ELSEVIER

Biochimica et Biophysica Acta 1573 (2002) 177–182

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Extracellular metabolisation of NADH by blood cells correlates with intracellular ATP levels

Karl Nadlinger *, Wilhelm Westerthaler, Danijela Storga-Tomic, Jörg G.D. Birkmayer

Department of Research and Development, Birkmayer Laboratories, Schwarzspanierstr. 15, A-1090 Vienna, Austria

Received 26 April 2002; received in revised form 20 August 2002; accepted 4 September 2002

Abstract

A new assay allowing quantitation of extracellular NADH metabolisation by intact blood cells was compared with the intracellular ATP/ADP ratio of these cells. The sensitivity, reproducibility and NADH specificity of this assay were determined. The diagnostic potential of this test was examined in a study with highly conditioned athletes. NADH consumption was measured before and immediately after maximum aerobic performance as well as 1 day later and was compared with the ATP/ADP level in these blood cells. A significant decline of cellular energy after aerobic performance was detected with both approaches to a similar extent ($P<0.01$). However, the extracellular NADH metabolisation assay (ENMA) is more convenient to perform than the determination of intracellular ATP/ADP. Due to its easy and versatile handling, a huge array of possible applications like monitoring the training efficiency of athletes, the fitness of senior citizens or the recovery from disease may be envisioned.

Keywords: NADH; ATP; Blood test; Sports

1. Introduction

Cellular energy is produced in mitochondria by oxidative phosphorylation, generating adenosine triphosphate (ATP). The key enzyme mediating the production of ATP is NADH cytochrome *c* reductase, also known as Complex I–III. This enzyme reduces cytochrome *c* via NADH. The reduced cytochrome *c* is oxidized further by cytochrome *c* oxidase (Complex IV) to form water.

Illustratively, the more energy a cell needs, the more NADH it contains. For example, heart cells have 90 μg/g; brain and muscle cells contain 50 μg/g tissue; liver cells contain 40 μg/g and red blood cells contain 3 μg/g tissue. Thus, the activity of NADH cytochrome *c* reductase is directly linked to the amount of NADH in the cell and reflects the energy producing capacity of a cell [1,2].

In a variety of diseases (the so-called mitochondrial diseases), energy production, in particular the activity of NADH cytochrome *c* reductase (Complex I–III), is decreased. This was demonstrated not only for brain and muscle tissue, but also in platelets [3–5] for patients with Parkinson's disease (PD) and Alzheimer's disease. A further demonstration of the link between NADH cytochrome *c* reductase activity and disease involves the toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a compound that irreversibly inhibits NADH cytochrome *c* reductase in certain brain areas leading to Parkinsonian-like symptoms [6]. These and similar findings have significant implications for investigations into the etiology of neurodegenerative diseases.

Azidothymidine (AZT), commonly used in the treatment of AIDS patients, also damages NADH cytochrome *c* reductase and causes a decrease of the cellular energy production [7]. By measuring the activity of NADH cytochrome *c* reductase in muscle tissue biopsies, it was demonstrated that AZT destroys the enzyme's activity, resulting in muscle atrophy.

In contrast to substances with an inhibitory effect on the activity of key enzymes related to cellular processes for the production of energy, there are also substances like the reduced form of nicotinamide dinucleotide (NADH) and nicotinamide adenine dinucleotide phos-

* Corresponding author. Tel.: +43-1-402-2367-39; fax: +43-1-408-9908.

E-mail address: K.Nadlinger@birkmayer.com (K. Nadlinger).

0304-4165/02/$ - see front matter
PII: S0304-4165(02)00421-X

phate (NADPH), which are able to enhance NADH cytochrome *c* reductase activity and consequently the cellular energy production. These two coenzymes have been proved to be useful in the treatment of PD [8]. Furthermore, NADH alleviates certain symptoms of Alzheimer's disease [9] and is able to supply additional muscular energy to healthy individuals [10].

Assay methods for the determination of NADH cytochrome *c* reductase activity were described for many tissues, in particularly for muscle, liver, brain and heart cells [11]. The utility of such enzyme-based assays can be extended to the use of coupled reaction schemes in which the distinct analyte does not directly participate in the enzyme catalyzed process giving rise to an analytical signal, but is indirectly linked to the process by a coupled reaction. Despite the use of a common enzyme cofactor in these analytical assays, all these procedures share a common trait in that they are limited to reactions not directly involved in cellular energy production. As a consequence, such reactions are dependent on the addition of both exogenous enzyme and cofactor, such as NADH, in order to generate an analytical signal. A number of specific limitations exist for those analytical procedures that are more directly related to cellular reactions. For example, all tissue-based assays require the sampling of tissue through biopsy or other even more invasive surgical procedures. For certain tissues like the brain, it is difficult if not impossible to obtain samples. Sometimes, samples can be taken only post mortem. Assays using platelets derived from a blood sample offer an advantage to methods using muscle or other tissue obtained by biopsy. However, using platelets to test the distinct enzyme activity depends on the level of purification of the platelets, which may cause an analytical error.

To overcome the drawbacks mentioned above, a method for the determination of energy-related enzyme activity has to be simple and easy to perform. Our extracellular NADH metabolisation assay (ENMA) fulfills this requirement. Here we describe a procedure for the determination of the relative energy producing capacity of blood cells by quantitating the amount of NADH that is metabolized to NAD^+ by these cells. The amount of NADH that is consumed by blood cells is compared to the ATP/ADP ratio of the same cells, which is regarded as a measure of the level of the energy of the cell.

Under the condition of hypoxia and lack of energy-yielding compounds, the reduced coenzymes of the respiratory chain no longer can be oxidized sufficiently. Hence, the aerobic energy output of the hypoxic cell is reduced, leading to a decreased functional capacity. The cellular $NADH/NAD^+$ ratio as well as the cellular NADH turnover reflect such an unfavourable cellular condition. As it is impossible to determine the mitochondrial or cellular $NADH/NAD^+$ ratio by certain blood test directly, only indirect methods such as, e.g., the determination of the lactate/pyruvate ratio in blood, can be employed to describe the redox state of the respiratory chain in a cell. Information about cellular NADH availability is important for the interpretation of conditions of hypoxia and disturbed mitochondrial function, which lead to an altered NADH turnover particularly under metabolic stress. These changes result in low ATP/ADP ratio. The extracellular NADH metabolisation by an in vitro assay can help to measure cellular NADH availability and provide valuable information for patients with metabolic disorders or for healthy individuals during and after intensive physical exercise.

2. Materials and methods

2.1. Standard procedure for the ENMA

Five hundred microliters of EDTA blood (used between 1 and 4 h after collection) are diluted with 1 × PBS (8 g/l NaCl; 0.2 g/l KH_2PO_4; 1.15 g/l Na_2HPO_4; adjusted to pH 8.0 with 6 M KOH) in a Centrisart I filter tube with a cut off range of 20.000 MW (Sartorius). Fifty microliters of NADH solution (8 mg NADH/ml 1 × PBS) are added to start the reaction. This mixture is incubated for 2 h at 37 °C. The reaction is stopped by centrifuging the tube for 10 min. The filtrate, which contains the NADH not consumed by the reaction, is collected and analyzed by HPLC. Two standards with 2 ml 1 × PBS containing (a) 25 μl or (b) 50 μl of NADH solution (described above) are incubated for the same time under the same conditions and are used to quantify the metabolized NADH amount.

2.2. Reproducibility of the assay

Two EDTA blood samples from each of three subjects were collected and a series of 20 analyses per subject (10 tests per sample) were performed.

2.3. Specificity of the assay

NADPH or ATP was used instead of NADH as described in the standard procedure to find out whether this test works specifically with NADH only.

2.4. pH value dependence

Standard assays were performed under different pH values ranging from 6.0 to 14.0.

2.5. Dependence on the test reaction temperature

In order to examine the effect on temperature of the assay, blood and the mix 1 × PBS containing NADH solution were preincubated for 1 h at 8, 25 and 37 °C, respectively. Then, the blood was mixed with the NADH solution and incubated for 2 h at these temperatures.

2.6. Cell dependence of the reaction

For testing the cell dependence on the reaction, 4 ml whole blood was centrifuged at 900 × *g* for 2 min and the serum was removed. Afterwards, the blood cells were washed three times with 5 ml of 1 × PBS. After the last wash, the blood cells were diluted 1:1 with 1 × PBS and used as enzyme probe for the standard procedure as described above.

In another approach, 250 μl of cell-free serum, corresponding to the serum amount of the test procedure, as collected by Vacutainer blood drawing, was used to test the cell-free reaction whether NADH metabolisation is also found in cell-free serum.

2.7. Dependence of the assay on the concentration of the blood cells

The standard procedure was performed with 2, 5, 10, 25, 50, 100, 200, 400, and 800 μl of blood, diluted to a final volume of 2 ml with 1 × PBS. Fifty microliters of NADH solution (8 mg/ml) was added to each of the different blood concentrations.

2.8. Stability of blood samples after collection

In order to find out how long blood samples are stable for obtaining comparable results, blood samples were kept at room temperature for 15, 105, 235 and 290 min after collection.

2.9. Effect of storage of blood samples

Blood was collected and divided into 2 ml portions, stored for 1 and 2 days at 8 °C and compared with the result of the assay using fresh blood. Intact blood cells are essential for reliable results. Freezing and thawing of blood samples lead to different results.

2.10. Practical application of the ENMA on blood samples obtained from athletes

The assay was used in a study with 14 highly conditioned endurance athletes who had a significant higher aerobic performance compared to normal healthy individuals. In these athletes, the ATP/ADP ratio in blood cells was determined before, immediately after and the next day after an exhaustive aerobic performance test on a treadmill. The ENMA was also performed at the same time intervals 10 weeks later.

2.11. HPLC of NADH

The method of Formato et al. [12] was adapted as follows. With a two pump Shimadzu LC10A System, NADH was detected using a Lichrospher RP18, 5 μm 250 × 4 mm (Merck) column by measuring the absorption at 340 nm with a diodearray detector (Shimadzu SPD-M10A). Profile of the eluent gradient in percent of mobile phase on pump 1: 0–1.50 min 100%; 2 min 80%; 5.50 min 75%; 8 min 25%, 13 min 40%; 15 min 0%; 15.2 min 100%, 19 min 100%, 21 min stop.

Mobile Phase for pump 1 consisted of 2720 mg tetrabutyl ammonium hydrogen sulfate; 0.1 M potassium dihydrogen phosphate; 1 ml sodium azide solution 1% (w/v), deionized water up to 1 l, pH value adjusted to 6.0 with 6 M KOH; the solution was degassed for 15 min by sonification. Mobile phase for pump 2 consisted of 2720 mg tetrabutyl ammonium hydrogen sulfate; 0.1 M potassium dihydrogen phosphate; 1 ml sodium azide solution 1% (w/v), 300 ml acetonitrile; deionized water up to 1 l, pH value adjusted to 6.0 with 6 M KOH; the solution was degassed for 15 min by sonification.

The result of the assay was calculated as percentage of NADH not metabolized by the blood compared to the 50 μl NADH standard (100% NADH, 0% NADH metabolized).

2.12. Alkaline extraction and measurement of ATP/ADP ratio

For alkaline extraction of ATP and ADP, the method by Stocchi et al. [13,14] was modified: In a Centrisart tube (cut off range 20.000 MW), 0.5 ml ice-chilled 0.5 M KOH was added to 0.5 ml fresh EDTA blood and vortexed. Five minutes later, 1 ml chilled distilled water was added. The mixture was vortexed and then centrifuged for 15 min at 2000 × *g*. The filtrate was collected, and ATP and ADP were analyzed by HPLC using the method described above but setting the diodearray detector at a wavelength of 254 nm. The ratio of ATP/ADP is expressed as μM ATP divided by μM ADP.

3. Results

3.1. Standard procedure results

Using the standard procedure, at least 5% of NADH was metabolized. After an exhaustive aerobic fitness testing, we found an NADH consumption between 80% and 100%.

3.2. Reproducibility

Serial tests ($n=20$) from two blood tubes, drawn from one subject at the same time were performed. A coefficient of variation (c.v.) of 6.1 ± 0.9% (three series) related to the oxidized NADH was found. Practically no difference between the two samples ($n=10$) from one person was found (c.v. of systematical error of pipetting and HPLC of approximately 2% is included).

3.3. Specificity of the reaction

NADPH or ATP are metabolized by whole blood only to a very limited extent (less than 5% of these two coenzymes are metabolized by this enzyme system).

3.4. Dependence on the pH value

The ENMA showed identical values in the range of pH 8.0 to pH 12.0. At pH lower than 7.0, NADH is degraded nonenzymatically, and at a pH above 12.0, blood cells get damaged.

3.5. Dependence of the assay on temperature

The highest NADH metabolisation rate was found at 37 °C. At 25 °C, only a slightly lower NADH metabolisation by the blood cells was observed. At 8 °C, a decline in the enzymatic reaction to approximately 50% was found.

3.6. Cell dependence of the reaction

The reaction of washed blood cells was in range of the results obtained by the test conditions. Serum showed different results. Commonly, only a low reaction (<5%) was found, but in some cases, a high turn over up to 30% of whole blood reaction was observed.

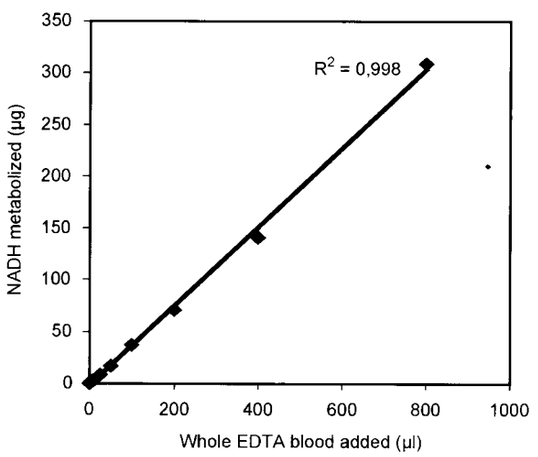

Fig. 1. Extracellular NADH metabolisation in dependence on the number of blood cells present. Blood was collected in EDTA containing vacutainer tubes. EDTA blood (2, 5, 10, 25, 50, 100, 200, 400 and 800 µl) was diluted with a phosphate balanced salt solution to a total volume of 2.0 ml. Four hundred micrograms NADH (200 µg/ml final concentration) was added to this suspension of blood cells and incubated for 2 h (120 min) at 37 °C. The content of NADH was determined after the incubation period by HPLC and by measuring the optical density at 340 nm. From the residual (not metabolized) NADH, the consumption of NADH by blood cells was calculated. The chart shows a linear correlation of the NADH consumed by increasing numbers of blood cells.

3.7. Dependence on the concentration of blood

The NADH metabolized by whole blood showed a nearly perfectly linear correlation in the tested range from 2 to 800 µl of blood (correlation coefficient R^2>0.99, shown in Fig. 1).

3.8. Stability of blood samples after collection

Within 4 h after collection, an increase of the reaction of about 10% was found.

3.9. Storage of blood samples

A strong increase of reaction was found when the blood samples were stored for 24 h at 8–10 °C.

3.10. NADH metabolisation of blood from athletes compared to their ATP/ADP ratio of blood cells

After exhaustive aerobic performance, the ATP/ADP ratio declines as well as the concentration of NADH not metabolized by the blood cells. This is an indication that blood cells consume more NADH after physical training than before the aerobic exercise. The ATP/ADP ratio decreased in 13 of 14 athletes (P<0.01) in the first phase of the study after exhaustive aerobic performance. In the second phase, a decrease of ATP/ADP ratio was also found in 13 of 14 subjects. In phase 1, on the day following exhaustive aerobic performance, the ATP/ADP ratio was higher in 13 of 14 athletes than immediately after exhaustive performance. Similarly, in the second phase of the study, all 14 subjects had a higher ATP/ADP ratio on the day following exhaustive performance. In terms of NADH metabolisation by blood cells, all 14 subjects showed an increased NADH consumption after exhaustive aerobic performance in phase 1 while 13 of 14 subjects showed an increase in the second phase. This single subject had a base value significantly lower than expected in comparison to the other athletes. On the following day, all 14 subjects showed a decreased NADH metabolisation in comparison to the value obtained immediately after exhaustive aerobic performance. In the second phase of the study, 12 of 14 subjects (P<0.01) exhibited a reduced NADH consumption.

If one compares the assay values from the day after exhaustive performance with the baseline value before exhaustive aerobic performance, the ATP/ADP ratio was lower in 10 (phase 1) and 11 (phase 2) of 14 subjects (P<0.05). The NADH metabolisation was still higher on the day after aerobic performance than at baseline in 12 (phase 1) and 13 (phase 2) of 14 subjects (P<0.01).

The mean values of the ATP/ADP ratio after exhaustive aerobic performance declined to 80% and 84% of the baseline. On the day after aerobic performance, it returns to 96% and 99% of baseline value. The residual NADH

Table 1
Extracellular NADH metabolisation and ATP/ADP ratio before, immediately after and 1 day after aerobic performance

	Before aerobic fitness test	After maximum aerobic fitness test	24 h later
First intervention			
μM ATP/μM ADP ratio	11.1 ± 1.9	8.9 ± 0.9	10.6 ± 1.4
Percentage of NADH not metabolized by blood cells	66.4 ± 25.6	4.5 ± 5.2	25.4 ± 16.9
10 weeks later			
μM ATP/μM ADP ratio	12.7 ± 1.8	10.7 ± 2.1	12.6 ± 2.6
Percentage of NADH not metabolized by blood cells	67.4 ± 19.2	7.6 ± 5.9	27.5 ± 14.6

Data show mean value ($n=14$) of the ATP/ADP ratio as well as of the residual NADH not metabolized by blood cells. Four hundred micrograms NADH was added to 0.5 ml EDTA blood before, immediately after and 1 day after exhaustive aerobic performance.

not metabolized by the blood cells decreases to 7% and 4%, respectively, of the baseline value. The day after aerobic performance, it returns to 38% and 40% of the baseline values (Table 1).

4. Discussion

The ENMA described in this paper shows excellent reproducibility. When blood is taken repetitively from one individual subject, the coefficient of variation is in the range of 6%. This is comparable to the most common assays in clinical chemistry (radioimmunoassays have higher coefficients of variation in most cases). It is very specific for NADH: NADPH and ATP show a slight reaction with intact blood cells but are metabolized to an extent of less than 5% as compared to NADH. The test values remain constant over a wide range of pH as well as of incubation temperature.

The NADH metabolisation is cell dependent. It correlates with the number of blood cells in a strictly linear manner. This implies that the amount of blood can be varied according to individual needs. The simple handling makes this test very useful for routine analysis.

However, the assay has to be performed immediately after drawing of blood; otherwise, alteration or lysis of blood cells may cause incorrect values. A certain disadvantage of this method is the termination of the enzymatic reaction. In order to stop the cells from consuming NADH, they have to be separated from the incubation mixture by filtration, which is usually done by separating the plasma from the blood cells by centrifuging through filters of certain mash size. Thus, it is required to keep an exact incubation time after adding NADH to the test system in order to achieve comparable results. The rather long incubation period of 2 h used in our study has been found to be an advantage as slight deviation from the incubation time does not lead to significant alterations of the results. The ENMA uses intact cells as the source of enzyme. Hence, it is of utmost importance to keep the cells intact. A longer storage of the blood samples or freezing and thawing or storing in a refrigerator lead to haemolysis. This causes other NADH metabolizing enzymes to leak out from the cell matrix, increasing the NADH consumption. Haemolytic blood samples cause considerable changes in the NADH consumption leading to falsely increased results. Due to this reason, only freshly drawn blood must be used.

What is the diagnostic relevance of this test? Our assumption was that subjects having a high NADH metabolisation have a lower endogenous NADH concentration at least in their blood and, hence, a lower ATP level. In order to test this hypothesis, we used our NADH metabolisation assay to test athletes before and after aerobic performance. It is well known that the ATP/ADP ratio changes after aerobic performance because ATP is consumed in the working muscles. Therefore, after aerobic physical exercise, more ADP is formed and the ratio of ATP and ADP declines. Normally, the ratio of ATP and ADP in human erythrocytes is 10:1 [15]. In highly conditioned athletes, this ratio is even higher. When highly conditioned athletes make a long distance (marathon) run, the ratio of ATP to ADP declines because ATP is consumed. After a resting period of 1 day, the ratio of ATP to ADP returns to the original baseline levels. Comparable results have been observed with the ENMA described in this paper: after extensive aerobic physical exercise, most of the endogenous NADH is consumed; hence, the blood cells need more exogenous NADH to restore their ATP pools. After a resting period of 24 h, the NADH consumption declines, implying that the subjects recover and regain their normal NADH pool. Hence, they need less exogenous NADH. In comparison to the ATP/ADP ratio, NADH metabolisation does not reach the original baseline value within one day. At phase 2 of the study (10 weeks later), the same baseline values as at phase 1 are obtained. Thus, an irreversible process can be excluded. The ENMA appears to provide a valuable monitoring tool for the physiological metabolic recovery of the organism.

The advantage of the ENMA in comparison to the ATP/ADP ratio determination is its simplicity and the much lower amount of blood cells needed for the determination. The exact determination of ATP/ADP ratio depends on the quality of the ATP/ADP extraction procedure as well as of the HPLC analysis. Both steps are very elaborate and time consuming and prone to methodical errors. In contrast, the ENMA could be easily adapted to a simple photometric test using only 5–10 μl of blood, and may be applicable to automatic clinic chemical analyzer.

Acknowledgements

We greatly appreciate the effort of Mag. Markus Wögerbauer for reviewing the manuscript and Yvonne Schindlegger and Mag. Stefan Wuketich for technical assistance.

References

[1] B. Alberts, D. Bray, J. Lewis, H. Raff, K. Roberts, J.D. Watson, Energy Conversion: Mitochondria and Chloroplasts in Molecular Biology of the Cell, 3rd ed., Garland Publishing, New York, 1994, pp. 653–720.

[2] A.L. Lehninger, Vitamins and Coenzymes in Biochemistry, 2nd ed., the John Hopkins University School of Medicine, Worth Publishers, New York, 1975, pp. 337–342.

[3] J.M. Cooper, V.N. Mann, D. Krige, A.H.V. Schapira, Human mitochondrial complex I dysfunction, Biochim. Biophys. Acta 1101 (1992) 198–203.

[4] Y. Mizuno, S. Otha, M. Tanaka, S. Takamiya, K. Suzuki, H. Oya, T. Ozamwa, Y. Kagawa, Deficiencies in complex I subunits of the respiratory chain in Parkinson's Disease, Biochem. Biophys. Res. Commun. 163 (1989) 1450–1455.

[5] J.M. Shoffner, R.L. Wafts, J.L. Juncos, A. Torroni, D.C. Wallace, Mitochondrial oxidative phosphorylation defects in Parkinson's Disease, Ann. Neurol. 30 (1991) 332–339.

[6] R. Bennecke, P. Strumper, H. Weiss, Electron transfer complex I and IV of platelets are abnormal in Parkinson's disease but normal in Parkinson-plus syndromes, Brain 116 (1993) 1451–1463.

[7] M.C. Dalakas, I. Lila, G.H. Pezeshkpour, J.P. Laukaftis, B. Cohen, J.L. Griffin, Mitochondrial myopathy caused by long-term zidovudine therapy, N. Engl. J. Med. 322 (1990) 1098–1105.

[8] W. Birkmayer, J.G.D. Birkmayer, C. Vrecko, B. Paletta, E. Reschenhofer, E. Ott, NADH bei Parkinson, New Trends Clin. Neuropharm. 4 (1990) 7–24.

[9] J.G.D. Birkmayer, NADH bei Alzheimer, Ann. Clin. Lab. Sci. 26 (1996) 1–8.

[10] L.M. Forsyth, H.G. Preuss, A.L. MacDowell, L. Chiazze, J.G.D. Birkmayer, J.A. Bellanti, Therapeutic effects of oral NADH on the symptoms of patients with chronic fatigue syndrome, Ann. Allergy, Asthma, & Immun. 82 (1999) 185–191.

[11] Y. Haten, D.L. Stiggall, Preparation and properties of NADH cytochrome *c* oxidoreductase (complex III), in: S. Fleischer, L. Packer (Eds.), Methods in Enzymology, Academic Press, New York, 1978, pp. 5–10.

[12] M. Formato, B. Masala, G. De Luca, The levels of adenine nucleotides and pyridine coenzymes in red blood cells from the newborn, determined simultaneously by HPLC, Clin. Chim. Acta 189 (1990) 131–138.

[13] V. Stocchi, L. Cucchiarini, M. Magnani, L. Chiarantini, P. Palma, G. Crescentini, Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells, Anal. Biochem. 146 (1985) 118–124.

[14] G. Leoncini, E. Buzzi, M. Maresca, M. Mazzei, A. Balbi, Alkaline extraction and reverse-phase high-performance liquid chromatography of adenine and pyridine nucleotides in human platelets, Anal. Biochem. 165 (1987) 379–383.

[15] D.L. Nelson, M.M. Cox, Principles of bioenergetics, in: A.L. Lehninger (Ed.), Principles of Biochemistry, 3rd ed., Worth Publishers, New York, 2000, pp. 501–502.