EBEC 2006 Colloquium D "New Methods and Devices" (24. July; 16:30-19:30)

Introduction to the Colloquium and to Part A

16:30-16:40 D1. **Erich Gnaiger (Innsbruck, Austria)** Features and applications of the OROBOROS Oxygraph-2k for high-resolution respirometry in bioenergetics, mitochondrial physiology, and diagnosis of OXPHOS deficiencies.

PART A: New multi-sensor devices: High-resolution respirometry, low oxygen, spectrophotometry, ROS-EPR, fluorescence

16:45-16:55 D2. **Natascha Sommer (Giessen, Germany)** Simultaneous remission spectrophotometry and high-resolution respirometry. Respiratory control by oxygen and redox states of mitochondrial cytochromes in living cells.

17:00-17:10 D3. **Susana Cadenas (Madrid, Spain)** Measurement of the control of cellular respiration by nitric oxide under normoxia and hypoxia: an instrument comparison including high-resolution respirometry.

17:15-17:25 D4. **Mikhail Vyssokikh (Moscow, Russia)** Decrease of oxygen content in closed polarographic chamber with cyanide-treated mitochondrial suspension relates to superoxide-anion radical formation. High-resolution respirometry application for direct measuring of ROS production rate.

17:30-17:40 D5. **Andrey Kozlov (Vienna, Austria)** Application of hydroxylamine based spin probes to detect mitochondrial reactive oxygen species in tissues and mitochondrial suspension.

17:45-17:55 D6. **Rodrigue Rossignol (Bordeaux, France)** Real-time investigation of mitochondrial functions using a highly sensitive coupled oxygraph-optic fiber system (OROBOROS, SAFAS and INSERM).

18:00-18:30 Coffee break

PART B: High-resolution scanning electron microscopy, gene therapy, proteomics

18:30-18:40 D7. **Charles Hoppel (Cleveland, USA)** Metabolic state-related structure of isolated cardiac mitochondria as seen by high-resolution scanning electron microscopy (HRSEM).

18:45-18:55 D8. **Emmanuel P. Dassa (Paris, France)** Alternative oxidase, a potential therapy for mitochondrial diseases.

19:00-19:10 D9. **Grégory Mathy (Liege, Belgium)** Applied proteomics: comparing mitoproteomes at the 21st century.

Up to 19:50 *Two more short contributions are welcome.*

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Abstracts Colloquium D

D1. Features and applications of the OROBOROS Oxygraph-2k for high-resolution respirometry in bioenergetics, mitochondrial physiology, and diagnosis of OXPHOS deficiencies.

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Approaches to mitochondrial and cellular respirometry have been transformed and extended in mitochondrial physiology, from specialized bioenergetics to integrative and clinical applications. Meeting rapidly increasing demands, the new OROBOROS Oxygraph-2k (O2k) covers major areas of interest: (i) Routine analysis of oxidative phosphorylation (OXPHOS) in diverse fields of research such as redox signaling, oxidative stress (1), ischemia-reperfusion (2), aging (3), degenerative and inherited diseases (4); apoptosis, cancer research and drug testing (5), and environmental stress. For the non-specialist, the O2k provides robustness and reliability of instrumental performance. Full software support by DatLab includes automatic oxygen calibration, continuous display of oxygen concentration and respiratory flux, and on-line tabulation of final results on OXPHOS activity during selected experimental sections. Standardized O2k quality controls eliminate interferences by instrumental artifacts. Long-term stability of the polarographic oxygen sensors ensures the O2k to be ready to use (no exchange of membranes and electrolyte for months). (ii) High throughput is required in research with cell cultures and pharmacological tests. User-friendly features make it possible to apply several O2k in parallel, each O2k with two independent chambers. (iii) Diagnosis of OXPHOS in living and permeabilized cells, small amounts of tissue from transgenic animal models, and human needle biopsies requires high-resolution respirometry to obtain accurate results even at high sample dilution. <1 mg fresh muscle tissue or <500.000 cells are required per chamber (10-fold less compared to conventional instruments). Multiple substrate/inhibitor titration protocols extend the information gained on various OXPHOS complexes in a single incubation. (iv) Prevalence of oxidative stress at air-level oxygen tensions demands a shift to physiological low-oxygen ranges for studying OXPHOS (6). (v) Multi-sensor applications (NO, H_2O_2, ion sensitive electrodes, light guides in the O2k chamber for spectroscopy, fluorescence), and sampling from the chamber (CS enzyme assays; ESR spin probes for ROS) extend the diagnostic power of high-resolution respirometry. Chamber design (avoiding oxygen-permeable materials such as Perspex or teflon-coated stirrers), instrumental concept (including integrated Peltier-temperature control, automatic Titration-Injection microPump TIP-2k), software features and methodological tests will be discussed, which make the O2k the unique instrument for high-resolution respirometry.

1. Stadlmann et al. (2002) *Transplantation* 74: 1800.
2. Kuznetsov et al. (2004) *Am. J. Physiol. Heart Circ. Physiol.* 286: H1633.
3. Hütter et al (2004) *Biochem. J.* 380: 919.
4. Pecina et al (2004) *Am. J. Physiol. Cell Physiol.* 287: C1384.
5. Renner et al (2003) *Biochim. Biophys. Acta* 1642: 115.
6. Gnaiger et al (2000) *Proc. Natl. Acad. Sci. USA* 97: 11080.

D2. Simultaneous remission spectrophotometry and high-resolution respirometry .
Respiratory control by oxygen and redox states of mitochondrial cytochromes in living cells

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Simultaneous determination of respiratory redox states and rates has been essential in contributing to our understanding of respiratory control in isolated mitochondria. To extend these concepts to intact cells, increased sensitivity is required in a combined detection system. In a closed chamber containing 5-10 Mio cells/ml (such as fibroblasts or promyeloid cells 32D) it takes about 60 s for the transition from 10 µM O_2 to zero oxygen. This raises the controversial issue of differences between results obtained at steady state conditions versus rapid transitions. Conventional gas/aqueous phase systems, on the other hand, have the problem of oxygen gradients at severe oxygen limitation. To address these methodological challenges, we compared mitochondrial oxygen kinetics derived from transitions with steady states obtained by continuous injection of air saturated aqueous medium (TIP-2k) into the chamber of an OROBOROS Oxygraph-2k (Austria). Redox states of mitochondrial cytochromes (cytochrome *b*, *c*, aa_3) were determined by measurement of difference spectra at wavelengths of 405-630 nm through a flexible glass-fibre light guide of the O2C spectrophotometer (LEA Medizintechnik, Germany). The apparent K_m for oxygen was 0.3-0.4 µM O_2 obtained from aerobic-anoxic transitions, and fully agreed with the steady state kinetics observed over 120-600 s of continuous oxygen injection. Oxygen concentrations were maintained at constant levels of 400 down to 50 nM O_2. A progressive reduction was monitored of mitochondrial cytochromes, corresponding to steady state respiration of 10-50 % of maximal rates. This combination of methods provides a valid reference for interpreting cytochrome spectra obtained in the intact organ during hypoxia and pathological states.

D3. Measurement of the control of cellular respiration by nitric oxide under normoxia and hypoxia: instrumental comparison including high-resolution respirometry

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At low oxygen levels, mitochondrial respiration is controlled by the nitric oxide (NO)-cytochrome *c* oxidase (COX) signaling pathway, since NO is a membrane-permeant second messenger and competitive inhibitor of COX (1). It is now well established that oxygraphs, with Teflon-coated stirrer bars and other plastic materials of high oxygen solubility, yield high rates of oxygen back-diffusion into the chamber when oxygen levels decline, causing artefacts of respiratory measurements. High-resolution respirometry with the OROBOROS Oxygraph-2k (O2k) reduces such back-diffusion by at least an order of magnitude, and incorporates automatic instrumental background corrections, treating the 'closed' chamber essentially as an open system with oxygen transport between the aqueous phase and the system boundary (2). For measurement of NO in experimental chambers, however, the same instrumental problem of gas exchange between hydrophobic plastic materials and the aqueous medium has not been addressed, despite the high partition coefficient of NO between aqueous and organic phases (3). To address these problems, we incorporated an NO sensor (ISO-NOP, WPI) into a Hansatech oxygraph chamber and a high-resolution respirometer (O2k), for simultaneous recording of respiration and NO. The NO sensor was calibrated by addition of known concentrations of KNO_2 under reducing conditions (KI/H_2SO_4) at 37 ºC and the

response of the NO sensor in terms of accuracy, stability and reproducibility of the signal was compared between the two chambers. Measurements were taken in 1 ml (Hansatech) or 2 ml (O2k) closed chambers at 37 ºC, using their standard Teflon- or PEEK-coated stirrer bars, respectively. The titanium stopper of the O2k chamber was replaced by a polyvinylidenfluorid (PVDF) stopper, including a second inlet (2 mm diameter) for the NO sensor in addition to the capillary used for extrusion of gas bubbles and titration of chemicals. The PVDF stopper showed identical characteristics to titanium in terms of minimum back-diffusion of oxygen in aerobic-anaerobic transitions, can be cleaned with 70 % and pure ethanol, and offers increased flexibility for accommodation of various additional electrodes for multi-sensor applications. We compared the response of the NO sensor in the determination of the release of NO from a chemical source (DETA-NO) and the endogenous release from controlled intracellular NO production. We determined the inhibition of respiration caused by NO under physiological oxygen concentrations using conventional and high-resolution respirometry (2).

1. Mason M.G. *et al.* (2006) Proc. Natl. Acad. Sci. USA 103: 708-713
2. Gnaiger E. (2001) Respir. Physiol. 128: 277-297
3. Liu X. et al. (1998) Proc. Natl. Acad. Sci. USA 95: 2175-2179

D4. Decrease of oxygen content in sealed polyarographyc system relates to superoxide-anion radical formation for rat heart mitochondria treated with inhibitors of respiration.

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It is known that at least significant part of intracellular reactive oxygen species (ROS produced by mitochondrial respiratory chain (1). In last thirty years the importance of ROS production was established for intracellular signaling, oxidative damage of DNA, programmed cell death and manifestation of number deceases concerned (2). Mainly, the out coming product is hydrogen peroxide and the rate of its production depends of respiration level and antioxidant enzymes activity in the cell. However, only superoxide anion radical is the product of pathway for one electron reduction of O_2 catalyzed by respiratory chain and hydrogen peroxide is just a product of disproportioning reaction catalyzed by superoxide dismutase or non-enzymatically (3). We encounter a difficulty to measure direct superoxide anion radical production rate without doubt since start of research on this field because the half time for this molecule is relatively small (seconds) by reason of its chemical instability (4). Ordinary the way out is to measure hydrogen peroxide as a product of disproportioning or compounds easily reacted with superoxide anion radical and of coarse number of methods exist are indirect. We tested and described the application of high-resolution respirometry for that purposes based on the oxygen concentration tracing for mitochondria treated with respiratory chain inhibitors such as rotenone, antimycine or cyanide ion in completely sealed polyrographyc cell. Data obtained for rate of superoxide anion radical production by oxygen content decline are in a good agreement with literature and compared with indirect method such as fluorescent detection of H_2O_2 production with AmplexRed probe or wih scopoletin. Facility of access for this method and future development of instruments give raises a possibility for further research of ROS production by mitochondria even within the cell.

(1) Chance, B., Sies, H., Boveris, A. (1979) Physiol Rev., **59**, pp527-605.
(2) Wallace, D.C. (1999) Science **283**, pp1482-1488.
(3) Loschen, G., Flohe, L., Chance, B. (1971) FEBS Letters, **42**, pp68-72.
(4) Fridovich, I. (1974) Adv.Enzymol., **41**, pp35-97.

D5. Application of hydroxylamine-based spin probes to detect mitochondrial reactive oxygen species in tissues and mitochondrial suspension.

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Three similar hydroxylamine-based spin probes (methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine = CMH, 1-hydroxy-3-carboxy-2,2,5-tetramethyl-pyrrolidine hydrochloride = CPH, 4-phosphono-oxy-2,2,6,6-tetramethyl-piperidine-N-hydroxyl = PPH) reacting with reactive oxygen species (ROS), first of all with superoxide ($O_2^{\cdot-}$), have different hydrophobicity increasing in the following order: PPH<CPH<CMH. Incubation with rat heart mitochondria (RHM) resulted in the formation of spin adducts with CPH, and CMH (but not PPH), which was not sensitive to superoxide dismutase (SOD) and correlated with hydrophobicity of tested spin traps. Levels of spin adducts were drastically increased by antimycin A and became sensitive to SOD, which decreased levels of PPH, CPH, and CMH adducts by 96%, 72% and 45%, respectively. None of the spin probes influenced respiratory control, P/O ratio, respiration rate in state 3 and 4 of RHM. Further experiments have shown that CPH can be applied to tissue homogenates, and that the resulting spin adduct, 3-carboxy-proxyl, is stable in such models. CPH can be infused into blood allowing detection of ROS in tissues frozen in liquid nitrogen after the infusion. We have found that in hearts obtained from old rats ROS generation was significantly increased compared to their young counterparts. Experiments with isolated RHM have shown increased $O_2^{\cdot-}$ generation in mitochondria from old rats compared to mitochondria obtained from young rats. Parallel experiments aimed at detecting H_2O_2, the dismutation product of $O_2^{\cdot-}$, using peroxidase and scopoletin failed to detect any difference between mitochondria from old and young rats. However, in the presence of antimycin A, both methods displayed similar results, an increase in ROS production. Our data suggest that (i) hydroxylamine-based spin traps are not toxic and sensitive probes for detection of ROS in mitochondrial suspension, tissue homogenate, and tissues; (ii) $O_2^{\cdot-}$ generated by intact mitochondria does not escape from mitochondria while in the presence of antimycin A $O_2^{\cdot-}$ leaks out to the medium and becomes available for SOD and hydrophilic spin traps; (iii) the increased levels of ROS in hearts from old rats originate from mitochondria.

D6. Real-time investigation of mitochondrial functions using a highly sensitive coupled oxygraph-optic fiber system.

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Mitochondria intervene in a large number of fundamental cellular processes that include energy production, apoptosis, calcium homeostasis and cell signaling. These different functions are likely to be interconnected with each other so that variations in mitochondrial energetics modulates the production of oxygen radical species, induction of gene expression, or the succeptibility to apoptosis, with possible implications in physiopathology. To study in real-time such changes requires the simulteanous measurement of different parameters representative of the key functions considered above. Moreover, to study living cells or small amounts of tissues necessitates accurate and reproducible standardized techniques with a fast time response. To this aim, we have connected a high-resolution oxygraph system with a

highly sensitive fluorometer using an optic fiber. This was developed in collaboration between INSERM (France), OROBOROS (Austria) and Safas (Monaco). Our system was also connected to an injection pump that allowed to perform inhibitor titration studies or the maintenance of a chosen steady-state of oxygen delivery. The instrumental design, test experiments, and preliminary results will be presented, obtained on human fibroblasts, looking more closely at the relationships between respiratory states and $\Delta\Psi$, ROS, NOS, matrix pH or redox potential (GFPs).

D7. Metabolic state-related structure of isolated cardiac mitochondria as seen by high resolution scanning electron microscopy (HRSEM)

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Osmium extraction of isolated cardiac mitochondria renders their membranes in stark relief when viewed by HRSEM. In our previous HRSEM study of cardiomyocytes in adult Sprague-Dawley rats it was found that subsarcolemmal mitochondria (SSM) have predominantly lamelliform cristae, whereas interfibrillar mitochondria (IFM) have mainly tubular cristae. When these respective populations are independently isolated, SSM are largely unchanged whereas the IFM have acquired a heterogenous (mixed) cristal structure. We asked if cardiac mitochondria morphologically reflected metabolic state as seen to be the case in hepatic mitochondria [Hackenbrock, J Cell Biol. 30:269, 1966]; to this end, we examined over 600 organelles by HRSEM. Under state 2, 60% of the SSM have lamelliform cristae; in state 3, 83% have tubular cristae; in state 4, 81% have lamelliform cristae. In the case of IFM, 72% of those in state 2 have tubular cristae; in state 3 69% have tubular cristae; in state 4, 77% have a mixture of cristae. In summary, with high oxidative rates and ATP production the cristae are predominantly tubular in both populations of cardiac mitochondria. When ADP becomes limiting with low oxidation and ATP production the cristae in SSM become lamelliform whereas in IFM there is a mixture of lamelliform and tubular cristae. We conclude that cristal architecture is closely associated with ATP production in cardiac mitochondria.

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D.8. Alternative oxidase, a potential therapy for mitochondrial diseases

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In most plants and in a number of microorganisms but not in mammals, the alternative oxidase (AOX) provides a by-pass of the cytochrome segment of the mitochondrial respiratory chain. This enzyme which is located at the inner surface of the inner mitochondrial membrane is activated under specific metabolic conditions to facilitate respiration. These conditions include the over-reduction of the quinone pool and the accumulation of pyruvate and other organic acids. Since these conditions commonly arise in cases of mitochondrial OXPHOS disease, we decided to test whether the expression of the recently discovered alternative oxidase from the ascidian *Ciona intestinalis* (1) can alleviate the deleterious consequences of a blockade of the respiratory chain in human cells. We therefore expressed

C. intestinalis AOX, both epitope-tagged and untagged, in cultured human cells (HEK 293) using the inducible Flp-In™ T-REx expression system. AOX was efficiently expressed, targeted to mitochondria and had no detectable deleterious effects on cell growth. The respiration of these cells became insensitive to cyanide but the cyanide-insensitive respiration was abolished by n-propyl gallate, a specific inhibitor of the alternative oxidase. Furthermore the AOX involvement in human cell respiration was enhanced by pyruvate as in plants. Finally, the AOX expression alleviated oxidative stress upon inhibition of the OXPHOS system (2). We are now in the process of stably expressing the AOX in COX-deficient human cells. On one hand we transfected the AOX construct in immortalized COX15-deficient fibroblasts. On the other hand, we used HEK 293 cells in which COX10 or SURF1 gene have been silenced by shRNA expressing vectors. The successful expression of *C. intestinalis* AOX in human cells (ref) constitutes a promising tool to further study the consequences of an OXPHOS dysfunction because it offers a unique possibility to disconnect electron flow through most of the respiratory chain from the phosphorylation process. In a longer term, allotopic expression of AOX may provide an effective therapy for currently intractable RC diseases. The first step in this endeavour should be the expression of AOX in whole organism models, *e.g.* mouse or *Drosophila*, exhibiting OXPHOS deficiency.

1. McDonald, A., and Vanlerberghe, G. (2004) *IUBMB Life* **56**, 333-341
2. Hakkaart, G. A., Dassa, E. P., Jacobs, H. T., and Rustin, P. (2005) *EMBO Rep* (advanced on line publication)

D9. Applied proteomics: comparing mitoproteomes at the 21st century.

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Proteomics represents now an integral part of the modern biochemist's toolbox. Organelle science does not escape proteomics and mitochondrial physiology is increasingly investigated using proteomic technologies according to the role of mitochondria in energy transduction, apoptosis and a growing range of pathologies. Two lines of research related to mitochondrial proteomes (or mitoproteomes) are currently acknowledged: (i) structural proteomics that focuses on the elucidation of mitoproteome components; (ii) comparative proteomics that studies adaptations in mitoproteome patterns in response to environmental modifications. Two-dimensional differential in-gel electrophoresis – 2D-DIGE – is the last-born technology available in gel-based comparative proteomics. 2D-DIGE takes advantage of differential fluorescence protein labeling and sample multiplexing to improve accuracy and reliability of comparative analyses. Accordingly, 2D-DIGE enables subtle changes in mitoproteomes under comparison to be identified and opens a new path toward the understanding of the mitochondrial plasticity in response to changes in the cell physiology.