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KINETICS OF LUCIFER YELLOW CH EFFLUX IN GIANT MITOCHONDRIA

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The fluorescent dye Lucifer yellow CH was microinjected electrophoretically into giant mitochondria isolated from mice maintained on a diet containing cuprizone. The dye was retained by the mitochondria, indicating that it was contained in a space bounded by a selectively permeable membrane. The labelling was reversible by reversing the polarity of the current. A study of the disappearance of the fluorescence indicates that the permeability of the mitochondrial membrane to the dye (probably the lithium and/or the potassium salts) ranges from 10^{-7} to 10^{-8} cm/s.

Introduction

Lucifer yellow CH is a water-soluble dye which has been used in electrophysiology to indicate the location of the electrode tip and to trace cellular processes or connections between cells. This dye is highly fluorescent and crosses cell membranes very slowly [1].

We previously reported on the electrophoretic microinjection of Lucifer yellow CH in the interior of isolated giant mitochondria and mitoplasts [2]. The dye was microinjected with negative current, generally $1.4 \cdot 10^{-9}$ A, for 200-ms intervals for a total period of 1.5 min from micropipettes estimated to be approximately 0.2 μm in tip diameter which were filled with 27 or 654 mM lithium salt of the dye. The dye is retained by either mitochondria or mitoplasts, suggesting that the electrode was in the internal mitochondrial space.

The present communication reports that: (a) the Lucifer yellow labelling is reversed when the polarity of the current delivered from the micropipette is reversed, and (b) the kinetics of the dye efflux after removal of the micropipette are at least approximately exponential. The latter permits calculating permeability constants which range from 10^{-7} to 10^{-8} cm/s.

Materials and Methods

Treatment with cuprizone

Cuprizone (bis-cyclohexanone oxaldihydrazone), (G. Frederick Smith Chemical Co., Columbus, OH) was purified by crystallization. 20 g of cuprizone were slowly added to 1000 ml of rapidly boiling ethyl alcohol (95%). After two-thirds of the alcohol had evaporated, the solution was slowly cooled to about 4°C. The white needle-shaped crystals were collected on filter paper and washed with 1000 ml of ethanol (95%) at room temperature, followed by air drying on the filter for about 30 minutes. The crystals were stored in the dark in a desiccator at room temperature.

Mice, 17 days old, (BLU:Ha(ICR)) of either sex, which had not been weened, were obtained weekly from Blue Spruce Farms, Inc., Altamont,

Abbreviations: Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-hydroxyethanesulfonic acid.

NY. Purified cuprizone was added to ground standard rodent chow (3 g of cuprizone/500 g of food) followed by mixing in a Waring blender and divided equally among four or five cages. The prepared mixture was replaced every other day. Mice were kept (four per cage) in stainless steel cages and supplied exclusively with deionized water.

Isolation of mouse liver mitochondria

Consistently good preparations (i.e., mitochondria in the range of 5–10 μm in diameter) were obtained from livers of mice maintained on a cuprizone diet for at least 4 days. The mice were killed by cervical fracture, followed by rapid removal of the liver and discarding of the gall bladder. The pH of all solutions used was adjusted with Tris. The liver was homogenized in cold 0.30 M sucrose, pH 7.0, followed by centrifugation at $121 \times g$ for 80 s. The supernatant was layered on top of 5 ml of ice-cold 0.5 M sucrose (pH 7.0) and spun at $1100 \times g$ for 3 min. The supernatant of this centrifugation was then centrifuged at $3000 \times g$ for 5 min, followed by resuspension of the pellet in ice-cold 0.30 M sucrose/5 mM Hepes, pH 6.6. The centrifugations were carried out with a refrigerated Sorvall centrifuge, model RC2B with an HB-4 swinging rotor. Stock mitochondrial suspensions were kept on ice in the dark.

Preparation of slides

Glass coverslips (35 × 60 mm, Carolina Biological) which had been cleaned and coated with gelatin were attached to a frame [3], using a mixture (Vaseline/lanoline/paraffin, 1 : 1 : 1). The coverslips had been previously cleaned thoroughly with detergent and cleaning solution (chromic and sulfuric acid) and thoroughly rinsed with glass-distilled water. The dry glass slides were coated with gelatin (G-2625, Sigma Chemical Co., St. Louis, MO), by placing them upright in a 1% solution of the gelatin for at least 5 min at 70°C, followed by draining and drying upright in air for at least 1 h.

Generally, a few drops of the stock mitochondrial suspension were placed on the slide. At no time was the suspension in contact with the Vaseline/lanoline/paraffin.

Electronics

The dye was delivered using the direct current source of an electrometer (W-P Instruments, New Haven, CT, model KS-700). Generally, a negative $2 \cdot 10^{-9}$ A pulse with a duration of 0.5–2 s was used. The current was monitored by a separate amplifier, a current-to-voltage converter (model 3308/12C, Burr-Brown Research Corp., Tucson, AZ), equipped with a feedback resistor (10^{-7} Ω, Victoreen Inc., Sheller-Globe Corp., Cleveland, OH). The output of the current-to-voltage converter was connected to an oscilloscope (model 5111, Tektronix, Beaverton, OR) equipped with a differential amplifier, model 5A19N, from Tektronix.

Microelectrodes

Generally, microelectrodes were pulled using a Brown-Flaming micropipette puller (Sutter Instrument Co., San Francisco, CA) using borosilicate glass tubing with dimensions of either 1.0 mm outer × 0.58 mm inner diameter or 1.2 mm outer × 0.60 mm inner diameter and containing a microfilament. The microelectrode tips were estimated to be below 0.2 μm in diameter. In some experiments, originally intended for another study, double-barreled electrodes (theta glass, R & D Optical Systems, Inc., P.O. Box 198, Spenceville, MD) were used. One side was filled with Lucifer yellow and the other with 2 M KCl.

The tips of the microelectrodes were filled with the dye by capillarity and a silver wire coated with AgCl was used to deliver the current from the electrometer to the microelectrode. The resistances of the microelectrodes were approximately 200–500 mohm, when tested in 0.3 M sucrose/10 mM KCl/5 mM Hepes, with a positive 10^{-9} amp pulse for 1 s.

We found that it was necessary to replace the microelectrode frequently after a successful implaement.

Microscopy

Mitochondria were viewed with a Zeiss 'Opton' inverted microscope equipped for differential interference microscopy [4] using a 40 × oil immersion objective (N.A. 0.85) (Carl Zeiss, Inc., New York, NY).

The fluorescent dye experiments were performed with the above optics but with a Zeiss '50' barrier filter in place of the upper Wallaston prism

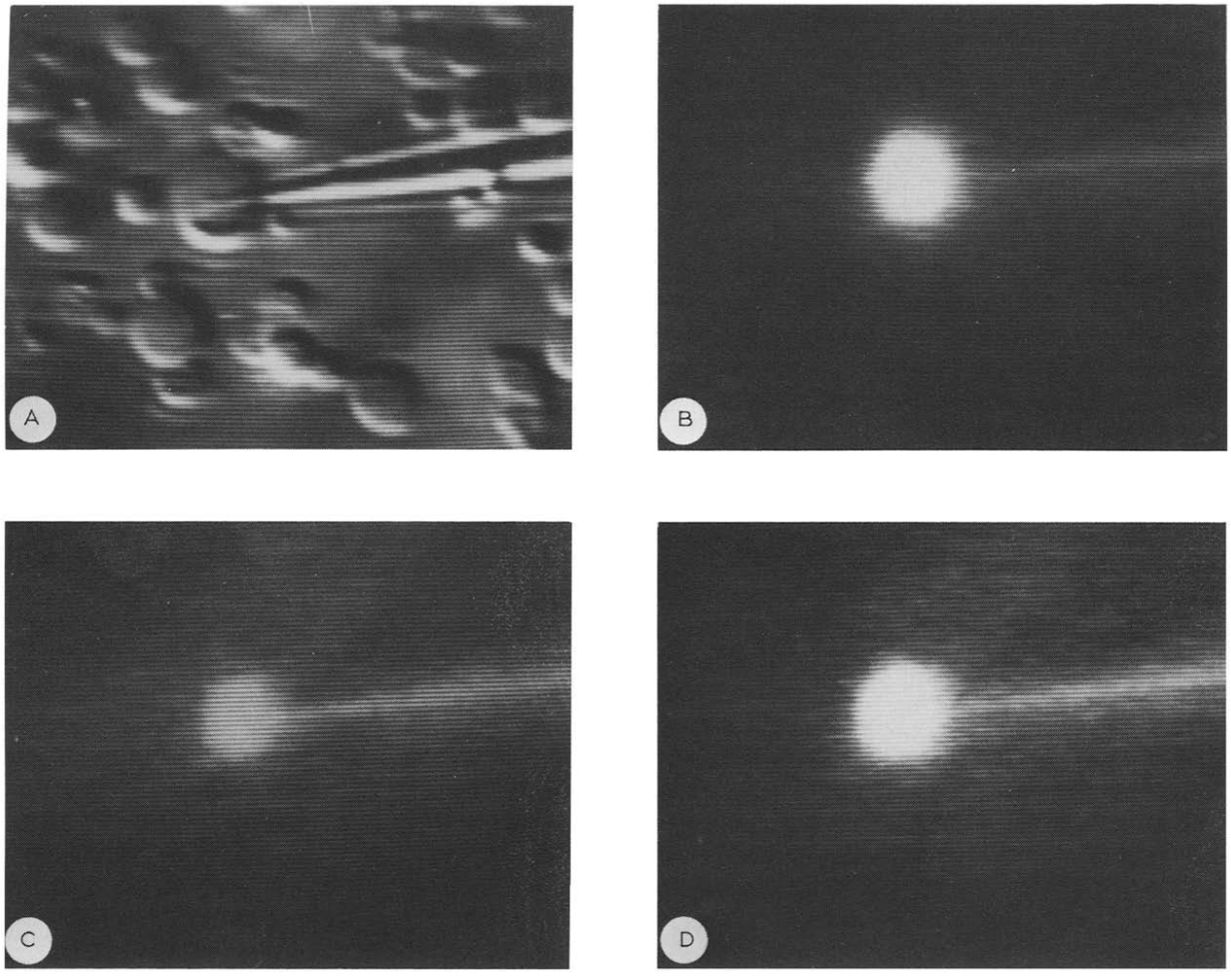

Fig. 1. The same mitochondrion is shown in Fig. 1A–D. A, Photograph of an image recorded on video tape using a television camera sensitive to low levels of light intensities. Mitochondrion impaled using microelectrodes made from theta glass (double barreled) and filled with 27 mM Lucifer yellow (CH) and seen with Nomarksi optics. Diameter of the mitochondrion, about 8 μm. B, Fluorescence optical image of the same mitochondrion after passage of negative current ($1 \cdot 10^{-9}$ amp for 1 s). C, Fluorescent optical image of a mitochondrion during the passage of positive current ($1 \cdot 10^{-9}$ amp for several seconds). The television camera was operated in the automatic mode so that the camera made an attempt to compensate for the decrease in the brightness of the image. D, Fluorescence image after the positive current was turned off.

and a Zeiss exciter filter (BG 12) under the field stop.

Fluorescent dye

Lucifer yellow (CH) (dilithium-6-amino-2-(hydrazinocarbonylamino)-2,3-dihydro-1,3-dioxo-1H-benz(de)isoquinoline-5,8-disulfonate) was supplied through the generosity of Walter Stewart of NIH (see Ref. 1).

Microspectrophotometer

For some experiments the decay in fluorescent intensity was followed with time using a microspectrophotometer. This instrument was loaned through the kindness of Felix Brogna, Farrand Optical Company, Valhalla, NY. Typically, the aperture was adjusted so that the measurement corresponded to the fluorescence in a circular spot on the center of the mitochondria, 2.5 μm in diameter.

Television camera

The fluorescence was viewed using a model 65, Mark II television camera sensitive to low intensities of light (Dage-MTI, Inc., Michigan City, IN) operated in the automatic mode, which maximizes the contrast. Images were recorded on 0.5 inch-wide video tape (Scotch, 3M, 3M Center, Saint Paul, MN). The images were photographed from the television screen with a Polaroid Camera with Polaroid film type 107.

Results and Discussion

Reversibility of Lucifer yellow distribution

The interpretation of the events which follow the injection of the dye require verification that the latter remains in solution inside the mitochondria. We have already shown that when a labeled mitochondrion is re-impaled with a conventional KCl-filled microelectrode, delivery of negative current 'chases' the label [2]. Since negative current delivers the anionic dye into the mitochondria, positive current should remove it and, in fact, we have been able to remove the dye simply by reversing the polarity of the dye-delivering electrode. These results are shown in Fig. 1A–D. The results shown in Figs. 1 and 2 are photographs of images recorded on video tape. An impaled mitochondrion, about 8 μm in diameter, shown in Nomarksi image in Fig. 1A, was filled with Lucifer yellow by passing a pulse of negative

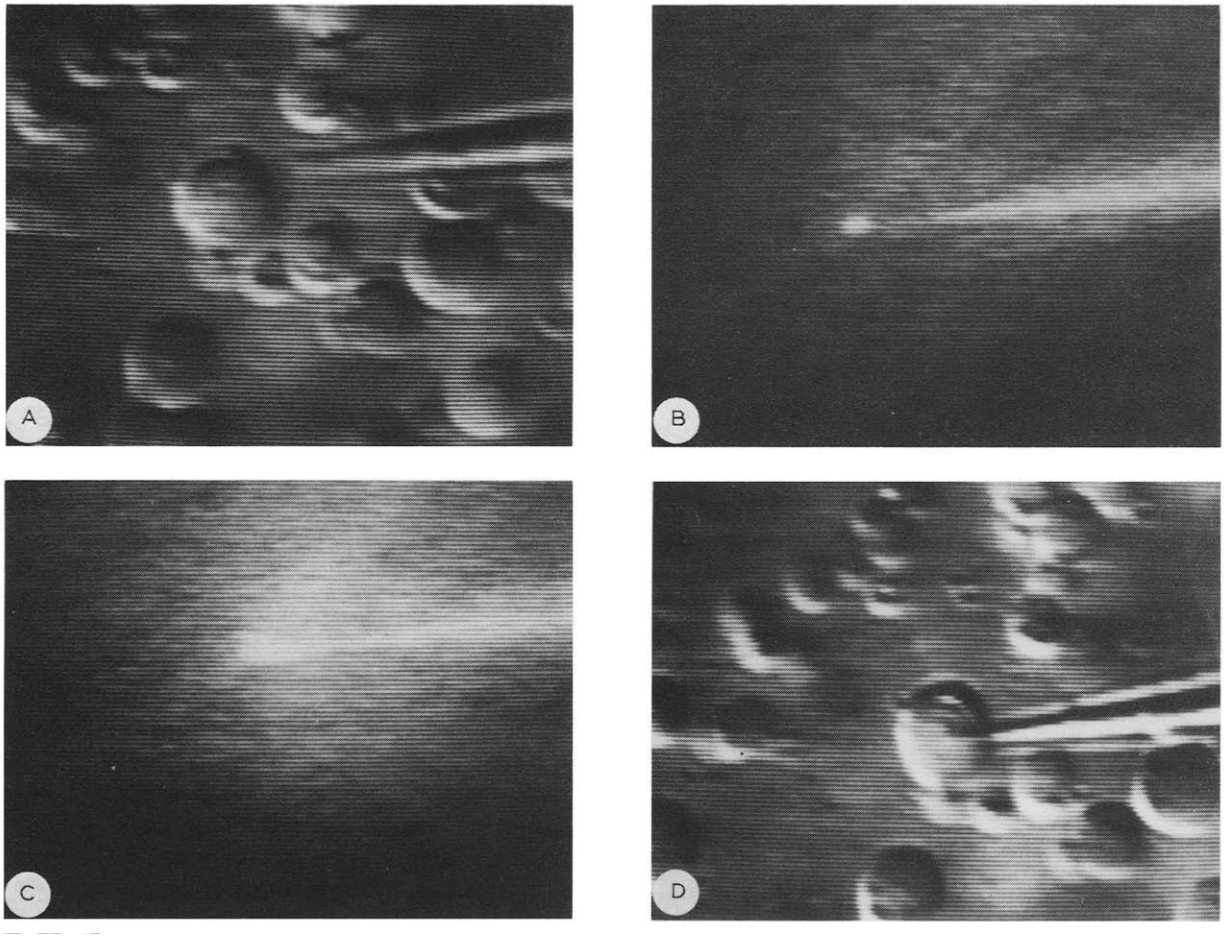

Fig. 2. Impalement failure. The same mitochondrion is shown in Fig. 2A–D. A, Nomarksi image before impalement with a microelectrode made from theta (double barreled) glass. Diameter of the mitochondrion, about 8 μm. B, Fluorescence image of a small bubble at the tip of the microelectrode. C, Fluorescence image taken during the passage of negative current ($1 \cdot 10^{-9}$ amp for several seconds). D, Nomarski image taken after cessation of negative current.

current (Fig. 1B) and essentially emptied of dye by the injection of positive current (Fig. 1C). Presumably, other anions are also removed by the positive current. The automatic adjustment of the video camera maximized the contrast so that the difference in contrast between Fig. 1B and C is much less than the actual fluorescence change. When the current was turned off, the dye moved down the microelectrode into the mitochondrion (Fig. 1D). The procedure did not alter the appearance of the mitochondrion (data not shown).

35% of the mitochondria failed to fill with the dye. This proportion coincides with the number of impalements considered unsuccessful in previous studies (see, e.g., Ref. 5 and 6). An example is shown in Fig. 2. Fig. 2A shows the video image of a mitochondrion viewed with Nomarksi optics. Fig. 2B shows the same mitochondrion after implaement and using fluorescence optics. A small fluorescent bubble is visible, as if the tip of the microelectrode was embedded in a pocket formed by the membrane. When tested the fluorescence was removed by positive current (not shown). In this case, the passage of negative current resulted in the transient appearance of fluorescence in the external solution adjacent to the mitochondria (Fig. 2C), supporting the idea that the tip resided in a pocket formed by the membrane. A Nomarksi photograph of the mitochondrion (Fig. 2D), taken a few seconds after Fig. 2C, shows that no obvious change in appearance of the mitochondrion had taken place (compare Fig. 2A and D). We interpret these results to represent an impalement failure.

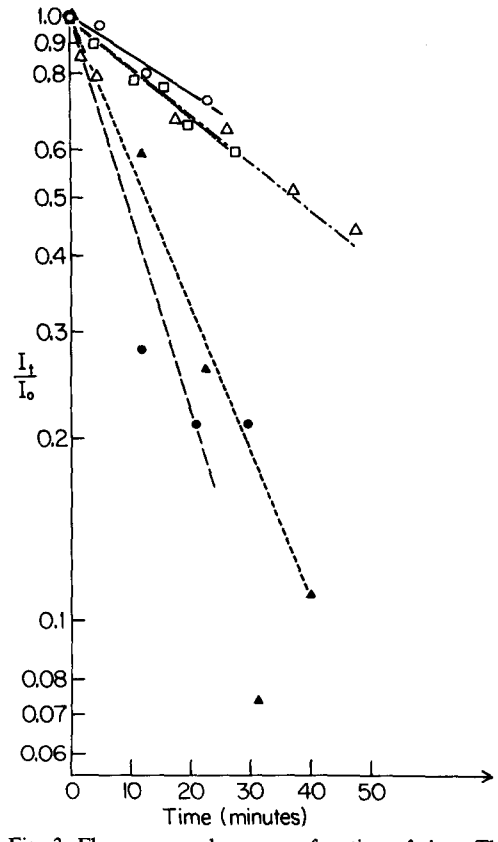

Fig. 3. Fluorescence decay as a function of time. The experiments were carried out with a microspectrophotometer. See Materials and Methods.

The appearance of the small fluorescent pocket may also occur in cases in which the mitochondrion eventually becomes labeled. The mitochondrion shown in Fig. 1 also showed a small bubble after impalement (data not shown), but the passage of negative current resulted in the complete labeling of the mitochondrion. However, in three other experiments no current was needed.

A total of 46 mitochondria were treated in this manner; out of these, 30 mitochondria became labelled whereas 16 did not.

Kinetics of the fluorescence decay

After the injection of the dye and withdrawal of the electrode, the intensity of the fluorescence was found to decrease with time. The fluorescence of

TABLE I

PERMEABILITY COEFFICIENTS CALCULATED FROM MEASUREMENTS OF FLUORESCENT DECAY USING THE LEAST-SQUARES METHOD

Medium: 0.3 M sucrose/10 mM KCl/5 mM Hepes (pH 6.8). Temperature, approx. 23°C. Each calculated permeability coefficient represents the results using at least four determinations of the decay in fluorescent intensity. The mitochondria were illuminated only during the time necessary for the measurement. The permeability constants were calculated from the experiments (Fig. 3).

Time observed (min)	Diameter (μm)	Permeability coefficient (cm/s)
47.5	6	$2.5 \cdot 10^{-8}$
23	7	$3.0 \cdot 10^{-9}$
27.5	7	$3.7 \cdot 10^{-8}$
40	6	$9.4 \cdot 10^{-8}$
29.5	6	$8.8 \cdot 10^{-8}$

each individual mitochondrion was measured directly using a microspectrophotometer (see Materials and Methods). The kinetics of the fluorescence decay are shown in Fig. 3. Each symbol represents a separate experiment. The decay appears to be at least approximately exponential and was interpreted to correspond to the diffusional efflux of the dye.

Permeability constants were calculated by a least-square analysis of the experimental points assuming that the mitochondria are perfect spheres, using the actual measured diameter for each calculation but without taking into consideration the convolutions of the inner membrane. For this reason, these values can only be considered estimates at the upper limit of the permeability constants. In our opinion, based on currently published electron micrographs [7], the surface area used in our calculation might have been underestimated by as much as a factor of 4. The permeability coefficients are summarized in Table I. The exposure to light may cause some bleaching. However, in these experiments, the exposure to light was for relatively short periods. Exposure to continuous light shows that the loss for the 1–1.5 min exposures would involve as much as 30% of the fluorescence for the duration of the experiment. This loss would not significantly affect our estimates of the permeability coefficients.

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