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Mitochondrial Filamentation: Some Methods of Isolation and Assay

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Abstract: *Working transiently but recurrently, during more than 40 years we managed to achieve the isolation of cellular mitochondria by differential centrifugation with less mechanical, thermal and chemical trauma; mostly from rodents' livers, kidneys and brains, and principally at moments of deep rest.*

The mitochondria present new substructures: filaments, cones and veils. Filamented mitochondria have different rates of oxidative phosphorylation loss, adenosine triphosphatase activity and glycolysis compartmentalization.

These preliminary findings could pave the way towards a more physiological consideration of mitochondria and represent important steps forward for an understanding of the nature, cause and treatment of very serious diseases among which we can count cancer and neural degeneration. The initial considerations of a deeper understanding of planetary life oxygen and physiological and metabolic death are also in sight.

I. Introduction

It could perhaps be the moment to provide some background information on the new field that is now slowly emerging in the physiology of life, disease, and death: Mitochondrial Filamentation.

During my post-doctoral stay with Britton Chance, in Philadelphia, at the end of 1968, I began my training with isolation of mitochondria. I was very struck by the great mechanical, thermic and chemical stress with which they were isolated. I was then an oncologist, who taught trough reading of books biochemistry and quantum physics to first year medical students, in Madrid. I had then no preparation in experimental biochemistry.

I had arrived at Chance's side, at the beginning of April 1968, almost by miracle, in order to study more physiologically with his fluorescence microscope of pyridine nucleotides, the cellular defect of cancerous cell respiration. Put forward by Otto Warburg in Germany along the decades of the thirties to the fifties of the 20th century. Following an initial study with this technique I wanted to get to the bottom of the problem of my interest by analyzing the effect of chemical carcinogens in the isolated mitochondria of rat's liver. On complaining about how little physiological was the method of isolating mitochondria, Chance first asked Albert Claude and then Sir Hans Krebs to make my acquaintance.

Claude said to me, in French so that I would understand him correctly, that morphologically speaking mitochondria could be isolated perfectly at room temperature. He did it in 1946. But these mitochondria were biochemically incapable. Sir Hans told me that certainly the trauma of isolating mitochondria was enormous. That my thinking of doing it more gently in order to do more justice to the study of Warburg, his former professor, was a very good idea. But that I did not ruin my life in a quest for something that might be impossible. That I work in the direction of isolating mitochondria more gently but only now and then while doing other easier things. There were enzymes, for example, such as the Na-K dependent Adenosine Triphosphatase of Jens Skou, whom I visited in 1976, which were affected by low temperatures. But at higher temperatures the cellular proteases destroyed the biochemical capacity of the mitochondrial membranes.

It was not until 1996 that my efforts, on and off since 1970, were successful in that direction: aided by my wife, Mariflor Blanco, and her inestimable attention to technical detail and that of a number of post graduate students. Then, a fortuitous power failure of the ceiling lamps, took me to oxygen production in anoxia and obscurity, with bicarbonate present, and to glimpse different applications for those more physiological studies in isolated mitochondria.

I now wish to draw your attention to the things that I consider key for isolation and assay of filamented mitochondria. Furthermore, I will mention certain applications for this new field. A new step forward in bioenergetics which promises, if it gets into the hands of authentic scientists with the right resources, a great harvest either for physiology and medicine as well as for society, in general.

Generic Advices on Filamented Mitochondria Isolation

I will point out the most generic things, necessary in my opinion, to obtain mitochondria more physiologically, filamented mitochondria, by differential centrifugation:

1) Sacrifice by nitrogen suffocation when still sleeping a young female rat or mouse fasted or well fed.
2) Homogenize its liver, gently, in a hypertonic sucrose medium with slightly basic pH, supplemented with trypsin inhibitor, EDTA and ATP, finally in no more than 40 milliliters, everything working at around a temperature of 14º C.
3) Centrifuge the 40 milliliters of the homogenate, filtered previously using gauze, in a 50 milliliter centrifuge test tube. This first centrifugation to remove the very heavy elements must not last more than five minutes nor exceed 900 g of acceleration. Everything at approximately 14º C.
4) The second centrifugation, for the supernatant, must not last longer than eight minutes and not exceed 2000g, at 14 º C, and must be done -using eight 50 milliliters test tubes -, i.e. with less than five milliliters per test tube. Depending on the desired purity of the filamented mitochondria of different sizes another centrifugation could be done, a rinsing one, again with a small volume, time and speed, at 14º C, using fewer test tubes to purify and concentrate them. In this case, mitochondria less filamented but purer will result. The mitochondria are conserved as almost dry pellets, or rather very concentrated, also at 14º C, until assay with various instruments.
5) The pipettes to manipulate the homogenate and supernatants, and most certainly the tips of the micropipettes used to add the filamented mitochondria, must be broad-tipped so that friction does not break up the filaments.

Naturally this advice is generic and essential but, in the hands of experts, it can be improved on. One needs an electronic scanning microscope to control if there is loss or gain in the grade of the mitochondrial filamentation, with some or other isolation and testing processes. I have always used the phase contrast optical microscope with maximum amplification mainly due to its simplicity. At times however, I searched for aid with scanning or transmission electron microscopy. Most instances simply with carbon shadowing.

Generic Advices on Filamented Mitochondria Assay

For the biochemical, biophysical or pharmacological testing of filamented mitochondria, I consider fundamental:

A) Minimal agitation in the cuvette, with perfect base lines, both at the beginning of the experiments, hyperoxia or normoxia, as well as at the end, anoxia.
B) Broad-tipped pipettes to add the filamented mitochondria.
C) Very oxygenated mediums defilament mitochondria and those with little oxygen filament them.
D) To detect oxygen production that arises in hypoxia and darkness, and all the physiological parameters of the filamented mitochondria, one must get close to the physiological constitution of the cytoplasm in the testing medium. Especially at least in carbon anhydride, potassium, sodium, NAD/NADH ratios and ratios of ADP/ATP/Phosphate.
E) In order to determine the aerobic glycolysis associated with filamented mitochondria, whether from pyruvate or glucose, this can be done with a few supplements as long as the preparations are no overly purified.
F) The pharmacological treatment of the animal - repercute first - on the filamented mitochondria isolated in the agents target organs.

II. Methods

Isolation of Mice Liver Filamentous Mitochondria

Two female albino mice of around 18 grams of weight each, fed ad libitum, are rapidly sacrificed at midmorning by neck stretching. About 3.5 grams of liver tissue are minced with scissors and the blood is washed out by an isolation medium composed of 350 mM sucrose, 1 mM potassium EDTA, 1 mM sodium ATP and 0.1 mg/ml trypsin inhibitor (pH 7.7, 14ºC). A mild homogenization of the minced and washed tissue is carried out manually in a glass homeginizer with a Teflon pestle in 40 ml of isolation medium maintaing a temperature of 14ºC. The resulting homogenate is centrifuged in a 50-ml tube at 2,750 rpm (900 x g) for five minutes at 14ºC. The supernatant obtained is likewise distributed by passing through cheesecloth into eight 50-ml tubes that are centrifuged at 3,750 rpm (1,730 x g) for six minutes at 14ºC. The resulting eight pellets are suspended in their own juices and are diluted with isolation medium to a final volume of 8 ml; this suspension is then divided into two 50-ml centrifuge tubes that are centrifuged at 4,500 rpm (2,420 x g) for six minutes. The resulting two pellets are suspended in their own juices and are combined to yeild approximately 1 ml of a suspension rich in heavy filamentous mitochondria that has a protein content of around 46 mg/ml (Lowry's method).

Isolation of Rat Brain Filamentous Mitochondria

Two female albino rats of around 160 grams of weight each, fed ad libitum, are rapidly sacrificed at midmorning by a blow to the neck. Their forebrains (approximately 2.6 grams of brain tissue) are rapidly extracted and the blood is washed out with an isolation medium composed of 350 mM sucrose, 2 mM potassium EDTA, 2 mM sodium ATP and 0.1 mg/ml trypsin inhibitor (pH 7.7, 12ºC). A mild homegenization of the washed tissue is carried out in 3 ml of isolation medium with a small, all glass homogenizer. This first thick homogenate is diluted with isolation medium for a final volume of 12 ml and is filtered through four layers of cheesecloth. This filtrate is then diluted to 30 ml

with isolation medium and is centrifuged in a 50-ml tube at 2,750 rpm (900 x g) for five minutes at 12ºC.
The supernatant is then distributed equally among six tubes of 50–ml that are centrifuged at 5,450 rpm (3,550 x g) for seven minutes at 12ºC. The reulting six pellets are suspended in their own juices and the suspension is diluted with isolation medium to a final volume of 8 ml which is divided into two 50-ml tubes that are centrifuged at 5,700 rpm (3,880 x g) for eight minutes at 12ºC. The resulting two pellets are suspended in their own juices and are combined to yeild approximately 1 ml of a suspension rich in heavy filamentous mitochondria that has around 38 mg of protein per ml (Lowry's method).

Future Possible Applications

Without wishing to be exhaustive, I should like finally to mention some applications which are opening up following these initial new findings in mitochondrial molecular physiology. I have simply begun these studies with some modest pilot experiments. It is really from now when matters pass to the detailed scrutiny of the most capable science.

This field is especially important in cancer, either in its nature, treatment, cause or diagnosis as well as the use of cancer as a therapeutic agent in spinal paralysis or neural blindness and deafness. For degenerative diseases, senility and rejuvenation, mitochondrial filamentation studies are obligatory. In the extension of metabolic life after physiological transitory death, for the increasing sporting prowess and in food production the manipulation of the number and type of mitochondria filaments could be very beneficial. Pharmaceutical modulation of mitochondrial filamentation also promises great benefits in the treatments of obesity, some of the secueles of anesthesic procedures and in acute and chronic schizophrenia. This new oxygen production by filamented or conified membranes, if found in many cellular life species, could be important for the net balance of planetary oxygen.

I still think that many more things will start to be better understood if a relaxed cooperative study is started, truly worldwide, in the next decades. Shortly, now, we will begin to understand in detail the casual clothing, recurrent, of these cellular dancers, masters of biological energy: the cones, veils and mitochondrial radial filaments of varying diameters, in different mitocondria, at various times. Studies should be undertaken, please, in several species to gain the adecuate perspective in the physiology of energy metabolism at large.

At the end of many more things, we will manage to learn about the incredibile efficiency of filamented mitochondria to make disappear, or appear, oxygen and ATP, according to the physiological needs of the many cellular adaptations that life endure. Among which, in my opinion, it could be counted well cured diseases and overcome physiological deaths. The disorder of this wonderful capability for different illneeses, above all some of those called rare diseases, will take more time.

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