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QUALITATIVE MEASUREMENTS OF THE MITOCHONDRIAL MEMBRANE POTENTIAL IN SITU IN EHRLICH ASCITES TUMOUR CELLS USING THE SAFRANINE METHOD

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Summary

1. A differential spectrum with a decrease in the absorbance at 524 nm appears when Ehrlich ascites tumour cells are added into a medium containing safranine. This spectral change is similar to that which occurs upon polarisation of the membrane of isolated mitochondria. The maximal change occurs within 15--20 min after cell addition. Additions of KCN, rotenone, antimycin A or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to the cell suspension do not reverse the response but result in a spectrum in which a peak appears at higher wavelengths (540 nm). This suggests that upon deenergization of cells safranine is transferred to a milieu of low polarity.

2. Addition of air to anaerobic cells or glucose to cells whose respiration is inhibited by rotenone, also produces a shift in the safranine spectrum typical to that which occurs upon energization of mitochondria. The response of glucose is insensitive to KCN but abolished by dicyclohexylcarbodiimide, suggesting that ATP produced by glycolysis is responsible for this spectral change.

3. If succinate is added to the cell suspension no response of this substrate is seen with intact cells. However, if cells are pretreated with dextran sulfate, which makes the plasma membrane permeable to small molecules a fast shift is produced upon succinate addition.

The results suggest that the spectral changes are signals from mitochondria within the cell. Thus by measuring these changes in the safranine spectrum information about the energy metabolism of cells and how mitochondria function in situ can be obtained.

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Introduction

Dye probes, the fluorescence or absorbance of which changes upon induction of electric potential changes in membranes, have received wide interest lately [1,2]. These probes provide useful tools for studying membrane potentials in small cells [1] or organelles [2] in which electrode techniques are difficult or impossible. Large shifts occur in the spectrum of safranine, a positively charged dye, upon induction of K^+ diffusion potentials with negative internal polarity across the membrane of liposomes by the aid of the ionophores valinomycin [3] or gramicidin [4]. Changes in the safranine spectrum of the same magnitude also occur when K^+ or H^+ diffusion potentials are induced by the aid of valinomycin or in the presence of a proton conducter, FCCP, across the membrane of the isolated mitochondria [5]. Similar spectral shifts can also be induced upon the addition of succinate and ATP to a mitochondrial suspension [5] or upon induction of Ca^{2+} diffusion potentials in the presence of an endogenous Ca^{2+} translocator in the mitochondrial membrane [6]. The extent of spectral change correlates linearily to the magnitude of membrane potential [5] at least up to 200 mV and thus accurate estimates of the mitochondrial membrane potential in different conditions can be obtained [5]. The mechanism by which the changes in the spectrum of the dye occurs appears to be an uptake into and aggregation at the membrane inner surface [3,7,8] of the membrane particles (liposomes or mitochondria). The mitochondrial membrane potential has also been measured with cyanine dyes [9]. However, since the response of the cyanines is very small at high potentials [9] due to the non-linear relationship of the extent of responses to the magnitude of the membrane potential [10], they are more suitable for measurements of potential changes in the plasma membrane of the cell [11].

As safranine reacts especially to high membrane potentials [5] and is very permeable to membranes [3] the aim of the present study was to test the possible applicability of the safranine method for studying the mitochondrial membrane potential in intact cells, without interference from potential changes in the plasma membrane.

Materials and Methods

Ehrlich ascites tumour cells obtained by courtesy of Dr. J.A. Räsänen (Aurora Hospital, Helsinki, Finland) were harvested for 6—7 days in Swiss Albino mice. The mice were decapitated and ascitic fluid was removed into sterilized centrifuge tubes. The cells were washed twice in 0.25 M sucrose/10 mM Hepes-Tris (pH 7.2) by centrifugation at $600 \times g$ for 5 min and suspended into 50% (packed cell volume/medium volume) in this medium. The viability of cells as measured with trypan blue exclusion ranged between 95—99%. If the cells were harvested longer than 7 days the amount of ascitic fluid increased with loss of cell viability.

The dextran sulfate treatment was carried out as described by Kasahara [12]. The cells were also in this case further washed once in the sucrose medium and suspended into 50% medium.

The basal experimental medium contained 140 mM NaCl, 6 mM KCl,

1.5 mM $MgCl_2$, 20 mM Tris, 10 mM Hepes (adjusted to pH 7.4 with HCl). The spectral changes of safranine were measured in an Aminco DW_2 spectrophotometer.

FCCP was kindly donated by Dr. P.G. Heytler, antimycin A and valinomycin were obtained from Sigma Chemicals Co., St. Louis, MO.; safranine was from E. Merck A.G., Darmstadt, F.R.G., and sodium dextran sulfate from Pharmacia Fine Chemicals, Uppsala, Sweden. All other reagents were commercial products of highest quality available.

Results and Discussion

Spectral changes of safranine in the presence of Ehrlich ascites cells

Addition of cells to the sample cuvette containing safranine results in a differential spectrum which consists of a decrease in absorbance at 524 nm as compared to the reference cuvette with safranine alone (Fig. 1). This spectral change is typical to that which occurs upon induction of membrane potentials in isolated mitochondria [5,6] and does not occur in the absence of safranine (not shown). There is a lag period of 2—5 min before the shift is produced. The response is completed within 15—20 min and is probably a result of mitochondrial respiration due to endogenous substrates (see below). Deenergization of cells with KCN (Fig. 1), rotenone, antimycin A or FCCP (not shown) does not reverse the response, but a new differential spectrum is produced within 1—2 min with a peak at higher wavelengths (547 nm) and a decrease in absorbance at about 495 nm. This suggests that the spectrum of safranine in cells with deenergized mitochondria differs from that in external medium (see below). There is also an increase in absorbance below 480 nm, which is also seen in the absence of safranine (not shown). The other changes in the

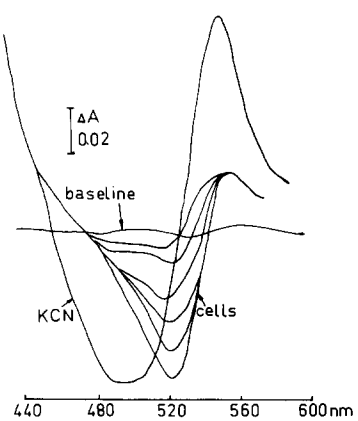

Fig. 1. Differential spectra of cells in the presence of safranine. Both samples and reference cuvette contained 20 μM safranine in the basal medium at 37°C (baseline). Thereafter 2% (v/v) of cells (cells) were added and the trace adjusted with high voltage because of the backround absorbance of the suspension and the differential spectrum was recorded with 2—3 min intervals. The largest change shown was recorded 15 min after addition of cells. Thereafter no change in the spectrum was observed. Addition of 2 mM KCN (KCN) as indicated.

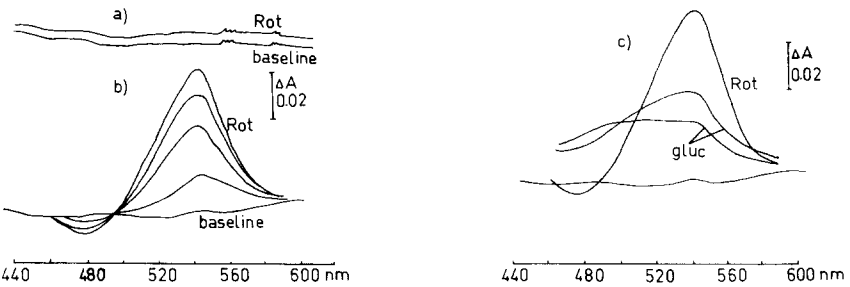

Fig. 2. Deenergization differential spectrum of Ehrlich ascite cells in the presence and absence of safranine. Conditions as in Fig. 1, except that both cuvettes contained 2% cells. The baseline was drawn after 15 min incubation at 37°C in the absence (a) or presence (b, c) of 20 μM safranine. Additions: 10 μM rotenone (Rot) and 5 mM glucose (c, gluc) as indicated. The spectrum was drawn within 1—2 min intervals after additions.

spectrum (at 524, 547, 495 nm) are not seen in the absence of safranine (not shown).

When cells are preincubated for 15 min in the presence of safranine and rotenone (Fig. 2b), KCN, antimycin A or FCCP (not shown) are added, a differential spectrum of the deenergization is seen. It consists of an absorbance peak at 540 nm and a small decrease in absorbance with a maximum at lower wavelengths (480 nm). Note that the differential spectra in Figs. 1 and 2 are not comparable since the former represents a differential spectrum of cells in the presence of safranine whereas the latter a differential spectrum of energized versus deenergized cells. The decrease in absorbance at lower wavelength (480 nm) probably represents a decreased amount of multimer transition (destacking) of the dye, i.e., reversal of the energized spectrum (cf. Ref. 3 and 7). The effects of rotenone (Fig. 2c), KCN or antimycin A (not shown) are partially reversed by glucose. No spectral changes are seen in the absence of safranine (Fig. 2a) in the present conditions. The transfer of the peak to higher wavelengths would suggest that the milieu in which safranine exists in cells with deenergized mitochondria is different from that of the external medium (water solution) in which the peak usually is near 524 nm [3,7]. Fig. 3 shows the spectrum of safranine in the external medium as compared to that in ethanol and butanol. It can be seen that the peak is transferred to higher wavelengths in organic solvents. This would suggest that upon deenergization of mitochondria inside the cell safranine is partially present in a milieu of low polarity, possibly associated with membranes.

If both cuvettes are anaerobic and air is subsequently bubbled into the sample cuvette a spectral shift is produced in 1—2 min (Fig. 4). This shift is very similar to that which occurs upon energization of or upon induction of diffusion potentials in isolated mitochondria [5,6]. A similar change also occurs for instance upon glucose addition to cells deenergized with rotenone (not shown). These experiments clearly prove the reversible nature of the spectral changes of safranine in the presence of intact cells.

Spectral changes of safranine as a function of time in the presence of Ehrlich ascites cells as measured with the dual wavelength method

When cells are added to the medium containing safranine a small change in

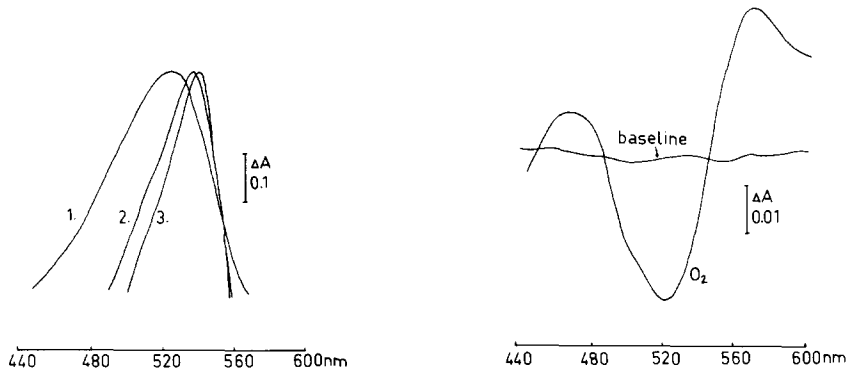

Fig. 3. Absolute spectra of safranine in the basal medium (1), ethanol (2) or butanol (3). Note that the extinctions are not comparable since they increase in the order basal medium > ethanol > butanol.

Fig. 4. Differential spectrum upon energization of anaerobic cells with air in the presence of safranine. The cells were preincubated in the presence of 20 μM safranine for 15 min. Thereafter N_2 was bubbled into both cuvettes for 3 min and the baseline was drawn. The mouths of the cuvettes were closed with parafilm. Air was subsequently bubbled into the sample cuvette and the spectrum was drawn (O_2) within 1—2 min. Other conditions as in Fig. 2.

absorbance is seen at the respective wavelength pair (524—484 nm) (Fig. 5). After a lag period a slow downwards deflection of the trace due to cell respiration occurs. This response is reversed upon rotenone addition (Fig. 5). Subsequent addition of glucose results in a very fast downwards deflection (Fig. 5b) less than 10% of which can be reversed by KCN. The signal is on the other hand completely reversed by addition of dicyclohexylcarbodiimide, an inhibitor of mitochondrial ATPase, which suggests that the spectral change in the presence of rotenone and glucose is mainly due to ATP production by glycolysis under the present conditions. In the presence of iodoacetate only a small nonsignificant response is seen upon glucose addition in the present conditions (Fig. 5a). These results are in agreement with Demetrakopoulos et al. [13] who recently

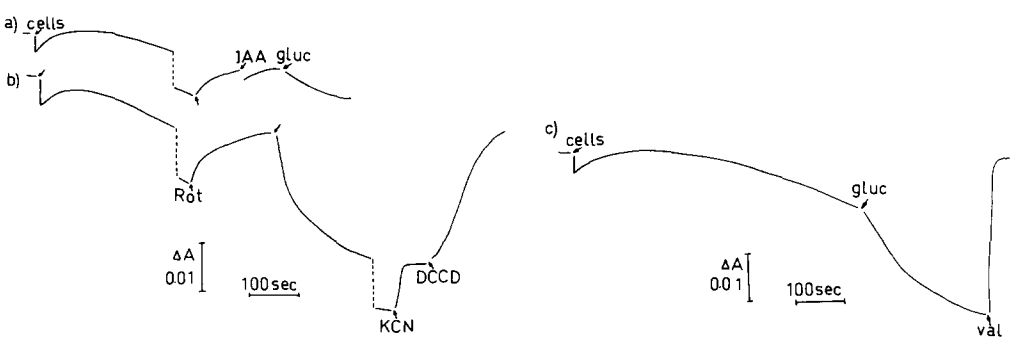

Fig. 5. Spectral changes in the presence of safranine as a function of time. Conditions as in Fig. 1. Wavelength pair 524—484 nm. Additions: (a) 2% cells (cells) 10 μM rotenone (Rot), 1 mM iodoacetamide (IAA) or 5 mM glucose (gluc) as indicated. (b) cells, rotenone, glucose, 5 mM KCN (KCN) and 200 μM dicyclohexylcarbodiimide (DCCD) as indicated. (c) cells, glucose and 200 ng/ml valinomycin (Val) as indicated. The dotted lines represent slow linear phases of the trace not shown. Downward deflection increased stacking (membrane potential).

reported that the ATP concentration of tumour cells deprived of glucose is very low. Furthermore, glucose addition to Ehrlich ascites cells results in a significant rise in mitochondrial ATP [14]. Note that the initial rate of the change induced by glucose is much faster when the endogenous response has been abolished with rotenone (Fig. 5b) than when glucose is added without prior deenergization with rotenone (Fig. 5c). This would suggest that the penetration of safranine into the cell is slow, but when it has penetrated a fast reshift in its spectrum is produced upon reenergization. This would also suggest that the very slow response during respiration due to endogenous substrates (Figs. 1 and 5) is not due to a slow penetration of safranine into the cell but due to a slow build-up of the mitochondrial membrane potential when the cells are diluted into the experimental medium. Fig. 5c shows that the safranine response is completely abolished by a large concentration of valinomycin, which further suggests that the mitochondrial membrane potential is measured and not that of the cell membrane, since the K^+ concentration outside the cell is low but should be high inside and hence a complete depolarisation of the mitochondrial membrane would be expected. The response of valinomycin is not seen with probes that measure the membrane potential of the plasma membrane [11] in the absence of high external K^+.

Effects of dextran sulfate treatment on the spectral changes of safranine in the presence of Ehrlich ascites cells

It has recently been shown [12,15] that treatment of Ehrlich ascites cells with dextran sulfate makes the plasma membrane permeable to small molecules but that it retains its impermeability to larger molecules. Fig. 6a shows that succinate has no effect on the safranine signal of intact cells. However, addition of succinate to cells treated with dextran sulfate leads to a very fast downwards deflection of the trace. This signal is unaltered by rotenone but completely abolished by KCN (Fig. 6b). These experiments clearly indicate that with intact cells the safranine signal is due to energy metabolism in the cell. It is not a response from mitochondria of broken cells, because succinate should pene-

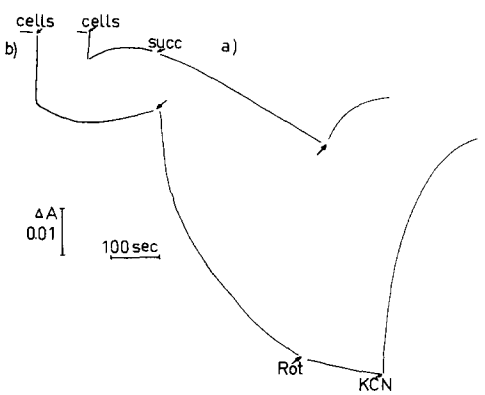

Fig. 6. Effect of dextran sulfate treatment of cells on spectral changes in the presence of safranine. Conditions as in Fig. 5. (a) normal cells and (b) cells treated with dextran sulfate. Additions: 2% cells (cells), 4 mM succinate (succ), 10 μM rotenone (Rot) and 5 mM KCN (KCN) as indicated. Downward deflection increased stacking (membrane potential).

trate only slowly across the cell membrane if it is intact which indeed seems to be the case.

It is proposed that safranine penetrates the cell membrane and is driven into the mitochondria by the membrane potential with negative intramitochondrial polarity. In the interior of the mitochondria stacking of the dye molecule occurs in a similar way as with isolated mitochondria [5].

It is concluded that the present results suggest that the safranine method, which has been employed for studies of membrane potentials in isolated mitochondria also enables qualitative measurements of the mitochondrial membrane potential in intact cells and thus this method provides an useful tool for studies on the energy metabolism of cells in suspension.

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