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^{31}P-NUCLEAR MAGNETIC RESONANCE ANALYSIS OF THE INTRACELLULAR pH IN THE SLIME MOLD DICTYOSTELIUM DISCOIDEUM

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SUMMARY. Amoebae of the slime mold **D. discoideum** were studied by phosphorus nuclear magnetic resonance. Under aerobic conditions, major intracellular phosphate compounds included phosphomonoesters, inorganic phosphate (Pi), ADP and ATP. Nucleotides were essentially as magnesium complexes. Two intracellular Pi resonances were clearly resolved and the corresponding pHs determined by the chemical shifts characteristics were 7.7 and 6.7. These intracellular pHs were strictly constant over an extracellular pH range between 5.0 and 7.5. The two cellular compartments defined by the Pi resonances were assigned to mitochondria (pH 7.7) and cytosol (pH 6.7) on the basis of their response to anaerobiosis or to carbonylcyanide-m-chloro phenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation, which equilibrate the two intracellular pHs.

Intracellular pH is thought to possibly regulate a wide range of cellular functions (1, 2). For example, the pH gradient across the inner mitochondrial membrane is a key factor in the phosphorylating activity of mitochondria. The matrix space is kept at a higher pH than the cytosol (3). A variety of techniques have been used to estimate intracellular pHs. In the cellular slime mold **Dictyostelium discoideum** (4, 5) intracellular pH has been measured using the pH-dependent fluorescence of fluorescein (6). Most of these techniques: fluorescent probes, weak acids or bases, determine some overall pH value which reflects both the pH itself and the respective size of the various subcellular spaces. ^{31}P-NMR can be used in a non-invasive way to assess the different pH values in the individual cell compartments (7-12).

We here report ^{31}P-NMR studies of phosphate and phosphate metabolites in the slime mold **D. discoideum**. A remarkable feature is the large intracellular pH gradient between the mitochondria and the cytosol observed in aerobic amoebae by ^{31}P-NMR.

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MATERIAL AND METHODS

D. discoideum, strain AX2 (ATCC 24397) was grown at 22°C in axenic medium (13) and harvested during the exponential phase of growth by low-speed centrifugation (800g, 4 min.). Cells were washed twice with ice-cold 20 mM morpholinoethanesulfonic acid (Mes) brought to the required pH with NaOH. The packed cell pellet was kept at 0°C for less than 1 hour prior to the preparation of the NMR samples.

D. discoideum cells were suspended at a mean cellular density of 2-3 x 10^8 cells/ml in 20 mM Mes-Na buffer (see legends to figures for specific conditions) and supplemented with 4% D_2O (v/v) and 25 ml were placed in a 25 mm NMR tube. To avoid cell sedimentation and to maintain aerobic conditions at the high cell concentrations required to collect the NMR signals, the suspension was simultaneously stirred and oxygenated with a steady stream of small oxygen bubbles via polyethylene tubing introduced through a stopper at the top of the NMR tube. Bubbling rate was adjusted to about 20 ml/min. A glass capillary tube containing 70 mM methylene diphosphonate in 30 mM tris(hydroxymethyl)aminomethane buffer, pH 8.9, and coaxial with the magnetic field was also added as a chemical shift reference (+ 16.4 p.p.m.) to the cell suspension in all the experiments. All chemical shifts were expressed with respect to orthophosphoric acid at 0 p.p.m.

Perchloric acid (PCA) extract was performed on cells (3.9 x 10^8 cells/ml) washed and suspended in 20 mM Mes-NaOH, pH 6.5. The suspension was bubbled with oxygen and the total volume was 20 ml. Rapid freezing of an aerobic sample was achieved by freezing small drops of the oxygenated suspension directly in liquid nitrogen. Beads were then crushed with 8% PCA. After thawing, the extract was centrifuged 10 min. at 40 000 rpm in a Beckman R40 rotor. The supernatant was neutralized with KOH and potassium perchlorate eliminated by centrifugation. The resulting supernatant was chromatographed through a Chelex-100 column (1cm x 7cm, Bio-Rad, 200-400 mesh). The eluted sample was adjusted to pH 7.3 with HCl and freeze-dried. The residue was suspended in 3 ml D_2O to give a pH(D) of 7.5 and transfered in a 10mm-NMR tube.

NMR spectra were acquired in the pulsed Fourier transform mode at a frequency of 81.01 MHz for phosphorus on a Bruker WM 200 wide-bore spectrometer. Interpulse intervals were 0.6 s and the flip angle was 52°. The spectra accumulated over the first 5-10 minutes were usually discarded because it took several minutes for the preparation to reoxygenate (the stored cell pellet was anaerobic) and equilibrate at 22°C in the magnet. The magnetic field was locked in the deuterium NMR signal from D_2O present in the suspension medium. Proton decoupling was not used.

The pH of the cell suspension was determined directly with a glass electrode in the NMR tube before introduction and also after removing it from the magnet.

RESULTS

^{31}P-NMR spectrum of **D. discoideum** amoebae. Fig.1 shows typical ^{31}P-NMR of respiring **D. discoideum** cells. The major resonances observed represented phosphomonoesters at a chemical shift position of 3.5 p.p.m., three inorganic phosphate peaks (int Pi, ext Pi) at 2.2, 1.2

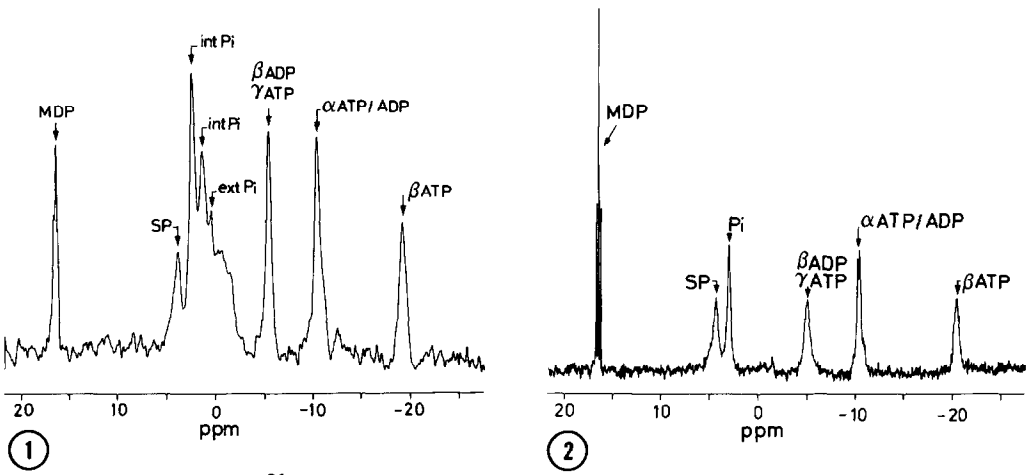


Fig.1 Typical ^{31}P-NMR spectrum of aerobic D. **discoideum** amoebae. Cells were washed and suspended in 20 mM Mes-NaOH, pH 5.65 to give a final cellular concentration of 2.9 x 10^8 cells/ml. The NMR signal was accumulated for 25.2 minutes. MDP = methylene diphosphonate (used as external chemical shift reference at +16.4 p.p.m.), SP = sugars phosphates (phosphomono-esters), α, β, γ, ATP/ADP = phosphates of nucleosides tri- and diphosphates, intPi, extPi = compartmentalized inorganic phosphate at different pHs (see Text).

Fig.2 ^{31}P-NMR spectrum of a perchloric extract of aerobic D. **discoideum** amoebae recorded during 2.1 hours. Experimental conditions as described under "Material and Methods". For abbreviations, see legend to fig.1.

and 0.25 p.p.m., the γ phosphate of ATP and the β phosphate of ADP at -4.8 p.p.m., the α phosphates of ATP and ADP at -10.6 p.p.m. and the specific resonance of the β phosphate of ATP at -18.7 p.p.m. Although other minor peaks were visible they cannot be identified with certainty but one can note the absence (or their peaks were below the level of detection) of phosphorylated compounds present in other cell types such as phosphocreatine or polyphosphates. With respect to the physiological stability of the cells during data collection, sugar phosphates slowly decreased but ATP concentration remained stable for extended periods (up to 4 hours). The concentration of external Pi rose slowly.

^{31}P-NMR spectra of perchloric acid extracts of aerobic amoebae showed the same compounds as intact cells, buts only one resonance corresponded to Pi (fig.2). In the Pi region, no compounds such as glycerophosphoryl choline or glycerophosphoryl ethanolamine (14) were detected in significant amounts.

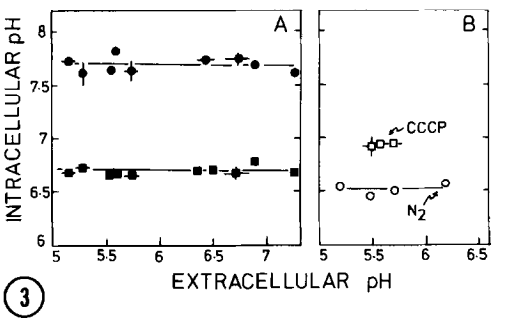
Effect of external pH on intracellular pH. The assigment of pH values can be determined from the positions of the Pi resonances as compared to a calibration curve (11). Multiple Pi peaks may represent subcellular compartments of different pH as well as resonance of Pi

from the extracellular space. External Pi (ext Pi) in the spectrum shown in fig.1 is characterized by resonance at 0.25 p.p.m. (external pH = 5.7). Internal pHs of 6.7 and 7.7 were determined from the resonances (int Pi) at 0.9 p.p.m. and 2.2 p.p.m. respectively. The cytosolic pH in **D. discoideum** was previously determined to be 6.2-6.4 using fluorescein as a pH dependent probe (6). In the amoebae, the vacuolar compartment was shown morphometrically to be quantitatively small. It represents 5-7% of the cellular volume (15, 16). As discussed in more detail later (see Discussion), it is the most plausible that the two NMR-visible compartments represent the cytosol and the mitochondria. As shown in the figure 3A, the two intracellular pHs stayed at strictly constant levels, pH 6.7±0.05 and pH 7.7±0.1 respectively, when the external pH varied widely over about 2 pH units (from pH 5.1 to pH 7.3).

Effect of anaerobiosis. Anaerobiosis was achieved by bubbling the cell suspension with nitrogen instead of oxygen as shown in fig.4. The ATP level was drastically decreased as shown by the total disappearance of the βATP resonance. It should be mentioned that oxygen limitation was already observed with air bubbling (fig.4). Only one internal pH was observed of about 6.5-6.6, thus more acidic than the intracellular pHs in oxygenated conditions but still different from the external pH (fig.3B). The pH gradient was restored by reintroduction of oxygen and simultaneously, ATP levels returned to the normal aerobic value (fig.4).

Effect of uncoupler of oxidative phosphorylation. Upon addition of 10 μM CCCP, an immediate decrease of ATP and a collapse of the two internal pHs to a single value of pH 6.9 was observed (fig.3B). Cellular proton gradients were not entirely destroyed as the internal pH value was intermediate between the two control aerobic pHs but distinct from the pH of the suspension medium.

Determination of intracellular ATP-Mg^{2+} and free magnesium. The percentage of ATP complexed to Mg^{2+} is linked directly to the chemical shifts of the phosphate resonances of ATP (17, 18). From data derived from spectra of aerobic amoebae (such as shown in the fig.1 or fig.4), it could be calculated that 91 ± 3% (n=12) of total ATP was present as ATP-Mg complex. Using a dissociation constant of 50 μM for the ATP-Mg complex, the level of free Mg^{2+} was then estimated to be 0.6 ± 0.3 mM (n=12).



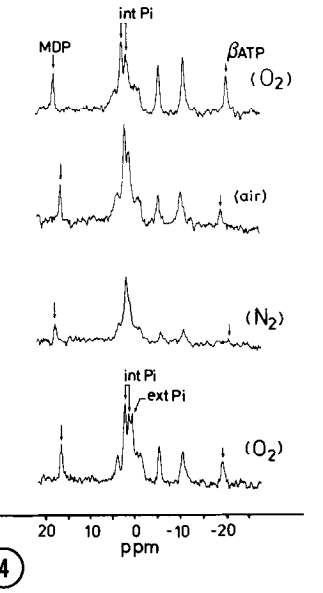


Fig.3 Intracellular pHs of **D. discoideum** amoebae as a function of external pH.
A) Cells were washed and suspended in 20 mM Mes-NaOH buffer at the indicated pHs and oxygen was bubbled through the suspensions. The cellular concentrations were between 1.8×10^8 and 3.8×10^8 cells/ml. The NMR signals were accumulated for periods between 3.6 and 25.2 minutes. The pH of the samples was measured before and after collecting the NMR data.
B) Similar experiments were performed either in the presence of 10 µM CCCP or with nitrogen bubbling instead of oxygen.

Fig.4 ^{31}P-NMR spectra of **D. discoideum** cells under different physiological conditions. Amoebae (3.2×10^8 cells/ml) washed and suspended in 20 mM Mes-NaOH buffer, pH 6.5, were put successively in the following conditions: (a) aerobic (oxygen bubbling) ; (b) partial limitation in oxygen (air bubbling) ; (c) anaerobic (nitrogen bubbling) ; (d) aerobic (recovery from anaerobiosis, oxygen bubbling). Spectra corresponded to accumulation times of 7.2 minutes except under nitrogen where it was only 3.6 minutes.

DISCUSSION

The purpose of the present study is to explore the use of ^{31}P-NMR in the slime mold **D. discoideum.** We have characterized the phosphate metabolites and measured intracellular pHs. Cells can be grown and used in large amounts and intracellular Pi, ADP, ATP and sugar phosphates are easily detected. The good physiological stability of the amoebae permits data collection for long periods. The existence of two phosphate resonances is in agreement with the compartmentalization of phosphate pools within the cell. The two peaks are assigned to Pi in the mitochondrial matrix and in the cytosol as discussed by Cohen et al. (8) in hepatocytes on the basis of the anticipated response, i.e. collapse of the pH gradient obtained after de-energisation by

CCCP, an uncoupler of oxidative phosphorylation, or by prevention of mitochondrial respiration by substitution of nitrogen for oxygen.

In aerobic amoebae, the mitochondrial matrix is more alkaline than the cytoplasm and the pH gradient is about 1 pH unit. Cytosolic-mitochondrial gradient of similar magnitude have been reported (3, 20, 21). The cytosolic pH in **D. discoideum** was determined to be 6.3 as measured with intracellularly trapped fluorescein (6). This value is in agreement with our NMR-derived value of 6.7 if one considers the marked acid load occuring during the intracellular hydrolysis of the fluorescein diacetate.

Similarly to many other cells, most of the ATP is present in **D. discoideum** as an ATP-Mg^{2+} complex. ^{31}P-NMR estimation of free Mg^{2+} lead to a value of 0.6 mM, comparable to the level in **Physarum polycephalum** (21).

In isolated mitochondria, matrix Pi is easily detected (22) but in many cell types, Pi in the mitochondrial compartment is considered to be NMR-invisible. In some cases, the amount of mitochondrial Pi could be quantitatively negligible or its resonance is so broadened that it is undetectable (22-24). The finding of an easy discrimination of cytosolic and mitochondrial Pi and of a large pH gradient suggests that **D. discoideum** amoebae are valuable cells to examine energetic aspects of mitochondrial function.

In most cells the cytosolic pH is modulated by changes in the external pH (19). In contrast, intracellular pHs in **D. discoideum** are kept strictly constant when the external pH is widely varied. The ability to control intracellular pH is likely to be an important attribute for free-living amoebae like **D. discoideum** that face large variations in the pH of their environment.

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