IOC-28. *Mitochondrial Physiology Network* 9.5: 1-10

International Course on High-Resolution Respirometry and MiP-Net 2004

15-21 Sept. 2004

Schröcken, Vorarlberg, Austria

Programme 15-18 Sept.

Wednesday, 15. September: Informal evening at Hotel Mohnenfluh.

Thursday, 16. September

08:30 – 11:45 Erich Gnaiger (Innsbruck, AT): www.oroboros.at - the Oxygraph-2k and hot topics in MiP. Introduction and practical approach.

14:00 -19:00 Eveline Hütter, Assegid Garedew, Brigitte Haffner (Innsbruck, AT): Oxygraph-2k: Instrument demonstration. Practical Session 1.

20:30 Tutorials.

Friday, 17. September

08:30 - 11:15 Hands-on experiments with the Oxygraph-2k. Practical Session 2.

12:00 – 18:00 Alpine walks and talks.

Fotos: Flemming Dela, Scott Gullicksen, Regitze Kraunsoe and Zeyneb Özbek with the tutors Evi Hütter, Assegid Garedew, Brigitte Haffner and Erich Gnaiger.


Top left: Evi Hütter demonstrating; top right: Flemmig Dela practicing with Oxygraph-2k.



Top left: Evi Hütter titrates inhibitors and uncouplers; middle: Scott Gulliksen checks the membrane application of the polarographic oxygen sensor, observed by Regitze Kraunsoe and Zeyneb Özbek, and guided by Brigitte Haffner; right: Evi Hütter oversees the Oxygraph-2k instrumental background competition.

21:00 Wine respiration.

Saturday, 18. September

08:30 - 12:00 Working Groups: High-resolution respirometry / DatLab Analysis.

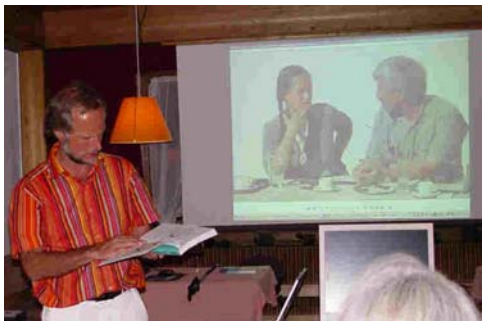
12:00 – 16:00 Alpine walks and talks/MiP-Net arrivals.


16:15 -17:30 **Erich Gnaiger** (Innsbruck Medical University, AT) High-resolution respirometry and mitochondrial physiology: Experimental titration regimes. *MiPNet-01*.

Mitochondrial Physiology Network MiP-Net Meeting 2004

Saturday, 18. Sept.

20:30 Eveline Hütter as program director, putting up the scientific program just before the opening of the meeting. She is assisted by Buno Fink, Brigitte Haffner, Zeyneb Özbek, Laszlo Wenchich and Susanna Cadenas.



21:30 **Erich Gnaiger** (Innsbruck Medical University, AT) MiP-Net Introduction: 10 years OROBOROS, a spin-off from science - Oxygraph-2k: from prototype to O2k MiP-NetAnalyzer – Slides from the 28th Oxygraph Course and before. *MiPNet-02*.

Left: Erich Gnaiger introduces MiP-Net 2004, looking 10 years back to the book BTK 1994: "*What is controlling Life ?*" and 1 year back to a slide with Evi Hütter and Steve Hand at MiP2003 (Foto: Mikhail Vyssokikh).

Sunday, 19. Sept.

Session I: **NO(S) - COX - and mitochondrial physiology.**
Chair: **Andrey Kozlov**

09:15-09:45 **Pedram Ghafourifar** (Marshall University, US) Mitochondrial nitric oxide synthase and mitochondrial respiration. *MiPNet-03*.

09:45-10:15 **Mikhail Vyssokikh** (Moscow State University, RU) Oxidative and nitrosative stress in rat kidney under conditions of ischemia/reperfusion. *MiPNet-04*.

10:45-11:15 **Bruno Fink** (Noxygen Science Transfer & Diagnostics GmbH, DE) Cyclic hydroxylamines as a tool for the diagnosis of mitochondrial function and dysfunction. ***MiPNet-05***.

11:15-11:45 **Susana Cadenas** (Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, ES) Questions on the cytochrome c oxidase/ guanylate cyclase branchpoint. ***MiPNet-06***.

11:45-16:00 MiP-Net Walks and Talks

Session II: **ROS and mitochondrial physiology.**
Chair: **Mikhail Vyssokikh**

16:15-16:45 **Heimo Mairbäuerl** (University of Heidelberg, DE) Oxygen consumption and ROS-formation by alveolar epithelial cells in hypoxia. *MiPNet-07*.

16:45-17:15 **Eveline Hütter** (Institut für Biomedizinische Alternsforschung, Innsbruck, AT) High-resolution respirometry for analysis of non-mitochondrial ROS production in ageing endothelial cells. *MiPNet-08*.

17:15-17:45 **Erich Gnaiger** (Innsbruck Medical University, AT) Oxyconformance in cellular respiration. *MiPNet-09*

Instrument session: **Instrument demonstrations after dinner**.
Chair: **Laszlo Wenchich**

Bruno Fink (Noxygen Science Transfer & Diagnostics GmbH, DE) Bench-Top ESR Spectrometer. *MiPNet-10*.

Erich Gnaiger (OROBOROS INSTRUMENTS, Innsbruck, AT) O2k MiP-NetAnalyzer. *MiPNet-11*.

Monday, 20. Sept.

Session III: **Mitochondrial Cellular Physiology.**
Chair: **Susana Cadenas**

09:15-09:45 **Zakaria Almsherqi** (National University of Singapore, SG) Potential role of myocardial UCP in regulating superoxide generation post acute myocardial ischemic insult in dogs. ***MiPNet-12***.

09:45-10:15 **Gino Heeren** (Univ. Salzburg, AT) MiPNet-13.

Session IV: **Diagnosis of Mitochondrial Function.**

10:45-11:15 **Andrey Kozlov** (L. Boltzmann Institut für experimentelle und klinische Traumatologie, Vienna, AT) Endotoxic shock and mitochondrial function in rats. ***MiPNet-14***.

11:15-11:45 **Laszlo Wenchich** (Charles University, Prague, CZ) Polarographic analyses of respiratory chain complexes in isolated muscle mitochondria and permeabilized muscle cells in children with mitochondrial disorders. ***MiPNet-15***.

12:00 OROBOROS INSTRUMENTS party: Walk from Hochkrumbach to Körbersee. **Cheese and Wine Reception at the Alpmuseum uf m Tannberg**.

Session V: **Respiratory and Mitochondrial Physiology in Greenland and on Top of the Alps.**
Chair: **Haimo Mairbäuerl**

21:00 **Robert Boushel** and **Cindy Wright-Paradis** (Concordia University, Montreal, CA) and **Erich Gnaiger** (Innsbruck Medical University, AT) Introduction, overview and slide show. *MiPNet-16*.

Participants

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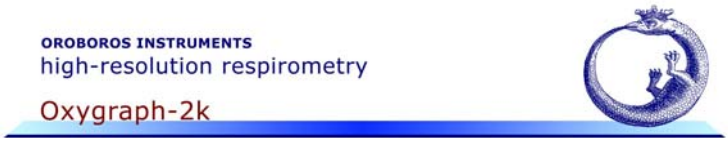

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Cooperation and Feedback in Science

Abstracts MiP-Net 2004

MiPNet-01 **Erich Gnaiger** (Innsbruck Medical University, AT) High-resolution respirometry and mitochondrial physiology: Experimental titration regimes.

MiPNet-02 **Erich Gnaiger** (Innsbruck Medical University, AT) MiP-Net Introduction.

MiPNet-03 **Pedram Ghafourifar** (Marshall University, US) Mitochondrial nitric oxide synthase and mitochondrial respiration.

MiPNet-04 **Mikhail Vyssokikh** (Moscow State University, RU) Oxidative and nitrosative stress in rat kidney under conditions of ischemia/reperfusion.

MiPNet-05 **Cyclic hydroxylamines a tool for the diagnosis of mitochondrial function and dysfunction.**
Fink Bruno, Noxygen Science Transfer & Diagnostics GmbH, Elzach, Germany
Atrial fibrillation is the most common cardiac arrhythmia. It is associated with a 5-6 fold increase in the incidence of stroke, due almost exclusively to thrombus formation in the left atrial appendage. We hypothesized that this decrease in $NO^{\bullet}$ may be due to increased superoxide ($O_2^{\bullet-}$) production and oxidative destruction of $NO^{\bullet}$. To address this hypothesis, we induced AF in pigs using rapid atrial pacing.

We studied reaction of $O_2^{\bullet-}$ with a new spin probe, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH), for analysis of $O_2^{\bullet-}$ production in suspension of cardiomyocytes and in isolated heart tissue. After 10 min incubation, the intracellular concentration of CMH in cells reached 18.1 %, while the content of 1-hydroxy-3-carboxy-2,2,5,5,-tetramethylpyrrolidine (CPH) was only 9.2%. Rate constant of CMH reaction with $O_2^{\bullet-}$ (1.2×10^4 $M^{-1}s^{-1}$) was 3.7-times higher than that of CPH**.** Intracellular $O_2^{\bullet-}$ production was measured from PEG-SOD inhibited formation of 3-methoxy-carbonyl radical ($CM^{\bullet}$). A 1.8-fold increase in LAA $O_2^{\bullet-}$ from 80 to 140 a.u./mg tissue/10 min was confirmed using ESR and spin probe CMH. Treatment of cardiomyocytes with lactate leads to 3-fold increase in $O_2^{\bullet-}$ production in LAA.

We conclude that atrial fibrillation is associated with increased $O_2^{\bullet-}$ and decreased NO• production in the left atrial appendage. Using new synthesized cyclic hydroxylamines CMH we detected intracellular $O_2^{\bullet-}$ production from mitochondria. High cell permeability and high reactivity with $O_2^{\bullet-}$ of CMH allow effective detection of low amounts of intra- and extracellular $O_2^{\bullet-}$.

MiPNet-06 **Questions on the cytochrome c oxidase/ guanylate cyclase branchpoint.**
Susana Cadenas, Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III (CNIC), Ronda de Poniente 5, Tres Cantos, 28760 Madrid, Spain
Nitric oxide is an intracellular messenger produced in a reaction catalysed by the enzyme nitric oxide synthase. The first well characterized target molecule for transducing the NO signal was the haem iron enzyme soluble guanylate cyclase. The major physiological actions of NO including relaxation of smooth muscle, neurotransmission and inhibition of platelet aggregation, are mediated by NO binding to the haem iron of soluble guanylate cyclase, causing a stimulation of cGMP production. Another target for NO

is cytochrome c oxidase, the terminal component of the mitochondrial respiratory chain, responsible for almost all cellular O2 consumption (1,2). By competing with oxygen for binding to cytochrome c oxidase, NO might be a physiological regulator of the oxygen sensitivity of respiration in tissues. Whether simultaneous activation of soluble guanylate cyclase and inhibition of cytochrome c oxidase occurs within cells, is a matter of debate. It has been recently suggested that guanylate cyclase is significantly more sensitive to being activated by NO than cytochrome oxidase is to being inhibited at physiological pO2 (3). We are working to determine the differential sensitivity of both targets at low pO2, a condition where oxidase inhibition is likely to become more relevant. We are using HEK 293 cells transfected with the inducible isoform of the nitric oxide synthase gene in order to endogenously produce controlled amounts of NO. We are currently involved in assessing cGMP production by soluble guanylate cyclase by immunoassay at different pO2, and in determining oxygen consumption al low pO2 concentrations using high resolution respirometry, in the presence of different amounts of NO. Work in progress will be presented.

[1] Brown G.C., Cooper C.E. (1994) Nanomolar concentrations of nitric oxide reversibly inhibit synaptosomal cytochrome oxidase respiration by competing with oxygen at cytochrome oxidase. *FEBS Lett.* 356: 295-298.

[2] Cleeter M.W.J., Cooper J.M., Darley-Usmar V.M., Moncada S. and Schapira A.H.V. (1994) Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory chain, by nitric oxide. Implications for neurodegenerative diseases. *FEBS Lett.* 345: 50-54.

[3] Bellamy T.C., Griffiths C., Garthwaite J. (2002) Differential sensitivity of guanylyl cyclase and mitochondrial respiration to nitric oxide measured using clamped concentrations. *J. Biol. Chem.* 277: 31801-31807.

MiPNet-07 **Heimo Mairbäuerl** (Medical Clinic, Section VII: Sports Medicine, University of Heidelberg, Luisenstr. 3 Geb.4100, 69115 Heidelberg, Germany) Oxygen consumption and ROS-formation by alveolar epithelial cells in hypoxia.

MiPNet-08 **High resolution respirometry to analyze non-mitochondrial ROS production in aging cells.**

Eveline Hütter[1], Hermann Unterluggauer[1], Erich Gnaiger[2], and Pidder Jansen Dürr[1]
[1]Institute for Biomedical Aging Research, Austrian Academy of Sciences, 6020 Innsbruck
[2]D. Swarovski Research Laboratory, Department of Transplantation Surgery, Innsbruck Medical University, 6020 Innsbruck. email: eveline.huetter@oeaw.ac.at

Oxidative stress is a major determinant of cellular aging. It was assumed over the past three decades, that reactive oxygen species (ROS) are produced „accidentally" in cells, as a consequence of an aerobic lifestyle. However, the discovery of enzymes, like the Nox family, with their predominant function of producing superoxide, indicates physiological implications of ROS in cellular processes as growth, apoptosis, or senescence [1]. We found oxidative stress-associated cell death in primary human endothelial cells [2]. Preliminary results make NADPH oxidases likely candidates for ROS production in senescent endothelial cells.

Mitochondrial and non-mitochondrial oxygen consumption were measured by high resolution respirometry with the OROBOROS® Oxygraph. The software DatLab (OROBOROS, Innsbruck, Austria) was used for data aquisition and analysis. Several inhibitors of the mitochondrial respiratory chain were used to distinguish between mitochondrial and non-mitochondrial oxygen consumption. Oxygen kinetic experiments were performed with fibroblasts [3] and endothelial cells, and the linear oxygen dependence of respiration in the high oxygen range was correlated to the proportional increase of rotenone and antimycin A [3], or cyanide insensitive oxygen consumption. To

establish measurements of NADPH-oxidase activity with the oxygraph, a pilot experiment with stimulated human peripheral blood mononuclear cells (PBMC's) was performed.

Oxygen kinetics of human primary cells were biphasic, which was explained by a mitochondrial hyperbolic component in the low-oxygen range and a linear increase of antimycin A-inhibited oxygen consumption in the high-oxygen range. Since antimycin A-mediated mitochondrial ROS production accounts maximally to 4% of state 4 respiration [4], probably the major proportion of inhibited respiration represents non-mitochondrial ROS production. Measurements of oxidative burst activity in PBMC's showed, that the analysis of NADPH oxidase-mediated superoxide production might be a new interesting application of high resolution respirometry.

[1] Lambeth JD (2004) Nox enzymes and the biology of reactive oxygen. *Nature Reviews* 4: 181-189.

[2] Unterluggauer H, Hampel B, Zwerschke W, Jansen-Dürr P (2003) Senescence-associated cell death of human endothelial cells: the role of oxidative stress. *Exp. Geront.* 38: 1149-1160.

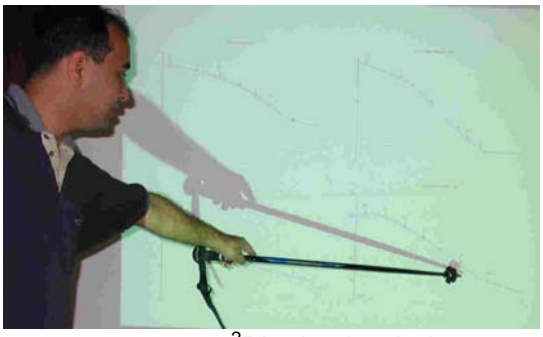
[3] Hütter E, Renner K, Jansen-Dürr P, Gnaiger E (2002) Biphasic oxygen kinetics of cellular respiration and linear oxygen dependence of antimycin A inhibited oxygen consumption. *Molec. Biol. Rep.* 29: 83-87.

[4] Boveris A (Reivich M, Coburn R, Lahiri S, and Chance B, Eds.) (1977) Mitochondrial production of superoxide radical and hydrogen peroxide. Tissue hypoxia and ischemia: 67-82.

MiPNet-09 **Erich Gnaiger** (Innsbruck Medical University, AT) Oxyconformance in cellular respiration.

MiPNet-10 **Bruno Fink** (Noxygen Science Transfer & Diagnostics GmbH, DE) Bench-Top ESR Spectrometer.

MiPNet-11 **Erich Gnaiger** (OROBOROS INSTRUMENTS, Innsbruck, AT) O2k MiP-NetAnalyzer.



MiPNet-12 **Potential role of myocardial UCP in regulating superoxide generation post acute myocardial ischemic insult in dogs.**

Zakaria A. Almsherqi[1], Malgorzata B. Slocinska[1], Liew Yu Ying[1], Nikolai Kocherginsky[2], Kostetski Iouri[2], Francis E. Sluse[3], Rachel Navet[3], Low Chwee Wah[1], Yuru Deng[1]

[1]Department of Physiology, Faculty of Medicine, National University of Singapore, Singapore; [2]Biophysics Laboratory, Division of Bioengineering, National University of Singapore, Singapore; [3]Laboratory of Bioenergetics, Institute of Chemistry, University of Liège, Belgium.

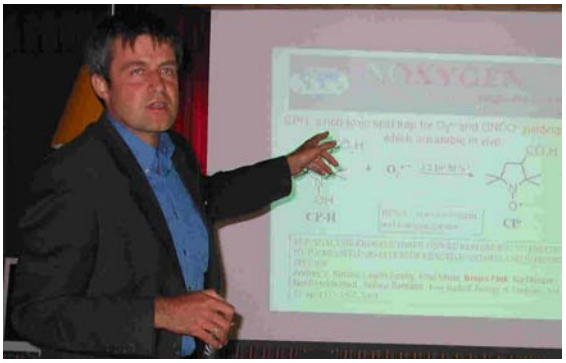
Uncoupling proteins (UCPs) uncouple respiration from oxidative phoshorylation by dissipating the transmembrane proton gradient. Thus, UCPs divert energy from ATP synthesis and this mild uncoupling would decrease the mitochondrial reactive oxygen species (ROS) production. In this study we show that myocardial UCP expression increases at the non-ischemic posterior wall of the left ventricle in response to the increased work demand post partial ischemic insult on the anterior wall. Increased UCP expression could be related to the decrease of ROS generation-detected at the coronary sinus-post left anterior descending artery ligation.

Eight mongrel dogs underwent left anterior descending artery ligation to induce regional ischemia on the anterior wall of the left ventricle. Haemodynamic parameters including heart rate, cardiac output, oxygen saturation and ECG were monitored over 24 hours. Blood samples were collected directly from the coronary sinus and mixed immediately with PBN spin-trap for superoxide detection. After 6 and 24 hours of

continuous ischemia, samples from non-ischemic posterior wall of the left ventricle were collected for bioenergetics and transmission electron microscopy (TEM) ultrastructural studies.

The bioenergetics data namely show that state 4 respiratory rate of isolated and FFA-depleted myocardial mitochondria from the non-ischemic posterior wall after 24 hours of ischemic insult to the anterior wall was almost doubled by adding 10 μM Palmitic Acid (PA). GTP inhibition of PA-induced respiration was increased from 4% to 7% and 33% after 6 and 24 hours of ischemic insult, respectively. This increase of PA-induced respiration could be due to an increase of UCP activity. The increased UCP activity over time could be correlated to the decrease of superoxide levels detected and the haemodynamic status. TEM ultrastructural studies revealed a transition of mitochondrial cristae from lamellar to zig-zag form (> 80%) after 24 hours of ischemic injury.

According to the state of mitochondrial respiration, the respiratory chain generates superoxide anions converted into hydrogen peroxide. Myocardial UCP which is able to modulate the coupling between the respiratory chain and ATP synthesis could be involved in decreasing mitochondrial free radicals generation. Our results show that as myocardial UCP activity augmented over time, superoxide level-detected at the myocardial venous return- decreases. We may hypothesis the participation of myocardial UCP as an early protective mechanism against ROS overproduction due to ischemic insult. Moreover, the concomitant transformation of inner mitochondrial membrane with these observations may shed a light on structure-function relationship in biological systems.



MiPNet-14 **Endotoxic shock and mitochondrial function in rats.**

Andrey V. Kozlov[1], Katrin Staniek[2], Susanne Haindl[1]; Christina Piskernik[2], Hans Nohl[2], Soheyl Bahrami[1]; Heinz Redl[1].

[1]Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria; [2]Research Institute of Pharmacology and Toxicology, University of Veterinary Medicine, Vienna, Austria.

Precise mechanism(s) causing multiorgan dysfunction in sepsis and endotoxic shock remain unclear. It has been commonly accepted that an excessive production of reactive oxygen and nitrogen species (RONS) in mitochondria is an important factor leading to organ dysfunction in a number of diseases. Therefore, the aim of this study was to clarify whether endotoxic shock results in an increased RONS production in rat liver and heart mitochondria (RLM and RHM, respectively) and disturbances in mitochondrial function. Sprague-Dawley rats weighing 280±21 g were divided into three groups: a control group receiving saline i.p. (control group; n=16), a group receiving 8mg lipopolysaccharide LPS/kg i.p.(LPS 8 group; n=8), and a group receiving 20 mg LPS/ kg i.p (LPS 20 group; n=8). The rats were sacrificed 16 h after injections and mitochondria were prepared. At this time the survival in control and LPS 8 groups was 100%, in LPS 20 group only 70%. Mitochondrial function was estimated using respirometry technique (WPI, USA, and OROBOROS, Austria), RONS generation was estimated by spin-trap CPH (NOXYGEN Ltd, Germany) and electron spin resonance. A decreased oxygen uptake in state 3 was observed in glutamate/malate respiring RHM. However, free radical generation was not affected in RHM obtained from both LPS 8 and LPS 20 groups as compared with controls. In contrast, RLM from LPS 8 group exhibited approx. 30% greater oxygen uptake in state 3, which was not accompanied by increased RONS generation. In LPS 20 group, RLM also had increased oxygen consumption in state 3 and additionally a decreased ADP/O ratio. This was accompanied by increased RONS production. The changes in RLM were observed to a similar extent with both glutamate/malate- and succinate-induced respiration, indicating that these changes do not originate from complex I of respiratory chain. The fact that increased RONS production, a decrease in ADP/O ratio and an increase in the mortality appeared simultaneously allows us to assume increased mitochondrial RONS generation and mitochondrial dysfunction as one of the mechanisms leading to organ failure in

endotoxic shock. Apart from this assumption is the increased oxygen consumption in state 3 observed in RLM in both LPS 8 and LPS 20 groups. This reflects more likely an increase in mitochondrial activity. *Acknowledgement*: The authors are thankful to Christine Kober, Anna Khadem, Manfred Albrecht, Mohammed Jafarmadar, and Carina Weber for excellent assistance, support, and useful discussions.

MiPNet-15 **Polarographic analyses of respiratory chain complexes in isolated muscle mitochondria and permeabilized muscle cells in children with mitochondrial disorders**

L. Wenchich, Z. Drahota[1], T. Honzik, M.Bohm, H. Hansikova, J. Houstek[1], J.Zeman
Center for Integrated Genomics, Department of Pediatrics, Charles University and [1]Institute of Physiology, Academy of Sciences, Prague, Czech Republic

Mitochondria in mammalian cells participate in a number of metabolic pathways, but it´s principal biological function is ATP production by oxidative phosphorylation. Both acquired or inherited disturbances of the mitochondrial energy providing system represent a large group of heterogeneous disorders in childhood. The biochemical approach to the diagnostics of mitochondrial diseases is mostly based on spectrophotometric and electrophoretic analyses of the respiratory chain complexes in muscle biopsies and fibroblasts. The aim of our study was to compare the results of spectrophotometric analyses of respiratory chain complexes in isolated muscle mitochondria with the polarographic analyses of the function of respiratory chain complexes in isolated muscle fibres or isolated muscle mitochondria.

Material and Methods In the years 1999-2004 the muscle biopsies were performed in 88 patients in the age from the neonatal period till adulthood with progressive hypotonia, muscle weakness, myopathy, encephalopathy, psychomotor retardation or progressive external ophtalmoplegia after informed consent of the patients or their parents. In all patients, activities of respiratory chain complexes I, II, III and IV and citrate synthase were measured spectrophotometrically in isolated muscle mitochondria. Polarographic studies of the oxygen consumption after several substrates, which can describe the function of all respiratory chain complexes were analysed in isolated mitochondria in 27 patient and in saponin-skinned muscle fibres in 61 patients.

Results Decreased activity of one or more respiratory chain complexes in muscle mitochondria were found in 57 from 88 patients using spectrophotometric methods and decreased oxygen consumption after one or more substrates were observed in 51 from 88 patients. The results of spectrophotometric analyses in isolated muscle mitochondria were in concordance with polarographic measurements in isolated mitochondria in 24 from 27 patients (89 %) and they were also similar to measurements in saponin-skinned muscle fibres in 39 from 61 patients (64 %), respectively.

Discussion and Conclusions: Both the spectrophotometric and the polarographic techniques provide different insight into the mitochondrial functions. In contrast to oxygen consumption, where the function of all respiratory chain complexes as well as coupling of oxidative phosphorylation can be analysed, the spectrophotometric methods allow to assess the maximal capacities of the individual respiratory chain complexes, but at the conditions that are different from "in vivo" situation. It would be optimal in the biochemical diagnostics of mitochondrial disorders to combine spectrophotometric measurements with the polarographic studies of the oxygen consumption in permeabilised cells or permeabilised tissue fragments which preserve mitochondrial integrity and intracellular structural and interorganellar communications. *This work was supported by grant GA UK 16/2004/C and LN 00a079 .*