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DIMETHYLAMINOSTYRYLMETHYLPYRIDINIUMIODINE (DASPMI) AS A FLUORESCENT PROBE FOR MITOCHONDRIA IN SITU

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SUMMARY

An investigation has been made on the properties of dimethylaminostyrylmethylpyridiniumiodine (DASPMI), its reaction with isolated pigeon heart mitochondria and its suitability as a vital stain for mitochondria in situ. DASPMI is a low toxicity specific vital stain for mitochondria in living cells. In vitro dye concentrations over 6 nmol/mg protein inhibit fast (state 3) respiration after a preincubation time of more than 5 min in the presence of substrate. No uncoupling was observed. Energization of pigeon heart mitochondria by addition of ATP or various substrates yields an average 8.5-fold increase in fluorescence intensity in relation to DASPMI-stained mitochondria that are under anoxia, substrate deficiency, or under the influence of respiratory inhibitors, or uncouplers. The alterations in fluorescence intensity are not primarily due to ion movements or pH changes. The amount of dye (2.96 ± 0.8 nmol) yielding maximal fluorescence response with 1 mg mitochondrial protein remains constant during energization of mitochondria.

As indicated by electron microscopic studies the observed changes in emission intensity may be related to changes in the fine structural organisation of cristae. A remarkable difference exists between isolated mitochondria and mitochondria in situ with respect to the reaction to cyanide. According to the reported results DASPMI will be a useful probe for the investigation of mitochondrial activities in living cells.

INTRODUCTION

During the last years much work has been dedicated to the study of biological membranes. Especially those of mitochondria were investigated by fluorescent probes (Ballard et al. [1], Bertina et al. [2], Kraayenhof [3], Vanderkooi et al. [4], Yaginuma et al. [5], Chang and Penefsky [6], Massari et al. [7]). The main advantage of this method lies in the information it yields relating to kinetics of changing membrane

Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; DASPMI, dimethylaminostyrylmethylpyridiniumiodine; DASPEI, dimethylaminostyrylethylpyridiniumiodine; MOPS, morpholinopropane sulfonic acid.

parameters at the molecular level without destroying normal function. In mitochondrial studies, this method is applied to isolated organelles or submitochondrial particles. In only a few cases were vitally stained cells examined. Chance and coworkers investigated living tissues by merocyanine-dye fluorescence. Absorbance changes of stained mitochondria or submitochondrial particles have been evaluated after janus green [8] or bromthymol blue treatment [9], but fluorescent methods have the advantage of being much more sensitive. Therefore, Kohen et al. [10–12] followed NADH-fluorescence in living cells after addition of substrate. Spatial resolution of the measurements was limited by sensitivity of the optical device (photon counting of the microscopical image) to about 20 μm^2. Differences of mitochondrial activities in various regions of a living cell cannot be accurately evaluated by this ingenious method. These may better be demonstrated by measurements of fluorescence, caused by irradiation of vitally stained organelles, because higher fluorescence intensities will be obtained. In addition, the use of artificial probes will provide further insight in the structure, charge, and ion distribution of membranes [13–15], it allows e.g. spectrophotometric measurements of intensity distributions in different cellular areas as well as a single organelle.

The goal of the experiments described here was to find a fluorescence probe suitable for the determination of metabolic activity in situ. About 30 different dyes have been tested with respect to their selectivity of staining mitochondria in situ. Their change of fluorescence as a function of the metabolic state of mitochondria has been studied. Of the dyes tested (including 1-anilinonaphthalene-8-sulfonate (ANS), atebrin, and ethidium bromide) only three exhibited sufficient specificity for mitochondria when applied to intact cells (either skin of teleost fish or endothelial cells from tadpole hearts in tissue culture; BHK 21-cells): rhodamine 6GO, 2-(*p*-dimethylaminostryryl)-1-methylpyridiniumiodine (DASPMI) and 2-(dimethylaminostyryl)-1-aethylpyridiniumiodine (DASPEI). Contrary to the observations of Haaker et al. [16] and Yaginuma et al. [5] on rhodamine 6G, rhodamine 6GO (differ only in commercial name) did not exhibit fluorescence response, when stained pigeon heart mitochondria changed their metabolic state. Both of the other dyes listed are chemically very similar and their properties as vital stains of mitochondria coincide. In most experiments DASPMI was used to determine whether it is suitable for evaluation of the metabolic state of isolated mitochondria and mitochondria in living cells.

MATERIALS AND METHODS

1. *Preparation of mitochondria*

Most experiments were done with isolated pigeon heart mitochondria, prepared according to the method of Chance and Hagihara [17]. Rat liver mitochondria have been used for a few experiments. They were prepared as for pigeon heart mitochondria but without nagarse treatment. Adult pigeons were decapitated, hearts were removed quickly and perfused with ice cold mannose/sucrose/EDTA-medium (0.22 M mannose, 0.075 M sucrose, 0.5 mM EDTA, pH 7.4). The tissue was minced and rinsed several times in mannose/sucrose/EDTA medium, treated 5 min with nagarse at 37 °C (pH held constant by the addition of tris buffer), homogenized and fractionated by differential centrifugation. After the final centrifugation step sedimented pigeon heart mitochondria were suspended in mannose/sucrose-medium to a final

protein concentration of 15–25 mg/ml. Protein concentration was determined by the Biuret method.

2. *Respiration measurements and fluorimetry*

Oxygen consumption was determined polarographically with a Clark type electrode: 10–100 μl mitochondria were suspended in 1.5 ml reaction medium (0.225 M mannose, 0.05 M sucrose, 20 mM morpholinopropane sulfonic acid (MOPS), 10 mM K_2HPO_4; pH 7.0). 10 μl substrate was added from a 1 M stock solution. 2 μM pentachlorophenol were used for uncoupling. All these reagents were of p.a. grade. Rotenone (about 90 %) and oligomycin (mixture of A and B) were obtained from Sigma. DASPMI was kindly provided by Dr. Sol Harrisson, who prepared the dye and proved the purity chromatographically. DASPEI was commercially obtained from Eastman, and used without further purification. A stock solution (0.5 g/ml) of the dyes was prepared for exact microsyringe-dosage. Fluorescence measurements were performed with a Hitachi fluorometer MPF 2A; at the emission side a Wratten No. 12 filter was inserted to diminish stray light effects.

3. *Determination of flurorescence intensity of mitochondria in living cells*

Intracellular mitochondrial activities were studied in cells of primary cultures of *Xenopus laevis* (Anura) tadpole hearts. These cells contain relatively few mitochondria, which can be clearly identified by phase contrast microscopy (Fig. 1) (Bereiter-Hahn and Morawe [18]).

The cultures from tadpole hearts were prepared in Rose chambers in a medium according to Wolf and Quimby (obtained from Gibco). Cells were stained by addition of 1.9 μM DASPMI. Two hours before beginning the measurements, the cultures

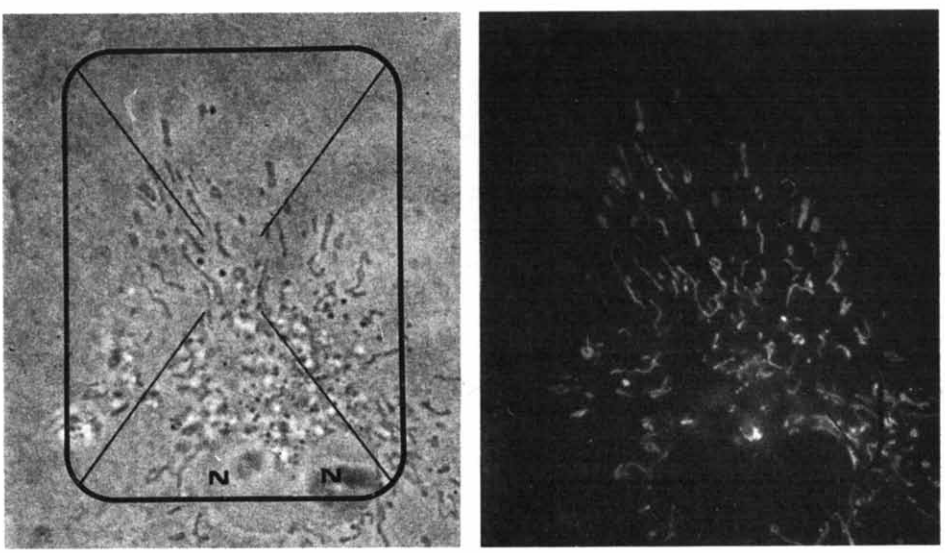

Fig. 1. Vitally stained mitochondria in a binucleate tadpole heart tissue culture cell (1.9 μM DASPMI). a, phase contrast; b, fluorescence microscopic image of the same cell, mitochondria exhibit marked fluorescence. (Excitation: HBO 100, filter BG 12, 3 mm; emission: filter K 520 nm;). N, nucleus.

were kept in balanced, glucose-free Hanks saline, brought up to the tonicity of the culture medium by addition of distilled water. The purpose of preincubation is to obtain defined substrate levels during the experiments. After these 2 h the medium was changed to Hanks saline supplemented with 8 mM sodium succinate. The action of cyanide and uncoupler were recorded 10 min after addition. Exposure of open culture chambers to a stream of highly purified nitrogen caused anoxia of the cells. The experiments were performed at room temperature 22–25 °C.

In living cells the fluorescence intensity was recorded by micrographs on Kodak recording film 35 mm (Fig. 1b). For analysis of the negatives a Quantimet 720 was used: the faintest fluorescence of mitochondria was indicated by the lowest grey level. The Quantimet detector was set to this level allowing the evaluation of the whole area covered by mitochondria. This area expressed in a number of picture points was set at "100". The data of the areas covered by structures of higher grey levels (corresponding to higher fluorescence intensities) were correlated to the ground level. Integration of all these "normalized" data reveals a measure of fluorescence intensity in area units × optical density of the film (= integrated density), which is independent of number and size of the observed mitochondria (corrected fluorescence intensity).

The determination of emission intensities by a photomultiplier would yield incorrect data because the extent of mitochondria covered area cannot be considered. The relative number of mitochondria per image field changes in the cells used.

RESULTS AND DISCUSSION

1. *Properties of DASPMI*

The dye is readily soluble in water and chloroform. The M_r is 351, its formula is given in Fig. 2. Maximal absorption wavelength of a 1 μM solution is 429 nm in water and 469 nm in chloroform, this means a difference of 40 nm due to solvent characteristics; maximal emission wavelengths (excitation at 467 nm): 557.5 nm in water, 555.0 nm in chloroform. Intensity of emission is 7.8 times as great in chloroform as in water. Neither absorption nor emission change occurs over a pH range from 4.0 to 8.5 (10 mM MOPS buffer).

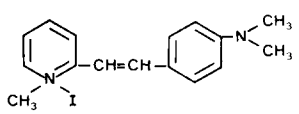

Fig. 2. Structural formula of 2(*p*-dimethylaminostyryl)-1-pyridinium-methiodine (DASPMI).

2. *Toxicity of DASPMI*

Whether or not a dye is suited for vital application depends upon its toxicity. The effect of DASPMI on the respiration of isolated pigeon heart mitochondria has thus been tested.

During an experimentation time of up to 20 min, 20 nmol DASPMI/mg protein do not influence state 4 respiration (β-hydroxy butyric acid, or malic and glutamic acid as substrates), whereas state 3 respiration is diminished in relation to incubation time of the dye in the presence of substrate (Fig. 3). The inhibition is independent of the amount of ADP (180–1080 μM) added. It depends on the dye concentration per

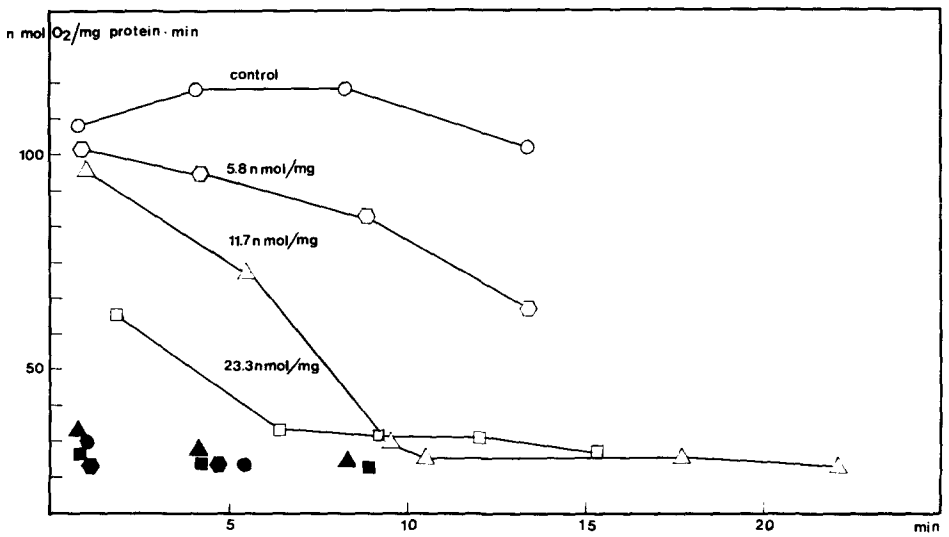

Fig. 3. Inhibition of respiration by DASPMI. Open signs, oxygen consumption in state 3 (280 μM ADP); filled signs, oxygen consumption in state 4. Dye concentrations for signs of the same shape as indicated. Time scale on abscissa indicates duration of DASPMI+substrate action; ordinate: oxygen consumption per mg protein · min^{-1}. Substrates: glutamate+ malate, 5 mM each, 0.3 mg mitochondrial protein (each point represents mean of three independent measurements).

mg mitochondrial protein, and the time after substrate addition. No inhibitory effect occurs when mitochondria are pretreated with DASPMI alone or with ATP. The dye does not uncouple (P/O ratios remain constant). Acceleration of respiration by 10 μM pentachlorophenol is obtained only in the range of the ADP response in the presence of DASPMI.

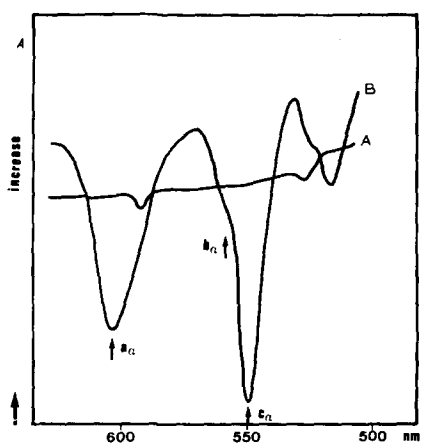

Fig. 4. Difference spectra of DASPMI-stained pigeon heart mitochondria. A, both cuvettes aerobic, 4.2 mg mitochondrial protein, 5 mM β-hydroxybutyric acid in 2 ml reaction medium. B, 8 min. after A: reference anaerobic, sample with 250 μM ADP. Oxidation of cytochromes is indicated by lack of absorbance in the regions of reduced cytochrome absorbance peaks. *A*, absorbance.

For experiments performed to clarify the site of DASPMI action, a dual beam spectrophotometer was used (Johnson Research Foundation). Mitochondria, in the following conditions, were compared with each other: (a) aerobic mitochondria with substrate (and ADP), reference without, sample with DASPMI; (b) DASPMI, substrate (and ADP) in both cuvettes, reference anaerobic, sample aerobic.

In the presence of DASPMI (8.5 nmol/mg protein) cytochromes *a*, *c* and *b* become oxidized on addition of ADP (Fig. 4), while the amount of NADH is enhanced, e.g. after a 3-min incubation of mitochondria in DASPMI 8.5 nmol/mg protein in the presence of substrate only 40 % to 50 % of the initial ADP induced oxidation is achieved. Flavoprotein is also oxidized. Therefore, the inhibitory effect is thought to be located at the site of NADH oxidation. This view is confirmed by undisturbed respiration in the presence of the dye, with succinate as substrate.

3. *Specificity of DASPMI as a vital stain for mitochondria*

Various tissue culture cells take up the dye from medium containing 1.9 μM DASPMI (or DASPEI) and incorporate it into their mitochondria. Fluorescence can be detected 5–10 min after addition of the probe to the culture. They dye is specifically accumulated in mitochondria (Fig. 1), as revealed by identification in the phase contrast microscope, and electron microscope. Stained cells remain morphologically unchanged for several days when kept in subdued light. When they have been intoxicated by prolonged (3 h) exposure to pentachlorophenol (10 μM) and substrate deficiency, cytosomes or vacuoles are also labeled.

4. *Reaction of DASPMI with isolated pigeon heart mitochondria*

Fluorimetry was performed with 1 to 8 nmol of the dye per mg protein (mean 2.6 nmol/mg); dye concentration in reaction medium: 1.2–5.7 μM (mean 2.4 μM). No pathological alterations have to be expected as the performance of the measurements normally did not exceed 6 min (compare Fig. 3).

TABLE I

CHANGES OF FLUORESCENCE INTENSITY OF DASPMI-STAINED PIGEON HEART MITOCHONDRIA

0, no effect; –, fluorescence intensity decreases to original level; *, fluorescence intensity decreases to an intermediate level.

Induction of fluorescence by:	Effect of:						
	Rotenone (3 μM)	KCN (1 mM)	Oligomycin (5 μM)	ADP	PCP (10 μM)	Potassium (1 μmol) and valinomycin (12 nM)	Anaerobiosis
Succinate (7.5 mM)	0	–	*	*	*	*	–
Malate+ glutamate (3.75 mM each)	–	–	0	*	*	*	–
ATP (0.4 mM)	0	0	–	0	–	*	

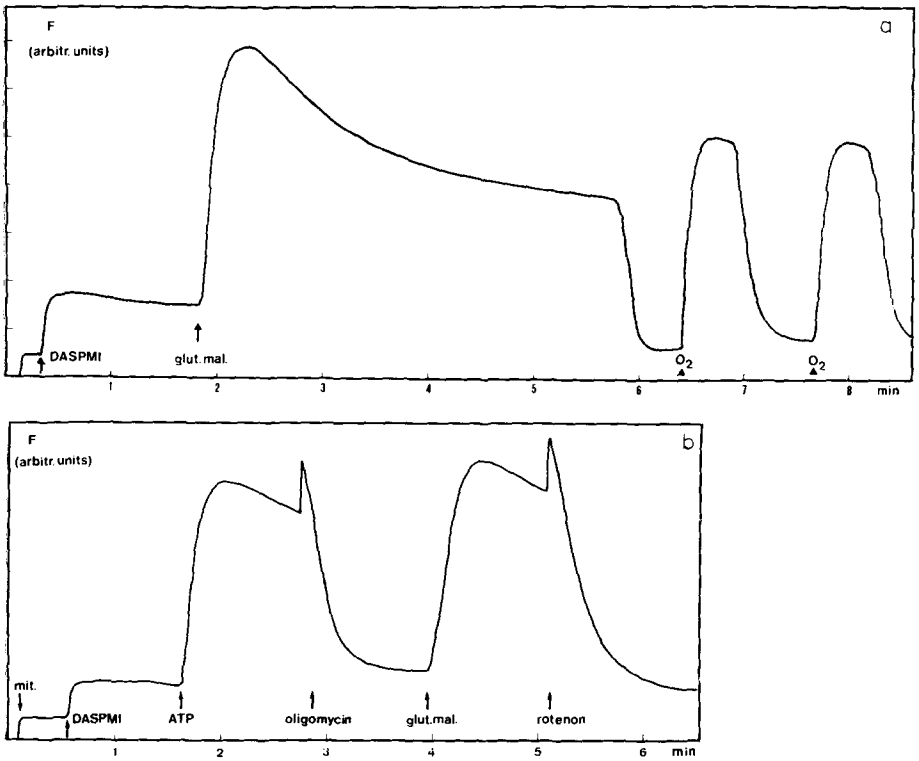

Fig. 5. Changes in fluorescence intensity (ordinate) in relation to the physiological state of pigeon heart mitochondria Excitation, 468 nm; emission, 550 nm. a, 1.5 ml reaction medium, 0.7 mg mitochondrial protein, 3.75 mM malate, 3.75 mM glutamate, "O_2" indicates addition of 5 ml air bubbled through the suspension by a fine needle. Spontaneous decrease of fluorescence after substrate induced enhancement is due to fading. b, 1.5 ml reaction medium, 1.15 mg mitochondrial protein, 400 μM ATP, 5 μM oligomycin, 3.75 mM malate, 3.75 mM glutamate, 3 μM rotenon, 4.8 μM DASPMI.

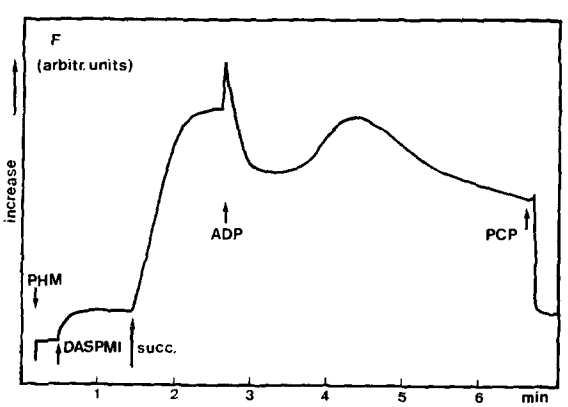

Fig. 6. Effect of ADP (267 μM) and PCP (2 μM) on succinate (7.5 mM)-induced fluorescence increase, 0.64 mg mitochondrial protein, 4.8 μM DASPMI.

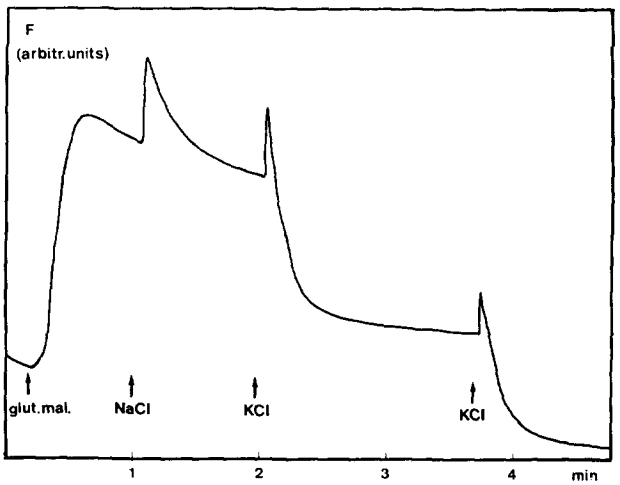

Fig. 7. Effect of sodium (5 μmol) and potassium (5 μmol each addition) on substrate (7.5 mM sodium succinate)-induced DASPMI (2.4 μM) fluorescence. Additions of substrate, NaCl and KCl indicated. Reaction medium contained Na_2HPO_4 instead of $K_2H_4PO_4$, 1.4 mg mitochondrial protein, 20 ng valinomycin. Excitation, 468 nm; emission, 550 nm.

Changes in fluorescence of DASPMI-stained pigeon heart mitochondria induced by addition of substrate or ATP are shown in Fig. 5. The range of intensity increase is about 8.6 times the fluorescence of mitochondria in the non-energized state, the maximum increase measured was $\times 12.5$. The wavelength of emitted light shifts from 547 nm (non-energized) to 554 nm (energized). The effects of various agents on the induction of fluorescence increase and its inhibition are listed in Table I. Addition of ADP (Fig. 6) diminishes fluorescence intensity (about 25 %) for a short time (until it is phosphorylated). Pentachlorophenol causes a decrease between 50 and 100 % of the substrate-induced enhancement for 15 s; when anaerobiosis is reached, reduction to the original ("non energized") level occurs.

CN^--induced fluorescence decrease clearly occurs more slowly than that observed after pentachlorophenol treatment or oxygen depletion; the de-energized level is reached between 60 and 120 s. Enhancement of fluorescence after addition of substrate depends, to a certain degree, on P_i concentration; to obtain maximal response at least 0.6 mM P_i are required. The fluorescence is not influenced by well permeating ions as thiocyanate or nitrate. Also, sodium or potassium have no effect on emission intensity. When potassium is added in the presence of valinomycin, a concentration-dependent fluorescence decrease is observed (Fig. 7).

The fluorescence intensity of EDTA-particles (kindly provided by Dr. C. P. Lee) corresponds to intensity of non-energized mitochondria. No significant response could be detected under conditions comparable to mitochondria treatment.

5. *Reaction of DASPMI with mitochondria in situ*

The in situ environment of mitochondria is never as clearly defined as a reaction medium of isolated mitochondria. Preincubation of tissue culture cells in glucose-free Hanks-saline should restrict cells on their internal energy sources, which are sufficient to keep mitochondria for more than 2 h in the energized state. Corrected

fluorescence intensity (see "Material and Methods") was assayed for each experimental condition in 5 different chambers. 10 image fields were analysed from each culture vessel. The lowest measurable emission intensity is about 60 units of integrated density, lower fluorescence intensities are set 50–60 units. This means very faint emission, too low to be recorded on photographic film.

Fluorescence intensity in aerobic cells in balanced saline reveals an 8- to 10-fold increase with respect to anaerobic cells (Fig. 8). Addition of external substrate induces no further increase in emission intensity indicating, that mitochondria are optimally supplied by intracellular sources. Insufficient permeation of succinate into the cells can be excluded: cultures treated with 10 μM pentachlorophenol for 3 h without substrate decrease their fluorescence intensity below the sensititvity limit of the method, while cells incubated in a succinate complemented medium remain on the level indicated in Fig. 8a.

Contrary to the reaction under anoxia, cyanide (KCN or NaCN 0.1–4 mM)

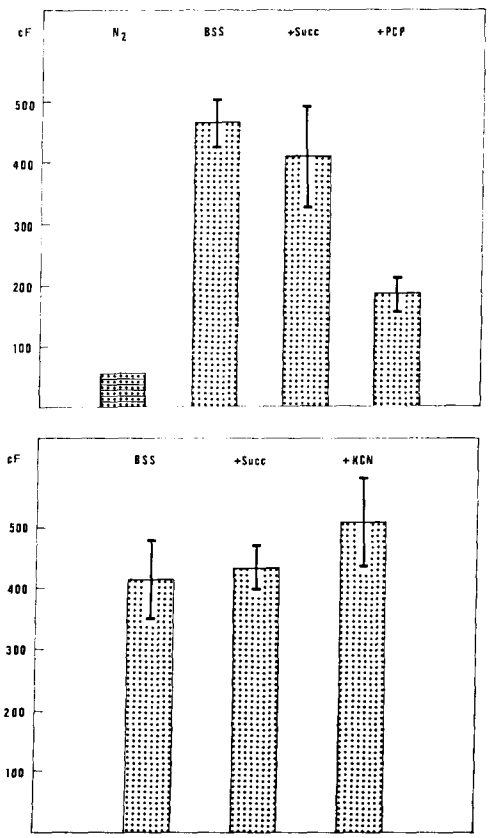

Fig. 8. Fluorescence intensity (cF, "corrected fluorescence", see Material and Methods) of DASPMI-stained mitochondria in living cells. 1.1 ml Hanks saline, 1.9 μM DASPMI. a, response to anoxia, substrate and pentachlorophenol. After 2 h treatment with oxygen-free N_2, emission intensity is beyond sensitivity limit of the method, therefore it is set "50" (see text). BSS, balanced salt solution (Hanks saline, glucose free); +Succ, BSS+10 mM succinate; +PCP, BSS+8 mM succinate+10 μM pentachlorphenol. Standard deviations indicated. b, response to cyanide (4 mM KCN).

slightly increases fluorescence intensity (Fig. 8b). This behaviour differs from the reaction of isolated mitochondria. Considering the occurrence of cyanide-insensitive respiration, especially of plant mitochondria [19–23] and of mitochondria of animal origin [24, 26, 27], heart mitochondria were isolated and tested from *Rana catesbiana*. Their respiration was fully sensitive. It seems highly probable that cyanide has an inhibitory effect on tadpole heart cells in tissue culture [18]. Due to the small amount of cells which are obtained by the primary culture technique used, direct measurements of oxygen consumption have not been successful.

6. *Bases for fluorescence changes of DASPMI*

Because DASPMI fluorescence is insensitive to pH variation NO_3^-, SCN^-, the responses listed in Table I may be due to dye transport in connection with mitochondrial ion translocations. Therefore, fluorescence of various amounts of energized and non-energized pigeon heart mitochondria were investigated. For both conditions a double reciprocal plot [6] of fluorescence intensity and mitochondrial protein content revealed a straight line (Fig. 9). Both curves intersect the abscissa at the same point, but differ in their slopes. This shows that the amount of mitochondrial protein, (absolute value of abscissa intercept; 1/0.6125 mg) required for half maximal fluorescence is not changed by a non-energized/energized transition. The increase of emission is due to an enhancement in quantum yield, as well as affinity of mitochondria to the dye. According to Fig. 11 3.56 nmol DASPMI give half maximal fluorescence response

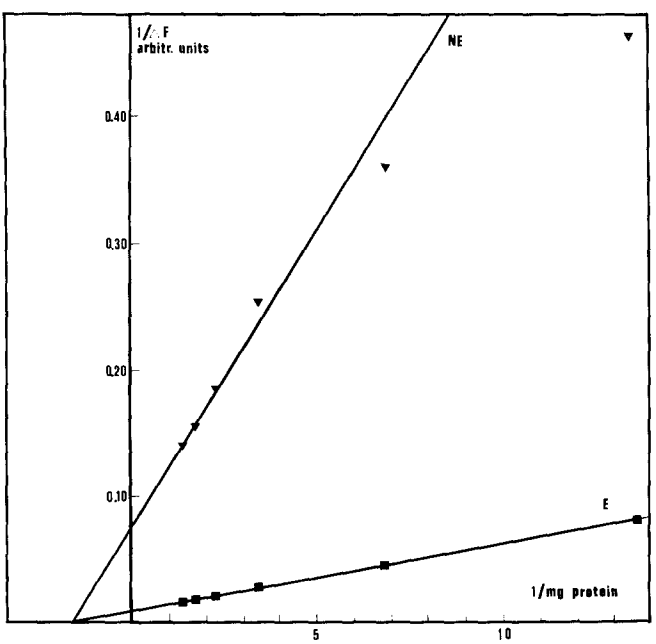

Fig. 9. Double reciprocal plot of fluorescence intensity of DASPMI (3.56 nmol) stained pigeon heart mitochondria and concentration of mitochondrial protein, in presence of 7.5 mM succinate as substrate (E) and without any substrate (NE). 468 → 550 nm, 3 μM rotenon. Low fluorescence intensities in curve NE are not determined as exactly as higher intensities, therefore these values deviate from the straight line.

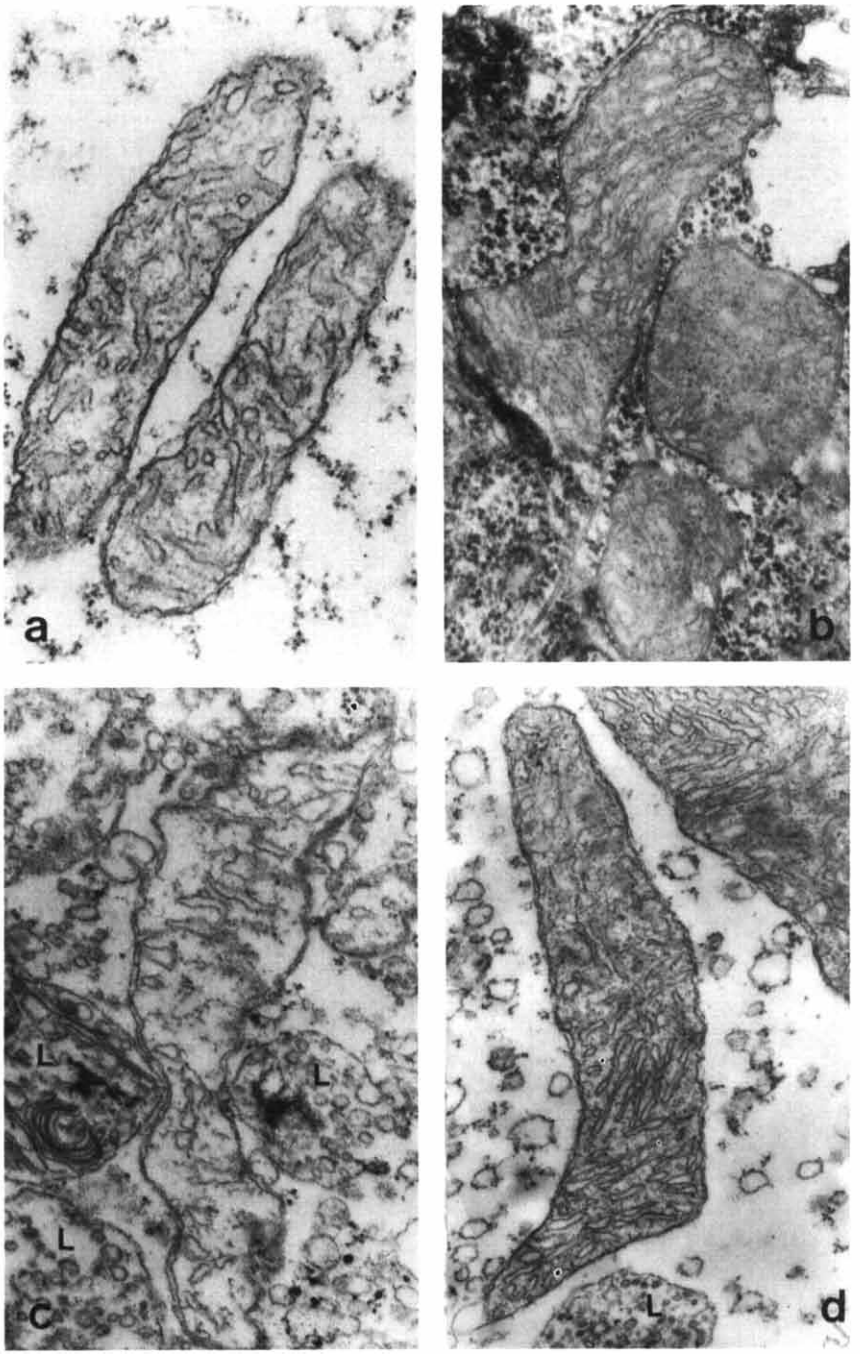

Fig. 10. Fine structural changes of DASPMI-stained mitochondria in tissue culture cells of Xenopus tadpole hearts. Cells preincubated 2 h in 1.9 μM DASPMI. Fixation, 1 % OsO_4, 0.65 % NaCl, 1.25 % $K_2Cr_2O_7$, adjusted to pH 7.2 by KOH. a, 5 mM succinate added to the saline; b, cells treated 2 h with oxygen-free nitrogen, normal culture medium: prefixation in 5 % glutardialdehyde in 0.1 M phosphate buffer, pH 7.2. Intermediate stages of the disappearance of the cristae. c, Mitochondria in cells treated with 5 μM pentochlorphenol for 10 min. Relative space obtained by cristae is diminished, several contacts to lysosomes (L), d, 8 min after addition of 4 mM KCN in Hanks saline, supplemented with 5 mM succinate; well developed cristae. Magnification, $\times$ 40 000 (a–c); $\times$ 35 000 (d).

when 0.6125 mg protein are added irrespective of their physiological state. Therefore, 1 mg mitochondrial protein causes maximal fluorescence by binding 2.96 ± 0.8 nmol DASPMI (mean of 3 titrations, each titration is the mean of 6 separate determinations for each protein concentration). The monophasic trace in fluorescence enhancement accompanying energization is in good accordance with this observation.

The ordinate intercept ratio in Fig. 11 is 1 : 8.6. Such an alteration in quantum yield (it can be concluded that at the ordinate intercept all dye is bound) would be expected for instance when an extreme change in the polarity of the environment of the dye molecules occurs, because similar differences are observed between water and chloroform solutions of the same DASPMI concentration. The time required for half maximal fluorescence response in isolated mitochondria, between 4 and 14 s depending on the inducing agent, is much longer than that for cytochrome oxidations in changing from anaerobic to aerobic conditions [9]. The rate, however, may be limited by transport of diffusion of the inducers into the mitochondria. A direct relationship between redox-state and fluorescence response cannot be established. A substrate-induced fluorescence rise lasts approximately half the time of gross changes in mitochondrial structure as indicated by swelling and shrinking (determined by nephelometry).

The preceding considerations lead to the conception that the response of DASPMI (and DASPEI) may indicate alterations at the molecular level accompanying transitions from non-energized to energized state. This was investigated for instance by Hoelzl-Wallach and Graham [27], who suggest that the change is manifested in fine-structural alterations of the inner mitochondrial compartment. Electron microscopical studies of tissue culture cells confirm this correlation qualitatively. Under normal aerobic conditions mitochondria exhibit a more or less vesicular aspect of their inner membrane (Fig. 10a) and an electron-lucent matrix (the "orthodox state", according to Hackenbrock [28]). Anaerobiosis induces either an electron dense matrix, and slighty enlargened intracristael spaces ("aggregated state") or a more diffuse inner compartment, and disappearance of cristae after prolonged anoxia (Fig. 10b). In mitochondria of pentochlorophenol-treated cells the cristae are clearly reduced; mitochondria often lie in the direct neighbourhood of multivesicular bodies or lysosomal vesicles (Fig. 10c). In cyanide treated cells mitochondria reveal well-developed cristae, less vesicular than in controls. In isolated organelles the fluorescence decrease on addition of cyanide is much slower than that after addition of uncoupler. In living cells no response could be detected.

These results confirm the view of a relationship between fine structure and DASPMI fluorescence intensity. This suggestion is in good accordance with the lack of fluorescence response in submitochondrial particles.

CONCLUSIONS

According to the experiments described here DASPMI exhibits a fluorescence behaviour and binding capacity similar to that of ethidium bromide [29], but it differs with respect to its charge response. The most important advantage of DASPMI is its high specificity as a vital stain for mitochondria as well as its low toxicity. DASPMI reactions of isolated mitochondria and in vivo coincide, but cyanide-treated material is an exception. The use of DASPMI as a probe for living mitochon-

dria in situ will be a valuable tool in studying metabolic activities of these organelles in their natural environment.

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