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Rapid spectrophotometric method for quantitation of cytochrome *c* release from isolated mitochondria or permeabilized cells revisited

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Abstract

This paper recalls the earlier work by Keilin, Margoliash and others at the beginning of the 20th century and shows how their results can be used for the rapid solution of new problems of modern science. It describes a rapid and simple spectrophotometric method for quantitative determination of cytochrome *c* release from isolated mitochondria or permeabilized cells induced by proapoptotic proteins. For this, the Soret (γ) peak at 414 nm in the spectrum of cytochrome *c* is used. The results of spectrophotometric assay of cytochrome *c* release are in accord with those of oxygraphic determination of cytochrome *c*-dependent respiration of isolated mitochondria and permeabilized cardiomyocytes.

1. Introduction

Intensive studies of the role of mitochondria in initiation of apoptosis by releasing cytochrome *c* in response to extramitochondrial stimuli, such as the action of the proapoptotic proteins Bax and Bid [1–4], make it important to use a rapid and reliable method for quantitation of the release of cytochrome *c*. In most biological studies the release of cytochrome *c* is determined by immunochemical methods, such as Western blot analysis or immunohistochemical analysis [5,6], both coming from cell biology. These methods are highly specific for cytochrome *c*, but cannot be considered quantitative and they are rather time-consuming. However, in the classical bioenergetic research early in the 20th century cytochromes, including cytochrome *c*, were studied in great detail by spectrophotometric methods [7]. Dmitry Zorov's group in Moscow and Manon's group in Bordeaux have already used this technique in studies of cytochrome *c* release by applying the method of Chance – recording the difference between reduced and oxidized cytochromes by dual wavelength double beam spectrophotometry [8,9]. This technique used the α-peak area of the cytochrome spectrum, which gives high resolution for different cytochromes. However, it requires expensive equipment and because of the low sensitivity ($\varepsilon = 18$ mM^{-1} cm^{-1}) a rather large amount of material or recording at low temperatures is required. There is, however, a very intensive γ or Soret peak in the spectrum of cytochrome *c* ($\varepsilon = 100$ mM^{-1} cm^{-1}) [7]. This provides an attractive possibility to try to use this peak for determination of the release of cytochrome

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c. We found in this work that this method can indeed be used for rapid and quantitative determination of the release of cytochrome *c* either from isolated mitochondria or from permeabilized cells when the release of other heme-containing proteins is practically excluded.

2. Materials and methods

Mitochondria were isolated from white Wistar rat hearts as described by Saks et al. [10].

Cardiomyocytes were isolated from adult rat hearts, permeabilized with saponin (50 μg/ml) during 30 min at 4°C and myosin extracted by a concentrated solution of KCl (800 mM) to produce the phantom cardiomyocytes as described by Kay et al. [11]. In some experiments skinned cardiac fibers prepared as described in [12] were used.

Cytochrome c release was studied in a medium with an ionic composition close to the muscle cell cytosol [13]. This solution (solution B) contains, in mM: CaK_2EGTA 1.9, K_2EGTA 8.1, $MgCl_2$ 1.4, KH_2PO_4 3.0, dithiothreitol (DTT) 0.5, MES 100.0 (pH 7.1), imidazole 20.0, taurine 20.0, pyruvate 5.0, bovine serum albumin 2 mg/ml.

Isolated heart mitochondria, at a final concentration of 4.2 mg/ml, or permeabilized phantom cardiomyocytes, 2.7 mg/ml, were incubated in 1 ml of this medium for 30 min at 25°C in the presence of different additions as shown in the legends to the figures. After that the respiratory parameters were measured by oxygraphy and samples were taken for cytochrome *c* determination. The total number of determinations was 30.

The cytochrome c spectrum was recorded by a spectrophotometer Uvikon 941, Kontron Instruments, UK. After incubation with Bax and/or Bcl-2 as described above, cells or mitochondria were centrifuged at $10\,000 \times g$ during 30 min. The supernatant was filtered through a 0.2 μm Millipore membrane. The optical density of clear supernatants was recorded against the medium as a reference from 390 to 600 nm.

Oxygen consumption rates were recorded using a high resolution Oroboros oxygraph, Anton Paar, Graz, Austria. All measurements were performed at 25°C in a solution containing, in mM: Mg-acetate 3.0, DTT 0.3, KCl 125.0, HEPES 20.0, glutamate 4.0, malate 2.0, KH_2PO_4 5.0, EGTA 0.4, BSA 2 mg/ml, pH 7.1 at 25°C. Data were analyzed by computer with the DatLab program which can generate two data curves: the oxygen concentration in the chamber of the oxygraph and the rate of oxygen consumption (V_{O_2}).

Protein concentration was determined using a BCA protein assay kit (Pierce, USA). Samples of mitochondria or cardiomyocytes in solution were taken from each step of the experiment.

Bax was used in two forms: full-length (Bax FL) and truncated (BaxΔTM) [14–16]. This latter does not contain the hydrophobic C-terminal domain and can be kept in a buffer without detergent, which could interfere in our results.

3. Results and discussion

Fig. 1A shows the spectrum between 390 and 600 nm of oxidized cytochrome *c* for concentrations between 1 and 10 μM. These spectra are identical to those described by David Keilin in his History of Cell Respiration and Cytochrome ([7], Chapter 14, Fig. 41). From the linear relationship between optical density at 414 nm and cytochrome *c* concentration shown in Fig. 1B we can calculate the extinction coefficient which was determined to be $\varepsilon = 100$

Table 1
Dose dependence of cytochrome *c* release from isolated rat heart mitochondria

Added concentration of BaxΔTM (μM)	Cytochrome *c* released (nmol/mg mitochondrial proteins)
Intact isolated mitochondria	0.04
+0.25	0.05
+0.5	0.06
+1	0.065
+2.5	0.097
+5	0.102
+25	0.216
Hypotonic medium	0.306

Hypotonic medium (60 mM KCl, 10 mM HEPES, pH 7.1) was used instead of solution B during incubation of mitochondria to completely destroy the outer mitochondrial membrane and then KCl was added to 200 mM concentration for extraction of cytochrome *c*. The results of one series of experiments are shown.

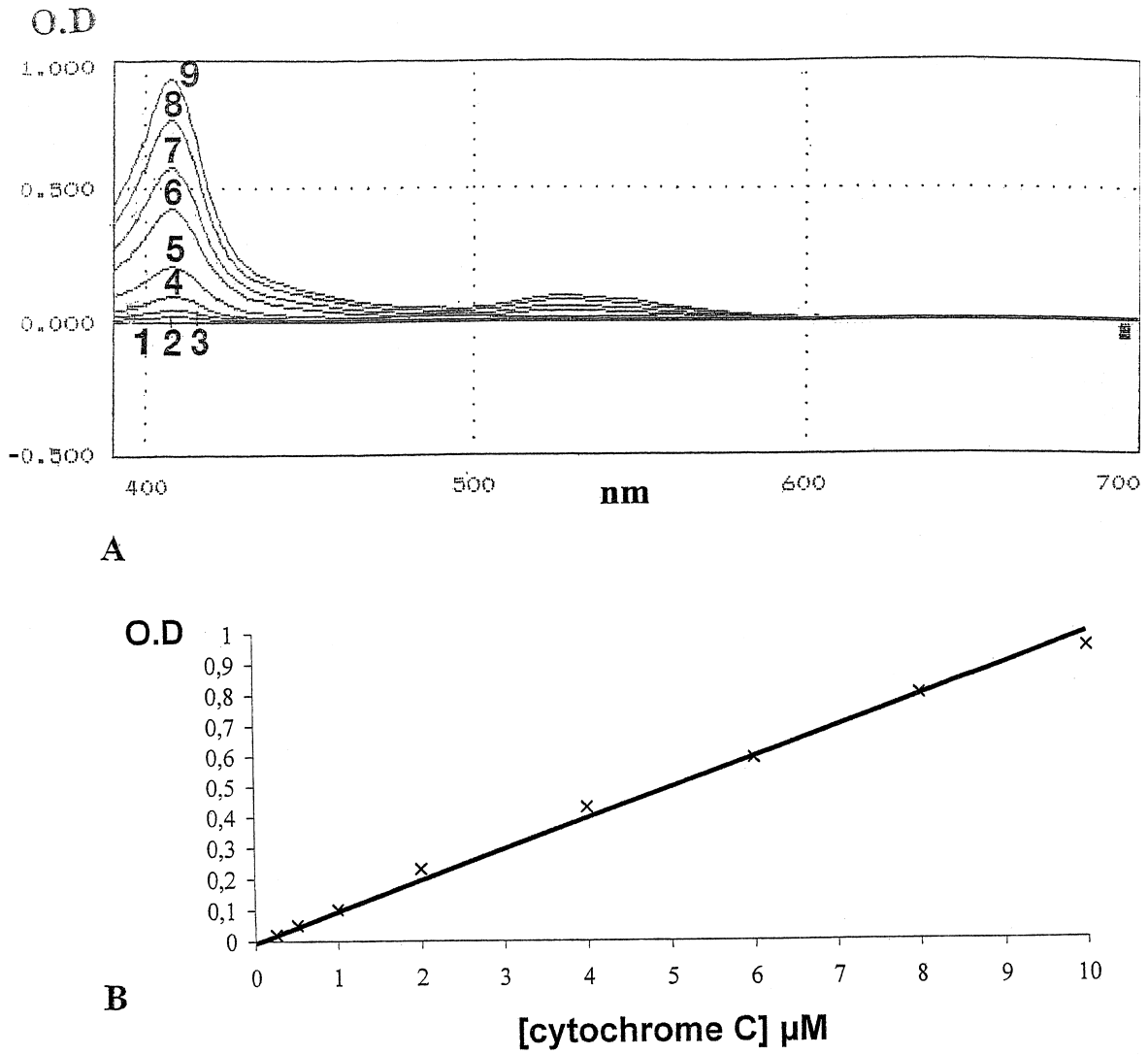

Fig. 1. (A) Oxidized cytochrome *c* spectra recorded between 390 and 600 nm. O.D.: optical density. Concentrations of cytochrome *c* used were: 1: 0; 2: 0.25 µM; 3: 0.5 µM; 4: 1 µM; 5: 2 µM; 6: 4 µM; 7: 6µM; 8: 8 µM; 9: 10 µM. (B) Calculation of extinction coefficient ε from data given in (A). The value of ε is found as the slope of the straight line.

mM^{-1} cm^{-1}, the same as found by Margoliash and Keilin [7]. This value of the extinction coefficient, which is a molecular property of cytochrome *c*, can now be used to calculate the concentration of cytochrome *c* in a medium from any measured value of optical density. Reduction of cytochrome *c* by dithionite increased the absorbance of all peaks [7]. In our experiments, it increased ε to 120 mM^{-1} cm^{-1} (not shown). However, the spectrum of the reduced form was not used in this study because of interference from dithionite in the optical density at low wavelengths around 400 nm.

Isolated heart mitochondria (not purified on Ficoll or Percoll gradients) usually contain a fraction of 'light' mitochondria with a damaged outer membrane. In the physiological salt solution (solution B) with high ionic strength this fraction releases a low quantity of cytochrome into the medium, as illustrated by recording 1 in Fig. 2. This figure shows the cytochrome *c* spectrum in the supernatant after sedimentation of mitochondria and Millipore filtration. Remarkably, incubation with Bax significantly increased the release of cytochrome *c* from the mitochondria, as seen in the increased Soret peak at

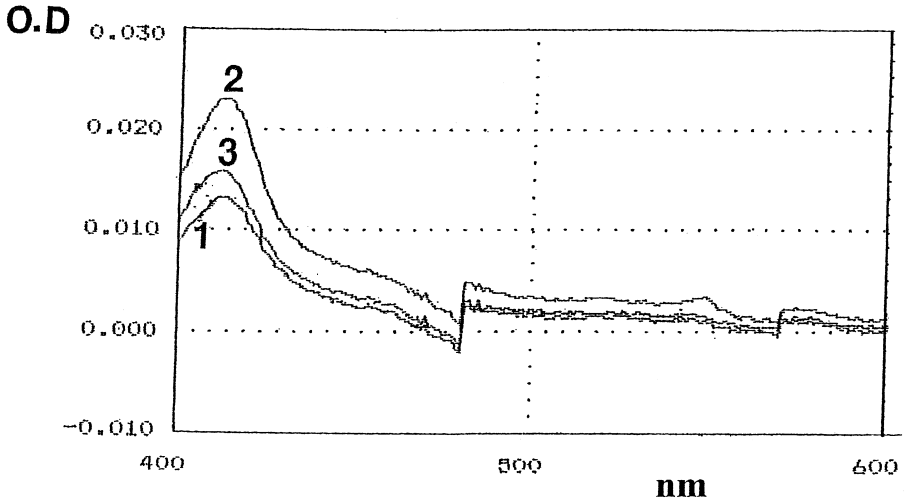

Fig. 2. Cytochrome *c* spectra in filtrated supernatants after centrifugation of isolated rat heart mitochondria incubated for 30 min in physiological salt solution with different additions. 1: control; 2: in the presence of BaxΔTM, 2.5 μM; 3: in the presence of the mixture of Bcl-2, 2.5 μM and Bax-GST, 2.5 μM. O.D.: optical density.

414 nm (recording 2 in Fig. 2). Bcl-2 almost completely inhibited the effect of Bax (recording 3 in Fig. 2). The cytochrome *c* release was dependent on the concentration of Bax (Table 1).

A more intact system than isolated mitochondria is the permeabilized cardiomyocytes – there is no light fraction of mitochondria in isolated cardiomyocytes permeabilized by saponin, due to the very mild treatment of the cells [12]. All mitochondrial outer membranes are intact and intracellular structures are well preserved [12]. To avoid any problems of Bcl-2 and Bax diffusion to the mitochondria, we used the phantom cardiomyocytes deprived of myosin but containing an intact mitochondrial–cytoskeletal system [11,12]. Recording 1 in Fig. 3 shows that there is no release of cytochrome *c* (or any heme-containing protein) into the medium from these permeabilized phantom cardiomyocytes during incubation in the physiological salt solution. However, incubation with Bax clearly releases cytochrome *c* from the phantom cardiomyocytes (recording 2 in Fig. 3). Using the extinction coefficient determined in Fig. 1B,

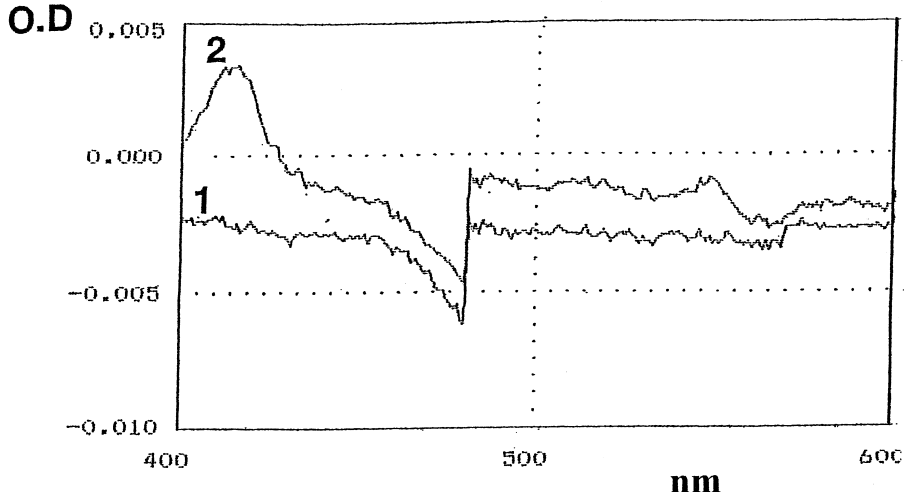

Fig. 3. Cytochrome *c* spectra in filtered supernatants after sedimentation of permeabilized phantom cardiomyocytes incubated in physiological salt solution for 30 min. Recording 1: control; recording 2: incubation in the presence of truncated Bax, 5 μM. O.D.: optical density.

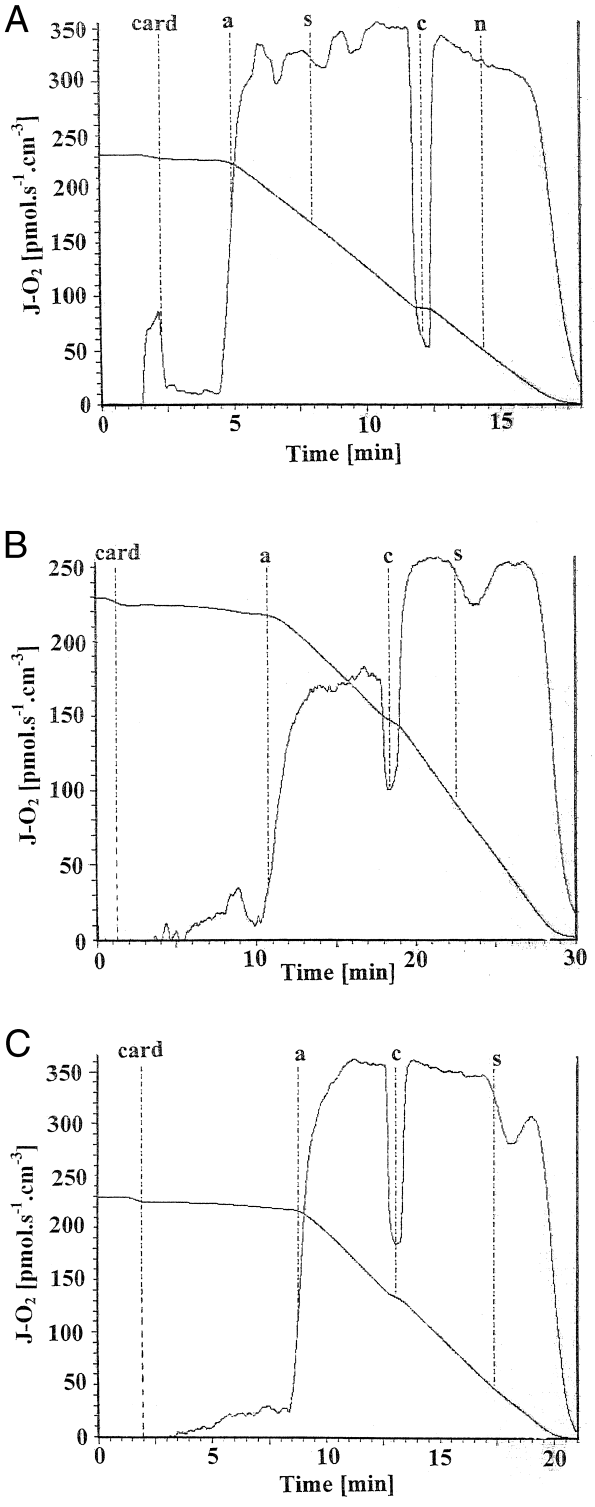

Fig. 4. Oxygraph recordings on the effects of cytochrome *c* release from mitochondria. All figures show two data curves: the time dependence of oxygen concentration in the system (curve 1) and its first derivation (curve 2) showing the direct rate of oxygen consumption. The mitochondrial substrates used were glutamate and malate. The cells (card) were introduced into the respiratory chamber. When 2 mM of ADP (**a**) was added, this induced maximal stimulation of the oxidative phosphorylation, succinate (**s**) is able to give the maximal activity of the respiratory chain, exogenous cytochrome *c* (**c**) (8 μM) does not modify the respiration except if the mitochondrial outer membrane is damaged, because in this case cytochrome *c* is detached from the outer face of the inner membrane in the presence of KCl and is released into the cytosol, the respiration can be then restored by addition of a high concentration of exogenous cytochrome *c*. NADH (**n**) does not have any effect on the intact cardiomyocytes. Panel A shows phantom cardiomyocytes incubated with Bcl-2 (2.5 μM), this protein has no effect on the respiration, this is a reference. Panel B shows cardiomyocytes incubated with 2.5 μM BaxΔTM, activation of the respiration by ADP is decreased and exogenous cytochrome *c* can increase but not fully restore Vm. This shows clearly that Bax releases cytochrome *c* from mitochondria because of changes in the outer membrane. Panel C shows that this effect of Bax is completely inhibited by an equimolar concentration of Bcl-2.
←

calculations show that under these conditions 0.017 nmol of cytochrome *c* is released per mg of cellular protein.

Bax-induced release of cytochrome *c* is evidenced by oxygraph recordings of respiration of permeabilized phantom cardiomyocytes. Fig. 4 shows the oxygraphic experiments we call 'the cytochrome *c* test' for determination of the state of the outer mitochondrial membrane [12]. This test consists in measuring the respiration rate in the presence of high saturating concentrations of ADP (2 mM) in KCl medium [11,12]. In this medium cytochrome *c* dissociation from the membrane is increased [13]; however, if the outer membrane is intact, cytochrome *c* participates in electron transfer, the respiration rate is high due to the rapid equilibrium between dissociation and association of cytochrome *c* in the mitochondrial intermembrane space, and addition of exogenous cytochrome *c* has no effect on the respiration rate. This is the case with phantom permeabilized cardiomyocytes (Fig. 4A). However, as shown in Fig. 4B, after incubation with 2.5 μM Bax, the addition of exogenous cytochrome *c* had a remarkable stimulating effect on respiration, showing that a fraction of cyto-

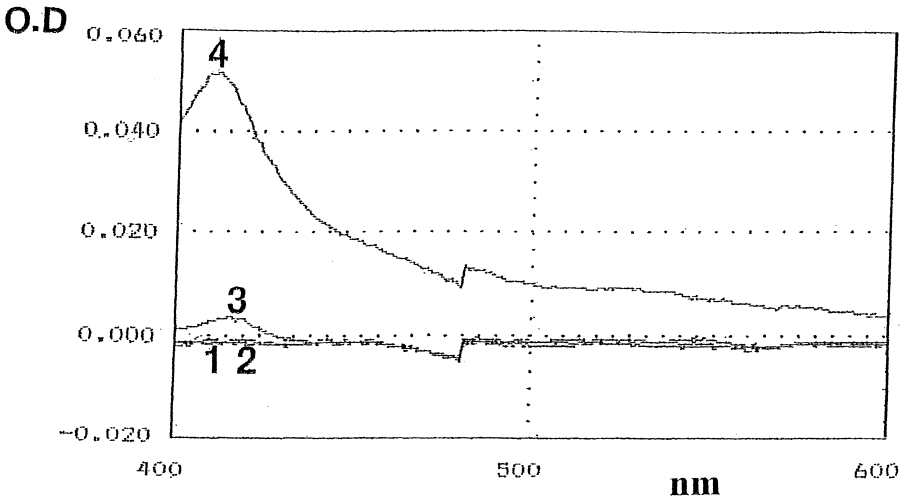

Fig. 5. Cytochrome *c* spectra in filtered supernatants after sedimentation of permeabilized phantom cardiomyocytes incubated in physiological salt solution for 30 min under different conditions. Recording 1: control; recording 2: with Bcl-2, 2.5 μM; recording 3: with truncated BaxΔTM, 5 μM (this is the same as recording 2 in Fig. 3, see the difference of the *y*-axis scale); recording 4: with Bax FL 5 μM.

chrome *c* had left the intermembrane space, and that exogenous cytochrome *c* could enter the intermembrane space and thus restore the respiration. However, no effect of the exogenous cytochrome *c* was seen if the cardiomyocytes were incubated with Bax in the presence of an equivalent amount of Bcl-2 (Fig. 4C), showing that Bcl-2 completely blocks the effect of Bax on mitochondria in phantom cardiomyocytes. The oxygraphic 'cytochrome *c* test' is another easy and rapid, but more indirect method of measuring the release of cytochrome from mitochondria, demonstrated in Figs. 2 and 3.

Thus, the results of this study show that recording of the Soret peak intensity in the cytochrome *c* spectrum in filtrates allows us to quantitatively study the effect of Bax on the release of cytochrome *c* from isolated mitochondria or permeabilized cells. This conclusion is further confirmed by the results shown in Fig. 5. This figure shows that in the presence of full-length Bax one can observe the release of very significant amounts of cytochrome *c*. Calculations show that in this case 0.153 nmol of cytochrome *c* per mg of cell protein is liberated. Taking the normal content of cytochrome *c* to be equal to 0.3–0.5 nmol/mg of mitochondrial protein and mitochondrial protein to represent about 40% of cell protein, practically all cytochrome *c* is liberated from the cell under these conditions.

The protocol for these experiments is simple and the measurements can be performed in 1 or 2 h. The estimations give us the maximal values of cytochrome *c* which could have been released, since it is not excluded that under some specific experimental conditions other compounds may be extracted or released from the mitochondria and this could contribute to the optical absorbance at 414 nm. In this case a combination of both spectrophotometric and oxygraphic methods described in this work will be helpful. Thus, for rapid screening of the effects of pro- and anti-apoptotic proteins on the release of cytochrome *c* the methods described in this paper are suitable.

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