage in this organism. Other cations apparently were not tested. In both cases, relatively high concentrations of cations (50 mM or greater) were required for morphogenetic induction. It would be worth testing the possibility that the mode of action of cations in these systems is identical to that proposed for *Geodermatophilus* particularly in the case of the closely related organism *Dermatophilus*.

An increase in intracellular pH could have a profound influence on the metabolism of an organism. Zarlengo and Abrams (10) showed that glycolysis is stationary-phase cells of *S. faecalis* was inhibited by a low intracellular pH. Glycolysis was restored immediately upon uptake of NH_3 or organic amines. Glycolysis also was restored, although less effectively, by K^+ and Na^+ uptake. These compounds were shown to raise the intracellular pH. In addition, Tempest et al. (8) found that the glutamate content of the free amino acid pools in *Aerobacter aerogenes* increased dramatically when the organism was grown in the presence of high NaCl concentrations. Glutamate dehydrogenase activity exhibited a marked pH dependence with an optimal pH of approximately 8. Na^+ uptake was accompanied by H^+ efflux, raising the intracellular pH. It was, therefore, proposed that the increase in glutamate in the presence of NaCl was due to enhanced activity of glutamate dehydrogenase. We propose that a metabolic event necessary for cellular differentiation in *Geodermatophilus* is enhanced by raised intracellular pH.

ACKNOWLEDGMENTS

We thank Richard M. Forbes and L. Leon Campbell for use of the atomic absorption spectrophotometer and pH-stat, respectively. This work was supported by Public Health Service training grant GM 510 from the National Institute of General Medical Sciences.

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