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NMR and pressure correlated analysis of metabolic changes in soft-X-rays irradiated yeast cells

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Abstract. An NMR technique for the identification of the energy related molecules, AMP, ADP and ATP, is suitably taylored to follow up the dynamics of the glycolitic metabolism in a suspension of yeast cells. Such a technique proves to be efficient to investigate soft X-ray radiation damage as complementary to other physical and chemical assessment.

PACS. 87.64.Hd EPR and NMR spectroscopy – 87.22.-q Physics of cellular and physiological processes – 87.50.Gi Ionizing radiations (ultraviolet, X-rays, γ-rays, ions, electrons, positrons, neutrons, and mesons, etc.)

The cell is a dynamical system far from thermal equilibrium which performs an extraordinary high number of tasks under the widest range of environmental conditions. Such tasks require a wealth of communications and interactions, typical of ordered structures within a population, catalytic driven chain reactions, exchange of energy and matter often in synchronized conditions.

Energetics is in particular a central issue, even in the most sophisticated and novel cases such as those regarding DNA structures and topoisomerases [1].

A synergetic approach to the cell system dynamics is particularly appealing from the biophysical point of view especially when a non-invasive monitoring is privileged in agreement with the increasing drift in this direction from literature [2], intended to unveil and control the actual mechanisms at work in cells.

Furthermore, the fact that the cell is substantially non inflationary, from the point of view of its energy currency (typically the ubiquitous adenylate pool) allows to link energy needs to the cell operations biunivocally.

This is also true when cells undergo various types of insults, including ionizing radiation. Since all the mechanisms operating in cells are at the very end related to metabolism (catabolism and anabolism) each of them providing also a feedback controlling the rates of processes, it proves to be very helpful to use a spatial disaggregation of enzymatic loops and this can be made by suitably selecting for instance the physical parameters of the insulting agent (it is usually performed by chemical agents).

A detailed investigation in terms of cell compartments involved is difficult; a fact which emphasizes the advantage of non invasive techniques on one hand, while it would be greatly beneficial to have in addition an insight into the cell to ascertain and localize the involved elementary mechanisms. The glycolitic metabolism of the cell is well suited for this purpose since it covers different facets of the problems:

- it is related to the whole cell and can be traced by non invasive techniques, as high lightened by some of us [3];
- its response can be studied by a physical parameter computerized on line recording measurement (pressure of produced CO_2): on the time scale of the second it can finely monitor (up to some days) the overall metabolic dynamics; the time step and the overall duration can be expanded or reduced by at least one order of magnitude;
- it permits to distinguish the energy response of the cell to different metabolites under different states of stress;
- it permits to trace a full population behaviours (collective properties), including, when possible, synchronized situations even looking at finite structures of time evolution such as (metabolic) oscillations.

If such a diagnostics is used in conjunction with careful controllable irradiation procedures mainly involving selected compartments of the cell [3,4], precious information can be obtained on specific given energetic pathways, typically the enzymatic chain reaction of glycolysis in yeast cells.

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The above technique can also benefit from cross-correlation with other substantially non invasive techniques such as Nuclear Magnetic Resonance (NMR) in order to increase the resolution of the basic mechanisms involved, as we shall explain here on. The above general considerations can be specialized for yeast cells (in particular the most popular strain of *saccharomyces cerevisiae*) which possesses several outstanding properties:

(i) they are simple eukaryotic cells, the nearest to the complex mammalian ones;
(ii) their metabolic pathways is a milestone in biology since the pioneering work of Pasteur and the following generation, where most biochemical details are elucidated;
(iii) their waste output (as we shall detail here on) is CO_2, a simple molecule which can be easily handled into chemical physical terms;
(iv) the suspending medium can be over simplified into simple pure water and sugar type nutrients.

Some of us [3] have shown that under given condition *saccharomyces cerevisiae* cells with the most standard nutrient (α-D-glucose) in the simplest culture medium (de ionized water) undergo, in absence of any external constraint, the anaerobic metabolism which consists in a chain reaction involving a dozen of enzymes [4]. Biologists' canonical wisdom have indicated that a key control of this pathway is an allosteric enzyme, the phosphofructokinase (PFK) one [5] and that the whole process typically occurs at the cytoplasmic region which borders the inner phospholipidic membrane, a compartment which can be hit by carefully controlled short range quasi-monochromatic soft-X-rays [3].

From such an approach many overall conclusions can be drawn, including fine aspects such as oscillatory behaviours, where however no details of the irradiation effects on the single steps of the Embden-Meyerhof pathway can be elucidated.

The energy output from the metabolism is actually the creation of the adenosine triphosphate (ATP) molecular unit through a reversible chain of reactions which in principle is of the type: ADP + Pi + $\delta E \leftrightarrow$ ATP, where δE is the energy involved and ADP and Pi are other molecular groups belonging to the cytoplasmic material. It is understood that the energy creating metabolism runs this ideal reaction in the right hand direction, creating a sufficient amount of ATP. When at any stage of the cell life energy is needed the reaction is run in the left hand direction with release of energy (which can be used for muscular mechanical work, to run a chemical endoergonic reaction, *etc.*) The ATP used is thus called energy currency, but was stored in the cell just in "sufficient" quantity, thus the analogy with a non-inflationary situation. Of course its concentration is in quasi-equilibrium, thus sustained by oscillatory behaviour as previously outlined.

In principle the approach [3] permits to ascertain that, upon irradiation, the metabolic process of glycol Isis still occurs and one can infer that overall it is enhanced/depressed by the enhancement/depression of the CO_2 output. However the shortcomings of such non invasive technique is that no details are discernible in the pathway, which might have changed at given points, while generating final identical outputs.

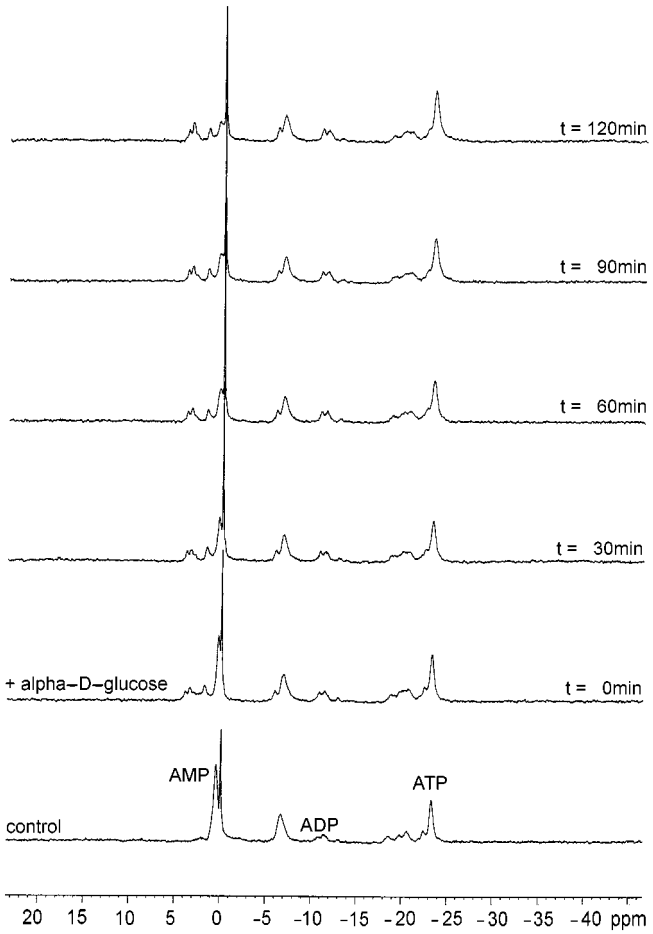

Fig. 1. Time evolution of AMP, ADP and ATP concentration is followed after the addiction of α-D-glucose to the control of yeast suspension in PBS (1 ppm = 162 Hz).

We have therefore cross-correlated the above results applying the NMR technique on the adenylate pool molecules which are particularly suited since they can be easily traced thank to the dynamics of the phosphorus atoms.

It is well-known that CO_2 production is correlated with metabolic processes such as respiration and fermentation, that implies production of ATP molecules where the energy, derived from carbon sources, is stored in form easily manageable for cells.

For instance fermentation implies the production of two CO_2 molecule and one ATP molecule from one sugar molecule. It is therefore possible to test this correlation among the different molecular species intervening in the metabolic process by cross-correlating different techniques each of them being a check for one molecular species.

A non invasive on line technique to detect and follow CO_2 production based on differential pressure sensors has been developed which is thoroughly described in

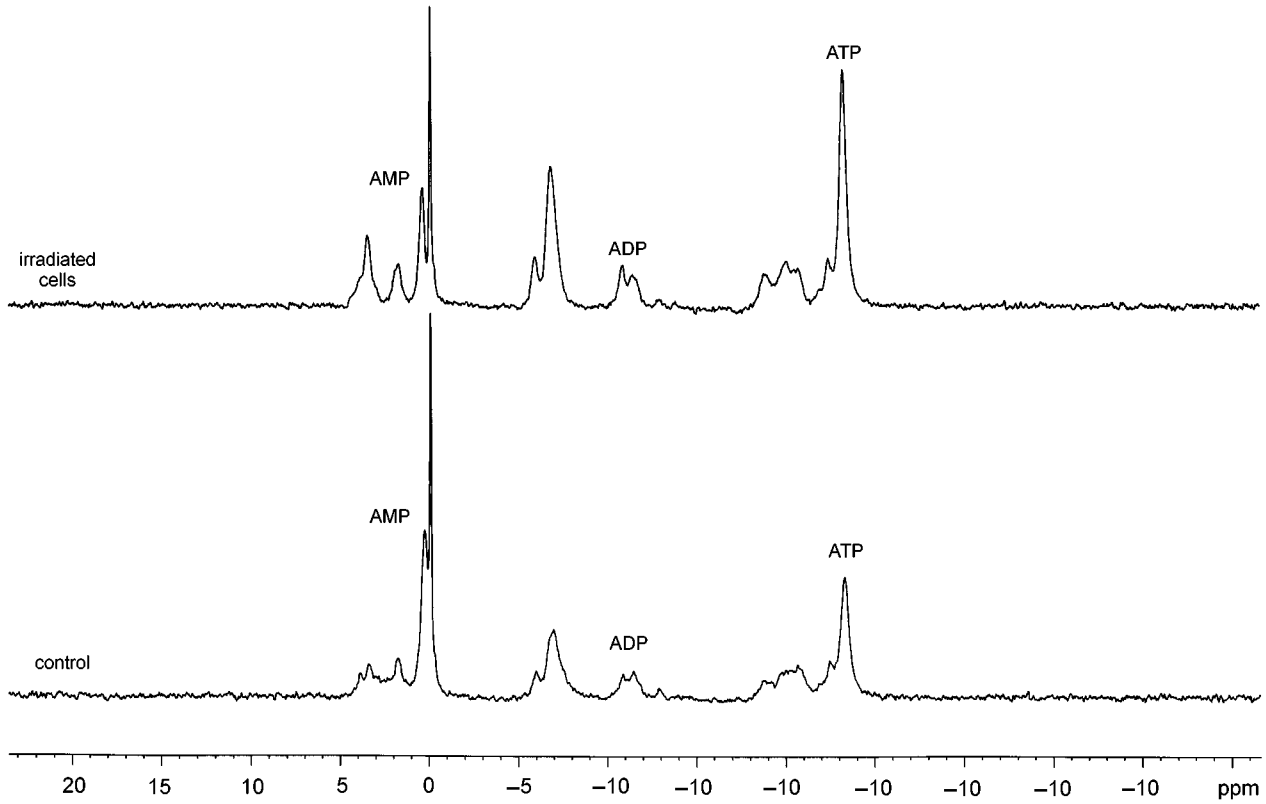

Fig. 2. NMR spectra of irradiated cells (100 Gy at $0.7 \div 0.9$ keV) after α-D-glucose addiction to the suspension. Enhancement of ATP is clearly visible *vs.* control.

reference [3]. The atmosphere in the sealed bottles has been analyzed by mass spectrometry making us sure that the pressure increase is exclusively due to CO_2 production.

NMR spectrometry is the suitable tool to monitor the evolution of adenylate molecular groups, so that it can consistently close the detailed balance of the chemical reaction when it is coupled to standard chemical determination of glucose consumption.

NMR is a spectroscopic method to observe nuclear spin reorientation in an applied magnetic field. It gives detailed information about the properties of molecules and their relationship with the environment [5].

Phosphorus-31 NMR spectroscopy has been used for monitoring intra cellular concentration phosphate metabolites in suspension of whole cells of *saccharomyces cerevisiae* during metabolic activity. NMR signals are sensitive to environment, so the peaks of ^{31}P in AMP, ADP and ATP are in different positions of the spectra [6,7].

The experiment consists in monitoring the kinetics of adenylate molecular groups (AMP, ADP, ATP) after addition of α-D-glucose at concentration of 20 g/l (2% w/v) to a suspension of yeast cells in PBS (Phosphate Buffered Saline) directly in the sample tube. Spectra have been taken every 30 minutes for 2 hours and, to decrease the speed of ATP's production, the sample was maintained at 283 K. pH values have been recorded during the experiment and there weren't significative changes from pH 6 of the suspending medium [3].

NMR measurements were performed with a Bruker AMX400 spectrometer whit 10-mm sample tubes operated at 162.0 MHz for ^{31}P observation. The spectra were taken with 2000 scans of 60° pulses with 0.35 s repetition rates.

Yeast's cells were grown in Sabouraud liquid medium containing neopeptone 1% (w/v) and D-glucose 2% (w/v). The cultures have been incubated in 500-ml flasks continuously area ted at 306 K in a thermal bath. Growth was monitored by cell counting at optical microscope with a Burker cell.

The cultures were grown to the mid logarithmic phase, at the initial concentration of 3×10^6 cells/ml $= 0.2$ g/l. They need 5 hours at 306 K to reach this phase with a final concentration of 3×10^7 cells/ml. The flasks were immersed in ice cold water and the cells were centrifuged at low speed and washed twice with 7 mM phosphate buffer, pH 6. The cells were sedimented and resuspended in an equal volume of the salt solution, and D_2O was added to 10% final concentration.

The peaks were assigned by adding standards to the sample. It has been observed that the amplitude in frequency of peaks from whole cells were larger than the amplitude of standards' peaks, in fact relevant molecules outside cells originated peaks with a higher resolution. The spread in frequency of observed spectra peaks is a natural consequence of the presence of AMP, ADP and ATP

inside the yeast cells where their signals are shielded by the membrane and others structures of cells.

The spectra of adenylate molecular groups kinetics show the transformation of AMP in ATP in real time after solution of α-D-glucose in the sample (Fig. 1). There is a decrease of AMP's peak and an increase of ATP's peaks, however from the spectra it is not clear if also ADP raises because part of ATP's signal sums to ADP's one. The chemical shift is not as significative as expected [7].

The reliability of the NMR investigation can be checked even during time evolution of the suspension by calculating the energy charge (EC) conservation, where EC={[ATP]+1/2[ADP]}/{[AMP]+[ADP]+[ATP]} according to [4].

NMR therefore can be used to investigate the dynamics of the energy requiring processes in the glycolitic metabolism of yeast, thus the effects of radiation with respect to controls. We here show, as an example, the case of the above cells when irradiated with soft X-rays in the range $0.7 \div 0.9$ keV, as described in [3] and established in Figure 2, where the cells are exposed to a dose of 100 Gy. The same spectra taken at different times (not shown here) exhibit a behaviour similar to that of Figure 2, with an enhancement of ATP peak. It can be concluded that the glycolitic metabolism is enhanced as an effect of the irradiation, as it has been shown elsewhere [3] for CO_2 production.

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