Effects of ionophores and metabolic inhibitors on the mitochondrial membrane potential within isolated hepatocytes as measured with the safranine method

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A difference spectrum with a peak of absorbance at 526 nm appears slowly upon addition of valinomycin or KCN in combination with oligomycin to a hepatocyte suspension in the presence of safranine. When the cells are incubated at 37°C in a medium containing safranine, a slow decrease in the absorbance occurs at the wavelength pair 524–484 nm. The change in absorbance is completed within 20–30 min after additions of cells to a medium containing safranine. At this time the safranine concentration of the outer medium is considerably decreased. The safranine signal is completely reversed by valinomycin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide or KCN in combination with oligomycin. None of these treatments have any immediate effects on cellular ATP concentrations or the $^{36}\text{Cl}^-$ equilibrium potential across the plasma membrane. In the presence of iodoacetate a slow reversal of the trace can be induced upon addition of KCN, but not of oligomycin alone. Rotenone, in combination with oligomycin, does not reverse the safranine signal except when both KF and iodoacetate are present, in which case a slow reversal is seen. A subsequent addition of duroquinone brings back the signal to the same level as in the presence of rotenone alone. The results indicate that the spectral response of safranine in the presence of isolated hepatocytes is a result of a slow penetration of safranine into intracellular mitochondria, where aggregation of safranine molecules occurs as a response to the mitochondrial membrane potential.

It is well established that an essential link between mitochondrial electron transport and ATP synthesis is the formation of a membrane potential ($\Delta\psi$) with negative intramitochondrial polarity (Boyer *et al.*, 1977). In isolated mitochondria, values of $\Delta\psi$ of the order of 180 mV (Rottenberg, 1973; Nicholls, 1974; Åkerman & Wikström, 1976) or more (Mitchell & Moyle, 1969) have been obtained during State-4 respiration by using different methods. On the other hand, at present little information is available on the relation of the overall energy metabolism in intact

cells to changes in the mitochondrial $\Delta\psi$. Efforts to measure the mitochondrial $\Delta\psi$ within cells have recently been performed with permeant radioactive tracers such as [^3H]TPMP⁺ in neuroblastoma cells (Deutsch *et al.*, 1979), isolated hepatocytes (Hoek *et al.*, 1980) and synaptosomes (Scott & Nicholls, 1979, 1980). With these methods crude quantitative estimates of $\Delta\psi$ have been obtained. By using the cationic membrane potential probe, safranine, qualitative changes in the mitochondrial $\Delta\psi$ in intact Ehrlich-ascites-tumour cells have also been measured in different metabolic conditions (Åkerman, 1979). The advantages of the safranine method are rapidity and the ability continuously to monitor changes in membrane potential as a response to various perturbations.

The aim of the present study was to test the applicability of safranine for studies on changes in the mitochondrial membrane potential within isolated hepatocytes under different conditions.

Abbreviations used: TPMP⁺, methyltriphenylphosphonium cation; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazide; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

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Experimental

Methods

Isolation and incubation of cells. Hepatocytes were prepared from fed adult male Wistar rats by the method of Seglen (1973) as modified by Moldeus *et al.* (1978) except that the cells were finally suspended in the experimental medium given below containing 4 mM-glucose. During the experiments the cells were stored at 20°C with constant slow mixing to prevent aggregation and sedimentation. The viability of the cells, as measured by Trypan Blue exclusion, was always higher than 85%. The basal experimental medium used contained 140 mM-NaCl/6 mM-MgCl₂/20 mM-Tris/10 mM-Hepes, adjusted to pH 7.4 with HCl. All experiments were carried out at 37°C.

Spectrophotometric measurements. The spectral changes of safranine were measured in an Aminco DW2 spectrophotometer. To prevent cell sedimentation and anaerobiosis, the suspension was mixed slowly at defined time intervals (about 2 min). When difference spectra were measured, both cuvettes were mixed at the same time.

Determination of cellular ATP content. Cells were incubated in conditions identical with those described above with constant slow mixing. Thereafter aliquots were taken into ice-cold HClO₄ to give a final acid concentration of 2.4% (w/v), followed by extraction for 5 min and centrifugation in an Eppendorf 3412 bench centrifuge for 2 min. The supernatant was separated and the acid was neutralized with a mixture of 3 M-K₂CO₃ and 1 M-Tris base. After recentrifugation ATP was determined in the supernatant by the method of Williamson & Corkey (1962).

Determination of the ³⁶Cl⁻ distribution ratio and intracellular water. Incubation conditions were identical with those above except that the suspension contained either 2 μCi of [³H]inulin/ml and 0.5 μCi of ³⁶Cl⁻/ml (1.2 mM) or 2 μCi of ³H₂O/ml and 0.5 μCi of [¹⁴C]inulin/ml. The cells were separated from the outer medium by centrifugation through a 1:1 (v/v) mixture of silicone oil (Corning 550) and dinonylphthalate. The samples were subsequently extracted with 12% (w/v) HClO₄, neutralized with 2.7 M-Tris base and counted for radioactivity.

Measurements of safranine uptake. The incubation conditions were identical with those described above. Aliquots were taken at defined intervals and centrifuged through oil as described above. The safranine content was measured from the absorbance of the supernatant at 524 nm.

Materials

FCCP was kindly donated by Dr. P. G. Heytler, DuPont, Wilmington, DE, U.S.A.; valinomycin,

oligomycin and duroquinone were purchased from Sigma; safranine was from E. Merck AG, Darmstadt, Germany. All the other reagents used were commercial products of the highest grade. FCCP, valinomycin, oligomycin, duroquinone and rotenone were added during the experiments from concentrated stock solutions in ethanol, never allowing the ethanol concentration to rise above 0.1%. In control experiments this ethanol concentration had no significant effect on any of the parameters studied.

Results

Spectral changes of safranine upon addition of KCN and valinomycin to a hepatocyte suspension

A difference spectrum with a peak of absorbance at about 526 nm appears slowly upon addition of inhibitors of mitochondrial energy transduction (oligomycin in combination with KCN) to respiring hepatocytes suspended in the presence of safranine (Fig. 1a). This difference spectrum is very similar to the absolute spectrum of the dimeric form of safranine (Colonna *et al.*, 1973) i.e. the spectrum of the dye in aqueous solutions. The difference spectrum seen upon addition of KCN and oligomycin is also similar to the expected reversal of the spectrum that occurs upon induction of membrane potentials in isolated mitochondria (Åkerman & Wikström, 1976). The K⁺-specific cation conductor, valinomycin, causes a spectral change similar to that seen with KCN plus oligomycin (Fig. 1b). No spectral changes are observed in the absence of safranine under the present conditions, indicating that the changes occur as a response to an interaction of the dye with the cells.

Effects of valinomycin and FCCP on the time course of the spectral changes of safranine in the presence of hepatocytes

When the cell suspension is diluted into the experimental medium containing safranine, a slow linear decrease in the absorbance of the dye at the respective wavelength pair (524–484 nm) occurs, as indicated by the downwards deflection of the trace in Fig. 2. The signal is unaffected by the addition of glucose or by mixing (Fig. 2). The trace is also virtually identical whether glucose is added or not (results not shown). The lack of an effect of mixing indicates that the response is not a result of cell sedimentation or aggregation. Subsequent additions of valinomycin or a proton conductor, FCCP, result in a fast reversal of the signal (Fig. 2). The maximal change in absorbance, which occurs approx. 20–30 min after the addition of cells, is also shown in Fig. 2.

The ATP contents of cells incubated for 30 min under the conditions of Fig. 2 are of the order of

Fig. 1. De-energization difference spectrum of safranin upon addition of KCN or valinomycin to a respiring hepatocyte suspension

Both cuvettes contained 0.26×10^6 cells/ml, suspended in the basal medium (see the Experimental section) at 37°C and containing $20 \mu\text{M}$ -safranin and 5 mM -glucose. The baseline was drawn after 5 min of preincubation. In (a) after addition of 5 mM -KCN and $8 \mu\text{g}$ of oligomycin/ml the difference spectrum was recorded after 5 (1), 10 (2) and 20 (3) min. Thereafter no further change was observed. (b) As in (a), except that 80 ng of valinomycin/ml was added (4, 5, 6).

Fig. 2. Effect of valinomycin and FCCP on the spectral changes of safranin in the presence of isolated hepatocytes. Experimental conditions were as described in Fig. 1. The cuvette contained $20 \mu\text{M}$ -safranin in the basal experimental medium at 37°C . Observations were made at the wavelength pair $524\text{--}484 \text{ nm}$. (a) Addition of 0.24×10^6 cells/ml (Cells), 2 mM -glucose (Glc) and 80 ng of valinomycin/ml (Val) as indicated; (b) addition of cells, glucose, mixing of the cuvette (Mix and arrows) and $1 \mu\text{M}$ -FCCP as indicated. (c) Cells were preincubated at 37°C for 30 min in the presence of 2 mM -glucose, followed by addition of safranin and incubation for 2 min, and then the addition of $1 \mu\text{M}$ -FCCP as indicated. (d) As in (c), with a 10 min incubation in the presence of safranin and addition of FCCP as indicated. ΔA_{max} denotes the total absorbance change when no further change in the trace could be observed.

1.9–2.5 $\mu\text{mol/g}$ wet wt. At 3 min after subsequent addition of FCCP or valinomycin very little change occurs in the ATP content, whereas after 10 min it has decreased to 10–30% of its initial value.

The $^{36}\text{Cl}^-$ equilibrium potential across the plasma membrane (32–45 mV) is not significantly affected by either FCCP or valinomycin up to 10 min after their addition. It should be noted that, in the present conditions, only a depolarization of the plasma membrane would accurately be detected by the $^{36}\text{Cl}^-$ distribution ratio.

It is obvious from the traces in Figs. 2(a) and 2(b) that the slow appearance of the de-energization difference spectrum in Fig. 1 does not reflect a slow de-energization but the slowness of the appearance of the safranin response in the reference cuvette.

It is of importance to know whether the slowness of the response in Figs. 2(a) and 2(b) upon dilution of the cell suspension is due to a slow build-up of the mitochondrial membrane potential or whether it is due to a slow penetration of safranin into the cells. Figs. 2(c) and 2(d) demonstrate that, if the cells are

diluted and preincubated at 37°C for 30 min (the time needed for the maximal response to occur), the response to FCCP depends upon how long the cells have been preincubated in the presence of safranin. This result would suggest that the slow signal is due to slow penetration of safranin into, or slow redistribution of the probe within, the cell.

Effects of metabolic inhibitors on the spectral changes of safranin in the presence of hepatocytes

KCN, when added alone, does not significantly affect the appearance of the safranin response (Fig. 3). If oligomycin is subsequently added a complete reversal of the signal is observed (Fig. 3). The same is the case if oligomycin is added in combination with KCN. No immediate effects on cellular ATP contents or on the $^{36}\text{Cl}^-$ equilibrium potential can be observed by addition of KCN in combination with oligomycin under the conditions of Fig. 3.

In the presence of iodoacetate no change in the appearance of the safranin response can be detected (Fig. 4) except for a reversal of the signal after

Fig. 3. Effects of KCN and oligomycin on the spectral changes of safranin in the presence of isolated hepatocytes. Conditions were as described in Fig. 2. Cells ($0.3 \times 10^6/\text{ml}$) were present from the beginning. (a) Addition of 8 μg of oligomycin/ml (Oligo) and 5 mM-KCN as indicated; (b) addition of KCN and oligomycin (Oligo) as indicated.

Fig. 4. Effects of iodoacetate, KCN and oligomycin on the spectral changes of safranine in the presence of isolated hepatocytes

Conditions were as described in Fig. 2. Parts of the slow traces are replotted from similar ones in Figs. 2 and 3 (time scale reduced 5-fold). Conditions: cells ($0.3 \times 10^6/\text{ml}$) and 1 mM-iodoacetate were added before the experiment followed by (a) 5 mM-KCN (●) or 8 μg of oligomycin/ml (Oligo) and 5 mM-KCN (Δ) as indicated. (b) Effect of mixing of the cuvette (Mix and arrows). The trace represents a continuation of the trace with iodoacetate alone (O) continued approximately 3 min after the last point shown on the left.

prolonged incubation when anaerobiosis occurs. In this case a fast reappearance of the signal can be induced by mixing the cell suspension. When KCN is added in the presence of iodoacetate a slow reversal of the trace occurs after a lag period (Fig. 4). With some glucose-deprived preparations a similar response can be seen even in the absence of iodoacetate in the presence of glucose (results not shown). Note, however, that this preparation is the same as that in Fig. 3, with which preparation no reversal can be obtained with KCN alone (see Fig. 3). The effect of oligomycin is similar in the presence or absence of iodoacetate (Figs. 3 and 4).

Rotenone affects the safranine response in a similar way as does KCN (Fig. 5). However, a subsequent addition of oligomycin has no further effect on the trace. This is the case also in the presence of iodoacetate. On the other hand, if both iodoacetate and KF are present a slow reversal of the trace can be observed (Fig. 5) upon addition of rotenone in combination with oligomycin. Duroquinone causes fast reappearance of the safranine signal to the same level as in the presence of rotenone alone. A complete reversal of the trace is then obtained by a subsequent addition of antimycin A (Fig. 5).

Safranine uptake by hepatocytes

When safranine is added to a hepatocyte suspension preincubated at 37°C for 30 min there is a significant uptake of the dye as judged from the decrease in the safranine concentration of the outer medium (Fig. 6). This uptake levels off between 20 and 40 min after the addition of safranine and is completely abolished by FCCP.

Discussion

The safranine method has previously been used for demonstration of membrane potentials in liposomes (Åkerman & Saris, 1976) and bacterial vesicles (Schuldiner & Kaback, 1975) as well as for quantitative determination of membrane potentials in mitochondria (Åkerman & Wikström, 1976; Brand *et al.*, 1978) and reconstituted mitochondrial enzyme systems (Wielburski & Nelson, 1978). The present results indicate that this method might be useful also for the qualitative determination of changes in the mitochondrial membrane potential within hepatocytes. This is of special interest because of the wide use of hepatocytes in metabolic studies (Seglen, 1976) as well as in toxicology (Moldeus *et al.*, 1978).

Fig. 5. Effect of iodoacetate, KF, rotenone, oligomycin, duroquinone and antimycin A on the spectral changes of safranin in the presence of isolated hepatocytes

Conditions were as described in Fig. 2. Parts of slow traces were replotted from similar ones as in Fig. 2 and 3 (time scale reduced 5-fold are shown). Conditions: 0.4×10^6 cells/ml, 1 mM-iodoacetate (O) and 10 mM-KF (▲) or 4 mM-glucose alone with cells (●) added before experiment. Additions: 6 μ M-rotenone (Rot), 8 μ g of oligomycin/ml (Oligo), 40 mM-duroquinone (DQ) and 0.2 μ g of antimycin A (AA)/ml as indicated. Control traces with no additions are indicated (Con).

Fig. 6. Safranin uptake by hepatocytes
Cells (1.25×10^6 /ml) were incubated for 30 min at 37°C in the basal experimental medium containing 5 mM-glucose. At time zero 20 μ M-safranin was

Source and mechanism of the spectral changes of safranin

The evidence that the safranin responses are from intracellular mitochondria and not from the cell membrane comes from the finding that valinomycin causes a complete fast reversal of the spectral change, as is the case with Ehrlich ascites tumour cells (Åkerman, 1979) and isolated mitochondria with high external K^+ (Åkerman & Wikström, 1976). Outside the cells the K^+ concentration is low, whereas it is high inside the cells, and therefore a polarization rather than a depolarization of the plasma membrane would be expected upon addition of valinomycin. A response indicating a polarization of the plasma membrane is indeed seen with cyanine dyes in such conditions (Laris *et al.*, 1976). On the other hand, a depolarization of the mito-

added and the safranin concentration of the outer medium was measured as described in the Experimental section. ●, Control conditions; (○), 1 μ M-FCCP added at time zero.

chondrial membrane would be expected (cf. Mitchell, 1966). Valinomycin has been shown to depolarize intracellular mitochondria (Hoek *et al.*, 1980; Scott & Nicholls, 1980), which is in agreement with the above proposal. Also, the very fast responses to inhibitors and FCCP, which have no immediate effect on cellular ATP contents or the $^{36}\text{Cl}^-$ equilibrium potential, would suggest that the mitochondrial membrane potential is measured. The reason why safranin measures the mitochondrial membrane potential in conditions where cyanine dyes measure the potential of the plasma membrane is probably due to the high solubility of safranin in aqueous solutions as well as to its easy permeation through membranes (Åkerman & Saris, 1976). The cyanines, on the other hand, are mainly associated with the membrane phase, with less in the aqueous bulk phase (Sims *et al.*, 1974; Waggoner, 1976; Waggoner *et al.*, 1977). The safranin response is also linear at much higher membrane potentials (Åkerman & Wikström, 1976) than that of the cyanines, the responses of which saturate above 100 mV (Laris *et al.*, 1975; Burkhardt, 1977).

The slow appearance of the safranin signal upon cell dilution is presumably due to a slow penetration of safranin across the plasma membrane or slow redistribution of the dye within cells. The slow uptake of safranin by the hepatocytes (Fig. 6) is in agreement with this.

Effects of metabolic inhibitors on the safranin response

The results with the combinations of inhibitors would suggest that hepatocytes are able to maintain their mitochondrial $\Delta\psi$ both by anaerobic glycolysis (partial insensitivity to KCN) and by oxidative metabolism (partial insensitivity to oligomycin) in agreement with previous studies on synaptosomes (Scott & Nicholls, 1980) or Ehrlich ascites tumour cells (Åkerman, 1979). In the first case the mitochondrial $\Delta\psi$ would be kept up by hydrolysis of glycolytic ATP according to the phosphorylation potential (ΔG_p). It should be noted that $\Delta\psi$ is not a linear function of ΔG_p but changes very little with changes in ΔG_p within the physiological range of this potential (Nicholls, 1974; Davis *et al.*, 1980). Therefore no immediate effect of KCN on $\Delta\psi$ would be expected. When glycolysis is blocked with iodoacetate the cells seem to depend on oxidative metabolism alone, as expected (sensitivity to KCN alone). The results with rotenone are of interest since no effect of oligomycin can be obtained in the presence of iodoacetate. It is therefore likely that shunting of reducing equivalents at the cytochrome *b* level still occurs through the *sn*-glycerol-3-phosphate dehydrogenase (Bücher & Klingenberg, 1958; Estabrook & Sacktor, 1958) or in some other way (Kadenbach *et al.*, 1960; Conover, 1961; Dallner &

Ernster, 1962; Gordon *et al.*, 1967a). When also F^- is present a slow reversal of the trace is obtained upon the addition of rotenone and oligomycin plus duroquinone brings back the trace to the same level as in the presence of rotenone alone. It is indeed known that various quinones, when added to cell suspensions, are able to shunt reducing equivalents into the mitochondrial respiratory chain (Kadenbach *et al.*, 1960; Conover, 1961; Dallner & Ernster, 1962; Gordon *et al.*, 1967a).

Unfortunately it is impossible to tell with the present experimental approach the extent of decrease in $\Delta\psi$ caused by different inhibitors, because the quantitative relation of the safranin response to the membrane potential within the cells is unknown.

As compared with Ehrlich-ascites-tumour cells (Åkerman, 1979) there are some differences with respect to responses to inhibitors. In the absence of glucose the safranin signal in the presence of tumour cells is totally abolished by rotenone (Åkerman, 1979) and in the presence of glucose the response is almost totally abolished by dicyclohexylcarbodi-imide, an inhibitor of the mitochondrial ATPase. This dependency upon glycolysis is in agreement with the results of Demetrakopoulos *et al.* (1978), who have shown that various transformed cells, in contrast to differentiated ones, have a very low ATP content in the absence of glucose. Another point of unknown significance is that the de-energization difference spectrum of safranin has its peak at higher wavelengths (540 nm) in the presence of the tumour cells (Åkerman, 1979) than with hepatocytes (526 nm, see Fig. 1), the wavelengths corresponding to the monomeric and dimeric forms of safranin respectively (cf. Colonna *et al.*, 1973).

The results thus would suggest that safranin penetrates the plasma membrane of the hepatocytes and is distributed into the mitochondria within the cell. In the mitochondria aggregation of the dye molecules occur (Colonna *et al.*, 1973) as a response to the mitochondrial membrane potential (Åkerman & Wikström, 1976) as is the case with isolated mitochondria (Colonna *et al.*, 1973; Åkerman & Wikström, 1976).

It is concluded that the safranin method, previously used for determination of membrane potentials in isolated mitochondria and for qualitative measurements of the mitochondrial membrane potential within Ehrlich-ascites-tumour cells, also appears to be applicable for similar studies with isolated hepatocytes. As compared with the TPMP⁺ method (Hoek *et al.* 1980; Scott & Nicholls, 1980) of measuring the mitochondria $\Delta\psi$ within cells, safranin has the advantage of rapidity and continuous monitoring. Therefore it may be useful for preliminary observations and for detecting transient changes (e.g. as a response to hormones and

inhibitors). Furthermore this method is unaffected by changes in the plasma membrane potential, which is not the case with the TPMP⁺ method, which involves simultaneous measurements of the plasma membrane potential and TPMP⁺ distribution ratios as well as the volumes of the intracellular aqueous spaces. On the other hand, the TPMP⁺ method is necessary if a quantitative measure of $\Delta\psi$ is required.

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