Biochem. J (1989) 257, 131–142 (Printed in Great Britain)

Calcium and proton activities in rat cardiac mitochondria

Effect of matrix environment on behaviour of fluorescent probes

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The ionic composition of the mitochondrial matrix, under both physiological and pathophysiological conditions, remains controversial. Although fura-2 and 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF), fluorescent probes for $[Ca^{2+}]$ and $[H^+]$ respectively, have successfully been loaded into mitochondria [Lukács & Kapus (1987) Biochem. J. **248**, 609–613; Davis, Altschuld, Jung & Brierley (1987) Biochem. Biophys. Res. Commun. **49**, 40–45], the adaptation of fluorescence-ratio spectroscopy to the study of the matrix ion content poses unique problems. In this report, we describe a method for successfully attaching viable rat cardiac mitochondria to glass coverslips, allowing continuous superfusion of isolated organelles during fluorescence microscopy. This technique obviated the need to correct for the accumulation of ion-sensitive and -insensitive fluorescent species of dye both within the matrix and outside of mitochondria in suspension in a cuvette, a particular problem with fura-2. By using this technique for superfusion of immobilized mitochondria, we found the pK_a of BCECF for H^+ at 25 °C shifted from 6.8 in buffer to 7.2 in rat cardiac mitochondria, with a marked hysteresis effect noted for intramitochondrial BCECF calibration curves. At higher pH, photobleaching of BCECF was enhanced. The dissociation constant (K_d) of fura-2 for Ca^{2+} was found to be 315 nM at 25 °C, pH 8.0, but only at $[Ca^{2+}]$ below 1 μM. At matrix $[Ca^{2+}] > 1$ μM, the K_d shifted into the micromolar range, an effect that appeared to be pH-dependent. Importantly, the matrix $[Ca^{2+}]$ was determined to be between 10 and 100 nM at perfusion buffer $[Ca^{2+}]$ below 500 nM, but rose rapidly at the higher extramitochondrial $[Ca^{2+}]$ reported to occur in ischaemic cardiac myocytes. Importantly, mitochondrial transmembrane H^+ and Ca^{2+} gradients both appeared to be maximal at perfusion buffer $[H^+]$ and $[Ca^{2+}]$ that approximate those of the cytosol of many resting cells.

INTRODUCTION

The composition of the ionic milieu within the mitochondrial matrix remains a controversial issue. As ion-flux rates across the inner mitochondrial membrane are largely dependent upon the maintenance of the transmembrane H^+ gradient by the electron-transport chain, continuous monitoring of matrix pH could provide important insights into homoeostatic mechanisms that determine mitochondrial-matrix ion content. Changes in mitochondrial-matrix free $[Ca^{2+}]$ have also been directly implicated in the regulation of the activity of several matrix dehydrogenases [1] and membrane-associated phospholipases A_2 [2]. However, the quantification of matrix $[Ca^{2+}]$ in isolated mammalian mitochondria remains problematic, as shown by the fact that recent estimates of intramitochondrial $[Ca^{2+}]$ in normally respiring organelles range from 30 nM [3] to 1 mM [4].

In addition to establishing the matrix $[Ca^{2+}]$ at extramitochondrial $[Ca^{2+}]$ that approximate to those of the cytosol in most cells, determination of the Ca^{2+} gradient across the mitochondrial inner membrane over a range of extramitochondrial $[Ca^{2+}]$ is of equal importance. This is due to the role that mitochondria may play in maintaining cellular Ca^{2+} homoeostasis in response to normal physiological stimuli [5–7], as well as during certain pathophysiological conditions, including ischaemia with or without reperfusion [8–10].

The introduction of new fluorescent dyes with high quantum yields and high selectivity for specific ion species has allowed quantification of cytosolic $[Ca^{2+}]$ and $[H^+]$ both in suspensions of cells and in single-cell preparations [11–13]. The availability of lipophilic esters of these dyes has provided a means by which dye can be rapidly loaded into cells with minimal effect on cell viability or transmembrane ion gradients. However, initial observations with the Ca^{2+}-selective fluorescent dye, quin2, suggested that free quin2, de-esterified by intracellular esterases, did not accumulate within intracellular organelles [14].

In contrast, there is now evidence that fura-2, a more recent fluorescent Ca^{2+} chelator, does enter certain intracellular compartments, including spindle poles of mitotically dividing cells [15], endocytotic vesicles [11] and mitochondria in intact cells [16]. There are recent reports, as well, of the successful loading of isolated mitochondria in suspension with either fura-2 [3,17–19] or, to a limited extent, quin2 [19]. Furthermore, the fluorescent H^+-sensitive probe, 2',7'-bis-2(carboxyethyl)-5(6)-carboxyfluorescein (BCECF), has been introduced into mitochondria following de-esterification of

Abbreviations used: BCECF, 2',7'-bis-2(carboxyethyl)-5(6)-carboxyfluorescein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; AM, acetoxymethyl ester; ex., excitation.

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the precursor, BCECF/AM (acetoxymethyl ester), by intraorganellar esterases [18].

In the case of both fura-2 and BCECF, changes in the fluorescence signal due to swelling of mitochondria or loss of dye due to photobleaching or leakage from the organelle should be accounted for if spectroscopic-ratio techniques are used. However, quantification of either matrix $[Ca^{2+}]$ or $[H^+]$ using these fluorescent dyes in suspensions of isolated mitochondria is made more complex by the presence of fluorescent, partially hydrolysed species of dye, as well as free, ion-sensitive fluorescent dye [20]. This becomes particularly complicated when several species of dye accumulate, both within the mitochondrial matrix and in the extramitochondrial space, due to leakage of dye from organelles. Calibration of a suspension of mitochondria, using either detergents or ionophores at the end of each experiment, presupposes that the fluorescence from extramitochondrial species of dye is either minimal or that their accumulation outside the organelle has been linear over time [21]. Neither assumption may be true for any given preparation. Ideally, continuous perfusion of the suspension medium would obviate the need to correct for extraorganellar accumulation of dye, but this poses obvious difficulties within a cuvette.

The application of fluorescence spectroscopy to research on mitochondrial function poses other problems that are peculiar to this organelle. Although no effect of pH on the affinity of fura-2 for Ca^{2+} has been described over the range of pH normally found in the cytosol [22], no thorough evaluation of the dye's ion affinities or spectral properties has been carried out at the higher pH ($\sim$ pH 8) present in normally respiring mitochondria. Similarly, the H^+-binding affinity of BCECF could change over the range of $[Ca^{2+}]$ thought to exist within the mitochondrial matrix. Another potential problem unique to mitochondria is the high ratio of membrane surface area to matrix volume compared with most cells. The possibility that less polar forms of a dye, complexed with their appropriate cation, might partition into membranes could affect accurate determination of matrix-ion composition.

Here the advantages and limitations of use of fura-2 and BCECF in isolated mitochondria are examined and a technique for immobilizing mitochondria with preservation of viability is described that also allows constant superfusion of these organelles on the stage of a fluorescence microscope. Values for intramitochondrial $[Ca^{2+}]$ were determined to be in the low nanomolar range (10–100 nM) in rat cardiac mitochondria isolated using standard techniques, and remain low even at extramitochondrial perfuson buffer $[Ca^{2+}]$ as high as 500 nM.

EXPERIMENTAL

Mitochondrial preparation

Heart and liver mitochondria were prepared from male Sprague–Dawley rats weighing approx. 225 g. All animals had been provided with chow and water *ad libitum* before they were killed. Heart mitochondria were prepared according to the method of Palmer *et al.* [23], with the following modifications: the initial homogenizations were performed using a Teflon pestle in a glass homogenization vessel, and all isolation procedures were carried out at pH 7.5 at 4 °C with 2 mM-EGTA. Mitochondria isolated following Nagarase (subtilopeptidase A; Sigma, St. Louis, MO, U.S.A.) treatment were used ('interfibrillar' mitochondria). Liver mitochondria were prepared as described by Broekemeier *et al.* [24], with the exception that 3 mM-Hepes/Na^+ was replaced by 3 mM-Mops/K^+. The initial homogenization of both heart and liver mitochondria was carried out at 4 °C in mannitol/sucrose buffer (Buffer I), containing 210 mM-sucrose/70 mM-mannitol/3 mM-Mops/K^+, pH 7.5 (heart) or pH 7.2 (liver). Sucrose and mannitol used in the preparation buffers were routinely freed of Ca^{2+} by passing them through an ion-exchange resin (Amberlite, MB-3). The final free $[Ca^{2+}]$ in each buffer, as determined by an ion-sensitive electrode, was 1 μM before the addition of Ca^{2+} chelators. A second buffer (Buffer II), used for final washes during the isolation of mitochondria from each tissue, contained 110 mM-KCl/20 mM-Mops/K^+, pH 7.5, and sufficient sucrose/mannitol (3:1) to adjust the osmolality to 295 mosM. Additional reagents were added as indicated below.

Preparation of Ca^{2+} buffers

Buffers used in the incubation and perfusion of isolated mitochondria of known $[Ca^{2+}]$ were prepared using EGTA-buffered solutions. The purity of the EGTA was determined to be 98.5% using the procedure of Bers *et al.* [25]. Ca^{2+} standard solutions were prepared with 1 mM-EGTA in Buffer II by adding appropriate amounts of a 100 mM-$CaCl_2$ standard solution. The free $[Ca^{2+}]$ was calculated according to the method of Fabiato & Fabiato [26], except that Ca^{2+}-, Mg^{2+}- and H^+-binding constants for EGTA were taken from Grynkiewicz *et al.* [22]. The free $[Ca^{2+}]$, when > 300 nM, was verified with a Ca^{2+}-sensitive electrode (Model 93-20; Orion Research, Boston, MA, U.S.A.) and a reference electrode (Model 90-01; Orion Research) containing 4 M-KCl and saturated AgCl. For loading mitochondria with fluorescent dyes, an incubation buffer was used consisting of Buffer II supplemented with 10 mM-potassium succinate, 1 mM-$MgCl_2$, 1 mM-potassium phosphate, 1 mM-EGTA/K^+, 1 μM-rotenone in ethanol and 1 mM-ATP (K^+). The perfusion buffer was similar to the incubation buffer, except that the ATP concentration was lowered to 0.1 mM. The pH values of Ca^{2+} buffers were adjusted with 10 M-KOH or 6 M-HCl as necessary.

Loading mitochondria with fluorescent dyes

Following the final wash at the end of the isolation procedure, mitochondria were resuspended in Buffer II at a protein concentration of 10 mg/ml at 4 °C [mitochondrial protein content was determined using the Biuret technique with 1.0% (w/v) deoxycholate] [24]. For loading of fluorescent dyes in to mitochondria, these organelles were resuspended at 6 mg/ml in incubation buffer. Either 5 μM-BCECF/AM or 5 μM-fura-2/AM were then added in dimethyl sulphoxide. The final dimethyl sulphoxide and ethanol concentrations in the incubation mixture did not exceed 0.5% (v/v). This is because, at a mitochondrial protein concentration of 1 mg/ml, some loss of respiratory control could be observed at concentrations of either vehicle above 1.0% (v/v). The final volume of this mitochondrial suspension was 250 μl. The organelles were allowed to load with dye for 5 min at 25 °C in a 1.5 ml Eppendorf microcentrifuge tube, followed by transfer of an aliquot ($\sim$ 50 μl) to a coated coverslip (see below). The mitochondria continued to load and hydrolyse the dye for a further 25 min at

25 °C before the suspension of mitochondria was diluted by washing the coverslip with 2 ml of incubation buffer. For fluorescence measurements made in a cuvette, the mitochondria were allowed to incubate at 25 °C for 30 min, followed by a 30 s centrifugation in a microcentrifuge and one wash in incubation buffer, with resuspension in that buffer at 6 mg of protein/ml. The final protein concentration in the cuvette was 0.5 mg/ml. Viable dye-loaded mitochondria could be kept at 4 °C either in suspension or attached to coverslips for up to 4–6 h at 4 °C.

Superfusion of immobilized mitochondria

To eliminate the effect of accumulation of hydrolysed and partially hydrolysed fluorescent dyes in the extramitochondrial space, as well as to allow rapid exchange of buffers with differing composition, a continuous perfusion technique was developed using isolated mitochondria immobilized on a glass coverslip. An adhesive polyphenolic protein (5 μg) derived from the marine mussel *Mytilus edulis* (CellTak; Biopolymers, Farmington, CT, U.S.A.) was spread evenly on a glass coverslip (12 mm in diameter, 1 mm thick). After drying in air, the coverslip was washed once with ethanol and then twice with distilled water, and then thoroughly dried before use. BCECF- or fura-2-loaded mitochondria, suspended in 50 μl of incubation buffer, were placed on the coated coverslip and attachment was allowed to occur for 25 min at 25 °C.

Coverslips containing attached mitochondria were transferred to a circular perfusion chamber (25 mm in diameter; 700 μl total volume), mounted on the stage of a Nikon epifluorescence microscope (Nikon, Diaphot). The perfusate was warmed to 25 °C using a Haake water bath. The microscope had a 40 × objective lens with 0.83 numerical aperture. Perfusion of attached organelles was begun at 2.6 ml/min using a peristaltic pump. An area of the coverslip with homogeneous attachment of organelles was selected for measurement of fluorescence. Although individual mitochondria could not be identified, what appeared to be clumps of organelles yielded the highest fluorescence signals.

Determination of intramitochondrial pH

Intramitochondrial pH was determined using the pH-sensitive fluorescent dye BCECF. Immobilized mitochondria containing BCECF on a coverslip were perfused with perfusion buffer. The microscope contained a 510 nm dichroic cut-off mirror with a 520–560 nm barrier filter, and a 5 nm band-pass emission filter centred at 530 nm was placed in front of the photomultiplier tube. An emission signal was obtained from the coverslip by alternating excitation wavelengths between 490 nm and 450 nm by a beam splitter at a frequency of 30 Hz. Following determination and subtraction of autofluorescence at each wavelength (obtained from coverslips with attached mitochondria that had not been loaded with dye), the ratio of the emitted fluorescence signal allowed the intramitochondrial pH to be calculated. No correction was required for the fluorescence of pH-sensitive dye in the extramitochondrial space, as dye that leaked out of the organelle (10 % per h at 25 °C) was diluted many-fold and washed away by constant perfusion of the chamber. Leakage of dye did not affect determination of intramitochondrial pH because the fluorescence of the 490 nm and 450 nm excitation signals were ratioed. Subsequently, the ratio of the emission signal of $(490/450)_{ex.}$ is referred to as the fluorescence ratio. Fluorescence signals were monitored continuously and integrated over 2 s intervals. After each experiment, the fluorescence ratio was calibrated by equilibrating the intra- and extra-mitochondrial pH at a minimum of three points. This was achieved by dissipating the transmembrane proton gradient by adding 5 μM-carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), 5 μM-nigericin and 6 μM-oligomycin to the perfusion buffer. To avoid photobleaching of BCECF, stock solutions were kept in the dark at −20 °C. Each sample was exposed to the excitation beam only while data were being acquired, and the excitation slit width was kept to a minimum (1.6–3.3 nm).

Determination of intramitochondrial $[Ca^{2+}]$

Intramitochondrial $[Ca^{2+}]$ was measured using the Ca^{2+}-sensitive fluorescent dye fura-2. A coverslip with immobilized mitochondria containing dye was placed in the circular perfusion chamber described above. The microscope contained a 400 nm dichroic cut-off mirror with a 400 nm barrier filter; a 20 nm band-pass emission filter centred at 500 nm was placed in front of the photomultiplier tube. The fluorescence signal was obtained from the coverslip by alternating excitation wavelengths between 340 and 380 nm at 30 Hz. Subsequently, the ratio of the emission signal of $(340/380)_{ex.}$ is referred to as the fluorescence ratio. As with BCECF, any fluorescence signal due to leakage of Ca^{2+}-insensitive or of Ca^{2+}-sensitive fura-2 into the extramitochondrial space (29 % per h at 25 °C) was minimal owing to the constant perfusion of the coverslip within the perfusion chamber.

Fluorescence ratioing allows measurements of intramitochondrial $[Ca^{2+}]$ to be independent of the concentration of dye in the matrix. The intramitochondrial $[Ca^{2+}]$ was calculated employing the equation from [22]:

$$[Ca^{2+}]_i = K_d(R-R_{min.})/(R_{max.}-R)\cdot c \qquad (1)$$

where K_d is the apparent dissociation constant for fura-2 and Ca^{2+}; c is the ratio of the fluorescence at 380 nm excitation of free to bound dye; R is the ratio of fluorescence at 340 nm to 280 nm excitation; and $R_{min.}$ and $R_{max.}$ are the ratios R of free (zero Ca^{2+}) and bound (saturating Ca^{2+}) dye respectively. At the end of every experiment, $R_{min.}$ and $R_{max.}$ were determined by perfusing with 4 μM-ionomycin (acid and Ca^{2+} salt respectively), 5 μM-CCCP and 6 μM-oligomycin in perfusion buffer. Autofluorescence was measured by quenching fura-2 using perfusion buffer containing 1 mM-Mn^{2+}; this fluorescence at 340 and 380 nm excitation was subtracted before ratioing. Determination of $R_{min.}$ and $R_{max.}$ at the end of each experiment was used to correct for unhydrolysed Ca^{2+}-insensitive fura-2 [20]. As with BCECF, to minimize photobleaching, stock solutions of fura-2 were kept in the dark and the excitation slit width was minimized (3–4.5 nm). Fluorescence signals were continuously monitored and integrated over 2 s intervals. The emission signal from dye-loaded mitochondria in suspension in a cuvette was captured front face on.

Determination of dissociation constants of fura-2 and BCECF for Ca^{2+} and H^+

The determination of dissociation constants for both BCECF and fura-2 in Buffer II was performed in 3 ml

cuvettes placed in a Spex Fluorolog-2 (Edison, NJ, U.S.A.) instrument equipped with a thermostatically controlled cuvette holder and a magnetic stirrer. BCECF (0.3–1 μM) was titrated in Buffer II containing 0–100 μM-Ca^{2+}; pH was confirmed using a H^+-sensitive electrode. Fura-2 (1 μM) was titrated at various pH values (6.5, 7.15, 7.7) in perfusion buffer containing known amounts of Ca^{2+}, that were confirmed using a Ca^{2+}-sensitive electrode. Similarly, dissociation constants for binding of Ca^{2+} and H^+ by fura-2 and BCECF respectively, were determined in isolated mitochondria attached to a coverslip using ionophores and oligomycin as described above. The K_d of intramitochondrial BCECF for H^+ was determined at 0 M-Ca^{2+}, and the K_d of intramitochondrial fura-2 for Ca^{2+} at pH 7.1 and 8.0. The K_d of fura-2 for Ca^{2+} was determined using a Hill plot and eqn. (1).

Fluorescence imaging of immobilized mitochondria

Fluorescence images of mitochondria loaded with BCECF or fura-2 were acquired using the same instrumentation described above, with the exception that a 100× oil-immersion lens (1.3 numerical aperture) was used and an SIT-Camera (Model 66, Dage MTI, Michigan City, IN, U.S.A.) was attached to the microscope. The image was digitized and then enhanced using imaging software from Spex, Edison, NY, U.S.A.

Chemicals and reagents

$CaCl_2$ standard solution (100 mM) was obtained from Orion Research. EGTA acid was purrissima grade from Fluka (Ronkonkoma, NY, U.S.A.). CellTak was obtained from Biopolymers. BCECF and fura-2 and their acetoxymethyl esters were purchased from Molecular Probes (Eugene, OR, U.S.A.). Ionomycin (Ca^{2+} salt and the free acid) was obtained from Calbiochem (San Diego, CA, U.S.A.). CCCP, nigericin, oligomycin and all other common chemicals were from Sigma and were reagent grade or better.

RESULTS AND DISCUSSION

Mitochondria in suspension: hydrolysis and leakage of fura-2

The leakage of unhydrolysed acetoxymethyl esters (AM) of both BCECF and fura-2 and of free dyes into the suspension medium could complicate the interpretation of fluorescence ratios over time if the time course for appearance of dye outside the mitochondria were not linear. This is a particularly troublesome problem with fura-2 because of the fluorescence of incompletely hydrolysed precursors.

To quantify the rate of appearance of fura-2 in the suspension media in the cuvette, liver mitochondria were loaded with fura-2/AM (48 μM) for 30 min at 25 °C, washed twice, and then resuspended at a final protein concentration of 0.5 mg/ml at 25 °C in medium containing succinate and rotenone. An aliquot was immediately taken to determine the total fluorescence yield of fura-2 and of Ca^{2+}-insensitive species at time 0. Aliquots were taken and centrifuged at successive time intervals, and the fluorescence at 340 nm excitation of the supernatant at 0 and 1 mM-Ca^{2+} was measured. The contribution of Ca^{2+}-sensitive and -insensitive fluorescent species was determined according to Scanlon *et al.* [20]:

$$F2_{ins.} = (z \cdot F_{min.} - F_{max.})/(z-1) \quad (2)$$

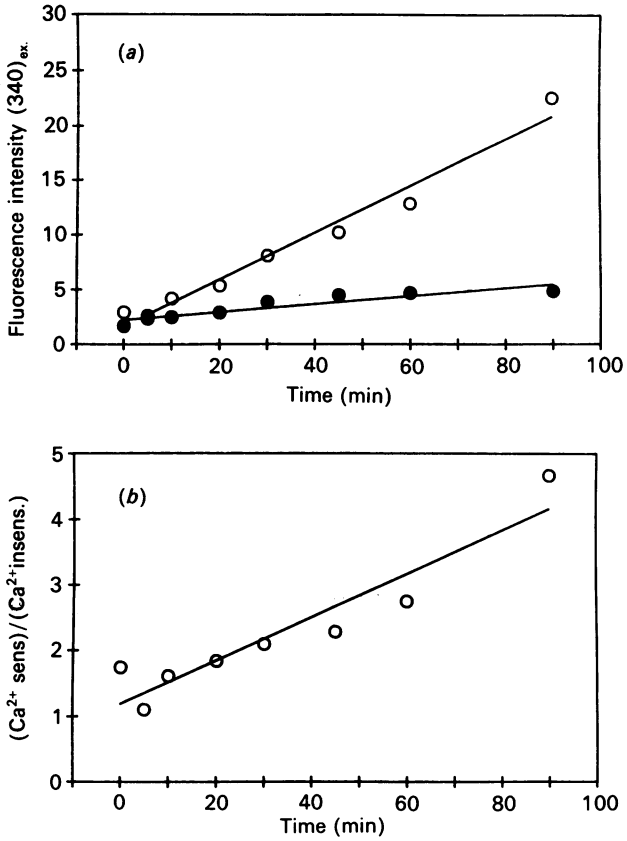

Fig. 1. Appearance of fluorescent Ca^{2+}-sensitive and Ca^{2+}-insensitive species of dye from liver mitochondria

(*a*) The time course of appearance of hydrolysed, Ca^{2+}-sensitive fura-2 (○), as well as Ca^{2+}-insensitive, partially hydrolysed species of fura-2 (●) into the extramitochondrial space is illustrated. Following incubation (for 30 min) of liver mitochondria (6 mg/ml) with fura-2/AM (48 μM), organelles were suspended in perfusion buffer at 25 °C containing succinate (10 mM) and rotenone (1 μM). At the indicated times, aliquots were removed, centrifuged, and fluorescence in the supernatant recorded at 340 nm excitation at 0 M- and 1 mM-Ca^{2+}. The contribution to fluorescence by Ca^{2+}-insensitive dye was calculated by the method of Scanlon *et al.* [20], eqn. 2 (see the Results and discussion section). (*b*) The time course of the change in the ratio of extramitochondrial Ca^{2+}-sensitive (Ca^{2+} sens.) to insensitive (Ca^{2+} insens.) species of fura-2 is shown over time for rat liver mitochondria in suspension.

where $F2_{ins.}$ is the fluorescence contribution of Ca^{2+}-insensitive dye at 0 M-Ca^{2+} ($F_{min.}$) and at 1 mM-Ca^{2+} ($F_{max.}$) in the supernatant at each time point; z represents the ratio of fluorescence at 340 nm excitation of the free acid at 0 and 1 mM-Ca^{2+} ($z = 3.28$). Fig. 1(*a*) shows the accumulation of extramitochondrial Ca^{2+}-sensitive and Ca^{2+}-insensitive species of fura-2 at successive time points following incubation of liver mitochondria with fura-2/AM, with centrifugation and resuspension at time 0 in perfusion buffer. Both Ca^{2+}-sensitive (fura-2) and Ca^{2+}-insensitive species of dye increased over time. Extramitochondrial Ca^{2+}-sensitive dye (fura-2) increased rapidly to a level higher than that originally present in mitochondria at time 0. This suggests either that ongoing de-esterification of incompletely

hydrolysed fura-2/AM and leakage of free dye from the organelles occurred following resuspension or that there was ongoing leakage of incompletely hydrolysed fura-2/AM out of liver mitochondria with subsequent cleavage by extramitochondrial esterases. With the assumption that total fluorescence is the sum of the fluorescence of Ca^{2+}-sensitive and Ca^{2+}-insensitive dye, the ratio of Ca^{2+}-sensitive fura-2 to all Ca^{2+}-insensitive fluorescent species in the supernatant could be calculated using eqn. (2). At time 0, the ratio of Ca^{2+}-sensitive to -insensitive dye was 1.8 and had increased to 4.8 by 90 min (Fig. 1*b*). Importantly, however, within the mitochondrial matrix, the ratio of Ca^{2+}-sensitive to Ca^{2+}-insensitive dye at time 0 was only 0.32.

Attachment of dye-loaded isolated mitochondria to a glass coverslip

A significant advantage was obtained when isolated mitochondria were studied in a chamber in which constant exchange of the extramitochondrial medium was devised. Such techniques are in common use for the study of intact cells in monolayers [27]. Cells are attached to optically transparent surfaces using a variety of attachment factors, including collagen, fibronectin, poly-D-lysine and others. By using standard attachment protocols, we attempted to adhere isolated heart mitochondria loaded with BCECF or fura-2 to glass coverslips using each of the above-mentioned matrices. Although some dye fluorescence could be detected, the fluorescence intensity was only slightly above autofluorescence, and the degree of attachment was quite variable. With the adhesive polyphenolic protein of the marine mussel *Mytilus edulis* (CellTak) [28,29], attachment was highly reproducible and resulted in good fluorescence yields with both dyes.

In Fig. 2(*a*), a phase-contrast microscopic image of isolated heart mitochondria attached to a coverslip with the marine mussel protein is shown. The mitochondria appeared to form a multilayered aggregate. The same area of the coverslip is shown in Fig. 2(*b*), but the emission light of BCECF, excited at 508 nm, was captured. Under these conditions, mitochondria are fully energized with succinate in the presence of rotenone. The possibility that multilayered aggregates exist was suggested here because the field was not evenly illuminated. This was supported by the observation that, after changing the plane of focus, another phase-contrast illuminated structure appeared. In Figs. 2(*c*) and 2(*d*), another coverslip is shown in which heart mitochondria attached to a coverslip were loaded with fura-2. For this illustration, fura-2 was excited at 340 nm and the emission light was captured: the perfusion buffer contained no free Ca^{2+}.

Spectra characteristics of BCECF and its calibration in mitochondria

Owing to the possibility that the polyphenolic adhesive protein that constitutes CellTak might inhibit normal mitochondrial respiration, it was important to verify that isolated, attached mitochondria could establish an H^+ gradient normally. However, before absolute quantification of intramitochondrial pH could be ascertained, the fluorescence characteristics of the dye within these organelles had to be determined. Heart, rather than liver, mitochondria were chosen because of the likelihood that contamination from other organelles that could hydrolyse the dye's acetoxymethyl ester would be lower.

Intramitochondrial BCECF showed a 3 nm red-shift of its excitation maximum from 505 nm obtained in incubation buffer (see the Experimental section) to 508 nm. The pK_a for BCECF in incubation buffer was 6.8, somewhat lower than that reported by Rink *et al.* [12] in a similar solution. $[Ca^{2+}]$, up to 100 μM, did not interfere with the BCECF signal over the pH range 5.0–9.0, as shown in Fig. 3. Within intact cells, virtually no change in the pK_a of BCECF for H^+ has been described [12,13]. Similarly, the parent compound of BCECF, 6-carboxyfluorescein, shows either no shift [21] or only a slight shift [30] in the pK_a when determined in buffer compared with intact cells. However, an appreciable shift in pK_a has been reported for this 6-carboxyfluorescein in mitochondria [21]. Our data with BCECF support these findings in that the pK_a shifts from 6.8 in incubation buffer to 7.2 within mitochondria (Fig. 3).

The calibration curve illustrated in Fig. 3 was obtained using intact, attached heart mitochondria perfused with buffer containing the H^+ ionophore, CCCP, which dissipated the H^+ gradient across the mitochondrial inner membrane, and the K^+/H^+ ionophore nigericin, which dissipated any residual membrane potential due to transmembrane K^+ gradients. Additionally, the perfusion buffer contained oligomycin to prevent any possible generation of an H^+ gradient due to reverse H^+/ATPase activity in the presence of ATP. ATP was routinely added to the perfusion buffer to help maintain membrane integrity via the acyl-CoA:lysophospholipid acyltransferase [31], and therefore minimize leakage of the dye. A field and a plane of focus that exhibited maximal fluorescence on each coverslip were chosen and were not changed throughout the course of an experiment. No correction for accumulation of extramitochondrial pH-sensitive dye was necessary with this technique. The calibration was begun by perfusing at pH 7.15 with ionophores and oligomycin, and then one of three protocols was followed: (1) the pH of the perfusing buffer was lowered to 6.20, followed by stepwise increments to pH 8.71 ('continuously ascending'); (2) the pH was raised to 8.71, followed by stepwise decrements to pH 6.20 ('continously descending'); or (3) the pH was raised to 8.71 and then lowered stepwise to 6.20; however, following each step down, the pH was raised again to 8.71 and a stable signal obtained before the next lower pH perfusion was begun ('alternating descending' technique). Each titration curve resulted in a different fluorescence-ratio signal at high pH, while all three converged to similar values at low pH. The greatest difference occurred between the 'continuously ascending' and the 'continuously descending' titration curves (curves 1 and 2 in Fig. 4), with the highest fluorescence ratio at pH 8.71 obtained for the 'continuously descending' experiment (curve 2), and the lowest ratio obtained for; the 'continuously ascending' titration (curve 1). When an 'alternating descending' protocol was followed (curve 3, Fig. 4), fluorescence-ratio values in the low to medium pH range were higher than for either of the other two titrations.

One possible explanation for these findings is the insertion of the protonated, less polar dye into the mitochondrial inner membane at low pH. It is assumed that, within the organelle, the fluorescence signal is a mixture of two pools of pH-sensitive dye, dye in the aqueous matrix and dye that has partitioned into the membrane. The reason for the observed hysteresis could

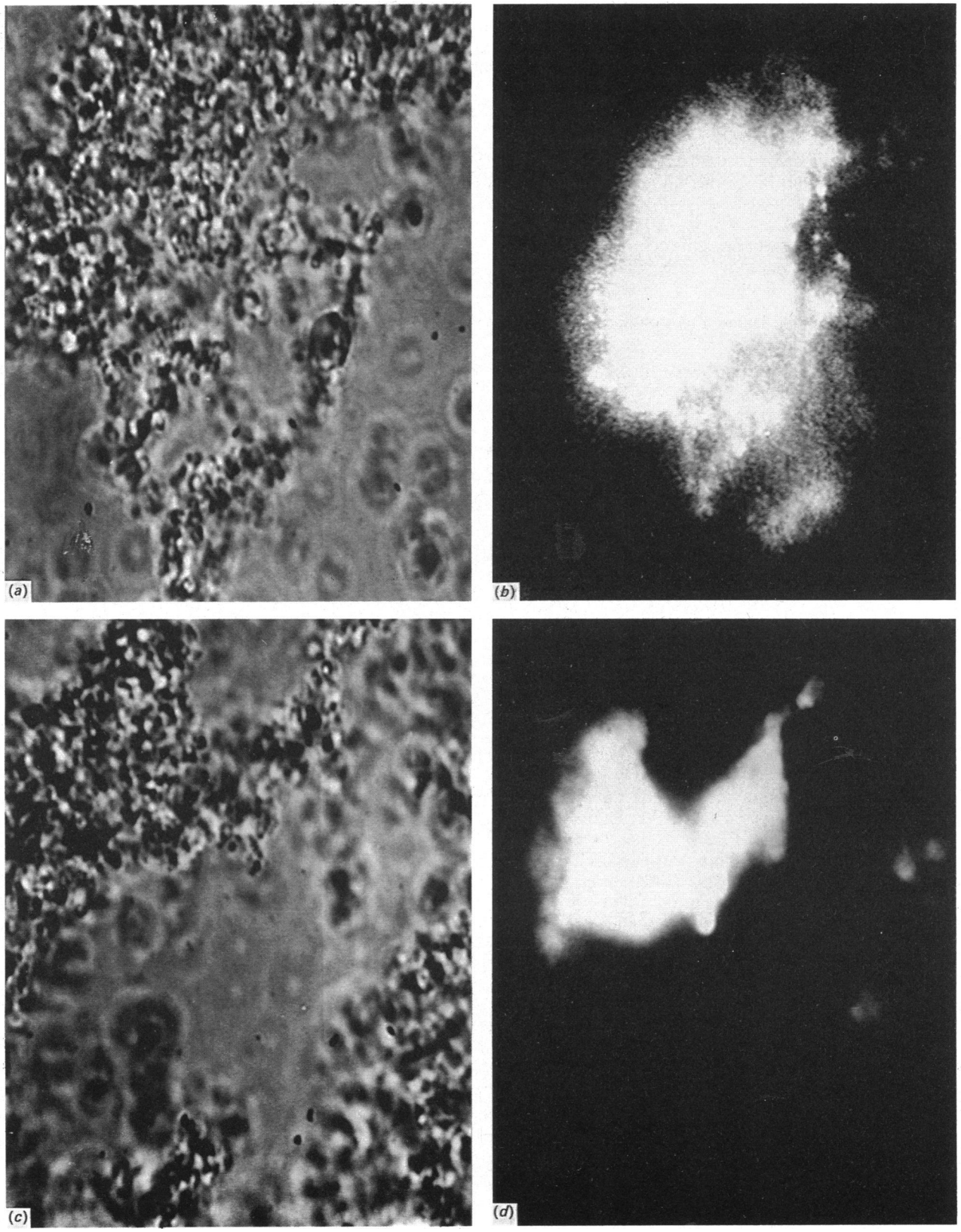

Fig. 2. Images of dye-loaded, immobilized cardiac mitochondria

Energized cardiac mitochondria were attached to coverslips and placed in a perfusion chamber on an epifluorescence microscope (100 × oil immersion lens). A fixed field and plane of focus were chosen, and the image was captured with an SIT-camera, and enhanced using digitizing software. The phase-contrast image (*a*) and the image of the emission light of BCECF-loaded mitochondria (*b*) from the same field and plane of focus, excited at 508 nm, are shown. Panels (*c*) and *d*) show a phase-contrast image (*c*) and the emission-light image (*d*) of fura-2 loaded mitochondria excited at 340 nm (0 M-Ca^{2+} in perfusion buffer). The excitation slit width used for obtaining images (*b*) and (*d*) was equivalent to 12 nm.

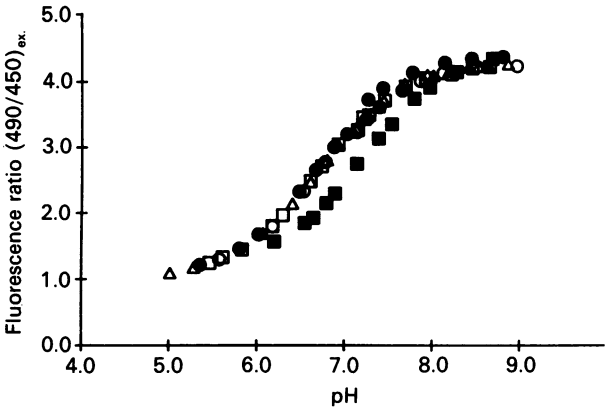

Fig. 3. BCECF calibration curves in buffer and in cardiac mitochondria

The ratio of fluorescence $(490/450)_{ex.}$ of BCECF (508 nm emission) is shown as a function of pH in Buffer II (see the Experimental section) at several $[Ca^{2+}]$ concentrations: 0 M-Ca^{2+} (1 mM-EGTA) (□), 1 μM-Ca^{2+} (△), 1.5 μM (▲), 3 μM (○), 100 μM (●). The pK_a of BCECF was 6.8 for these titrations, regardless of the $[Ca^{2+}]$. However, the curve for isolated rat cardiac mitochondria was obtained with mitochondria loaded with BCECF and attached to a glass coverslip, and titrated with perfusion buffer containing CCCP (5 μM), nigericin (5 μM) and oligomycin (6 μM) at various pH values (■). An 'alternating descending' protocol was followed (see the Results and discussion section). The pK_a of BCECF in cardiac mitochondria was determined to be 7.2.

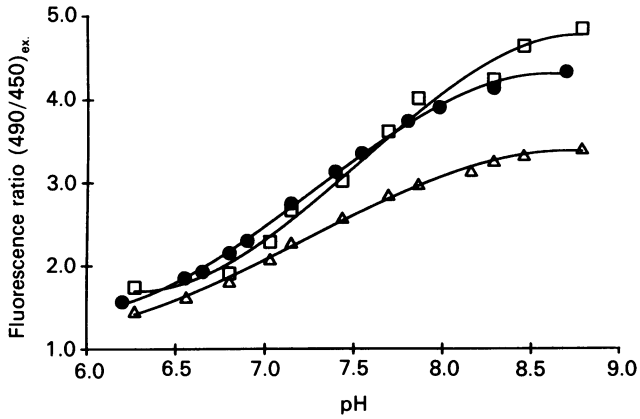

Fig. 4. Hysteresis effect of BCECF in mitochondria

Rat cardiac mitochondria were immobilized on a glass coverslip and titrated with perfusion buffers of various pH values containing H^+ and K^+ ionophores (see legend to Fig. 3 and the Experimental section). The ratio of fluorescence $(490–450)_{ex.}$ of BCECF (508 emission) is shown as a function of pH. In curve 1 (△), the pH of the perfusing buffer was lowered to 6.20, and then increased stepwise to 8.71 ('continuously ascending'); in curve 2 (□), the initial pH of the perfusion buffer was 8.71, and this was lowered in stepwise decrements to 6.20 ('continuously descending'). When these two titrations were performed sequentially on the same preparation of mitochondria, a marked hysteresis effect was observed as illustrated. In curve 3 (●), the initial pH of the perfusion buffer was 8.71, and this was decreased in steps to 6.20, but between each pH fall, the preparation was again transiently perfused at pH 8.71 ('alternating descending' protocol).

be that the kinetics of dissociation of a less polar compound, such as a protonated dye, from the membrane are considerably slower than the movement of dye into the membrane, as is the case for other lipophilic compounds [32,33], leading to trapping of fluorescent dye in the membrane at low pH. The reason this effect is so marked in mitochondria, but not in most cells, is possibly the high surface-to-volume ratio of the mitochondrial matrix. To minimize the accumulation of membrane-associated dye, all subsequent calibrations were performed with the 'alternating descending' titration procedure and acidification below pH 7.0 for more than several minutes was avoided.

Photobleaching of BCECF in immobilized mitochondria

A potential problem with fluorescent probes is photobleaching, a photochemical reaction that changes the characteristics of the dye. A fluorescent probe loaded into immobilized mitochondria on a coverslip is exposed to a significantly larger amount of incident light over a longer period of time than organelles in suspension in a cuvette. As a result, rapid photobleaching could occur depending upon the intensity of the excitation beam and the duration of time the dye is exposed. Ideally, the product of photobleaching would itself be non-fluorescent and the routine ratioing of two fluorescence signals would account for the decline in concentration of original fluorescent species as reported recently for BCECF in immobilized Swiss 3T3 cells [13]. As BCECF trapped within mitochondria behaves differently from when within intact cells, at least in terms of its pK_a, it is possible that the photobleached product of BCECF could also have different spectral qualities within this organelle.

As changes in pH over a fairly wide range were anticipated during the course of energizing and studying immobilized mitochondria, the effect of photobleaching was examined over the pH range from 6.2 to 8.7. Heart mitochondria loaded with BCECF were immobilized on a coverslip and exposed to excitation beams with a relatively high light intensity (wide slit width). A fixed field and plane of focus were chosen and, starting at a high pH, mitochondria were perfused in the 'alternating descending' protocol described above. Photobleaching under these conditions was monitored for 90 min. The actual exposure time to a lower pH never exceeded 2 min, in order to avoid irreversible compartmentalization of dye (see above). At the end of the titration, a different, unexposed field on the same coverslip was moved into the light beam, and a perfusion at pH 8.3 was begun. The fluorescence-ratio signal of BCECF in this previously unexposed field was almost identical with the fluoresence ratio in the initial field exposed at the beginning of the pH titration.

Photobleaching resulted in a much greater rate of decline in the fluorescence ratio at the alkaline pH characteristic of energized mitochondria. The ratio decline at high pH was underestimated somewhat by the alternating exposure to perfusion buffers containing ionophores at a low pH ('alternating descending' protocol). Conversely, the decline at low pH was overestimated due to the alternating exposure to buffers at higher pH. Ignoring these complications, calibration curves for BCECF could be constructed from pH 6.2 to pH 8.7 that were both uncorrected and corrected for the effects of photobleaching. If, at each pH during the

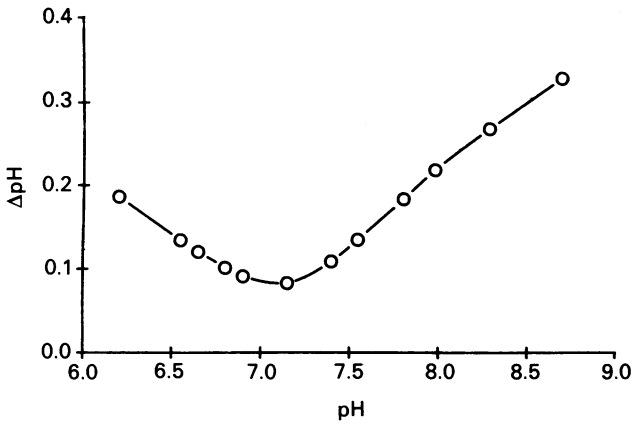

Fig. 5. pH-dependent photobleaching of BCECF

Photobleaching of BCECF was studied using the 'alternating descending' protocol (see the Experimental section). Photobleaching caused a decline in the fluorescence ratio $(490/450)_{ex}$. The slope of the decline was pH dependent. The difference (ΔpH) between calibration curves corrected and uncorrected for photobleaching is illustrated here. Note that while the effect is greatest at high pH, the absolute magnitude of this effect (i.e. the ΔpH range on the ordinate) will depend on the extent of photobleaching for any given instrumental setting, an effect that can be minimized by reducing the incident light or time of exposure.

titration, the value for the fluoresence ratio obtained from a calibration curve corrected for photobleaching was subtracted from the value obtained from the uncorrected calibration curve, the differences could be expressed as the pH change (ΔpH) that was due to photobleaching, and plotted as a function of pH as shown in Fig. 5. Photobleaching was most pronounced at alkaline pH, where the concentration of free, non-protonated dye is higher. The alkaline milieu appears to catalyse a photochemical reaction to a product with a higher contribution to fluorescence at 450 nm excitation. At H^+ concentrations close to that in the cytosol of most cells, around pH 7.0, the ratioing technique will largely correct for any effect of photobleaching of BCECF trapped in mitochondria, in agreement with observations in intact cells by Bright *et al.* [13]. The apparent increase in the induced error at pH values below 7 is due to the sigmoidal nature of both the corrected and uncorrected calibration curves in this pH range.

A first approach to minimize the effect of photobleaching is to reduce the indirect light by using neutral density filters or by narrowing the slit width of the excitation beams as much as possible. An additional method is to limit the amount of time the field of interest is exposed to excitation light to only that amount necessary to acquire data. If these methods prove impractical due to instrument limitations, the effect of photobleaching can be estimated and a correction made. One can assume the pH-sensitive reaction to be:

$$H\text{–}BCECF \rightleftharpoons H^+ + BCECF^-$$

in which the amount of free (non-protonated) dye can be calculated by mass action.

As total BCECF ($BCECF_0$) = H–BCECF + $BCECF^-$, the mole fraction x of free BCECF can be calculated as: $x = [BCECF^-]/[BCECF_0] = K_a/(H^+ + K_a)$

At any given pH, the mole fraction of free BCECF present within the mitochondrial matrix can be correlated with the rare of photobleaching, P, expressed as the slope of the decline in the fluorescence ratio with time at that pH. This is accomplished by fitting the data to the exponential equation:

$$P = ae^{-bx} \quad (3)$$

where a and b are constants representing the effect of the two light beams at 450 and 490 nm and must be determined for each instrument setting and for each preparation. This correlation suggests that it is the free dye that is most susceptible to photobleaching; thus, the photobleaching effect would be most pronounced at high pH.

These two limitations of BCECF for work in mitochondria, low pH insertion of dye into membranes and high pH photobleaching, can both be minimized by avoiding prolonged acidification of dye-loaded organelles and by limiting the intensity and/or time of exposure to incident light. Nevertheless, both effects must be considered in order to achieve experimental quantification of matrix pH.

Viability of immobilized mitochondria

Once the spectral characteristics of BCECF within mitochondria attached to a glass coverslip and observed with fluorescence microscopy were determined, it was necessary to prove that the mitochondria were still viable and capable of normal respiration. To study individual components of mechanisms responsible for establishing and maintaining the transmembrane H^+ gradient, mitochondria were loaded with BCECF under non-energizing conditions; that is, the organelles were warmed to 25 °C with BCECF/AM in the absence of any substrate. When perfused at pH 7.15, as shown in Fig. 6, the initial intramitochondrial pH was 7.0, a slight acidification of the matrix pH, probably due to the de-esterification of BCECF/AM resulting in formation of free dye, acetate and formaldehyde [34]. The subsequent addition of 16 mM-pyruvate and 4 mM-malate to the perfusion buffer resulted in a rise in intramitochondrial pH that could subsequently be reversed by perfusion with rotenone, known to block Complex I of the electron-transport chain. The failure of pyruvate and malate to raise the pH above 7.18 may be due to the absence of any added Ca^{2+} in the perfusion buffer with, consequently, a low matrix $[Ca^{2+}]$ and diminished activity of the Ca^{2+}-sensitive matrix pyruvate dehydrogenase. Addition of succinate in the presence of rotenone resulted in a rise in internal pH to 7.6. Complex IV could be blocked reversibly by addition of KCN to the perfusion buffer. Removal of KCN and its washout resulted in an increase in intramitochondrial pH in the presence of succinate to 7.8. Attempts to reverse the effects of KCN by perfusion with an electron acceptor, such as ferricyanide, were confounded by interference of this compound with the BCECF fluorescence signal (results not shown). Following establishment of a plateau at pH 7.8 with the succinate perfusion, CCCP and nigericin were added to abolish the transmembrane H^+ gradient. Although CCCP interfered slightly with the BCECF fluorescence signal, this was determined to be negligible at the concentrations used in these protocols; nigericin did not interfere with the BCECF signal. Although a maximal intramitochondrial pH of only 7.8 was achieved in this

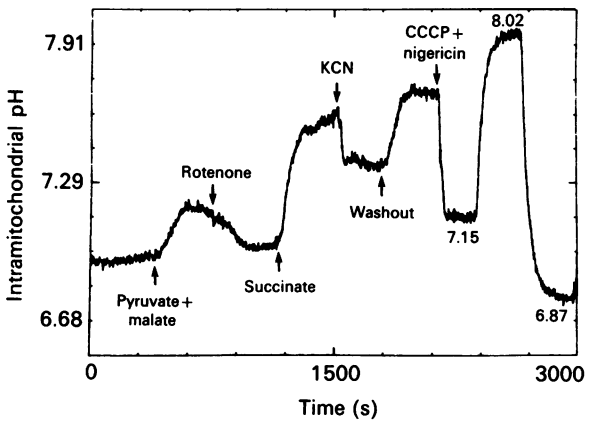

Fig. 6. Matrix pH response of isolated rat cardiac mitochondria attached to glass coverslips to metabolic substrates and inhibitors

An emission record of the ratio of fluorescence $(490–450)_{ex.}$ of BCECF is shown as a function of time; a correction has been made for autofluorescence at each excitation wavelength. Isolated rat cardiac mitochondria (6 mg/ml) were incubated with BCECF (5 μM) under de-energizing conditions (i.e. no exogenous substrate; see the Experimental section) and were attached to glass coverslips using the attachment polyphenolic adhesive protein from the marine mussel, *Mytilus edulis* (CellTak). Loading with BCECF lowered intramitochondrial pH to 6.99 (the perfusion buffer pH was 7.15). Addition of pyruvate (16 mM) and malate (4 mM) to the perfusion buffer (no succinate, no rotenone) increased intramitochondrial pH to only 7.18; subsequent addition of rotenone resulted in a gradual reduction to an intramitochondrial pH of 7.04. Following addition of succinate to perfusion buffer containing rotenone, intramitochondrial pH rose to 7.64. Addition of KCN reduced intramitochondrial pH to 7.4, an effect that was rapidly reversible (to pH 7.8) upon washout of this reagent in the presence of succinate. CCCP and nigericin (5 μM) and oligomycin (6 μM) were then added, with a collapse in the transmembrane H^+ gradient to zero. The preparation was subsequently calibrated by perfusion at pH 8.02 and 6.87.

experiment when dye was loaded into energized mitochondria in the presence of succinate and phosphate, the final intramitochondrial pH was routinely between 7.8 and 8.1 at a perfusion buffer pH of 7.15.

Spectral characteristics of fura-2 and its calibration in mitochondria

Accurate assessment of $[Ca^{2+}]$ with fura-2 presupposes knowledge of the dissociation constant for binding of Ca^{2+} to the dye under the relevant experimental conditions. Dissociation constants for fura-2 are reported to fluctuate over a wide range from 130 nM [15] to 774 nM [35]. This suggests that fura-2 is sensitive to its chemical environment. Parameters such as temperature, ionic strength [36] and viscosity [35] all have an impact on the properties of fura-2. Although pH is often assumed not to be an important factor (Grynkiewicz *et al.* [22] demonstrated no appreciable effect between pH 6.75 and 7.05), this may not be the case at matrix pH values of 8.0 or higher that are typical of fully energized mitochondria. Calibration curves at various pH values were carried out initially with fura-2 in a cuvette in Buffer II supplemented

Table 1. Dissociation constants for Ca^{2+} binding of fura-2

Values in parentheses represent number of observations at each pH. Perfusion buffer contained 110 mM-KCl/20 mM-Mops/K^+/1 mM-$MgCl_2$, and sufficient mannitol/sucrose (3:1) to yield 295 mosM; all perfusions were carried out at 25 °C. Rat heart mitochondria were immobilized on a glass coverslip; the perfusion buffer contained 4 μM-ionomycin, 5 μM-CCCP, 6 μM-oligomycin at 25 °C.

	$K_d \pm$S.D. (nM) derived from eqn. (1)	K_d (nM) derived from Hill plot	
		340 nm	380 nm
Perfusion buffer			
pH 6.5	310±16 (11)	304	310
7.1	312±34 (11)	297	323
7.7*	370±56 (5)	299	357
7.7†	842±161 (5)	422	504
Mitochondria			
pH 7.1‡	313±19 (9)	–	–
7.1§	1570±1593 (3)	–	–
8.0‡	318±20 (4)	–	–
8.0§	5540±6740 (4)	–	–

* Values below 2 μM-Ca^{2+}.
† Values above 2 μM-Ca^{2+}.
‡ Values below 1 μM-Ca^{2+}
§ Values above 1 μM-Ca^{2+}.

with 1 mM-Mg^{2+} and 1 mM-EGTA. The excitation spectra of fura-2 were recorded in response to buffers of known $[Ca^{2+}]$, verified as described in the Experimental section. Dissociation constants of fura-2 for Ca^{2+} binding were determined at 340 nm and 380 nm excitation by using either a Hill plot or eqn. (1).

In buffer alone, as noted in Table 1, the mean K_d values were in the 297–323 nM range at pH 6.5 and 7.1, somewhat higher than that described by Grynkiewicz *et al.* [22]. At pH 7.7, however, the average K_d shifted to an even higher value, as calculated using eqn. (1) or by a Hill plot of a fura-2 calibration curve at 380 nm excitation. As shown in Fig. 7(*b*), the shape of the calibration curve at pH 7.7 changed, and the K_d was clearly dependent on the $[Ca^{2+}]$. At pH 7.7, the K_d increased from 450 nM at 2 μM-Ca^{2+} to 900 nM at 15 μM-Ca^{2+}.

A similar but even more pronounced effect was found when fura-2 was calibrated within rat cardiac mitochondria immobilized on a glass coverslip. Mitochondria were perfused with perfusion buffer containing Ca^{2+} and H^+ ionophores, as described in the Experimental section, over a range of $[Ca^{2+}]$ from 0 to 200 μM at two pH values, 7.15 and 8.0. The excitation spectra did not show a sharp isosbestic point due to leakage of dye from mitochondria, necessitating the use of eqn. (1) to calculate K_d, which uses the fluorescence ratio $(340/380)_{ex}$. When the fluorescence ratio was plotted versus the log of the $[Ca^{2+}]$, even when subtracting $R_{min.}$ and normalizing to the same $R_{max.}$, as shown in Fig. 7(*a*), the curves did not overlap. Indeed, the common representation of fura-2 calibration curves [37] as depicted in Fig. 7(*a*) can be misleading, as the K_d for Ca^{2+} binding is not at the $[Ca^{2+}]$ midway between $R_{min.}$ and $R_{max.}$, and it does not consider the fact that factor '*c*' in eqn. (1), the fluorescence ratio at

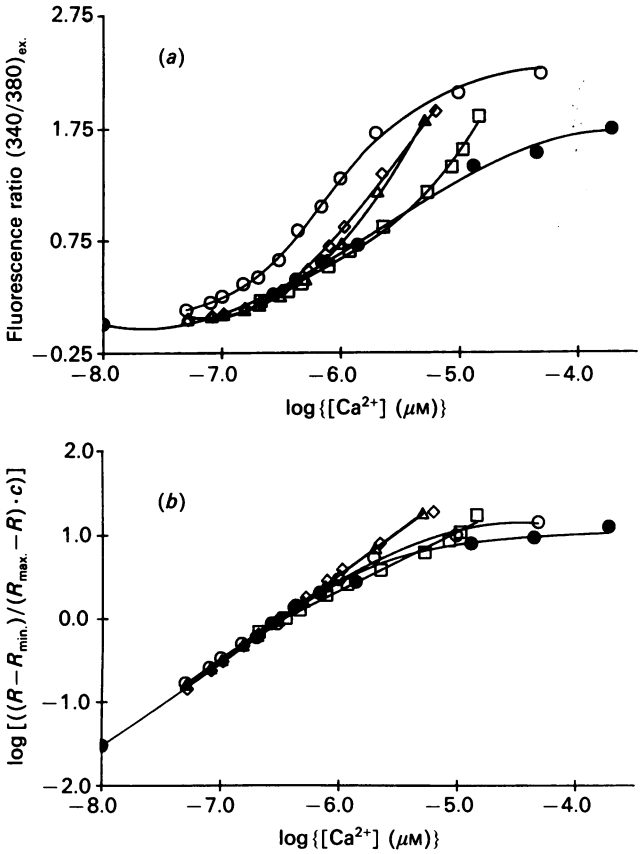

Fig. 7. Calibration curves for fura-2 in buffer and in cardiac mitochondria

(*a*) The ratio of fluorescence $(340/380)_{ex.}$ of fura-2 is shown over a range of $[Ca^{2+}]$ (0–15 μM) in Buffer II supplemented with 1 mM-Mg^{2+} at pH 6.5 (△), at pH 7.1 (◇) and at pH 7.7 (□). Also, the fluorescence ratio is shown for the fura-2 signal from isolated rat cardiac mitochondria attached to a glass coverslip, and over a range of $[Ca^{2+}]$ (0–200 μM) at pH 7.15 (○) and at pH 8.0 (●). Curves were normalized with respect to $R_{min.}$ and $R_{max.}$ (see Results and discussion section). (*b*) The data from panel (*a*) were linearized and replotted using the modified eqn. (1) (see the Experimental section), as $\log[(R-R_{min.})/(R_{max.}-R)\cdot c] = \log([Ca^{2+}]) - \log(K_d)$, where $R_{min.}$ and $R_{max.}$ are the ratios of fura-2 fluorescence at zero and saturating Ca^{2+}, respectively, and c is the ratio of fluorescence from 380 nm excitation of free to bound dye. Note the marked deviation from linearity of the fura-2 curve in solution at pH 7.7 at $[Ca^{2+}]$ concentrations above 1 μM, and of the fura-2 curves in mitochondria at both pH 7.15 and 8.0.

380 nm excitation of free to bound dye, differs with each curve. However, by constructing a Figure derived from eqn. (1) that plots $\log[(R-R_{min.})/(R_{max.}-R)\cdot c]$ versus $\log([Ca^{2+}])$, as shown in Fig. 7(*b*), below 1 μM-Ca^{2+} there is no large discrepancy in the shape or position of the calibration curves at any pH for dye in mitochondria or in buffer. At higher $[Ca^{2+}]$, even on this double log plot where small discrepancies may not be readily apparent, a clear deviation from linearity was evident, particularly for those curves at pH 8.0 obtained within mitochondria. This is reflected by a dramatic increase in K_d at $[Ca^{2+}]$ above 1 μM, as noted in Table 1.

The deviation from linearity of the curve for pH 7.7 in Fig. 7(*b*) suggests that the Ca^{2+}–dye complex-formation ratio has changed toward 2:1. Another possibility is that Ca^{2+} has some direct effect on the fluorophore that changes its spectral properties at higher pH, as might be the case if at higher pH a low-affinity binding site were unmasked. If this were the case, no isosbestic point or a shift in the isosbestic point with varying $[Ca^{2+}]$ would be expected as the pH increased. However, the isosbestic point remained unchanged for fura-2 in buffer alone at any pH from 6.5 to 7.7. Finally, a lowering of the true $[Ca^{2+}]$ due to lower solubility of Ca^{2+} in solution at high pH, is unlikely to be the cause of the apparent deviation in K_d. The experiments described here were all performed at free $[Ca^{2+}]$ 5 orders of magnitude below the solubility product [38], and buffer $[Ca^{2+}]$ were checked with a Ca^{2+} electrode.

While a change in the 1:1 complex-formation of the dye and Ca^{2+} at high pH seems the most likely explanation for the properties of fura-2 in buffer solution, the reason for the augmentation of the effect within mitochondria is less clear. The greater emission intensity of fura-2, excited at 340 nm, at higher $[Ca^{2+}]$ might be expected to result in more scatter light from dye within mitochondria attached to a coverslip compared with dye in solution. This, plus an increase in the inner-filter effect [39] with high Ca^{2+} activity at 340 nm excitation, in which emitted light is absorbed by the dye itself, could lead to an underestimate of the $340/380_{ex.}$ ratio. This might explain the drift toward higher values for the K_d for Ca^{2+} in mitochondria and, therefore, the large standard deviations obtained at pH 8.0 (Table 1).

At matrix free $[Ca^{2+}]$ below 1 μM, regardless of pH, fura-2 is an accurate and reversible indicator of $[Ca^{2+}]$ in isolated, immobilized organelles. The fact that apparent changes in fura-2 affinity for Ca^{2+} at higher pH were present, albeit less pronounced, in buffer alone suggests that the observed effects of pH on the chelation and spectral properties of fura-2 were not artifacts of the immobilization technique. For all further calculations of matrix $[Ca^{2+}]$, a K_d of 315 nM was used.

Photobleaching of fura-2 in immobilized mitochondria

Photobleaching was not as severe with fura-2 compared with BCECF under the experimental conditions described here. There did not appear to be any significant contribution due to accumulation of a fluorescent photobleached product of fura-2. Nevertheless, the same precautions were routinely used as noted above for work with BCECF and as recommended by Becker & Fay [40], to avoid excessive bleaching of dye. Unlike BCECF, which demonstrated a lack of reversibility and a marked hysteresis effect, due to dye movement into the mitochondrial inner membrane, this did not occur with fura-2.

Ca^{2+}-dependent mitochondrial transmembrane Ca^{2+} gradient

Immobilized rat cardiac mitochondria, loaded with fura-2 in 0 M-Ca^{2+} buffer (1 mM-EGTA) in the presence of succinate and rotenone as described in the Experimental section, were perfused at pH 7.15 with buffers at a range of $[Ca^{2+}]$ from 200 nM to 2 μM-Ca^{2+}. Ca^{2+} uptake occurred via the electrophoretic Ca^{2+} uniporter, and fura-2 reported the matrix $[Ca^{2+}]$. As mitochondrial autofluorescence at 350 nm is primarily due to NADH and changes in the NAD^+/NADH ratio during the

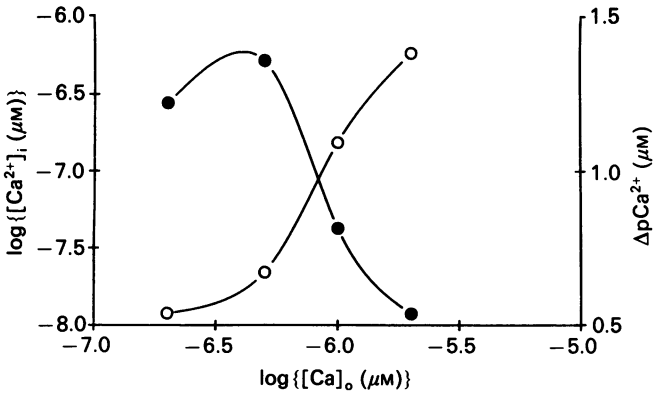

Fig. 8. Transmembrane Ca^{2+} gradient in cardiac mitochondria

Isolated rat cardiac mitochondria, attached to coverslips, were perfused with perfusion buffer for 10 min (see the Experimental section) at pH 7.15 at a number of free $[Ca^{2+}]$ of 200 nM, 500 nM, 1 μM and 2 μM. The ratio of fluorescence $(340/380)_{ex.}$ has been converted, using eqn. (1) (see Table 1; K_d = 315 nM), to $[Ca^{2+}]$ within the matrix ($pCa^{2+}]_i$; ○). The transmembrane $[Ca^{2+}]$ gradient at each ($pCa^{2+}]_o$, the difference between intraorganelle and buffer $[Ca^{2+}]$, is also shown (ΔpCa^{2+}; ●).

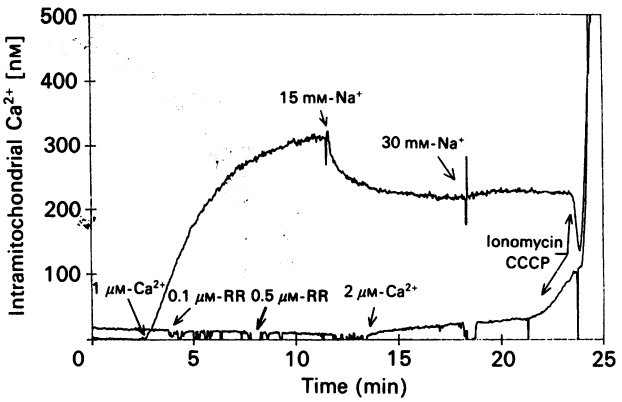

Fig. 9. Matrix Ca^{2+} response of isolated mitochondria to Na^+ and to Ruthenium Red

Isolated rat cardiac mitochondria, loaded with fura-2 and immobilized on a glass coverslip, were perfused initially with perfusion buffer containing no Ca^{2+} at pH 7.15 at 4 °C. At the indicated times, the mitochondria were superfused with perfusion buffer containing 1 μM-Ca^{2+}, followed by buffer containing 1 μM-Ca^{2+} with 15 mM-Na^+ and then with buffer containing 1 μM-Ca^{2+} and 30 mM-Na^+ (upper trace). In the lower trace, the perfusion buffer superfusing a second coverslip with attached mitochondria was supplemented with 0.1 μM- and then 0.5 μM-Ruthenium Red (RR). After an additional 10 min, sufficient Ca^{2+} to yield a final Ca^{2+} activity of 2 μM was added, but only a small increase in matrix $[Ca^{2+}]$ was observed. In both traces, the addition of 4 μM-ionomycin and 2 μM-CCCP caused a sharp rise in the Ca^{2+} signal, resulting in equilibration of the matrix Ca^{2+} activity with that of the perfusion buffer.

perfusion could affect calibration of the fura-2 signal, the $[NADH^+]$ was held constant by using succinate in the presence of rotenone for all experiments. As shown in Fig. 8, the free $[Ca^{2+}]$ after 10 min perfusion was very low at perfusion buffer $[Ca^{2+}]$ that approximated the cytosolic $[Ca^{2+}]$ in many resting cells. Even after perfusion with 200 nM-Ca^{2+} for 20 min, the matrix $[Ca^{2+}]$ did not exceed 100 nM. The maximal ratio of extramitochondrial to intramitochondrial $[Ca^{2+}]$ was 23 at a perfusion buffer $[Ca^{2+}]$ of 500 nM. At perfusion buffer Ca^{2+} above 500 nM, matrix $[Ca^{2+}]$ rose rapidly during the 10 min perfusion. The transmembrane Ca^{2+} ratio declined to only 3 at a perfusion buffer free-$[Ca^{2+}]$ of 2 μM.

Although these data indicate that matrix $[Ca^{2+}]$ may be lower than the cytosolic $[Ca^{2+}]$ by as much as an order of magnitude, it should be emphasized that these organelles were isolated and loaded with fura-2 in 1 mM-EGTA in the absence of any added Ca^{2+}. Nevertheless, the transmembrane Ca^{2+} gradient remained high in energized organelles even at perfusion buffer $[Ca^{2+}]$ of 500 nM. At higher $[Ca^{2+}]$ in the superfusion buffer, however, the transmembrane Ca^{2+} gradient rapidly declined (Fig. 8), although no Na^+ was present in the medium to initiate Ca^{2+} efflux by Na^+/Ca^{2+} exchange.

Although Ca^{2+} uptake was not measured directly, the rise in matrix Ca^{2+} activity indicated by the change in the fura-2 signal probably reflects Ca^{2+} uptake via the Ca^{2+} uniporter, since Ruthenium Red prevented virtually any change in response to 2 μM-extramitochondrial Ca^{2+} (Fig. 9). As expected, the addition of 15 mM-Na^+ to the superfusion medium reduced matrix $[Ca^{2+}]$, presumably by stimulating Na^+/Ca^{2+} exchange (Fig. 9). One possible explanation for the low matrix $[Ca^{2+}]$ found in these experiments is that the immobilized mitochondria, while able to generate a pH gradient, were unable to generate the membrane potential necessary to drive Ca^{2+} uptake via the uniporter. Another is the possibility that the attachment protein matrix itself somehow inhibited uniporter activity, and both these possibilities will be investigated. Nevertheless, the rapid rise in matrix $[Ca^{2+}]$ when perfused with Ca^{2+}-containing buffer, and its inhibition by Ruthenium Red, is consistent with a membrane potential-dependent uniporter mechanism.

These observations may have implications for the functional role of mitochondria in the response to ischaemia and reoxygenation or other cellular insults in which a rise in cytosolic $[Ca^{2+}]$ appears to occur during the development of cellular injury. A rise in cytosolic $[Ca^{2+}]$ could initiate a sudden release of accumulated matrix Ca^{2+} by abruptly decreasing the transmembrane Ca^{2+} gradient, and by other means, including activation of membrane-associated phospholipase A_2 activity [41]. In addition, the cytosolic acidification that accompanies ischaemia could also diminish the membrane potential across the mitochondrial inner membrane by reducing the transmembrane H^+ gradient. Such a change in the membrane potential could compromise the ability of mitochondria to buffer cytosolic Ca^{2+}, and perhaps lead to a release of previously accumulated Ca^{2+} into the cytosol.

Thus with the technique described above for constant superfusion of immobilized mitochondria, both BCECF and fura-2 are useful probes of matrix $[H^+]$ and $[Ca^{2+}]$, provided that the effects of the matrix environment on the behaviour of both dyes is recognized. Importantly, both H^+ and Ca^{2+} gradients across the mitochondrial inner membrane appear to be greatest at concentrations of both ions in the perfusing buffer that approximate those in the cytosol of myocardial cells

under normal physiological conditions. Attachment of mitochondria to a glass coverslip allows constant superfusion of these organelles, obviating the need to consider the contribution to total fluorescence of extramitochondrial species of dye. It is not known precisely how mitochondria are attached using the polyphenolic adhesive protein secreted by the mussel *Mytilus edulis*. Their viability seems unaffected, however, since normal transmembrane pH gradients can be achieved and maintained in the presence of oxygen and substrate.

This work was supported by grants HL19259 from the National Institutes of Health and a Clinician-Scientist Award from the American Heart Association to R. A. K. We thank Dr. M. Carey, Dr. J. Schinkel and Dr. T. Brock for their help and thoughtful analysis of portions of this work, and Dr. Donghee Kim of the Cardiovascular Division of Brigham and Women's Hospital who developed the perfusion apparatus and microscopic intrumentation in our laboratory for observation of fluorescent probes in isolated cells. His advice and encouragement were invaluable to the successful completion of this project.

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Received 4 May 1988/18 July 1988; accepted 24 August 1988