Calcium Transport and Proton Electrochemical Potential Gradient in Mitochondria from Guinea-Pig Cerebral Cortex and Rat Heart

By DAVID G. NICHOLLS

Department of Psychiatry, University of Dundee, Ninewells Medical School,
Dundee DD2 1UD, Scotland, U.K.

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A method is described for the preparation of 'free' and 'synaptosomal' brain mitochondria from fractions of guinea-pig cerebral cortex respectively depleted and enriched in synaptosomes. Both preparations of mitochondria have a low membrane H^+ conductance, a high capacity to phosphorylate ADP, and a capacity to accumulate Ca^{2+} at rates limited by the activity of the respiratory chain. Ca^{2+} transport by 'free' brain mitochondria is compared with that of heart mitochondria. The Ca^{2+} conductance of 'free' brain mitochondria was at least 20 times that for rat heart mitochondria. Ca^{2+} uptake by brain mitochondria increased the pH gradient and decreased membrane potential, whereas little change occurred during the much slower uptake by heart mitochondria. In the presence of ionophore A23187, dissipative Ca^{2+} cycling decreased the H^+ electrochemical potential gradient of brain mitochondria from 190 to 60 mV, but caused only a slight decrease with heart mitochondria, although the ionophore lowered the pH gradient and increased membrane potential. The Ca^{2+} conductance of 'free' brain mitochondria is distinctive in showing a hyperbolic dependency on free Ca^{2+} concentration. In the presence of Ruthenium Red, a rapid Na^+ -dependent Ca^{2+} efflux occurs. The H^+ electrochemical potential gradient is maintained during this efflux, and membrane potential increases, with both 'free' brain and heart mitochondria. The Na^+ requirement for Ca^{2+} efflux appears not to be related to the high Na^+/H^+ exchange activity but may represent a direct exchange of Na^+ for Ca^{2+} .

The concentration of free Ca^{2+} in the cytoplasm is firmly established as the regulator of muscle contraction and is implicated in the processes of neurotransmission and exocytosis (Katz & Miledi, 1965; Douglas, 1974). The extent to which heart mitochondria contribute to the regulation of cytoplasmic Ca^{2+} is being evaluated (Scarpa & Graziottini, 1973; Affolter *et al.*, 1976; Crompton *et al.*, 1976*a,b*). As mitochondrial Ca^{2+} transport shows considerable variation between tissues (Carafoli & Lehninger, 1971; Wikström *et al.*, 1975; Bygrave *et al.*, 1975; Crompton *et al.*, 1976*a,b*), it is not valid to extrapolate results obtained with heart or liver mitochondria to assess the corresponding physiological role of mitochondrial Ca^{2+} transport in neurotransmission or exocytosis.

The present paper attempts to examine the kinetics of Ca^{2+} transport by mitochondria from guinea-pig

cerebral cortex, in parallel with heart mitochondria.

Studies of Ca^{2+} transport by mitochondria from the brain should satisfy three criteria. Firstly the mitochondrial preparation must represent as homogeneous a population as possible; secondly the mitochondria should be shown to be bioenergetically competent during Ca^{2+} transport; and thirdly, if, as shown below, the C_mCa^{2+} of the membrane is sufficiently high for the respiratory chain to be rate-limiting during Ca^{2+} uptake, a valid form of kinetic treatment is required to obtain information on Ca^{2+} transport rather than on the activity of the respiratory chain (Heaton & Nicholls, 1976).

Of the published studies (Otsuka *et al.*, 1965; Yoshida *et al.*, 1966; Tower, 1968; Stahl & Swanson, 1969; Tijoe *et al.*, 1970; Carafoli & Lehninger, 1971; Diamond & Goldberg, 1971; Lazarewicz *et al.*, 1974; Vickers & Dowdall, 1976), few satisfy the initial criterion, and no studies have distinguished between mitochondria from fractions enriched or depleted in synaptosomes. Published techniques for preparing mitochondria of synaptosomal origin from the rat have relied on hypo-osmotic exposure to disrupt the synaptosomal membrane (Lai & Clark, 1976; Lai *et*

Abbreviations used: $\Delta\bar{\mu}_{H^+}$, proton electrochemical potential gradient; ΔE , membrane potential; ΔpH , transmembrane pH gradient; C_mH^+ , effective proton conductance of the membrane; C_mCa^{2+} , calcium conductance of the membrane; Tes, 2-[(2-hydroxy-1,1-bis(hydroxymethyl)-ethyl)amino]-1-propanesulphonic acid.

al., 1977). In the present paper this is circumvented by a method involving selective digitonin treatment. By using these preparations, the Ca^{2+} conductance of the mitochondrial inner membrane is compared with that of rat heart mitochondria under parallel conditions. It is concluded that mitochondria from cerebral cortex have a very much higher Ca^{2+} conductance than have heart mitochondria, and that their hyperbolic dependency of conductance on free Ca^{2+} concentration makes it feasible that they play a role in regulating low Ca^{2+} activities during neurotransmission.

Experimental

Materials

All radioactive isotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ionophore A23187 was a gift from Dr. R. L. Hamill, Eli Lilly and Co., Indianapolis, IN, U.S.A. Ficoll was obtained from Pharmacia, Uppsala, Sweden, and was exhaustively dialysed against water before use. Enzymes and Ruthenium Red were supplied by Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K. Ruthenium Red was recrystallized before use (Fletcher *et al.*, 1961). All other reagents were of analytical grade. Unless specifically stated, anions were added as the sodium salts. The Ca^{2+} content of sucrose stock solutions was minimized by equilibration with Dowex 50W (X8) before addition to media (Heaton & Nicholls, 1976).

Heart mitochondria

Mitochondria were prepared from the hearts of male Wistar rats (150–250 g) that had been maintained with free access to food and water. Hearts were homogenized with 10 strokes of a motor-driven Potter–Elvehjem homogenizer after tissue disruption in a Silverson tissue disintegrator (10 s at 25% of full power). The preparation medium contained 70 mM-sucrose, 210 mM-mannitol, 10 mM-Tes and 0.1 mM-EGTA, pH 7.4. Mitochondria were prepared by the method of Scarpa & Graziottini (1973). EGTA was omitted from the final washing and resuspension media.

Non-synaptosomal ('free') mitochondria from guinea-pig cerebral cortex

Mitochondria were prepared by a modification of the method of Cotman & Matthews (1971). Guinea pigs of either sex (Duncan–Hartley strain), aged 4–8 weeks, were allowed access to food and water *ad libitum*. For each preparation, two animals were killed by cervical dislocation, and cerebral cortices were rapidly excised and cooled to 0°C. The cortices were homogenized in a total of 60 ml of isolation

medium (320 mM-sucrose, 5 mM-Tes, 0.5 mM-EDTA, pH 7.4 at 0°C) with 10 strokes of a 1.9 cm diameter Teflon pestle rotating at 500 rev./min with a radial clearance of 0.25 mm. The homogenate was centrifuged in an MSE-18 8 × 50 ml angle rotor (5 min at 900 g_{max}). The pellet was resuspended in isolation medium, re-centrifuged, and the combined supernatants were centrifuged (10 min at 17000 g_{max}). The 'high-speed' supernatant was decanted, and the crude mitochondrial pellet resuspended in 10 ml of isolation medium, 5 ml being layered on top of each of two discontinuous gradients each comprising 4 ml of isolation medium containing 12% (w/v) Ficoll ($\rho = 1.085 \text{ g/ml}$), 1 ml of isolation medium containing 9% (w/v) Ficoll ($\rho = 1.075 \text{ g/ml}$) and 4 ml of isolation medium containing 6% (w/v) Ficoll ($\rho = 1.065 \text{ g/ml}$). The gradients were centrifuged in an MSE-25 6 × 16 ml swing-out rotor for 30 min at 75000 g_{max} . The 'myelin', 'synaptosomal' and 'free mitochondrial' fractions (Cotman & Matthews, 1971) formed respectively above the 6% Ficoll medium in a double band within the 9% Ficoll layer and as a pellet. The pellet was resuspended in 250 mM-sucrose/10 mM-Tes, pH 7.2, centrifuged (10 min at 8000 g_{max}) and finally resuspended in the same medium at a protein concentration of about 20 mg/ml.

'Synaptosomal' mitochondria from guinea-pig cerebral cortex

The 'synaptosomal' fraction from the discontinuous gradient described above was resuspended in 22 ml of isolation medium. The fraction formed as two bands within the $\rho = 1.075 \text{ g/ml}$ step of the gradient, and these bands were pooled for subsequent manipulations (see Lai *et al.*, 1977). To this resuspension was added an equal volume of isolation medium containing a suspension of digitonin (0.15 mg/mg of synaptosomal protein) that had been obtained by 2 min sonication at 6 μm amplitude. The mixture was vortex-mixed vigorously, left at 0°C for 5 min, and then subjected to very brief sonication (MSE 150 W ultrasonic disintegrator, 10 s at 6 μm amplitude). The sonicated preparation was centrifuged (5 min at 900 g_{max}) to sediment any excess digitonin micelles. Portions (11 ml) of the supernatant were then layered on 3 ml of isolation medium containing 12% (w/v) Ficoll ($\rho = 1.085 \text{ g/ml}$). The gradients were centrifuged in a swing-out rotor (60 min at 75000 g_{max}). The supernatant, the boundary at the interface and the pellet were collected separately. The pellet was resuspended in 10 ml of 250 mM-sucrose/10 mM-Tes and centrifuged (10 min at 800 g_{max}) before being finally resuspended in the same medium at a protein concentration of 20 mg/ml.

Other methods

Enzyme assays were performed at 30°C in a Cecil

CE272 recording spectrophotometer. Lactate dehydrogenase (EC 1.1.1.27) activity was assayed in an incubation mixture containing 50 mM-sodium phosphate, 1 μ M-rotenone, 0.17 mM-NADH, 4 mM-sodium pyruvate and 0.1% (v/v) Triton X-100. NAD⁺-linked glutamate dehydrogenase (EC 1.4.1.3) activity was assayed (Schmidt, 1963) in a medium containing 200 mM-ammonium acetate, 10 mM-Tes, 10 mM-2-oxoglutarate, 1 mM-EGTA, 1 μ M-rotenone, 1.5 mM-ADP, 0.17 mM-NADH and 0.1% (v/v) Triton X-100. Adenylate kinase (EC 2.7.4.3) was assayed in 50 mM-Tes, 5 mM-MgCl₂, 10 mM-glucose, 1 mM-ADP, 1 mM-NADP⁺, hexokinase (EC 2.7.1.1) (5 units/ml of incubation) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (5 units/ml of incubation), pH 7.6. (A unit of enzyme activity is that catalysing the transformation of 1 μ mol of substrate/min.) Cytochrome oxidase (EC 1.9.3.1) activity was assayed (Sottocasa *et al.*, 1967) in an oxygen-electrode chamber at 30°C in an incubation mixture containing 50 mM-potassium phosphate, 50 μ M-cytochrome *c*, 16 mM-ascorbate, 1 μ M-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 0.1% (v/v) Triton X-100, pH 7.5. Protein was determined by the biuret method (Gornall *et al.*, 1949).

Rotenone-insensitive NADH-cytochrome *c* reductase (EC 1.6.99.3) activity was assayed as previously described (Kuylenstierna *et al.*, 1970). Acetylcholinesterase (EC 3.1.1.7) activity was assayed by the method of Ellman *et al.* (1961).

Respiration was determined with a Clark-type oxygen electrode; the volume of the incubation chamber was 0.3 ml. The basic incubation medium for respiratory and radioisotopic experiments contained 75 mM-NaCl, 10 mM-Tes (sodium salt), 32 μ M-albumin (bovine fraction V from Sigma), 5 mM-sodium pyruvate and 5 mM-malate, pH 7.0, 30°C, with further additions as noted.

The components of the proton electrochemical potential gradient ($\Delta\bar{\mu}_{H^+}$) across the inner membrane were determined as previously described (Nicholls, 1974), with the modification that the volume of each sample taken for filtration was decreased from 400 to 100 μ l. Incubation mixtures (1 ml) contained 50 μ M-⁸⁶RbCl (0.05 μ Ci/ml), 100 μ M-[¹⁴C]methylamine (0.1 μ Ci/ml) and 100 μ M-[³H]acetate (1 μ Ci/ml). The sucrose-impermeable space of heart and brain 'free' mitochondria was determined as previously described (Heaton & Nicholls, 1976), in a medium containing 75 mM-NaCl, 10 mM-Tes and 32 μ M-albumin, pH 7.0, at 30°C. Values of 0.33 and 0.64 μ l/mg of protein respectively were found. Ca²⁺ transport was determined radioisotopically by using ⁴⁵Ca²⁺, with ³H₂O as an extra-matrix marker. Samples were filtered through Sartorius cellulose nitrate filters (0.6 μ m pore size) as previously described (Heaton & Nicholls, 1976).

Results

Non-synaptosomal (or 'free') mitochondria were prepared essentially by the method of Cotman & Matthews (1971). It was found that the densities of the Ficoll step-gradient had to be modified to optimize the yield and purity of the mitochondrial fraction when adapting this method for the preparation of mitochondria from guinea-pig cerebral cortex (see the Experimental section).

Fig. 1 is a histogram, plotted by the method of de Duve (1975), of specific activities and protein recoveries of fractions during the preparation of 'free' brain mitochondria. The distribution of cytochrome *c* oxidase activity (results not shown) essentially paralleled that of glutamate dehydrogenase, justifying the use of the latter in this tissue as a mitochondrial marker enzyme. With respect to the total homogenate, 20% of the cytochrome oxidase

Fig. 1. Preparation of 'free' mitochondria from homogenates of guinea-pig cerebral cortex

Enzyme specific activities and protein recoveries, determined as described in the Experimental section, are plotted by the method of de Duve (1975). LSP, 'low-speed' pellet; HSS, 'high-speed' supernatant; My, 'myelin' fraction of density gradient; Syn, 'synaptosomal' fraction of density gradient; Mi, 'mitochondrial' pellet of density gradient.

activity was recovered in the free mitochondrial fraction, and 35% in the 'synaptosomal' fraction. In contrast, the distribution of adenylate kinase activity (Fig. 1) most closely parallels that of lactate dehydrogenase, suggesting that in guinea-pig cortex this enzyme is predominantly located in the cytoplasm, thus vitiating its use as a marker of the mitochondrial intermembrane space. From the mean results for nine experiments, the recovery of glutamate dehydrogenase in the 'free' mitochondrial fraction was $25 \pm 10\%$ of the total homogenate activity, whereas the synaptosomal fraction accounted for a further $31 \pm 9\%$. The 'free' mitochondrial protein was 15 ± 2 mg per two guinea pigs (nine determinations).

Acetylcholinesterase activity was monitored as a measure of the microsomal and plasma-membrane content of the fractions. With respect to the activity in the crude mitochondrial pellet, $60 \pm 11\%$ was recovered in the synaptosomal fraction and only $2.6 \pm 0.3\%$ in the 'free' mitochondrial fraction, which is thus largely free of plasma membrane or microsomal contamination.

To isolate mitochondria from the synaptosomal fraction it is necessary to devise a means of selectively rupturing the synaptosomal membrane without at the same time damaging the mitochondrial outer, and more particularly the mitochondrial inner, membranes. Published methods for the isolation of

'synaptosomal' mitochondria from whole rat fore-brain rely on hypo-osmotic lysis of the synaptosomal fractions at high pH (Lai & Clark, 1976; Lai *et al.*, 1977). In view of the risk of some deterioration in the bioenergetic properties or ion content of the mitochondria during this prolonged hypo-osmotic treatment, an alternative method was used (see the Experimental section) that utilized selective lysis of the synaptosomal membrane by low concentrations of digitonin based on the method of Zuurendonk & Tager (1964) for the isolation of mitochondria from liver cells.

Digitonin-treated synaptosomes (see the Experimental section) were centrifuged on a Ficoll single-step gradient such that residual synaptosomes remained at the boundary, whereas released 'synaptosomal' mitochondria formed a pellet at the bottom of the tube. Results are shown in Fig. 2.

In the control (Fig. 2a), very little release of lactate dehydrogenase into the soluble fraction occurs, and such glutamate dehydrogenase activity as appears in the pellet is heavily contaminated with lactate dehydrogenase. Brief sonication (Fig. 2b) increases the release of lactate dehydrogenase into the soluble fraction, but again there is considerable contamination of the pellet. Similar results (not shown) were obtained after digitonin treatment in the absence of sonication. In contrast, digitonin together with brief

Fig. 2. Preparation of 'synaptosomal' mitochondria from the 'synaptosomal' fraction from guinea-pig cerebral cortex. The 'synaptosomal' fraction, obtained as described in the Experimental section, was subjected to various treatments: (a) 'control', vigorous vortex-mixing; (b) 'sonicated', vortex-mixing and brief sonication; (c) 'sonicated+digitonin', vortex-mixing and sonication after exposure to 0.15 mg of digitonin/mg of synaptosomal protein. After these treatments, suspensions were layered on Ficoll isolation medium (ρ 1.095 g/ml) (see the Experimental section) and centrifuged as described in the Experimental section. S, supernatant (solubilized activities); B, boundary (intact synaptosomes); P, pellet, 'synaptosomal' mitochondria. Sonication was for 10 s at a peak-to-peak amplitude of $6 \mu\text{m}$.

sonication of the synaptosomal suspension (Fig. 2c) results in almost complete release of lactate dehydrogenase, very few intact synaptosomes remaining at the boundary, and a pellet containing a high glutamate dehydrogenase specific activity with only marginal contamination with lactate dehydrogenase.

The mean recovery of glutamate dehydrogenase (six determinations) in the pellet was $16 \pm 3\%$ with respect to the homogenate, or $51 \pm 6\%$ of the activity of the synaptosomal fraction. Protein was $16 \pm 5\text{ mg}$ per two guinea pigs (six determinations).

The possibility that a proportion of the 'synaptosomal' mitochondria resulted from entrapment of 'free' mitochondria within the 'synaptosomal' layer of the first density gradient was examined, firstly by gentle resuspension of the synaptosomal layer and application of it to a second density gradient. Less than 15% of the glutamate dehydrogenase activity of the original synaptosomal layer was found in the new pellet. If rotenone-insensitive NADH-cytochrome *c* activity is taken as a marker of the mitochondrial outer membrane (Kuylenstierna *et al.*, 1970), the overt activity of this enzyme in the synaptosomal fraction is a measure of the extent of contamination with 'free' mitochondria. Specific activities of 2.8 and 44 nmol/min per mg of protein were found for the synaptosomal and 'free' mitochondrial fractions, values similar to those previously published (Lai *et al.*, 1977). However it must be borne in mind that the total protein content of the synaptosomal layer is much greater than that of the 'free' mitochondrial pellet (Fig. 1). Thus of the total overt rotenone-insensitive NADH-cytochrome *c* reductase activity, some 30–40% apparently resides in the 'synaptosomal' layer. If this activity is a reliable

marker for mitochondrial outer membranes, and in liver it is additionally located in the microsomal fraction (Kuylenstierna *et al.*, 1970), then this implies that of the 16 mg of protein of the 'synaptosomal' mitochondria, up to 6 mg could have originated as contaminating 'free' mitochondria. For this reason the fraction is strictly enriched in mitochondria of synaptosomal origin.

Acetylcholinesterase activity was recovered quantitatively after the digitonin treatment. $74 \pm 9\%$ of synaptosomal activity was found in the 'soluble' fraction (see legend to Fig. 2), $23 \pm 1.0\%$ was recovered at the boundary, and only $3.5 \pm 1.1\%$ pelleted with the mitochondria.

Although the dual localization of adenylate kinase described above prevents direct quantification of the intactness of the mitochondrial outer membrane by using this enzyme as a marker, a specific activity for the enzyme of 104 nmol/min per mg of protein in the pellet of 'synaptosomal' mitochondria compares favourably with a specific activity of 190 nmol/min per mg for 'free' mitochondria, and extends the observation of Zuurendonk & Tager (1974) that limited digitonin treatment can disrupt cytoplasmic membranes without extensively damaging the mitochondrial outer membrane. Corresponding specific activities for rotenone-insensitive NADH-cytochrome *c* reductase were respectively 44 and 33 nmol/min per mg of protein for 'free' and 'synaptosomal' mitochondria.

Respiration of 'free' and 'synaptosomal' mitochondria

Pyruvate and malate have been shown to be an effective substrate pair for 'free' and 'synaptosomal' mitochondria from rat forebrain (Clark & Nicklas,

Fig. 3. Respiratory rates in State 3 and during dissipative Ca^{2+} cycling in the presence of ionophore A23187. 'Free' brain mitochondria (FBM), 'synaptosomal' brain mitochondria (SBM) or rat heart mitochondria (RHM) (all 1 mg of protein/ml of incubation mixture) were incubated in the basic medium (see the Experimental section) with the addition of 1 mM- MgCl_2 for traces (a)–(d). In traces (a) and (b), 1 mM- P_i was additionally present. Additions: 0.3 mM-ADP; 0.6 μM -oligomycin (oligo); 0.4 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP); 45 μM - Ca^{2+} ; ionophore A23187 (A23187), 4.8 nmol/mg of protein; 1 mM-EGTA.

1970; Lai & Clark, 1976; Lai *et al.*, 1977). In Fig. 3 the equivalent guinea-pig cortical mitochondria are shown to have a low State-4 (Chance & Williams, 1956) respiration, indicative of a correspondingly low membrane proton conductance, and to demonstrate State-3 respiration close to the uncontrolled rates achieved with carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, indicating a high rate of ATP synthesis. Both preparations thus satisfy the classic criteria for 'tightly coupled' oxidative phosphorylation. Under the conditions of Figs. 3(a) and 3(b), with pyruvate and malate as substrates, the respiratory rate in State 3, that after oligomycin addition, and that after addition of proton translocator were respectively 55, 5 and 70 nmol of O/min per mg of mitochondrial protein for 'free' mitochondria and 50, 11 and 68 nmol of O/min per mg of mitochondrial protein for 'synaptosomal' mitochondria. With 2 mM-succinate as substrate the corresponding rates for the synaptosomal mitochondria were 50, 7.5 and 50 nmol of O/min per mg of mitochondrial protein.

Ionophore A23187 catalyses a $\text{Ca}^{2+}/2\text{H}^{+}$ exchange across the mitochondrial inner membrane (Reed & Lardy, 1972), and in concert with the native Ca^{2+} uniport, induces a cycling of Ca^{2+} across the membrane, driven by $\Delta\bar{\mu}_{\text{H}^{+}}$ (Heaton & Nicholls, 1976; Hutson *et al.*, 1976). When the ionophore is present in a sufficient excess, the rate of cycling is limited by the kinetics of the native uniport, and this provides a means for investigating the kinetics of the uniport under steady-state conditions (Heaton & Nicholls, 1976). Fig. 3 (traces *c* and *d*) shows that although the addition of Ca^{2+} to the 'free' or 'synaptosomal' brain mitochondria causes little stimulation of State-4 respiration, the subsequent addition of ionophore A23187 causes a large increase in respiratory rate. As the increased rate is completely sensitive to the addition of EGTA (Fig. 3), it is clear that the increased respiration is due to Ca^{2+} cycling, rather than to depletion of mitochondrial Mg^{2+} , particularly as the medium contains 1 mM- MgCl_2 . What is most significant is that the respiratory rates in the presence of Ca^{2+} plus ionophore A23187 are at least equal to those after the abolition of respiratory control by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Fig. 3). This implies that in the steady state the rate of Ca^{2+} transport by the native uniport is limited by the rate of respiratory-chain-dependent H^{+} extrusion, rather than by any property of the carrier itself.

This contrasts sharply with the behaviour of rat heart mitochondria under parallel conditions (Fig. 3e), where the respiratory stimulation caused by excess of ionophore A23187 and Ca^{2+} is far lower than that caused by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. As Mg^{2+} has been shown to inhibit Ca^{2+} uniport by heart mitochondria (Sordahl, 1974;

Crompton *et al.*, 1976c), Mg^{2+} was omitted in Fig. 3 (trace *e*). This result is therefore consistent with a rate of Ca^{2+} transport limited by the activity of the uniport rather than respiratory-chain-driven H^{+} extrusion.

As 'free' and 'synaptosomal' brain mitochondria appear to be similar in terms of the activity of their Ca^{2+} uniport, a detailed comparison was made between Ca^{2+} transport by 'free' brain and heart mitochondria.

The differential rates of Ca^{2+} transport indicated in Fig. 3 were confirmed by the classic direct technique of $^{45}\text{Ca}^{2+}$ uptake in the absence of ionophore A23187 (Fig. 4). Although the uptake of $^{45}\text{Ca}^{2+}$ into 'free' brain mitochondria was essentially complete before the first sample was taken, Ca^{2+} transport into heart mitochondria occurred at extrapolated initial rates of respectively 35 and 50 nmol of Ca^{2+} /min per mg of protein in the presence and absence of 1 mM- MgCl_2 .

Clearly, although $^{45}\text{Ca}^{2+}$ -uptake studies are a valid investigative method for heart mitochondria, little information can be obtained in this way from the 'free' brain mitochondria, which resemble liver mitochondria (Heaton & Nicholls, 1976) in that the activity of the respiratory chain limits the rate of Ca^{2+} transport (Fig. 3).

The driving force for Ca^{2+} uptake (the Ca^{2+} electrochemical potential difference $\Delta\bar{\mu}_{\text{Ca}^{2+}}$) via a uniport is given by eqn. (1):

$$\Delta\bar{\mu}_{\text{Ca}^{2+}} = 2\Delta E - 59 \log \left(\frac{[\text{Ca}^{2+}]_{\text{in}}}{[\text{Ca}^{2+}]_{\text{out}}} \right) \quad (1)$$

Fig. 4. Accumulation of $^{45}\text{Ca}^{2+}$ by rat heart mitochondria and guinea-pig 'free' brain mitochondria. Mitochondria (1 mg of protein/ml of incubation) were incubated in the basic medium with the addition of $^3\text{H}_2\text{O}$ (2 $\mu\text{Ci/ml}$): 50 μM - $^{45}\text{CaCl}_2$ (0.05 $\mu\text{Ci/ml}$ of incubation) was added after 1.5 min, and mitochondrial $^{45}\text{Ca}^{2+}$ measured after membrane filtration. 'Free' brain mitochondria, 1 mM- MgCl_2 present in incubation ($\circ$); heart mitochondria, no MgCl_2 ($\square$); heart mitochondria, 1 mM- MgCl_2 present ($\bullet$).

Fig. 5. Ca^{2+} uptake and its influence on the components of the proton electrochemical gradient of rat heart mitochondria and 'free' guinea-pig mitochondria in the presence and absence of ionophore A23187

For each experiment, mitochondria (1 mg of protein/ml of incubation mixture) were incubated in the basic medium with the addition of 1 mM-KCl and 0.5 μM -valinomycin. After 1.5 min incubation, 50 μM - CaCl_2 was added. For the study of Ca^{2+} uptake, $^3\text{H}_2\text{O}$ (1 $\mu\text{Ci}/\text{ml}$ of incubation mixture) was present, and $^{45}\text{CaCl}_2$ (0.05 $\mu\text{Ci}/\text{ml}$) was added at 1.5 min. For the study of the components of $\Delta\bar{\mu}_{\text{H}^+}$, isotopic indicators of ΔE and ΔpH were present as described in the Experimental section. $\square$, Ca^{2+} accumulated; $\triangle$, $\Delta\bar{\mu}_{\text{H}^+}$; $\bullet$, ΔE ; $\circ$, $-59\Delta\text{pH}$. (a) and (c), rat heart mitochondria; (b) and (d), 'free' brain mitochondria, 1 mM-MgCl₂ in incubation. In (c) and (d), ionophore A23187 (4.8 nmol/mg of mitochondrial protein) was added together with CaCl_2 .

As no information is available as to the magnitude of ΔE during Ca^{2+} uptake by either heart or 'free' brain mitochondria, the studies summarized in Fig. 5 were performed. Ca^{2+} uptake and the components of $\Delta\bar{\mu}_{\text{H}^+}$ were determined in parallel for heart and brain mitochondria: 1 mM-MgCl₂ was present in the incubation for brain mitochondria only. It should be noted that valinomycin, required for the determination of ΔE (Nicholls, 1974), is present.

The slow uptake of Ca^{2+} by heart mitochondria (Fig. 5a) causes little perturbation to ΔE , $-59\Delta\text{pH}$ or $\Delta\bar{\mu}_{\text{H}^+}$, ΔE being maintained close to 115 mV during the time course of the experiment. Apparently, compensatory ion movements across the inner membrane can occur to counteract the increased pH gradient generated by the net extrusion of H^+ by the respiratory chain during Ca^{2+} uptake.

The changes in ion gradients produced by the much faster uptake of Ca^{2+} by 'free' brain mitochondria (Fig. 5b) are more significant, there being a rapid decrease in ΔE as Ca^{2+} enters, and a corresponding increase in $-59\Delta\text{pH}$ as net H^+ extrusion occurs. It should be noted that Ca^{2+} uptake is essentially complete by the time of the first determination of the components of $\Delta\bar{\mu}_{\text{H}^+}$ and that in any case the time resolution of the isotope distribution is insufficient to follow such a rapid transient accurately. With time, compensatory ion movements occur, $-59\Delta\text{pH}$ decreasing and ΔE increasing back towards their initial values.

The influence of Ca^{2+} transport on $\Delta\bar{\mu}_{\text{H}^+}$ was also studied in the presence of excess ionophore A23187 (Figs. 5c and 5d). When the ionophore is present in sufficient excess, the Ca^{2+} distribution across the inner membrane is clamped at the value for electrochemical equilibrium of the ionophore (eqn. 2):

$$\frac{[\text{Ca}^{2+}]_{\text{in}}}{[\text{Ca}^{2+}]_{\text{out}}} = \frac{[\text{H}^+]_{\text{in}}^2}{[\text{H}^+]_{\text{out}}^2} \quad (2)$$

Fig. 5(c) confirms the interpretation of Fig. 3(e), that the activity of the native Ca^{2+} uniport of the heart mitochondria, rather than the respiratory chain, is

rate-limiting during the dissipative Ca^{2+} cycling. Thus only a slight decrease occurs in $\Delta\bar{\mu}_{\text{H}^+}$ when excess ionophore A23187 is present, the rate of Ca^{2+} cycling, and hence H^+ re-entry, being limited by the low activity of the native Ca^{2+} uniport (Heaton & Nicholls, 1976). It is striking, however, that simultaneous addition of Ca^{2+} and ionophore causes an increase in ΔE and a decrease in $-59\Delta\text{pH}$ owing to an efflux of endogenous Ca^{2+} or Mg^{2+} in exchange for H^+ . The effect of ionophore A23187 on the components of $\Delta\bar{\mu}_{\text{H}^+}$ is therefore to produce an electroneutral conversion of ΔpH into ΔE , and it is remarkable that the native Ca^{2+} uniport is so inactive that this effect of ionophore A23187 is still apparent under these conditions. As ΔpH remains negative (i.e. alkaline in the matrix), Ca^{2+} activity in the matrix should be lower than in the medium (eqn. 2), which is consistent with the negligible uptake under these conditions.

As dissipative Ca^{2+} cycling by 'free' brain mitochondria in the presence of ionophore A23187 is limited only by the rate of uncontrolled respiration (Fig. 3), this implies that $\Delta\bar{\mu}_{\text{H}^+}$ is lowered in these mitochondria below the value for respiratory control. Fig. 5(d) shows that under these conditions $\Delta\bar{\mu}_{\text{H}^+}$ is decreased from 190 to 60 mV, and that ΔpH reverses, becoming acidic in the matrix. This accounts (eqn. 2) for the significant concentration of $^{45}\text{Ca}^{2+}$ in the matrix (Fig. 5d). With time, $-59\Delta\text{pH}$ becomes less negative, and this is reflected in the decrease in matrix Ca^{2+} .

From the data of Fig. 5 and parallel respiratory traces (not shown) it is possible to calculate Ca^{2+} conductances for the inner membranes of heart and 'free' brain mitochondria (Table 1). Under the conditions of the experiment, $C_{\text{M}}\text{Ca}^{2+}$ values for 'free' brain mitochondria and heart mitochondria are respectively 4.1 and 0.2 nmol of Ca^{2+} /min per mg of protein per mV of $\Delta\bar{\mu}_{\text{Ca}^{2+}}$. Thus the brain mitochondria have a 20-fold greater conductance to Ca^{2+} .

Table 1. Apparent Ca^{2+} conductance of the inner membrane of rat heart mitochondria and guinea-pig 'free' brain mitochondria. The incubation conditions were exactly as described in Fig. 5; 45 μM -CaCl₂ was present, and ionophore A23187 (4.8 nmol/mg of protein) was added as indicated; 1 mM-MgCl₂ was present for brain mitochondria only. Respiration, and the components of $\Delta\bar{\mu}_{\text{H}^+}$, were determined after a preincubation of 1.5 min. The stoichiometry $\rightarrow\text{H}^+/\text{O}$ was taken to be 8 for pyruvate plus malate (Nicholls, 1977). $C_{\text{M}}\text{Ca}^{2+}$ was calculated as previously described (Heaton & Nicholls, 1976).

	Heart mitochondria		Brain mitochondria	
	Without ionophore A23187	With ionophore A23187	Without ionophore A23187	With ionophore A23187
Respiration (nmol of O/min per mg of protein)	20.9	38.2	37	135
$\Delta\bar{\mu}_{\text{H}^+}$ (mV)	190	180	190	60
$C_{\text{M}}\text{Ca}^{2+}$ (nmol of Ca^{2+} /min per mg of protein per mV)	—	0.20	—	4.11

Dependency of Ca^{2+} conductance on free Ca^{2+} concentration

Liver mitochondria show half-maximal Ca^{2+} conductance at 23°C when the external free Ca^{2+} activity is 4.7 μM , and, in addition, the dependency is clearly sigmoid (Heaton & Nichols, 1976). Such sigmoidicity has frequently been observed in initial-uptake-rate studies of a variety of mitochondria (Bygrave *et al.*, 1971; Scarpa & Graziotti, 1973; Vinogradov & Scarpa, 1973; Bygrave *et al.*, 1975; Reed & Bygrave, 1975). When $C_{\text{M}}\text{Ca}^{2+}$ is determined

Fig. 6. 'Free' brain mitochondria: effect of activity in the medium of Ca^{2+} on the Ca^{2+} conductance of the inner membrane

(a) Mitochondria (1 mg of protein/ml of incubation mixture) were incubated in the basic medium with the addition of 0.5 mM-KCl, 0.5 μM -valinomycin, 1 mM-MgCl₂ and 5 mM-nitrotriacetate, and the further addition of CaCl_2 to give free- Ca^{2+} concentrations in the range 0.3–14 μM , allowance being made for the endogenous Ca^{2+} content of the mitochondria, determined by atomic absorption to be 9.8 nmol/mg of protein. $C_{\text{M}}\text{Ca}^{2+}$ was determined from $C_{\text{M}}\text{H}^{+}$ in the presence and absence of ionophore A23187, exactly as previously described (Heaton & Nicholls, 1976), assuming a stoichiometry of $8 \rightarrow \text{H}^{+}/\text{O}$ for proton extrusion during the oxidation of pyruvate and malate (Nicholls, 1977). (b) $C_{\text{M}}\text{H}^{+}$ in the absence of ionophore A23187 as a function of free Ca^{2+} concentrations; conditions were as for Fig. 6(a).

for 'free' brain mitochondria at 30°C (Fig. 6a), hardly any sigmoidicity is apparent. Thus a $C_{\text{M}}\text{Ca}^{2+}$ of 0.5 nmol of Ca^{2+} /min per mg of protein per mV is attained by brain mitochondria when free Ca^{2+} is only 0.8 μM , whereas liver mitochondria, admittedly at 23°C, require 5 μM - Ca^{2+} to achieve the same conductance. Under the conditions of Fig. 6(a) the activity of Ca^{2+} was not increased sufficiently to saturate the conductance, and thus a concentration for half-maximal conductance could not be accurately calculated, although a value in the region of 4 μM is obtained by extrapolation. In contrast with liver mitochondria (Heaton & Nicholls, 1976), the effective H^{+} conductance did not increase as free Ca^{2+} was lowered (Fig. 6b).

Efflux of Ca^{2+} from 'free' brain mitochondria

When Ruthenium Red is added to heart mitochondria that have accumulated Ca^{2+} , there is an efflux of Ca^{2+} that depends on the presence of Na^{+} in the medium (Crompton *et al.*, 1976b), and this has been interpreted as being due to an antiport exchanging Ca^{2+} for Na^{+} , the exact stoichiometry being uncertain. As Fig. 7(a) shows, such an efflux occurs also with 'free' brain mitochondria, an efflux rate of 8 nmol of Ca^{2+} /min per mg of protein being reached under the conditions used. However, before such an efflux can be interpreted unambiguously as a Na^{+} -for- Ca^{2+} exchange, it is essential to establish that the mitochondria remain bioenergetically competent, and that Ca^{2+} loss is not due to mitochondrial damage.

To investigate this point, the components of $\Delta\bar{\mu}_{\text{H}^{+}}$ were monitored during the efflux of Ca^{2+} shown in Fig. 7(a). Results for heart and 'free' brain mitochondria are shown in Figs. 7(b) and 7(c) respectively. In both cases no decrease in $\Delta\bar{\mu}_{\text{H}^{+}}$ is associated with the Ca^{2+} efflux, confirming that Ca^{2+} loss is not due to mitochondrial damage. Indeed Ca^{2+} efflux is paralleled by a decrease in $-59\Delta\text{pH}$ but a compensatory increase in ΔE , the changes being most marked for the 'free' brain mitochondria. The efflux therefore causes alterations in the components of $\Delta\bar{\mu}_{\text{H}^{+}}$ that are the inverse of those occurring during Ca^{2+} uptake (Fig. 5). As befits the greater efflux rate from brain mitochondria, changes occurring with heart mitochondria are less pronounced.

The overall effect of Ruthenium-Red-induced Ca^{2+} efflux is therefore a Ca^{2+} -for- H^{+} exchange, the H^{+} entry being manifested in the decreased pH gradient across the inner membrane. With heart mitochondria, Na^{+} entry has been observed concomitant with Ca^{2+} efflux (Crompton *et al.*, 1976b). As the mitochondrial inner membrane has an active $\text{Na}^{+}/\text{H}^{+}$ exchange (Mitchell & Moyle, 1969; Douglas & Cockrell, 1974), these overall ion move-

Fig. 7. Efflux of Ca^{2+} from rat heart mitochondria and 'free' brain mitochondria in the presence of Ruthenium Red: influence on the components of the proton electrochemical potential gradient

Mitochondria (1 mg of protein/ml of incubation mixture) were incubated in the basic medium with the addition of 0.5 mM-KCl and 0.5 μM -valinomycin. After incubation for 1.5 min, 50 μM - CaCl_2 was added, and the mitochondria were allowed to accumulate Ca^{2+} for a further 2.25 min: 1 nmol of Ruthenium Red/mg of mitochondrial protein was then added. For the experiment with 'free' brain mitochondria, 1 mM- MgCl_2 was additionally present. $^{45}\text{CaCl}_2$ transport and the components of $\Delta\bar{\mu}_{\text{H}^+}$ were determined as described in the Experimental section. (a) Efflux of $^{45}\text{Ca}^{2+}$ from (□) 'free' brain or (■) heart mitochondria; (b) heart mitochondria and (c) 'free' brain mitochondria; (Δ) $\Delta\bar{\mu}_{\text{H}^+}$, (●) ΔE , (○) $-59\Delta\text{pH}$.

Fig. 8. Possible mechanism for the exchange of Ca^{2+} against H^+ and Na^+ across the mitochondrial inner membrane

- (a) $\text{Ca}^{2+}/n\text{H}^+$ exchange linked to Na^+/H^+ exchange;
(b) direct $\text{Ca}^{2+}/n\text{Na}^+$ exchange.

ments could be due to the combined effects of a Na^+/H^+ antiport with either a $\text{Ca}^{2+}/\text{H}^+$ or a $\text{Ca}^{2+}/\text{Na}^+$ antiport (Fig. 8), the stoichiometries of the hypothetical $\text{Ca}^{2+}/\text{H}^+$ or $\text{Ca}^{2+}/\text{Na}^+$ antiports being undefined. The Na^+ -dependence for Ca^{2+} efflux would be due in the case of a $\text{Ca}^{2+}/\text{H}^+$ antiport to the need to dissipate an excessive internal acidification by exchange against external Na^+ , K^+ being ineffective because of the much lower K^+/H^+ exchange activity (Douglas & Cockrell, 1974). The Na^+ -dependency for a $\text{Ca}^{2+}/\text{Na}^+$ exchange would, of course, be direct. To distinguish between these mechanisms for brain

mitochondrial Ca^{2+} efflux, the experiment depicted in Fig. 9 was performed. As shown previously for heart mitochondria, efflux of Ca^{2+} occurred in a Na^+ -based but not a K^+ -based medium (Crompton *et al.*, 1976b). However, when a high K^+/H^+ exchange activity was induced by the addition of nigericin, efflux of Ca^{2+} was still inhibited. This clearly demonstrates that the Na^+ requirement is not to initiate an electroneutral exchange of Na^+ against H^+ , and supports the proposal of a more direct $\text{Ca}^{2+}/\text{Na}^+$ exchange (Crompton *et al.*, 1976b).

Discussion

The preparations of 'free' and synaptosomally derived guinea-pig cerebral-cortical mitochondria described in the present paper display acceptable degrees of purity, as judged by enzymic assay (Figs. 1 and 2), and of bioenergetic integrity as judged by their low State-4 respiration and high rates of State-3 and uncontrolled respiration (Fig. 3). The use of brief sonication and low digitonin concentrations is clearly an effective method for the isolation of 'synaptosomal' mitochondria, and avoids exposure of released mitochondria to hypo-osmotic conditions (Lai & Clark, 1976; Lai *et al.*, 1977).

Studies of Ca^{2+} transport by brain mitochondria (Otsuka *et al.*, 1965; Yoshida *et al.*, 1966; Tower, 1968; Stahl & Swanson, 1969; Tijoe *et al.*, 1970; Carafoli & Lehninger, 1971; Diamond & Goldberg, 1971; Lazarewicz *et al.*, 1974; Vickers & Dowdall,

Fig. 9. Conditions for the efflux of Ca^{2+} from 'free' brain mitochondria in the presence of Ruthenium Red

Mitochondria (1 mg of protein/ml of incubation mixture) were incubated at 30°C and pH 7 in a medium containing 1 mM-Tes (potassium salt), $32\text{ }\mu\text{M}$ -albumin, 5 mM-potassium pyruvate, 5 mM-potassium malate, 1 mM- MgCl_2 and $2\text{ }\mu\text{Ci}$ of $^3\text{H}_2\text{O}$ /ml. Further additions were (□) 75 mM-NaCl, (●) 75 mM-KCl or (○) 75 mM-KCl plus $0.1\text{ }\mu\text{M}$ -nigericin. Then $50\text{ }\mu\text{M}$ - $^{45}\text{CaCl}_2$ ($0.05\text{ }\mu\text{Ci}/\text{ml}$) was added at 1.5 min, and 1 nmol of Ruthenium Red/mg of protein at 3.75 min.

1976) have largely centred on the so-called 'massive loading' conditions with uptake in excess of 500 nmol of Ca^{2+}/mg of protein in the presence of P_i . The physiological relevance of these conditions has, however, long been in question (Lehninger *et al.*, 1967), and the atractylate-insensitive effect of ATP (Tijoe *et al.*, 1970; Lazarewicz *et al.*, 1974) facilitating Ca^{2+} uptake by brain mitochondria is likely to be due to chelation of Ca^{2+} lowering the free cation concentration below values lethal for the mitochondria.

In the present paper a detailed comparison has been made between Ca^{2+} uptake and efflux by 'free' brain mitochondria and heart mitochondria. Heart mitochondria have been proposed to be capable of regulating the free- Ca^{2+} concentration in the sarcoplasm (Crompton *et al.*, 1976a), and the purpose of the present work is to establish to what extent a similar role can be ascribed to mitochondria from the brain. Although it is unlikely that appreci-

able Ca^{2+} transport across the mitochondrial inner membrane could occur during the 1 ms of the initiation of membrane depolarization and neurosecretion, it is feasible that mitochondria possessing a highly active Ca^{2+} -uptake mechanism may be capable of restoring initial Ca^{2+} concentrations in preparations for subsequent impulses.

The properties of the isolated 'free' brain mitochondria allow the possibility of such a role. Thus the mitochondria transport Ca^{2+} across the inner membrane at rates only limited by the ability of the respiratory chain to expel charge-compensating protons (Heaton & Nicholls, 1976). This contrasts with heart mitochondria, which under identical conditions have a 20-fold lower conductance to Ca^{2+} . It is most significant that a sigmoid dependency of Ca^{2+} transport on free- Ca^{2+} concentration, which appears to be a feature with many mitochondria (Bygrave *et al.*, 1971; Scarpa & Graziottini, 1973; Vinogradov & Scarpa, 1973; Bygrave *et al.*, 1975; Reed & Bygrave, 1975; Heaton & Nicholls, 1976), with the possible exception of mitochondria from smooth muscle (Wikström *et al.*, 1975), is not shown by 'free' brain mitochondria, which instead demonstrate a hyperbolic relationship (Fig. 6). As a consequence, 'free' brain mitochondria are much more capable of responding rapidly to low Ca^{2+} concentrations than are, for example, liver mitochondria, where little transport occurs of Ca^{2+} into the matrix if the external free- Ca^{2+} concentration is less than $2\text{ }\mu\text{M}$ (Reed & Bygrave, 1975; Heaton & Nicholls, 1976).

Na^{+} -dependent Ca^{2+} efflux from heart mitochondria has been proposed (Crompton *et al.*, 1976b) as a mechanism for the regulation of cytoplasmic Ca^{2+} concentrations in this tissue, a steady state being established between uptake by the Ca^{2+} uniport and efflux. As demonstrated in Figs. 7 and 9, 'free' brain mitochondria possess such a pathway. Uptake and efflux occur at similar rates in heart mitochondria (Figs. 5 and 7), whereas for 'free' brain mitochondria (Figs. 5 and 7), the initial rate of Ca^{2+} uptake greatly exceeds the initial efflux rate after addition of Ruthenium Red. The steady-state Ca^{2+} distribution would thus be expected to be less sensitive to changes in the activity of the Na^{+} -dependent Ca^{2+} efflux than for heart mitochondria.

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