ATP in different biological systems

New applications of intracellular ATP determination by bioluminescent method in red blood cells and microbial systems

1 Introduction and aims

1.1 Introduction

1.1.1 Significances of ATP in different biological systems

1.1.1.1 Relationships between red blood cell ATP content, iongradient and integrity

In spite of the well known significance of ATP in the energy dependent life processes, the role of ATP in maintaining cellular integrity is poorly understood. The crucial role of ATP and ATP metabolism in maintaining the functional and structural integrity of circulating red blood cells can hardly be questioned. The age distribution of cells is not going to change during in vitro incubations, parameters measured parallel with ATP content can give newer insights into unrevealed connections or further direct evidences for the role of ATP in maintaining structural and functional integrity of red blood cells. As a matter of fact parallel with the development of blood banking, the role of ATP and ATP metabolism in maintaining the functional and structural integrity of stored human red blood cells have been confirmed and proved unequivocally in the last decades [1-4].

Despite of a lot of good publications is not clear how many ATP molecule is needed minimally to maintain living processes of red blood cells.

1.1.1.2 Relationship between intracellular ATP content and viability of testmicrobes on TLC plates of direct bioautography

Bioautographic TLC is able to detect antimicrobial effects in situ after separation of a mixture. This is an unique chromatographic procedure. Bioautographic methods (contact, immersion, and direct) are based on the biological – antibacterial, antifungal, antitumor, antiprotozoal etc. – effects of the substances on testmicrobes under study [5]. The steps of direct bioautography are performed on the chromatograpic plates, the testmicrobes grow directly on the developed TLC plate. Direct bioautography is a powerful method to detect antimicrobial activity of a single compound in a complex mixture of chemicals [5-18]. There are other areas of its application for detection of antimicrobial compounds too [10-12].

In the first step of this technique a sample, which contains compounds with antimicrobial effects, is separated on two TLC plates. After separation of these molecules, one of the plates is used for chemical, the other is used for microbiological detection and identification procedures.

Originally, agar gel (agar-overlayer method) was an essential component in bioautography. TLC plates were employed only for separation and after that they were covered with an agar layer containing the test bacterium. However, the usage of agar-overlayer method has considerable disadvantages. Firstly, separated extracts diffuse slowly from the plate into the agar. During this time the test microbes start to multiply

before the antimicrobial compounds can reach and kill them. Secondly, the excessive dilution of the active compounds moving from the spot on TLC sorbent layer to the test microbes in agar layer considerably reduces the sensitivity of the test. Thirdly, it presents low contrast after development. There were long incubation time of TLC plate carried out in humid atmosphere, TLC layers could be soaked for example in case of polyamide, cellulose layers.

In our experiments we used the direct bioautographic method without agar gel overlayer.

We cultivated the testmicrobes in broth and immersed the developed chromatoplates directly into this broth culture. The broth medium captured by silica beads of a TLC plate after dipping the plate into a microbial culture supplies the nutrient source for the test microbes. There is no doubt that the use of optimized culture parameters to maintain good viability for testmicrobes on TLC plates is essential for correct bioautographic evaluation.

1.1.2 ATP measurements in different biological systems

The ATP determination is **one of the best methods to check viability of eukaryotic cells and prokaryotic** microbes **[19, 20],** all microbes have ATP content.

Firefly (*Photinus pyralis*) bioluminescent method is quick, sensitive and reproducible.

Application of photon counting by Strehler [21], Lundin [22] és McElroy [23] made a new way for ATP measurements.

If the external conditions (concentration of alimentary substances, temperature etc.) change then ATP content changes too in different biological systems (red blood cells, microbes). These ATP level changes can be followed by intracellular bioluminescent methods. It opens the door to us to study problems as demonstrated in earlier chapters (1.1.1.1. : red blood cells integrity, 1.1.1.2.: optimal living conditions of testmicrobes in direct bioautography)

1.2 Aims

1.2.1 How much ATP is needed to maintain metabolic activity of human red blood cells

To monitorize and compare ATP content, hemoglobin release, blood glucose, sodium-potassium distribution, water content and mean corpuscular volume (MCV) of red blood cells stored at 4^{o}C and 37 ^{o}C for 110 hours.

Despite of a lot of good publications it is not clear how much ATP is needed to maintain living processes and structural integrity of red blood cells.

It was the reason why, we decided to make an ATP depletion model **without supplemented compound causing ATP depletion** for study of relationship between ATP and integrity of human red blood cells.

1.2.2 Increasing of effectiveness of direct bioautography

Direct bioautography was proposed to make so reliable, sensitive and fast as possible. Living conditions of test microbes on TLC plate have hardly studied. This was why **we studied factors influencing of effectiveness of direct bioautography** regularly:

a.) suitability of TLC **adsorbent** under optimal and non optimal conditions
b.) suitability of **mobil phase** commonly used in TLC
c.) **metabolic activity of microbes** (bacteria, fungi) to ensure optimal living conditions in broth culture before dipping of a TLC plate into it and on TLC plate after dipping.

d.) **incubation time** of TLC plate for different types of microbes.

Measurement of microbial intracellular ATP can be realized on TLC plate, so informations about living conditions of testmicrobes can be received. By this way the direct bioautographic method can be improved.

1.2.2.1 To frame a test method to follow the dissolution of an antibiotic ingredient

The release of an active agent from a dosage form is a basic and substantial parameter of a pharmaceutical preparation. The **commonly used analytical detection methods** based on UV absorption can measuree the dissolved **amount of an active ingredient** from a delivery system however it **is not able to give any information about biological activity of the released drug (antibiotics)**.

Our aim was to frame a dissolution test method that can follow the antibiotic release by detecting of changes in the antibacterial activity as a function of time.

2 Material and methods

2.1 ATP depletion model in red blood cells

The CPD (citrate-phosphate-dextrose) blood samples were first divided into two sterilized plastic bags [24] and were incubated parallel either at 4^{o}C or at 37^{o}C for 110 h.

2.2 Monitoring of characteristic metabolic parameters in red blood cells during ATP depletion

Maintaining the steril conditions, total blood samples were taken from the plastic bags at 6th and 12th hours of incubation and there after at every following 12 hours intervals. The total blood K^+, Na^+, ATP and cell counts or after centrifugation for plasma and cell measurements separately.

The plasma sodium, potassium and calcium measurements were performed by flame photometry. The ATP measurements were carried out by Berthold Luminometer with a standard addition technique too [25-27] using ATP bioluminescence CLS II kit (No 1 639 695 Boehringer Mannheim, Germany). Glucose levels were determined by a glucose oxidase-peroxidase (GOD / POD) method. Plasma and total blood hemoglobin was determined by the modified Drabkin method.

2.3 Optimization of metabolic activity of testmicrobes in direct bioautography

Chemicals

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma (St.Louis, Missouri, USA), Triton X-100 a nonionic detergent from Merck (Darmstadt, Germany) and ethanol from Reanal (Budapest, Hungary).

Microorganisms, media and antimicrobial agents

The test strains were obtained from the American Type Culture Collections: *Bacillus subtilis,* ATCC 6633, *Micrococcus luteus,* ATCC 9341, *E. coli*, ATCC 25922, *Candida albicans,* ATCC 90028. Mueller - Hinton broth, pH=7,3$\pm$0,2 (Difco Laboratories, Detroit, Michigan, USA) was supplemented with 5% glucose in case of fungus. 0,01-10 ng Cefazolin (Totacef) from Bristol-Myers Squibb S.p.a. Sermoneta (Latina, Italy) as antibacterial-, 0,01-20 µg fluconazol (Mycosyst) and 0,01-4 µg fungizon (Amphotericin B) were used as antifungal testagent,

Mueller-Hinton broth in a 500 mL flask was inoculated by broth suspension of test microbe.

Determination of colony forming unit (c.f.u.) was carried out by tube dilution method and the growth of microbes was followed by photometer (Perkin-Elmer C4B) at 600nm.

Bioautography

TLC sorbents for our assays: TLC DC - Alufolien Silica gel 60 F254 (Art. 1.05554, Merck, Darmstadt, Germany), DC - Alufolien Aluminiumoxid $60F_{254}$,HPTLC-Alufolien Cellulose (Art. 16092, Merck), DC-Alufolien Polyamid $11F_{254}$ were used and cut into sheets of 10 x 10 cm. In our experiments the silica TLC plate gel was used generally.

Sample application modes: without development a series of 0.01-200 ng Cefazolin or other antimicrobial drug was dropped and dried as a test substance on chromatoplates.

Determination of bacterial viability in immersion suspension

Test strains were grown in Mueller-Hinton broth and kept in a shaker (New Brunswick Co., INC New Jersey, USA) at 37 ^{o}C. The microbial number was followed in a spectrophotometer (Perkin Elmer C4B) by determining of the OD at 600nm. The microbial viability in the immersing microbial suspension was determined by a bioluminescent ATP method [28-30] and the protein content by a Protein-Dye Binding (Coomassie Brilliant Blue G-250) assay [31].

Determination of microbial viability on TLC plate

The test strains were grown in Mueller-Hinton broth at 37 ^{o}C. TLC plates were immersed into the given microbial suspension. The microbes were growing on TLC plates further at 37 ^{o}C in a moisturized chamber. The TLC plates were one by one processed at different time points. After incubation one part of their silica surface (6 cm^2) was scratched for ATP and protein determination. The inhibition spots of cefazolin on the unscratched surface of TLC plates were visualised by MTT as part of the bioautographic process.

In the samples the microbial viability was determined by a bioluminescent ATP assay [28, 30]. The protein content was determined after *Bradford* [31] by a Protein-Dye Binding (Coomassie Brilliant Blue G-250) assay.

Scanning Electron Microscopy (SEM)

The electron micrographs were taken with a Jeol JSM 6300 (Japan) electron microscope. Microbes on TLC plates were fixed with glutaraldehyde (2.5%) followed by dehydration in acetone. Samples were treated with Jeol fine coat (Ion Sputter JFC-1100) [33,34].

3 Results and discussion

3.1 ATP depletion in human red blood cells

Human red blood cells were incubated for several days at 4^{o}C and 37 ^{o}C, and ATP, glucose, K^+, Na^+, hemoglobin, water content, MCV, pH, Ca analysed in time-sequences. At 37 ^{o}C total ATP and glucose decreased parallel. Significant and probably irreversible loss of intracellular potassium was seen only when cell ATP content decreased by about 90% [35]. Later this result was cited and confirmed Austrian scientists in their studies on blood preservations [36]. Our results were also used during application of leucocyte depleted whole blood [37].

The release kinetics of K^+ was sigmoidal with a steep increase after 48 hours of incubation, while ATP loss showed an exponential-like kinetics. However, sufficient glucose remained for the metabolic activity of red blood cells even at the termination of

the experiment. In other words, the complete depletion of ATP could not be explained by the lack of glucose and/or phosphate.

Interestingly, hemoglobin did not leak out of cells immediately even after complete ion equilibrium with the surrounding medium. This means that the death of red blood cells did not cause an immediate release of hemoglobin and other intracellular proteins. Release of hemoglobin started only after 96 hours of incubation.

Maximum speed of changes of the examined parameters was found at different time intervals. For example the maximal speed of concentration changes for glucose was found at 12 - 24 hours of incubation, at 24 - 36 hours for ATP, at 48 - 60 hours for K^+ - Na^+ and after 96 hours for hemoglobin.

Our data suggest that loss of ATP affects the whole macromolecular structure of the red blood cells and not only single entities, such as the Na^+-K^+-ATPase. It is correspond with the results of other studies [37-39].

3.2 Bioluminescent measurement of ATP is suitable method to follow metabolic activity of testmicrobes on TLC plates used in direct bioautography

ATP values were referred to total microbial protein content [40]. Others worked with overnight-culture (post log phase) [41,42]. Optimal living conditions of testmicrobes were maintained on TLC plates to reach log phase. We used broth culture of testmicrobes **growing in log phase** for immersion of TLC plates. **So incubation time of TLC plates in water vapour-chamber at 37^o were reduced highly. After shorter incubation time destruction of sorbent layer could be avoided. Consequently, it might become possible to use adsorbents other than silica, e.g. cellulose, polyamide** [43] **etc**. **Application areas of direct bioautography were increased.** Hirherto these layers were detached as a result of soaking because of the agressive layer pre-conditioning process required for the microbial detection method. Our proposed method does not require the pre-incubation of layers in a humid atmosphere. As we know cellulose TLC-layers are good for separation of highly polar compounds (for example carbohydrates, carbonic acids, amino acids…). For separation of chiralic compounds a TLC plate with cellulose layer having natural chirality is more suitable.

In earlier direct bioautographic methods the rate of living and not living microbes, and the role of secunder metabolites from microbes with post log phase were not analysed problems.

In our studies optimal living conditions of testmicrobes were very different. Therefore it is **important to determine these special conditions for testmicrobes**. *E. coli* (Gram-negatíve bacterium)*, M. luteus, B. subtilis* (Gram-pozitív bacteria) and *C. albicans* (buding fungus) microbes were studied for direct bioautography in our experiments [44-46].

3.3 Scanning electron micrographs of bioautograms

Scanning electron microscopy of bioautograms was firstly used to visualise microbes trapped to silica particles of the TLC plate surfaces. The shape and size of microbes reflect their viability and the effect of antimicrobial chemicals e.g. The micrograph of TLC bioautogram shows elongated pseudohyphae of *Candida albicans* strain they can be influenced by an antifungal compound (fluconazole). This method (SEM) can be used for detection of bacteria on TLC plates as well.

3.4 Our new method (MDD: Microbiologically Detected Dissolution) is suitable to follow changes of biological activity in delivery system of antibiotics

As we know this is the first study for determination of dissolved antibiotic and fungicidic drug in vitro dissolution tests. This optimized MDD method [49, 50] is a new one for studying of drug delivery system.

We studied the advantages of this new test method in a model system using test compounds: ciprofloxacin, clarithromycin, doxycycline, nystatin. They were chosen from different microbiologically active antibiotic groups (fluoroquinolones, macrolides, tetracyclines, fungicides).

This method can be extended to other antibacterial and even to antifungal compounds used in clinical practice.

3.5 BioAréna^{TM} : realization of optimized TLC (OPLC) and optimized bioautography

BioAréna^{TM} is a complex bioautographic system combining results of direct bioautography and professional TLC (OPLC) [8, 51-54]. This method can follow metabolic activity of testmicrobes and antimicrobial effects on TLC layer.

Our results were summarized in book chapters, published by **Springer [18]** and by **Elsevier: Encyclopedia of Analytical Science, 2005, Bioassays: Bioautography [55]**.

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Publications used as a basis for the present thesis

Articles(see p.7)
Book chapters (see p.7)
Abstracts published in journals with impact factors (see p.8)
Oral presentations, posters related to the thesis (see p.8)
Other publications (see p.8)

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