NANOCALORIMETER FOR MEASURING THE HEAT PRODUCTION IN MITOCHONDRIA

Measurements of energy transformation and dissipation in mitochondria by the calorimetric method were carried out in the second half of the last century. However, to date no specialized calorimeter has been developed for this purpose. Selection of compounds providing uncoupled mitochondrial respiration without damaging the respiratory chain components, for use in pharmaceutical compositions of drugs, development of new neuroprotectors, nephroprotectors require precise measurements of heat release by different mitochondrial uncouplers of oxidative phosphorylation. A capillary differential nanocalorimeter for studying energy transformation and dissipation in the mitochondria has been created in the IBI RAS. The instrument meets the above requirements. The principal advantage of the nanocalorimeter is that it has thermal bridges for the thermostating the mitochondria injection. In the thermal bridges mitochondria acquire the desired temperature for a few seconds. Mitochondria are introduced uniformly along the entire length of the calorimeter chamber by means of a dispensing needle. This provides mixing of mitochondria with the sample without great energy consumption and thermal noise. Precision measurements of thermal power of the processes of transformation and dissipation of energy in the mitochondria are carried out at an absolute error less than 50 nW.

INTRODUCTION

Still in 1978, the paper [1] based on the study of heat energy arising at respiration of mitochondria demonstrated that “the energy balance of reactions in a system of energy transformation can be directly measured by means of calorimetry”. Said results are founded on an experimental substantiation of equality between the measured energy of substrate oxidation by mitochondria and the known heat of combustion of the used substrate. The data was obtained on two different substrates, both on a succinate and a glutamate. An evaluation of the ATP synthesis was made on the basis of difference of measured heat energy in the cases of conjugated and uncoupled respiration of mitochondria. These important scientific results were obtained with the application of a method of differential thermal analysis originating in the 1960’s and representing a predecessor of precise calorimetry. In this case, the energy calculation was made thanks to the use of an original calibration to convert measured temperature differences into energy units.

Later on, papers describing studies of heat produced by mitochondria started to be published, said studies being performed with laboratory calorimetric set-ups solving specific tasks. The paper [2] deal with calorimetric measurements under conditions of a limited oxygen content in the calorimetric chamber.

The paper [3] reports a search for conditions upon which rice mitochondria release more or less energy. Modern similar set-ups, such as those described in papers [4, 5], Omega [6], VP-ITC, iTC200, Auto iTC200 (Microcal, USA) are not foreseen to measure heat power in the processes of energy transformation and dissipation in mitochondria due to the uncoupled respiration of mitochondria during a long period (more than 10 min) for equalizing temperature of the additive to that of the sample under study. Nevertheless, new tasks for finding compounds providing for uncoupled respiration of mitochondria without damaging the respiration chain components, to be used in pharmaceutical compositions of drugs [7], the development of new neuroprotectors [8–10], new nephroprotectors [10] require new calorimetric instruments for precise measurements of heat output in mitochondria under the effect of different mitochondrial uncouplers of oxidative phosphorylation.

THEORETICAL PRINCIPLES OF BUILDING A NANOCALORIMETER

Theoretical bases developed by the authors for building a nanocalorimeter relate to the key problems
as follows: a method of separating the sensitive volume of calorimetric chambers; a method of equalization of the additive concentration in the sensitive volume of a calorimetric chamber; a method of injecting additives into a calorimetric chamber.

**Method of isolating the measurement volume**

To provide a theoretical accuracy for separating the sensitive volume of calorimetric chambers in a differential titration calorimeter, a heat model of a calorimetric chamber has been developed and studied [11]. In said model, an ideal heat contact of the calorimetric chambers with a passive heat-conducting bridge having an ideal heat contact with the isothermal shield the temperature of which is kept constant thanks to an automatic control system. It is shown that the passive heat-conducting bridge eliminates the influence of heat effects in the area of said bridge, that is equal to the value of a recorded signal of heat power arising in the sensitive volume of the calorimetric chambers. The proposed technical realization of the heat-conducting bridge practically nullifies the influence of the heat effect in the area surrounding the bridge. The considered error is evaluated via the value of the coefficient $K$ calculated by the equation (1) and is as high as 0.003%.

$$K = \left[\frac{2\alpha \cdot L \cdot r_1 + \frac{n \cdot r^2_{wire} \cdot (\lambda_{const} + \lambda_{cop})}{l_{wire}}}{\ln \frac{R_{br}}{r_2} + \frac{2\lambda_{br} \cdot h \cdot \left(\frac{r_2^2 - r_1^2}{r_1^2}\right) \cdot \lambda_c + \frac{r^2_{wire} \cdot \lambda_{lig}}{d}}{r_1^2}}\right] \times \frac{\frac{1}{1 + \frac{2l_{wire} \cdot \alpha \cdot L^2 \cdot r_2 + n \cdot r^2_{wire} \cdot (\lambda_{const} + \lambda_{cop})}{2l_{wire} \left(\frac{r_2^2 - r_1^2}{r_1^2}\right) \cdot \lambda_c + \frac{r^2_{wire} \cdot \lambda_{lig}}{d}}}}.$$  \hspace{1cm} (1)

where $\alpha$ is a convective heat exchange factor; $r_1$ is the inner radius of a calorimetric chamber; $r_2$ is the outer radius of a calorimetric chamber; $L$ is the height of the sensitive volume; $h$ is the height of the heat-conducting bridge; $R_{br}$ is the heat-conducting bridge radius; $n$ is the number of branches of a copper-constantan measuring thermopile arranged between the chambers; $r_{wire}$ is the radius of conductors for the thermopile branches; $l_{wire}$ is the length of each branch of the thermopile; $\lambda_{const}$ is the thermal conductivity coefficient of constantan; $\lambda_{cop}$ is the thermal conductivity coefficient of copper; $\lambda_c$ is the thermal conductivity coefficient of the chamber material, $\lambda_{br}$ is the thermal conductivity coefficient of the heat-conducting bridge material; $\lambda_{lig}$ is the thermal conductivity coefficient of liquid; $d$ is the distance between the heat-conducting bridges.

**Equalization of the additive concentration in the sensitive volume of the calorimetric chamber**

For the titration calorimeter, capillary calorimetric chamber have been selected for evaluating the efficiency of mixing the additive and the sample in such a chamber. The time of equalizing the additive concentration in the volume of the sample as a result of concentration diffusion has been chosen as a criterion and is determined in accordance with the suggested calculated relation (2):

$$\tau = \frac{R^2_{br}}{2D_k} \ln \left[\frac{1}{1 - \frac{V_d}{V_k}}\right],$$  \hspace{1cm} (2)

where $V_d$ is the volume of the band (titrant dose); $R_{br}$ is the radius of the band; $V_k$ is sensitive volume of the calorimetric chamber; $D_k$ is the coefficient of titrant diffusion in the sample; $k$ is the coefficient of equalization of the titrant concentration in the sample.

This relation is substantiated experimentally on a capillary calorimeter according to a registered heat process of interaction of nicotinamide adenine dinucleotide (a reduced form of NADN) with lactate dehydrogenase (LDH) [12].

The additives are injected with the following procedure: when introducing a dose, the syringes are translated longitudinally for the whole length of the calorimetric chambers with a worm translational mechanism controlled by a stepping motor for translating syringes. Simultaneously, the pistons are translated by a screw mechanism controlled by a stepping motor for translating pistons. Due to a different value of translations for both screw mechanisms, the syringes dispense a determined volume of reagents. The stepping motors are computer-controlled. In this case, the additive is distributed in the whole length of the working volume of the calorimetric chamber as a fine round-section jet. In the course of an experiment, the additive can be injected repeatedly.

The calorimeter provides as well the mechanical stirring of reagents in the calorimetric chambers thanks to oscillations of dispensing needles with an amplitude and a frequency chosen depending on the experiment conditions. This operation mode prevents formation of precipitations while studying mitochondria.
Method of introducing a dispensing needle into the calorimetric chamber of a nanocalorimeter

Open calorimetric chambers were used first in our DASM-1 Differential adiabatic scanning microcalorimeter [13]. This technical solution was highly appreciated in the research practice and was applied in the present nanocalorimeter. An additive is introduced into calorimetric chambers via open ends of the chambers with dispensing syringes. The nanocalorimeter is provided with a system of heat-conducting bridges, and the additive when crossing the last after injection acquires the temperature of the sample under study, which enables one to inject an additive having a temperature corresponding to the storage temperature, i.e. without any previous thermostatting. In this case, the rate of injection can achieve the rate of 13.7 mm/s which enables to carry out the operation of additive injection into the nanocalorimeter within 5 s. The paper [14] provides for a theoretical substantiation of the considered operation mode of the nanocalorimeter. It was experimentally shown that said mode of injecting additives does not introduce any parasitic heat into the measurement volume of the calorimetric chamber, which allows, when measuring energy transformation and dissipation in mitochondria, to introduce mitochondria into the calorimetric chambers as additives.

This fact provides for recording the heat effect power without any time delay, from the moment of injecting mitochondria into the calorimetric chamber, and represents an important advantage of the nanocalorimeter enabling accurate measurements of heat power in the process of energy transformation and dissipation in mitochondria. In the case of an experiment when mitochondria are injected directly into a calorimetric chamber and are kept in the last for the time of acquiring desired temperature, the measurement accuracy can decrease since the viability of mitochondria changes in the case of a long stay of the last at a temperature of about 26 °C. Nevertheless, no quantitative evaluation of such a process is found in literature. The opportunities of our nanocalorimeter provide for carrying out such an evaluation. The reagents used for testing the instrument were from Sigma.

**BRIEF DESCRIPTION OF THE CALORIMETRIC UNIT OF THE NANOCALORIMETER**

The calorimetric unit of the nanocalorimeter is illustrated in Fig. 1. It shows elements the building of which has taken into consideration the requirements of the previous theoretical chapter of the paper.

![Diagram of the calorimetric unit of the nanocalorimeter](image-url)

**Fig. 1.** Calorimetric unit of the nanocalorimeter for measuring heat production in mitochondria.
1 — passive heat-conducting bridge;
2 — active heat-conducting bridge;
3, 4 — calorimetric chambers; 5 — silver brazing; 6, 7, 8 — thermostatting shields; 9 — holder; 10, 11 — dispensing needles
The heat-conducting bridges 1 and 2 are linked to capillaries 3 and 4 with the provision of a reliable heat contact thanks to silver brazing, the heat contact of the passive heat-conducting bridge 1 with a shield 6 being provided by a mechanical compression of the elements. A reliable heat contact between the chambers 3 and 4 and the heat-conducting bridges 1 and 2 provides for an accurate separation of the working volume as a result of preventing heat from the passive heat-conducting bridge 1 to get into the measurement volume of the calorimetric chambers. Said heat is drained off to the heat-conducting bridge 1 body and further to a thermostating shell composed of shields 6–8. In this way, the measured signal is supplemented only with heat released in the measurement volume. No heat exchange exists between the calorimetric chambers and the shield 6 since the temperatures of the calorimetric chambers 3, 4 and of the shield 6 into which they are enclosed are equal. The present solution does not require the use of any separated regulator of the calorimetric chamber temperature, which prevents the origination of any heat noise in the measured signal due to the operation of said control system.

The structure of the calorimetric unit provides coaxial arrangement of dispensing needles 10, 11 mounted on a holder 9 and that of the calorimetric chambers 3, 4, which excludes any shocks of the dispensing needles against the walls of the calorimetric chambers, that could give rise to heat noise that reduces the signal-noise relation while measuring heat power. This fact provides the adequacy of the time of reagent mixing to calculated time, thanks to concentrating diffusion. Mixing reagents in the calorimetric chamber by the method of concentrating diffusion is an efficient one. For example, testing a calorimeter for binding Ba$^{2+}$ with 18-Crown-6 with the only use of said mixing method provides for an accurate value of the binding parameter [15], and a necessary high baseline reproducibility is achieved thanks to the operation of heat-conducting bridges that minimize parasitic heat flows into the measurement volume of the calorimetric chambers.

**TESTING THE NANOCALORIMETER FOR THE INTERACTION Ba$^{2+}$—18-CROWN-6**

The power scale factor was determined on the first peak of the termogram for binding Ba$^{2+}$—19-Crown-6 [16] that is given in Fig. 2. In this case, the peak power was as high as 5.229 μJ. Said energy is contained in a rectangular shape signal having the height 4.526.26 nV and the duration 96 s, which gives a power scale factor equal to 83 nV/μW.

![Fig. 2. Thermograms of binding Ba$^{2+}$ with 18-Crown-6. Sample: 156 μl of 18-Crown-6 (0.02 M); additive: 1.6644 μl of Ba$^{2+}$ (0.1 M). Temperature: 25 °C. Mixing: needle oscillating at 18.5 Hz](image-url)
**NANOCALORIMETER SPECIFICATIONS**

Power scale factor — 83 nV / 1 μW;

Measurement of investigated processes of energy transformation and dissipation in mitochondria with absolute error of no more than — 50 nW;

Measurement volume of a calorimetric chamber — 156 μl;

Volume of added mitochondria in suspension (titrant) — 1 to 10 μl;

Isothermal mode of the nanocalorimeter operation: working temperature range — 15 to 50 °C;

Mechanical mixing of reagents in the calorimetric chambers with oscillating dispensing needles at a frequency — 1 to 20 Hz.

The nanocalorimeter of the present design is a modification of the KTD-2156 calorimeter [14]. This nanocalorimeter is characterized by a higher sensitivity thanks to a high-sensitive calorimetric unit designed specifically for the same, which has provided that the new nanocalorimeter meets modern requirements needed to measure heat power of the processes of energy transformation and dissipation in mitochondria.

**RESULTS OF THE CALORIMETRIC MEASUREMENTS**

For carrying out calorimetric measurements, liver mitochondria in rats, Wistar line, were separated by differential centrifugation according to a standard procedure. The separation medium contained 0.3 M of saccharose, 10 mM of HEPES (pH 7.4), 1 mM of EGTA. The mitochondria were washed in the separation medium without EGTA. The incubation medium contained 125 mM of KCl, 3 mM of KH$_2$PO$_4$, 1 mM of MgSO$_4$, 10 mM of HEPES pH 7.4 [17].

Fig. 3 illustrates curves representing time dependence of heat power released as a result of integrating heat power signals: the curve 1 was when adding 6.6 μl of defrosted mitochondrial suspension into the 156 μl sensitive volume of the calorimetric chamber containing incubation medium and hydroxybutyrate (8 mM). The calorimetric chambers temperature was 26 °C.

![Fig. 3. Heat power obtained as a result of integrating heat power signals after injecting defrosted mitochondrial suspension into the calorimetric chamber, depending on the substrate variety. Curve 1 is integrated heat power for the succinate substrate; curve 2 is integrated heat power for the hydroxybutyrate substrate.](image-url)
CONCLUSIONS

The nanocalorimeter is promising for solving modern problems to search for compounds providing for uncoupled respiration of mitochondria without damaging the respiration chain components, for use in pharmaceutical compositions of drugs, development of new neuroprotectors, nephroprotectors. It can be used for accurate measurements of heat power released by mitochondria under the effect of various uncouplers of oxidative phosphorylation.

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