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Intracellular pH topography of *Penicillium cyclopium* protoplasts. Maintenance of ΔpH by both passive and active mechanisms

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The intracellular pH distribution in protoplasts of *Penicillium cyclopium* has been studied using the recently developed fluorescent probe microscopic technique. The technique gives detailed pH maps of the interior of the protoplasts with the exception of vacuoles (no fluorescence signal from vacuoles was observed). In the cytoplasm two separate layers were distinguished: a thinner outer layer with acidic pH (around 5) and the larger core region with near neutral pH. The pH of the core region is decreased by the addition of uncouplers, inhibitors of respiration and during the uptake of L-phenylalanine. These compounds do not change the pH of the surface layer, which is, however, acidified by addition of vanadate, an inhibitor of the proton pump of the plasmalemma. We suggest that the pH of the surface layer is maintained by the combined effects of a Donnan distribution of protons (bound to postulated anion binding proteins) and the proton extrusion via the plasmalemma proton pump. This mechanism explains the protection of the cytoplasmic core of acidophilic eukaryotes from the influence of the usually acidic environment.

Introduction

Penicillium cyclopium acidifies the growth medium by excreting acids (mainly citric) and protons (in exchange for NH_4^+). Similarly to other acidophilic microorganisms the cell keeps the average intracellular pH approximately neutral as long as the external pH stays above a certain critical value (pH 3 in our case) [1]. The recently developed fluorescent probe technique which gives pH maps of the cell interior [2] was expected to shed some light on the nature of these pH-controlling systems. This seemed to be essential since the ΔpH between the bulk phases probably does not reflect the true proton gradient across the plasma membrane [3].

Because of the resolution limits of the optical microscope the hyphae (diameter approx. 1 μm) cannot simply give reliable data about intracellular pH distribution and therefore protoplasts (diameter approx. 10 μm) were used. The protoplasts were stable, easy to work with and as they have no cell wall no binding of fluorescein to the cell wall could interfere.

During the last few years a controversy has arisen whether the near neutral intracellular pH in acidophilic bacteria is maintained mainly by chemiosmotic or by passive means [4–8].

In the following study we present some data suggesting the combined roles of acid production,

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Mes, 4-morpholineethanesulfonic acid; TPP^+, tetraphenylphosphonium ion.

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Donnan equilibrium and active proton pumping in the pH control in an acidophilic eukaryote.

Materials and Methods

Organism and growth conditions

Penicillium cyclopium Westling SM 72 was maintained on agar slants containing 275 mM glucose, 35 mM ammonium tartrate, 0.9 mM KH_2PO_4, 0.67 mM KCl, 0.62 mM $MgSO_4$, 0.18 mM $FeSO_4$, 0.06 mM $ZnSO_4$, Cu, Co, Mn and Mo as trace elements, pH adjusted to 5.0 with H_2SO_4. After 4-day growth conidia were brushed off and inoculated into liquid media of either the same composition (submerged cultures on rotary shakers at 120 rpm) or with glucose replaced by sucrose plus glucose (both 56 mM; for emerged cultures in Petri dishes). Agar slant and derived liquid cultures intended for protoplast preparation contained 0.35 M NaCl. All cultures were grown at 24° C.

Preparation of protoplasts

After 2-day emerged growth mycelial disks, each representing 0.6 cm^2 of the culture area, were cut out and floated in Petri dishes on the surface of the protoplasting medium. The latter contained in sodium maleate buffer (50 mM, pH 4.5), 0.1 M NaCl, 0.3 M Na_2SO_4, 10 mM $CaCl_2$, 10 mM $MgCl_2$ and 10 mg/ml of the cell wall lytic enzyme preparation (see below). After 14 h incubation at 24° C protoplasts were separated from the medium and purified by flotation in a stepped density gradient made up from the following solutions (from bottom to top): I: 2 ml of protoplasting medium mixed with 4 ml 1.1 M sucrose in 50 mM Mes (pH 6.6).

II. Mixture of 3 ml 1.1 M sucrose and 7 ml 1.1 M sorbitol, both in 5 mM Mes (pH 6.6).

III. 1 ml 1.1 M sorbitol in 5 mM Mes (pH 6.6).

After centrifugation (4000 × *g*, 15 min) protoplasts collected at the interphase between layers II and III.

Preparation of cell wall lytic enzymes

Penicillium purpurogenum was cultivated on the same media as described for *Penicillium cyclopium* except that ammonium tartrate was replaced by 35 mM ammonium sulphate and glucose by 10 g/l of a cell wall preparation from *Penicillium cyclopium*. This preparation was obtained by sonication of a suspension of submerged grown hyphae (10% fresh weight) in 2% SDS solution, followed by several washings: first by centrifugation (5000 × *g*, 25 min) and resuspension in 0.5 M NaOH, than by suction filtration and resuspension in distilled water (3 times), 60% ethanol, 96% ethanol and ether. It was finally dried in the air.

After 6-day growth conidia of *P. purpurogenum* were inoculated (approx. 10^7 spores/ml) into a liquid culture medium of the above composition. Submerged cultures were grown on rotary shakers at 24° C. After 4-day growth 25-ml portions of spore derived cultures were transferred each to 250 ml of fresh medium and further cultivated. After 4-day growth a yellow-red culture filtrate was obtained to which 500 mg/l of bovine serum albumin was added. Protein was harvested by ammonium sulphate precipitation (90% saturation) followed by flow dialysis against distilled water and lyophilization of the salt free product.

Incubation of protoplasts with fluorescein diacetate and inhibitors

50 µl of a suspension of purified protoplasts was mixed with 50 µl of 100 mM sodium maleate buffer of the desired pH containing 1 M sorbitol and 27 mM glucose. To this mixture 0.5 µl of a 20 mM stock solution of fluorescein diacetate in glacial acetic acid was added 5 min prior to the microscopic examination or to the addition of inhibitor, uncoupler or transportant.

pH measurement

Fluorescein molecules liberated in situ (from fluorescein diacetate by enzymic reaction) were used as the pH-sensitive fluorescent indicator [9]. In this technique the pH-dependent ratio of the two fluorescent forms of the dye is followed using selective excitation. Two fluorescence microphotographs of the same cell are taken at different excitation wavelengths, together with a phase contrast picture as reference. Usually it took about 1 min to complete this triplet of photographs which is necessary for the determination of a pH map.

Computerized comparative densitometry of the two fluorescence pictures then yields the dif-

ferences in densities (i.e., local concentrations of the particular form of the dye) in different places, point by point, convertible through a calibration curve to the local pH values [2].

All necessary corrections required by the imperfection of the density curve of the film and the bleaching of fluorescein were made. If local pH values within the same picture are compared the present method is capable of resolution of pH differences to 0.1 unit in the pH range between 5 and 7. The spatial resolution is given by that of the optical microscope (200 nm at maximum). When comparing different pictures or when looking for absolute pH values the pH scale as a whole may be shifted up to 0.3 units in either direction due to discontinuities of the used photographic material and photometric procedures. For the purpose of this publication the continuous pH scale had to be reduced to the 9-step gray scale shown in the figures. All pictures shown represent typical results observed with the great majority of cells.

The pH maps obtained as described above are two-dimensional projections on the film plane of a three-dimensional object. Therefore each individual pH-point gives an average of the pH values of all fluorescein-containing compartments along a light path, i.e., a cylindrical columnlike section of the cell (Fig. 1).

Leakage of fluorescein from the protoplasts did not significantly interfere with the pH measurement. Within 25 min after addition of fluorescein

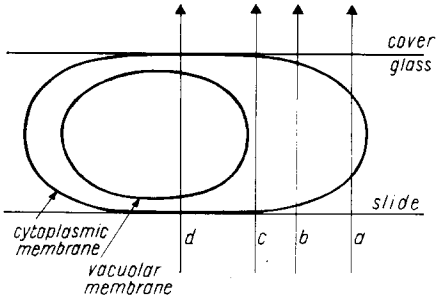

Fig. 1. Schematic cross section of a protoplast during microscopic examination. The indicated light paths average the pH signals of cellular regions characterizing a typical protoplast, e.g. (a) the periphery of the cytoplasm; (b) a section with the dominance of the central cytoplasmic area; (c) a section including the cytoplasm near the vacuole; (d) thin cytoplasmic layers between tonoplast and plasmalemma plus the vacuolar interior.

diacetate there was no measurable increase of background fluorescence.

Results

Origin of fluorescence signals

The pH map of a typical protoplast and the corresponding phase contrast picture are shown in Figs. 2a and 2b, respectively. Different findings indicate that the large central vacuole of the protoplast does not significantly contribute to the measured fluorescence signals.

First, isolated vacuoles do not fluoresce when incubated with fluorescein diacetate or fluorescein. Similar findings were reported with vacuoles from higher plants [10]. It is not yet clear whether the vacuolar membrane has low permeability for fluorescein diacetate or the enzymes splitting fluorescein esters are present in the cytoplasm only. The latter was suggested from cell fractionation studies with *Dictyostelium* [15]. Another reason might be that the vacuolar interior is acidic enough to prevent fluorescence, as judged from the strong trapping of neutral red in our object.

Second, if the vacuole does not fluoresce after the incubation of protoplasts with FDA the light paths crossing peripheral cytoplasm only and those which cross peripheral cytoplasm plus vacuolar interior (sections a and d of Fig. 1) should yield identical pH values even under conditions which differently influence the pH of the vacuole and the cytoplasm. Indeed, in our experiments both regions yielded identical or at least closely related pH signals under various metabolic conditions including the influence of respiratory inhibitors, uncouplers and transportants (Figs. 2a, 3, 4). (Small deviations from this identity most probably indicate different amounts of cytoplasm layered between cytoplasmic and vacuolar membrane. As can be imagined from Fig. 1 regions containing only very thin layers of cytoplasm between vacuole and cytoplasmic membrane occur especially in cases where the vacuole is flattened over a large part of the protoplast s diameter. The pH of such regions shows the best conformity with the pH seen at the peripheral cytoplasmic regions; e.g., Figs. 2a, 3, 4a).

For both reasons it seems justified to conclude

that the vacuolar pH does not significantly contribute to the cellular pH map, which should therefore solely represent the pH distribution within the cytoplasm. (The pH of nucleus and mitochondria is not resolved in the present study. The size of these organelles is near to or below the optical resolution of the used microphotographs with an optical magnification of about 800-fold).

pH-distribution in the cytoplasm

The cytoplasmic area appears divided into two layers with clearly different pH: a thin acidic surface layer with a pH near 5 situated just beneath the cytoplasmic membrane and a thick core region with a pH near 7. The gradient between both layers is rather steep with about 1.5 pH units per μm.

This intraplasmic pH distribution is independent of external pH in the used pH_{ext} range from 3 to 6 (compare Figs. 2a, 3 and Table I, lines 1–3). (Above and below this range protoplasts become unstable). Thus, the ΔpH between the cytoplasm and the outside bulk solution is determined solely by the pH_{ext} value.

Influence of inhibitors and of amino acid transport on intracellular pH

The addition of the uncoupler CCCP (1.5 μM) or the respiration inhibitor azide (100 μM) to the protoplast suspension gives similar effects: the

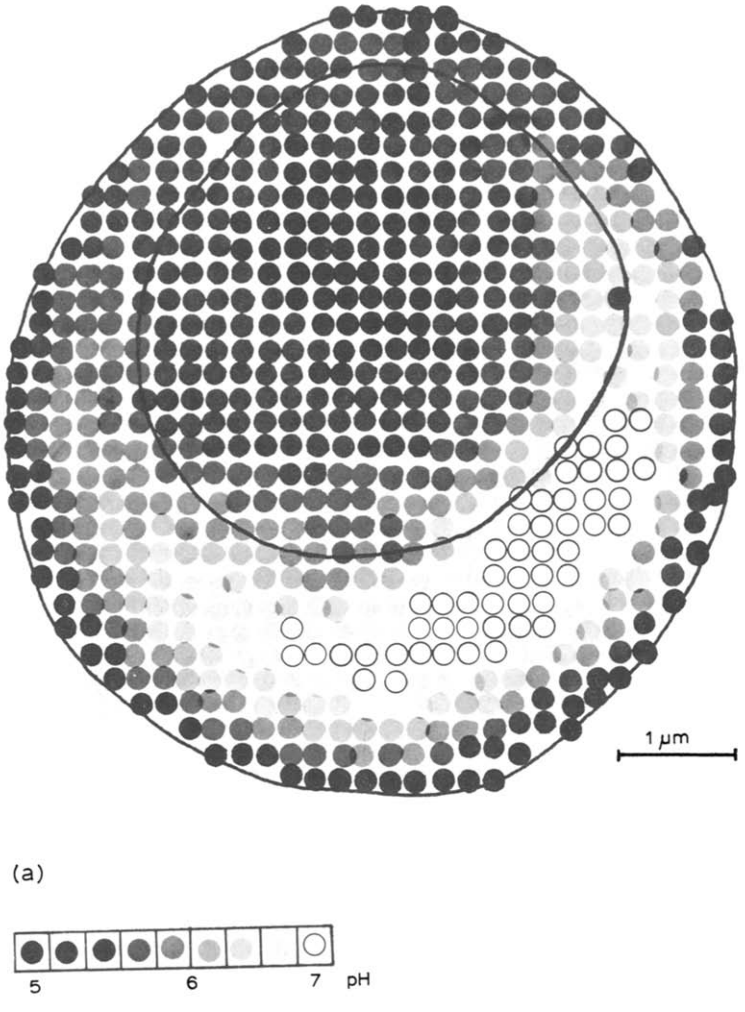

Fig. 2a. Typcial pH map of a protoplast at pH_{ext} = 3.5.

'core region' of the cytoplasm becomes more acidc, whereas the pH of the 'surface layer' is not significantly affected. As a result the pH map becomes more homogeneous (Figs. 4a, b). Again, the pH maps are independent of external pH in the pH_{ext} range between 3 and 6 (cf. Table I).

Earlier experiments have demonstrated that both protoplasts and intact hyphae are able to accumulate externally added amino acids. The uptake process is accompanied by a transient decrease of the average cellular pH, as measured by the distribution of bromophenol blue [12]. This finding was confirmed by the pH maps. Addition of 1 mM phenylalanine to the protoplast suspension causes a significant acidification of the 'core region' (the acidification is smaller than those triggered by uncouplers) with no significant effect on the pH of the 'surface layer'.

A summary of the average pH of both surface and core cytoplasm under different conditions is given in Table I.

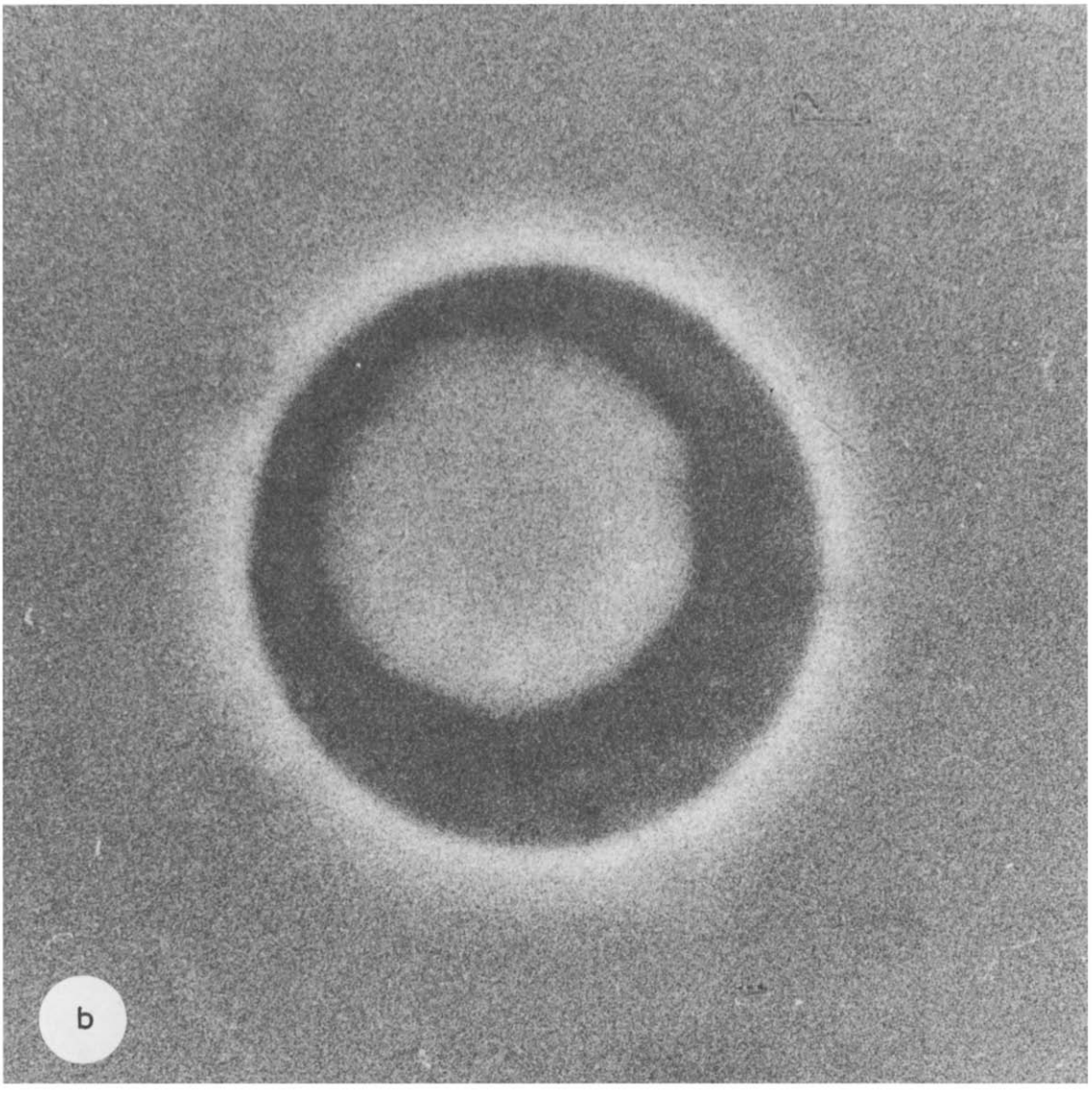

Fig. 2b. Phase contrast microphotograph of the protoplast used for the pH map of Fig. 2a.

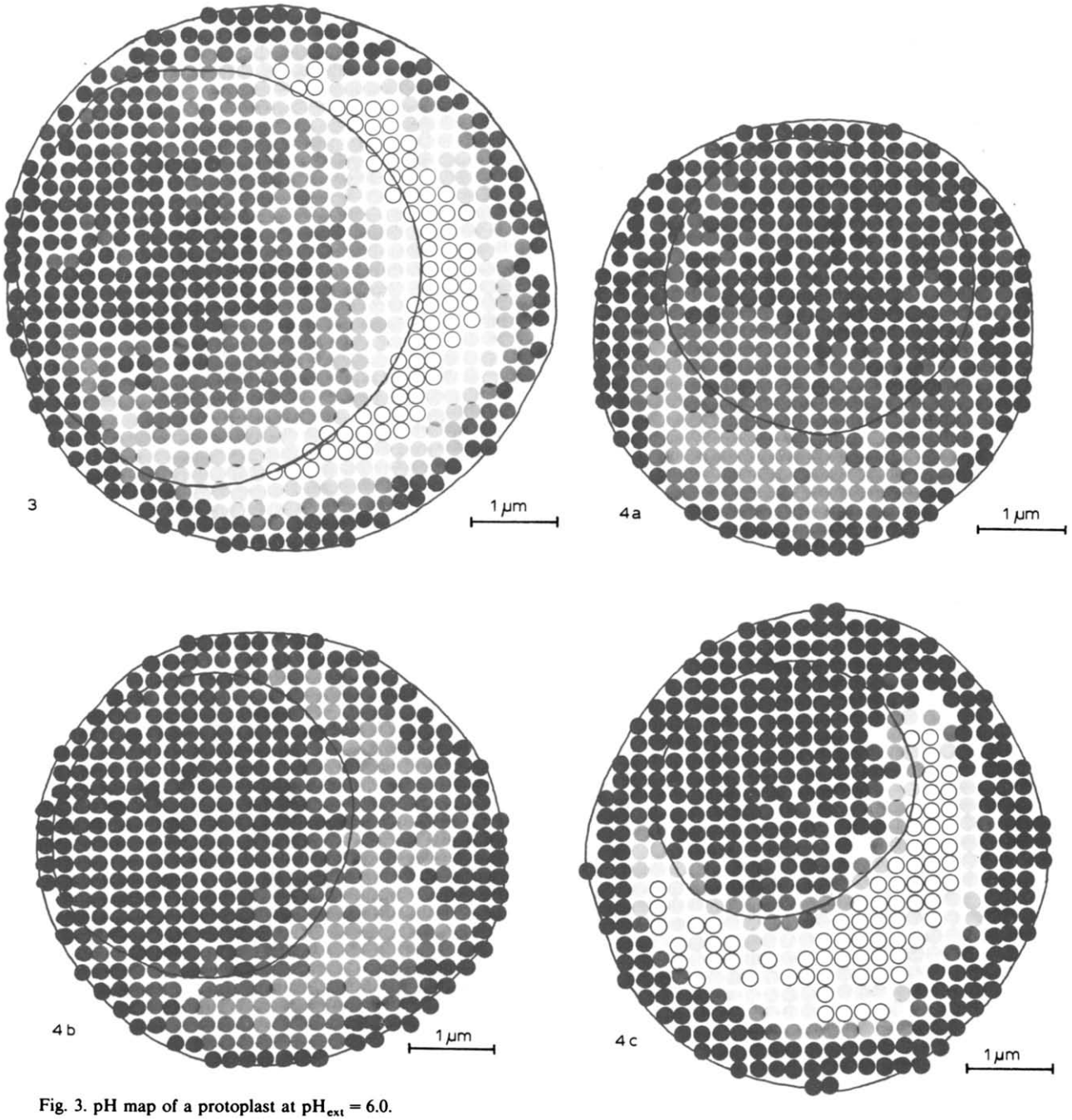

Fig. 3. pH map of a protoplast at $pH_{ext} = 6.0$.

Fig. 4. pH maps of protoplasts under the following conditions: (a) 1.5 µM CCCP, 5 min, $pH_{ext} = 4.5$; (b) 1.5 µM CCCP, 5 min, $pH_{ext} = 6.0$; (c) 100 µM Na_3VO_4, 20 min, $pH_{ext} = 4.5$.

Discussion

The remarkable intracytoplasmic pH gradient shown in the present object is much steeper than the gradual centrifugal decline of the cytoplasmic pH found in yeast cells by the same method [3]. This may be attributed to the physiological situation of a growing culture of *Penicillium cyclopium*:

TABLE I

COMPARISON OF MEAN pH VALUES IN THE CORE REGION AND THE PERIPHERAL REGION OF THE CYTOPLASM UNDER DIFFERENT EXPERIMENTAL CONDITIONS

pH_{core} and $pH_{surface}$ give the average pH of squares of 0.16 μm^2 (4 units in the gray field frame) which are situated on a line crossing the greatest distance between vacuole and plasmalemma. pH_{core} is taken at $\frac{1}{3}$ of this distance of the vacuolar side, $pH_{surface}$ at the cellular periphery. The data are derived from different protoplast preparations as well as from different microscopic pictures of the same preparation. The figures are presented as means ± S.D. n = number of pH maps.

Treatment	pH_{ext}	pH_{core}	$pH_{surface}$	n
Control	3.5	6.8±0.21	5.4±0.25	6
	4.5	6.9±0.20	5.4±0.28	6
	6.0	6.9±0.18	5.5±0.22	5
CCCP				
(1.5 μM; 5 min)	3.5	5.9±0.30	5.4±0.28	4
	6.0	6.0±0.25	5.5±0.20	4
N_3^-				
(100 μM; 5 min)	4.5	5.9±0.28	5.6±0.25	4
Vanadate				
(100 μM; 20 min)	4.5	6.8±0.25	5.0±0.25	4
Phe				
(10 mM; 10 min)	4.5	5.9±0.19	5.6±0.25	3

the cells have to manage an inwardly directed H^+-gradient (pH_{ext} below 4) established by the cellular excretion of acids [1]. Our data show that there is no mass influx of protons under such conditions even during the lack of ATP or in the presence of a protonophore. The protection of core cytoplasm from acidification is most probably achieved by the acidity of the outer cytoplasmic surface which is maintained mainly through passive mechanisms, i.e., uninfluenced by energy poisons.

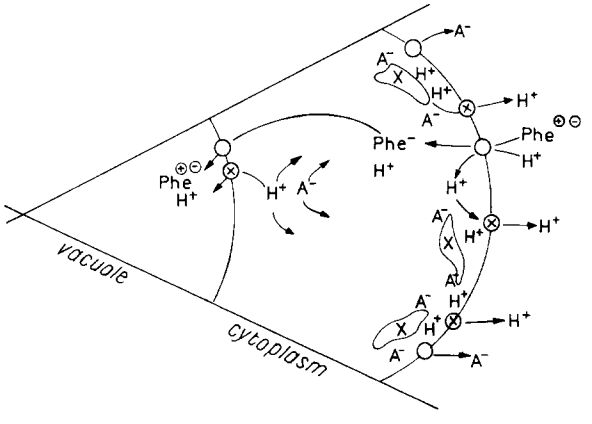

Fig. 5. Hypothetical scheme of mechanisms governing the distribution of protons in *Penicillium cyclopium* protoplasts. A^-, anions (mainly citrate); ×, anion binding protein; ○, transport system for anions or Phe, respectively; ⊗, H^+-transporting ATPase.

First, a minute influx of protons may immediately establish a positive diffusion potential which prevents a significant H^+ uptake. Accordingly, the lipophilic cation TPP^+ is practically not taken up into protoplasts unless a chaotropic anion (SCN^-) is added (Table II). Hence it is reasonable to conclude that the membrane potential is near zero or even positive and that the cytoplasmic membrane has a high resistance to ionic fluxes which otherwise could compensate for a hypothetical H^+ influx. Both properties can protect the cytoplasmic core region from acidification and thus aid in the adaptation of the cells to their usual highly acidic growth milieu.

Second, there is no significant loss of protons from the 'surface layer' if $pH_{out} > pH_{in}$ and CCCP is added. Together with the data mentioned above, this points to a Donnan distribution of H^+ based on the presence of a high concentration of immobile anionic charges in the surface layer compared with that of diffusible anions. A similar role of Donnan distribution, positive $\Delta\psi$ and low ionic membrane conductance is widely believed to be the basis of ΔpH maintenance in acidophilic bacteria [4–7].

The protons accumulated in the 'surface region' are obviously not acquired from the outer medium but are of cellular origin. This follows from (i) the fact that at external pH 6.0 the proton concentration of the 'surface layer' exceeds that of the outer medium and (ii) the hyperacidification of the 'surface layer' seen in the presence of vanadate.

This conclusion is in line with Sanders et al. [8] who found in *Neurospora* that the H^+-substrate for the plasmalemma H^+-pump originates from within the cell and the acidification caused by uncouplers and inhibitors represents no H^+ influx but metabolic perturbation. Many eukaryotic microorganisms synthesize and excrete organic acids, some of them in considerable amounts [13]. In *Penicillium cyclopium*, under the conditions used for the preparation and incubation of protoplasts

TABLE II

EQUILIBRIUM DISTRIBUTION OF [^{14}C]TPP$^+$ IN PROTOPLAST SUSPENSIONS MEASURED BY MICRODIALYSIS

50 μl of a suspension of purified protoplasts were mixed with 50 μl of 100 mM sodium maleate buffer (pH 3.5) containing 1 M sorbitol and 27 mM glucose and dialyzed against the latter solution in a microchamber [14] separated by a Visking 0.02 mm membrane (flow rate 1 ml per min). After 15 min 2.5 μl (approx. 10 kBq) of a $4 \cdot 10^{-6}$ M solution of tetra[U-^{14}C]phenylphosphonium bromide (Amersham) was added and the radioactivity of the dialysate was followed in 100 μl aliquots. If indicated, the TPP$^+$ solution contained NaSCN to give a final concentration of 10^{-4} M. Data are given in % of the maximum radioactivity reached in the dialysate of a sample containing 100 μl of the above buffer without cells. Uptake of TPP$^+$ is indicated by a depression of this value.

Treatment	[^{14}C]TPP in dialysate
Control (no cells)	100
Protoplasts	99
Protoplasts + NaSCN	71
Protoplasts, boiled 10 min	98
Protoplasts, boiled 10 min + NaSCN	100

(i.e. presence of glucose, absence of NH_4^+), citric acid was the major acid to be excreted into the medium [1]. The acidification of the medium stopped immediately after the addition of energy inhibitors including CCCP and azide.

Summarizing, it seems that there are three processes involved in the maintenance of the intracellular pH distribution, as schematized in Fig. 5:

(i) the metabolic production of organic acids (i.e., of anions togehter with free protons) which might occur both in the 'surface' and the 'core' region;

(ii) the noncovalent binding of the organic anions to specific proteins (or other macromolecules) in the surface layer. In this way an anion gradient between 'surface' and 'core' region would be established, which must give rise to a gradient of free protons via charge compensation. In this way the slope of the intracellular pH gradient would be predetermined;

(iii) the excretion of protons from the 'surface layer' via the H^+-translocating ATPase of the cytoplasmic membrane. This step (which may be coupled for charge compensation with the efflux of citrate or the uptake of cations [1]) would regenerate the citrate and/or proton binding capacity of the surface region and thus maintain a steady flow of H^+ from the site of production to the binding area. Therefore, the pH of the 'surface layer' is a steady state resulting from a joint action of a Donnan distribution of protons and the rate of transport via the plasmalemma H^+-pump. This view is consistent with all observed effects of inhibitors.

In the presence of CCCP or azide the net excretion of H^+ by active transport is inhibited due to the increased H^+-permeability of the membrane and the breakdown of oxidative energy supply. Consequently, the flow of acids to the 'surface region' ceases and further short continuation of acid production would lead to the observed acidification of the 'core region'. In contrast to CCCP and azide, vanadate is known to inhibit the H^+ extrusion without depleting the energy sources [11] (the ATP level should indeed transiently increase because of the reduced consumption by the pump). The selective decrease of the near plasmalemma pH caused by vanadate emphasizes the involvement of the H^+-ATPase in the steady-state H^+ distribution and further suggests that the H^+ fixing capacity of the surface region can be enlarged in the presence of energy sources, probably via an increased binding of organic anions. The latter possibility might be of regulatory significance under physiologic conditions: an increased production of organic acids would not disturb the cytoplasmic core pH but confront the H^+ pump with increased substrate concentrations thus giving rise to an increased rate of H^+ extrusion.

Furthermore, the existence of an acidic surface region established by a Donnan potential and active H^+-efflux would explain an earlier finding concerning the accumulation of amino acids in this fungus. The uptake of phenylalanine by H^+-symport causes only a small acidification of the cells which is not accompanied by a measurable alkalinization of the outer medium. This was interpreted as a very tight recycling of the cotransported proton [12]. Our present observation that the pH of the surface layer is not changed during the uptake, likewise points to an exchange of the incoming H^+ for a 'surface' proton. The entry of

the other proton coming from the dissociation of the COOH-group of phenylalanine is of course not prevented by the diffusion potential and would therefore proceed to the core region, thus causing the observed acidification (Fig. 5).

The hypothesis presented here certainly needs further support (e.g., broader spectrum of inhibitors and transportants, simutlaneous monitoring of changes of pH_{ext} and $\Delta\psi$). Namely, the nature of the postulated binding sites for anions remains to be established (e.g., is there some connection to the ATPase?). Be that as it may, the physiological advantages of such an acidic layer are obvious for a cell which needs to protect the cytoplasmic core from the influence of a highly acidic environment: (i) it prevents a mass influx of H^+ during a low energy situation or in the course of H^+-symports and (ii) it increases the velocity of H^+ pumping by increasing the substrate concentration of the plasmalemma proton pump and at the same time facilitates the operation of this pump by reducing the transmembrane H^+ gradient. It appears that the total H^+-gradient existing between the cytoplasmic core region and the acidic environment of the cell consists of two steps: an intracytoplasmic one, which is under direct cellular control, and a transmembrane step, whose maintenance is economized by the intracytoplasmic gradient.

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