Biochimica et Biophysica Acta, 591 (1980) 251—265

BBA 47867

CALCIUM UPTAKE BY TWO PREPARATIONS OF MITOCHONDRIA FROM HEART

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(Received September 18th, 1979)

Key words: Ca^{2+} uptake; Oxidative phosphorylation; (Cardiac mitochondria)

Summary

Ca^{2+} transport and respiratory characteristics of two preparations of cardiac mitochondria (Palmer, J.W., Tandler, B. and Hoppel, C.L. (1977) J. Biol. Chem. 252, 8731—8739) isolated using polytron homogenization (subsarcolemmal mitochondria) and limited Nagarse exposure (intermyofibrillar mitochondria) are described.

The Nagarse procedure yields mitochondria with 50% higher rates of oxidative phosphorylation than the polytron-prepared mitochondria in both rat and dog. Rat hear intermyofibrillar mitochondria contain 50% more cytochrome aa_3 than the polytron preparation, whereas in the dog, cytochrome aa_3 content is not significantly different. Cytochrome oxidase activities and cytochrome c, c_1 and b contents were comparable in both populations of rat and dog heart mitochondria.

The V of succinate-supported Ca^{2+} accumulation for Nagarse-prepared mitochondria from rat heart was 1.8-fold higher than the polytron-prepared mitochondria. In dog heart, the Nagarse preparation showed a 3.0-fold higher V for Ca^{2+} uptake compared to the polytron preparation. A lower apparent affinity for Ca^{2+} was demonstrated in the intermyofibrillar mitochondria for both species (K_m is 2—2.5-fold higher). The Hill coefficient was 1 both mitochondrial types. Subsarcolemmal mitochondria from both species were treated

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mops, 4-(*N*-morpholino)-propanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethyl ester)-*N,N'*-tetraacetic acid.

with Nagarse to determine the role of this treatment on the observed differences. Nagarse did not alter any kinetic parameter of Ca^{2+} uptake.

The properties of these mitochondria with reference to their presumed intracellular location may pertain to the role of mitochondria as an intracellular Ca^{2+} buffering mechanism in contractile tissue.

Introduction

The role of heart mitochondria in beat-to-beat Ca^{2+} cycling has been the subject of extensive debate. Whether mitochondria are able to supplement the activity of Ca^{2+} uptake and release by the sarcoplasmic reticulum poses a question of physiological interest. Although it is generally agreed that free cellular Ca^{2+} is in the range of 0.1—10 μM [1—3], compartmentalization of mitochondrial adjacent to membranous components of the cell may result in alterations in the concentration of ionized Ca^{2+} as well as Mg^{2+} [4], the latter cation being capable of markedly inhibiting Ca^{2+} uptake by heart mitochondria [5,6]. In the heart, mitochondria from beneath the sarcolemmal membrane may play a role in transmembrane Ca^{2+} fluxes, whereas the mitochondrial population associated with the myofibrillar elements could conceivably be involved in excitation-contraction coupling. A recent report describing the isolation of two such populations of mitochondria from heart [7] that appear to differ in both respiratory activity and various enzymatic activities provided a means to test the preceding hypothesis.

An analysis of Ca^{2+} uptake in two populations of mitochondria isolated from rat and canine heart has been carried out at high, mid-range and physiological Ca^{2+} concentrations. Differences in rates of uptake as well as in kinetic characteristics of uptake are described.

Materials and Methods

Materials. The chelometric dye, arsenazo III, was obtained from Sigma Chemical Co. and purified by DEAE-cellulose chromatography as described by Kendrick [8]. Using an atomic absorption Ca^{2+} standard, the K_d for the Ca^{2+}-dye complex was determined under the following assay conditions: 1.67 mM succinate, 5 μg rotenone, 73 mM KCl, 20 μM arsenazo (dye concentration determined at 655 nm, $E = 2.8 \cdot 10^4\ M^{-1}$), 1.67 mM potassium phosphate, pH 7.25, and 0.25 M sucrose, 10 mM Hepes, pH 7.25 in a final volume of 3 ml. In the presence of 73 mM KCl, at total final Ca^{2+} concentrations from 5 to 20 μM, the average K_d was calculated to be 10.8 ± 0.38 μM. Formation of the Ca^{2+}-dye complex was linear with respect to Ca^{2+} added up to 13 μM Ca^{2+} at 20 μM arsenazo III. Antipyrylazo III, recrystallized twice from 40% ethanol in H_2O at 50°C [9], was a gift of Dr. Eugene Barnes. Antipyrylazo (50 μM) was employed in a Ca^{2+} concentration range from 7.2 to 38.8 μM. A K_d value of 95 μM [9] was used to determine free Ca^{2+} present in the uptake medium. Tetramethylmurexide (tetramethyl purpurate) obtained from Calbiochem (San Diego, CA) or murexide (K and K Laboratories, Plainview, NY) were employed in final concentrations of 50 μM for uptake measurements over a

Ca^{2+} concentration range from 13.3 to 133 μM. Nagarse was purchased from Enzyme Development Corporation (New York, NY).

Mitochondrial isolation and assay. Mitochondria were isolated from rat hearts and from 5 to 8 g of dog left ventricle according to the procedure of Palmer et al. [7]. Heart tissue was trimmed to remove extraventricular muscle and placed in cold isolation medium A (220 mM mannitol, 70 mM sucrose, 5 mM Mops, pH 7.4). The tissue was rinsed, blotted dry and weighed. The hearts were finely minced and brought to a final concentration of 10 g tissue/100 ml of isolation medium A plus 2 mM EGTA and 0.2% bovine serum albumin. Rat heart tissue was homogenized with a Polytron tissue processor (Brinkman Instruments) for 20 s at a rheostat setting of 8. Dog ventricular tissue was homogenized with a Polytron for 25 s at a rheostat setting of 10. The homogenate was centrifuged at 500 × *g* in a Sorvall RC-2B (SS-34 rotor) for 10 min. The low-spin pellet was re-homogenized after re-suspension to the original volume in isolation medium A plus 2 mM EGTA and 0.2% bovine serum albumin and centrifuged at 500 × *g*. The two supernatants were combined and the mitochondrial pellet obtained by centrifugation at 3000 × *g* for 10 min. The polytron mitochondria were washed twice in isolation medium A in the absence of EGTA and suspended in this medium at a protein concentration of 25 mg/ml for calcium uptake studies. These mitochondria represent the mitochondria located between the intercalated disks and the sarcolemmal membrane and are termed subsarcolemmal mitochondria [7].

The washed low spin polytron pellet was resuspended in isolation medium B, containing 100 mM KCl, 50 mM Mops, pH 7.4, plus 2 mM EGTA and 0.2% bovine serum albumin. Nagarse was added to a final concentration of 5 mg/g wet weight tissue, directly in the Potter-Elvehjem homogenizing vessel. After one pass with the pestle, the homogenate was immediately diluted 2-fold with isolation medium B and centrifuged at 5000 × *g* for 5 min. The pellet was resuspended in the original volume of isolation medium B and sedimented at 500 × *g* for 10 min. The low spin pellet was washed twice with isolation medium B and the supernatants combined and centrifuged at 3000 × *g* for 10 min. The pellet was washed twice in isolation medium A in the absence of EGTA and resuspended in this medium at a final concentration of 25 mg/ml for calcium uptake studies. Nagarse treatment of the low speed polytron pellet has previously been shown to isolate mitochondria located between the myofibrils [7]. Therefore, these mitochondria represent the intermyofibrillar population. Protein was determined by the biuret procedure [10]. The protein yield for the subsarcolemmal mitochondria was 10 mg/g wet weight and 38 mg/g wet weight for the intermyofibrillar mitochondrial fraction. Respiratory parameters, using 7.5 mM glutamate-3.75 mM malate as substrate, were determined on a Yellow Springs Instruments Model 53 oxygen monitor (Yellow Springs, OH) at 30°C.

Cytochrome oxidase activities of rat and dog heart mitochondrial fractions were determined using the procedure of Schnaitman et al. [11]. Cytochrome oxidase was assayed polarographically at 30°C in a reaction mixture containing 75 mM potassium phosphate, pH 7.2, 3.75 mM sodium ascorbate, 300 μM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD), 0.5 mg mitochondria and 30—150 μM cytochrome *c* (horse heart Type III, Sigma

Biochemical Co.). Corrections for baseline oxygen consumption were made and results expressed as μmol/min/mg. Total cytochrome content of mitochondria prepared from rat and canine heart was estimated as described by Williams [12], on an Amino DW-2® spectrophotometer, using the split beam mode.

Toluene treatment of heart mitochondria was performed as described by Matlib and Srere [13]. Citrate synthase activities of intact and the toluene-treated mitochondria (in the presence of 9.5% polyethylene glycol) were followed as previously described [13]. Carnitine acyltransferase was determined on intact and toluene-treated mitochondria (8.5% polyethylene glycol) in the presence and absence of 0.04% Triton X-100 using the forward isotope exchange technique [14].

Electron microscopy. Heart mitochondria (500 μg protein) were fixed in suspension in 220 mM mannitol, 70 mM sucrose, 5 mM Mops, pH 7.4, for 30 min at 4°C with 1.5% glutaraldehyde. The mitochondria were pelleted in a Beckman microfuge and rinsed once with 0.05 M phosphate buffer, pH 7.35. The pellets were dislodged and placed in 0.05 M phosphate with two subsequent rinses in the same buffer. The pellets were post fixed in 2% osmium tetroxide in 0.05 M phosphate for 30 min, rinsed twice in water, and rapidly dehydrated through increasing concentrations of ethanol up to 100% ethanol. The pellets were rinsed two times in 100% ethanol and left in ethanol overnight at 4°C. The pellets were brought to room temperature and rinsed again in 100% ethanol. Pellet were oriented and embedded in Epon in flat silastic molds. Thin sections were cut on an ultramicrotome with a diamond knife so that the full thickness of the pellet was sampled. These sections were stained with a saturated solution of uranyl acetate in 50% ethanol and post stained with lead citrate and examined in a Philips 201 electron microscope at 80 kV.

Calcium uptake measurements. Calcium uptake by the mitochondria was followed on an Aminco-Chance® dual beam spectrophotometer and on an Aminco DW-2® stopped flow spectrophotometer for initial rates in a millisecond time span following Ca^{2+} injection. The initial rates of uptake determined over a free Ca^{2+} concentration range (see Materials) from 0.93 to 5.7 μM were no different at each Ca^{2+} concentration with either stopped flow or dual wavelength measurements in the presence of 20 μM arsenazo III as the Ca^{2+} indicator. The reaction was monitored using the wavelength pair 685—675 nm. For middle-range Ca^{2+} concentrations (7.2—38.8 μM), 50 μM Antipyrylazo III was employed at a wavelength pair of 642—600 nm. For measurements of Ca^{2+} uptake over a Ca^{2+} concentration range from 13 to 133 μM, 50 μM tetramethyl murexide was employed at 542-518 nm wavelength pair (or 50 μM murexide at 542—507 nm).

The composition of the uptake medium is described under Materials, and after a 3 min equilibration period, Ca^{2+} uptake was initiated by the addition of Ca^{2+}, in the presence of 2 mg mitochondrial protein at 30°C.

Results

The rates of phosphorylating respiration with glutamate-malate as respiratory substrate, in both rat and dog intermyofibrillar mitochondria, were 1.5

times greater than those observed with the subsarcolemmal mitochondria in agreement with published observations [7]. For rat heart subsarcolemmal and intermyofibrillar mitochondria, Q_{O_2} (state 3) values were 327 ± 37 and 488 ± 45 natoms oxygen/min/mg, respiratory control ratios were 5.9 ± 1.2 and 9.5 ± 1.4, and ADP/O ratios were 3.37 ± 0.28 and 3.19 ± 0.16, respectively. For dog heart subsarcolemmal and intermyofibrillar mitochondria, the Q_{O_2} values (state 3) were 250 ± 12.4 and 373 ± 20.2, respiratory control ratios were 4.3 ± 0.27 and 5.97 ± 0.19 and ADP/O ratios were 2.9 ± 0.147 and 3.08 ± 0.195, respectively.

In order to assure intactness and purity of the mitochondria preparations, electron microscopy was performed on the polytron and Nagarse-prepared mitochondria from dog heart (Figs. 1A and B). The mitochondria from both preparations were well-preserved and devoid of contaminating cytoplasmic material. Mitochondria from both populations were similar in morphology. Higher magnification of representative mitochondria from both groups demonstrated intact membranes, in agreement with the previous polarographic evidence of respiratory control and approximation of theoretical ADP to oxygen ratios.

Cytochrome oxidase activities of the two mitochondrial populations isolated from rat and dog heart were determined. At cytochrome *c* concentrations of 100—150 μM, no significant differences between rat or dog heart subsarcolemmal and intermyofibrillar mitochondrial activities were observed, i.e., 0.832 ± 0.043 μmol/min/mg and 0.886 ± 0.046 μmol/min/mg, respectively (also, Palmer, J.W., personal communication).

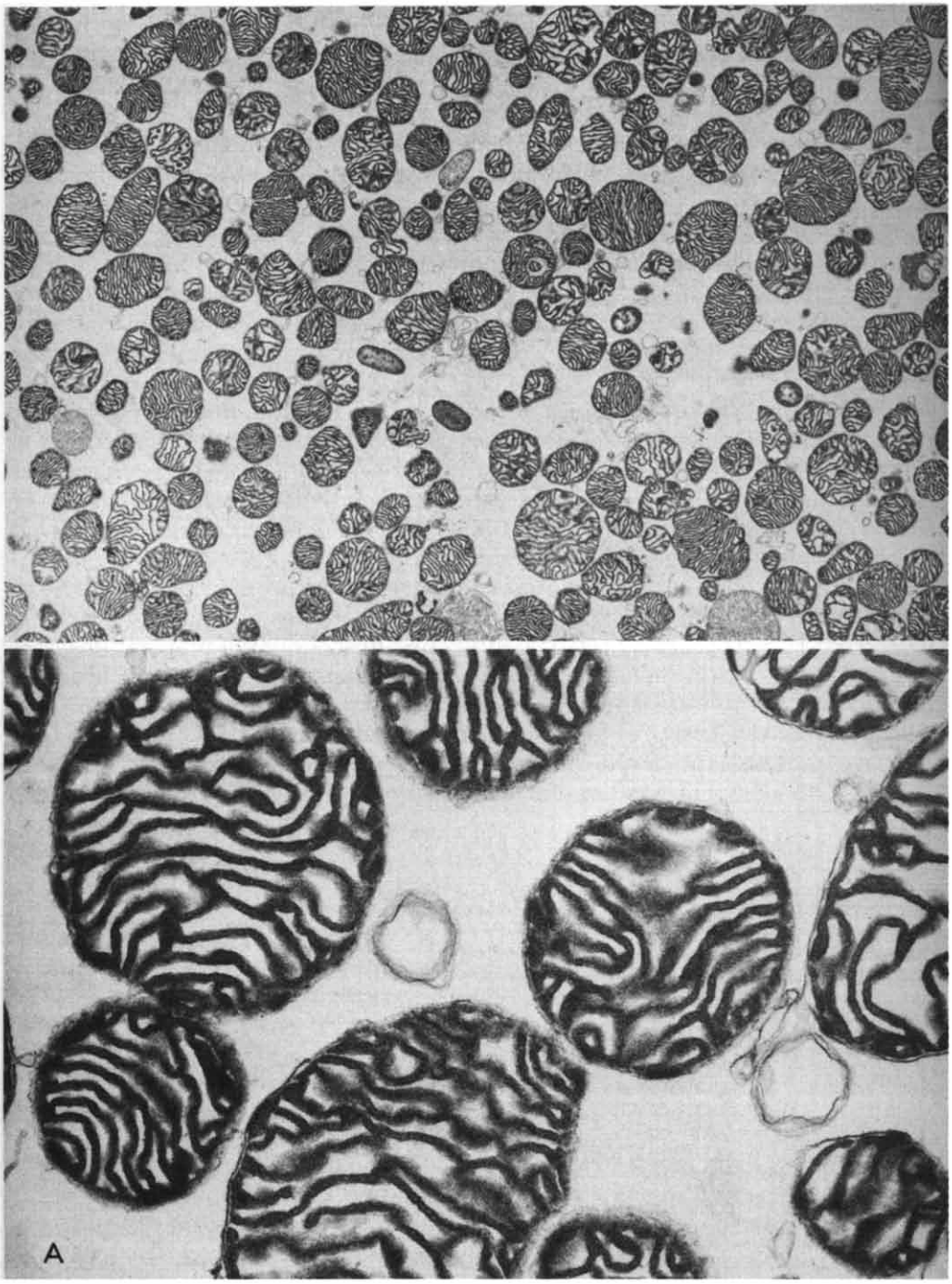
We wished to determine if depletion of a cytochrome component, e.g., cytochrome *c*, might be responsible for the differences in respiratory activity observed. The cytochrome content of isolated mitochondrial preparations from rat and dog heart is shown in Table I. No differences between cytochrome *c*, c_1 and *b* content were apparent in the rat heart mitochondrial preparations. However, in rat heart, cytochrome aa_3 content was 1.5-fold higher in the Nagarse preparation, despite similarities in cytochrome oxidase activities. In contrast, the cytochrome content of mitochondria isolated from canine heart

TABLE I

CYTOCHROME CONTENT OF RAT AND DOG HEART MITOCHONDRIA

Cytochrome	Cytochrome content (nmol/mg)	
	Polytron cytochrome	Nagarse preparation
I. Rat heart		
c	0.614 ± 0.004	0.539 ± 0.019
c_1	0.187 ± 0.009	0.194 ± 0.034
b	0.629 ± 0.038	0.635 ± 0.065
aa_3	0.670 ± 0.034	1.035 ± 0.152
II. Dog heart		
c	0.302 ± 0.047	0.318 ± 0.026
c_1	0.092 ± 0.011	0.108 ± 0.011
b	0.43 ± 0.049	0.428 ± 0.032
aa_3	0.496 ± 0.063	0.548 ± 0.046

was not statistically different between the Nagarse and polytron preparations. Thus, despite the apparently similar cytochrome contents between the two mitochondrial populations from dog heart, the intermyofibrillar mitochondria



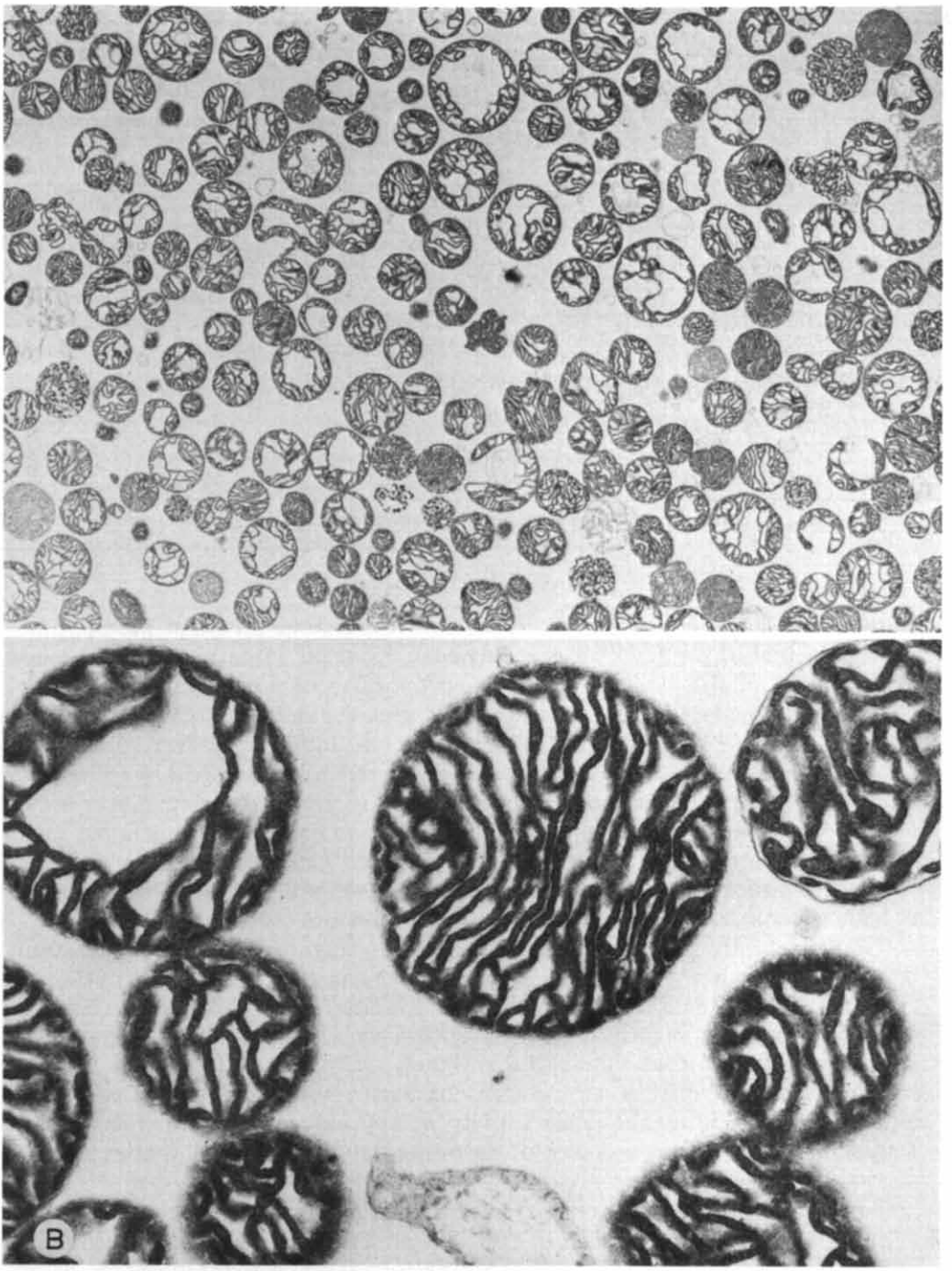


Fig. 1. Electron micrographs of isolated dog cardiac mitochondria. A. Polytron-prepared mitochondria: upper figure = 2.0 X 3000 magnification; lower figure = 2.0 X 20 000 magnification. B. Nagarse-prepared mitochondria: upper figure = 2.0 X 3000 magnification; lower figure = 2.0 X 20 000 magnification.

TABLE II

CARNITINE ACYLTRANSFERASE AND CITRATE SYNTHASE ACTIVITIES IN TWO PREPARATIONS OF RAT HEART MITOCHONDRIA

Carnitine acyltransferase activity was determined in the presence of 48 μM palmitoyl-CoA, 1.6 mM [^{3}H]-carnitine (1570 dpm/nmol) and 0.2 mg mitochondria after 2 min at 30°C. Citrate synthase activity was determined in an assay medium containing 100 μM 5,5′-dithio-bis-(2-nitrobenzoic acid), 250 μM acetyl-CoA, 500 μM oxaloacetate and 0.3 to 0.5 mg mitochondria in 1 ml. The reaction was started with oxaloacetate after obtaining a rate for acetyl-CoA hydrolase activity.

	Activity	
	Polytron preparation	Nagarse preparation
A. Carnitine acyltransferase (nmol palmitoylcarnitine/mg)		
Untreated mitochondria	4.4	3.6
Toluene-treated mitochondria + 8.5% poly(ethylene glycol)	8.9	8.0
Mitochondria + 0.04% Triton X-100	—	8.5
Toluene-treated mitochondria + 8.5% poly(ethylene glycol) + 0.04% Triton X-100	—	9.1
B. Citrate synthase (nmol citrate/min/mg)		
Untreated mitochondria	9.3	9.8
Toluene-treated mitochondria + 8.5% poly(ethylene glycol)	53.4	79.9

demonstrated higher respiratory rates than the subsarcolemmal preparations. Since kinetics of electron transfer has been reported to be related to the rate of activation of the carriers and to the proportion of nonactivated respiratory chain components [15], it is not likely that the total cytochrome content reflects a rate-limiting factor in oxygen consumption. Rather, these data suggest similar degrees of purity of the isolated mitochondrial preparations. Since the intermyofibrillar mitochondria were isolated using Nagarse, we examined the effect of Nagarse on respiratory rates of subsarcolemmal mitochondria. Treatment of polytron (subsarcolemmal) mitochondria with Nagarse (25 μg/mg protein, assuming 20% protein/gm wet weight heart) did not result in higher respiratory rates, in agreement with previous studies [7].

Toluene treatment of isolated mitochondria provides a means of measuring latent enzyme activity in the normal environment and concentration of enzymes in the inner membrane-matrix fraction [13]. After correction for acetyl-CoA hydrolase activity, citrate synthase activities were 50% higher in the intermyofibrillar mitochondria (Table II), cf., Palmer et al. [7]. Since toluene-treatment was effective in exposing latent citrate synthase, it was subsequently employed to examine the total activity of the external and inner membrane carnitine acyltransferase. Assay of carnitine acyltransferase activities demonstrated similar equilibrium concentrations of acylcarnitine formed in the intact and toluene-treated mitochondrial populations (Table II). To assure that total activity was expressed, Triton X-100 was added to the treated mitochondria and no further increment in acylcarnitine formation was observed. No differences in palmitoylcarnitine formation was observed. No differences in carnitine acyltransferase activities have been found previously between the two mitochondrial populations [7]. In agreement with the data above, the intermyofibrillar mitochondria oxidize 20 μM palmitoylcarnitine 1.5 times faster (280

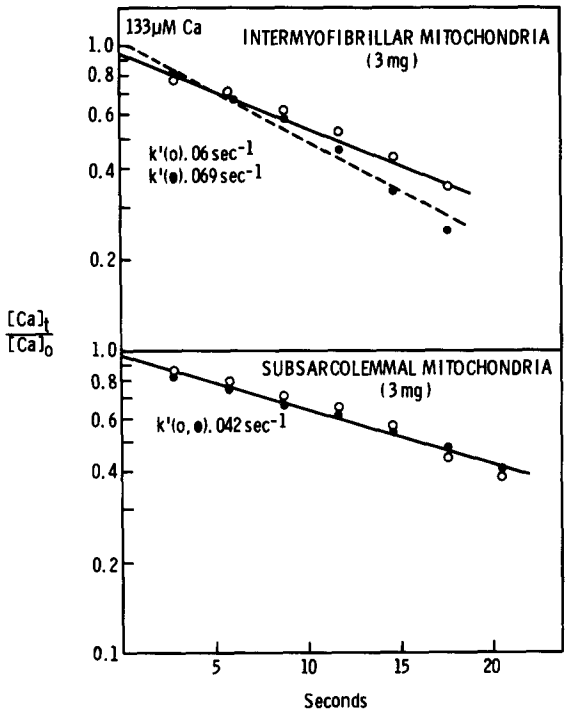


Fig. 2. Effect of Nagarse treatment of isolated rat heart mitochondria on Ca^{2+} uptake. Respiration-dependent Ca^{2+} disappearance from the standard assay medium, pH 7.25, containing 1.67 mM phosphate was measured in the presence of 50 μM murexide after addition of 133 μM Ca^{2+}. $[Ca]t/[Ca]_0$; Ca^{2+} concentration at time t: Ca^{2+} concentration at zero time. Control, isolated mitochondria (○); treatment of isolated mitochondria with 25 μg Nagarse/mg (●).

natoms oxygen/min/mg) than the subsarcolemmal population (187 natoms/min/mg).

These results demonstrate that the two mitochondrial populations are well-coupled and intact and not depleted of cytochrome components. Their enzymatic characteristics are reproducible as well, so that these preparations are suitable for studies on Ca^{2+} uptake.

With murexide as the Ca^{2+} indicator, overall rates of Ca^{2+} disappearance from the assay medium were measured (Fig. 2). At 133 μM Ca^{2+} added, the apparent first order rate constant for Ca^{2+} uptake was 50% higher in the intermyofibrillar mitochondria (0.060 s^{-1}) than in the subsarcolemmal mitochondria (0.042 s^{-1}). In order to assure that preparation of intermyofibrillar mitochondria using Nagarse did not alter the response of the organelles to Ca^{2+}, the resuspended mitochondrial pellets from both populations were treated with 25 μg Nagarse/mg protein followed by 2-fold dilution and immediate centrifugation as described in Methods. No significant effect on the apparent first order rates of Ca^{2+} disappearance was observed in either mitochondrial population treated with the enzyme (Fig. 2).

We wished to be certain that the changes in Ca^{2+}-dye absorbance can be shown to be a direct consequence of energy-dependent Ca^{2+} uptake by the mitochondria. Preincubation of mitochondria with antimycin A and rotenone

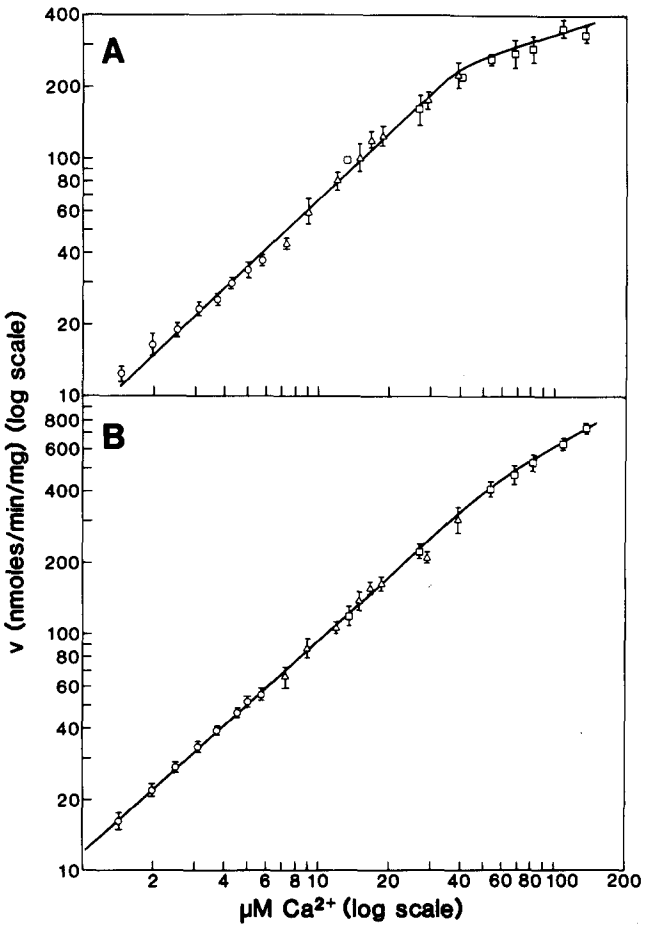


Fig. 3. Initial rates of Ca^{2+} uptake in dog heart mitochondria. Succinate-supported Ca^{2+} uptake was measured as described in Materials and Methods. A. Polytron-prepared mitochondria. B. Nagarse-prepared mitochondria. ○, Arsenazo III; △, antipyrylazo; □, tetramethylmurexide. Each point represents the average of 5 to 10 separate experiments ± S.E.M. The abscissa represents the calculated initial free Ca^{2+} concentration.

was carried out for 3 min at 30°C. After addition of 13.3 μM total Ca^{2+}, no change in the dye complex absorbance over time was evident when compared to 13.3 μM Ca^{2+} added to mitochondria under Ca^{2+} uptake conditions. Thus, the rate of change in the Ca^{2+}: arsenazo absorbance using dual beam spectrophotometry does not appear to be a consequence of passive Ca^{2+} binding to the mitochondrial membranes. In addition, at these wavelengths (685—675 nm), mitochondrial swelling (measured in the absence of arsenazo) does not contribute to the absorbance changes observed under Ca^{2+} uptake conditions.

Initial velocities of Ca^{2+} uptake by canine heart mitochondria were followed over a wide range of Ca^{2+} concentrations using the dye systems arsenazo, antipyrylazo and tetramethylmurexide (Fig. 3A and 3B). A linear slope of zero order Ca^{2+} uptake versus Ca^{2+} concentration was observed for both the polytron and Nagarse preparations, irrespective of the indicator dye employed. Hill

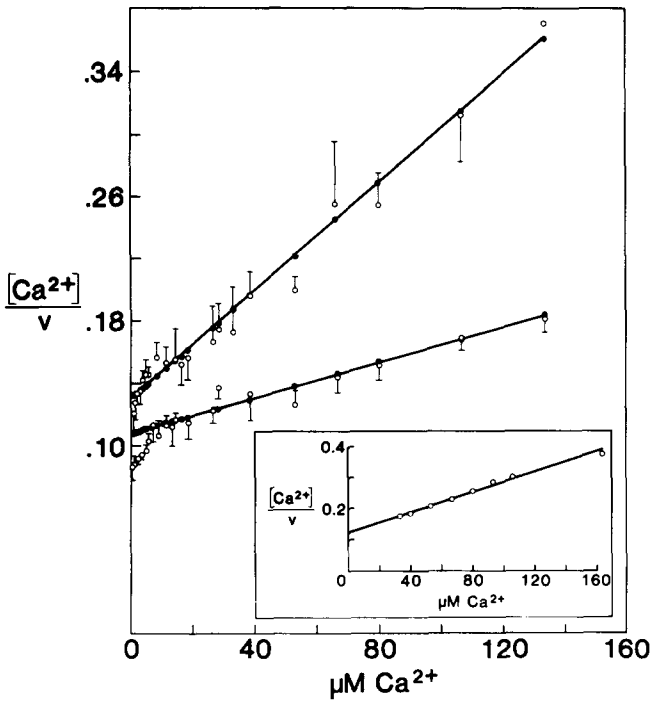


Fig. 4. Mitochondria Ca^{2+} uptake kinetics. A Hanes-Woolf plot of the data in Fig. 3 was used to derive kinetic constants. The line shown was obtained by reiterative analysis of the data [16]. Polytron-prepared mitochondria: upper trace. Nagarse-prepared mitochondria: lower trace. Inset: Hanes-Woolf plot of Ca^{2+} uptake rates from polytron mitochondria exposed to Nagarse (25 μg/mg mitochondrial protein).

plots of the velocity data were derived from Fig. 3 (0.93—133.3 μM Ca^{2+}). The slopes of the lines and the K_{app} values for Ca^{2+} uptake for intermyofibrillar and subsarcolemmal mitochondria, respectively, are 0.953 ($r = 0.999$) and 1.002 ($r = 0.996$); 190.5 μM and 72.4 μM. No cooperativity in Ca^{2+} uptake can be seen at any Ca^{2+} concentration or with either mitochondrial preparation.

Derivation of the kinetic constants, K_m and V, for mitochondrial Ca^{2+} uptake is shown on the Hanes-Woolf plot in Fig. 4. The slopes of the lines were obtained by reiterative analysis [16]. The V for subsarcolemmal mitochondria, obtained from the slope of the line ($r = 1.00$) is 577 nmol/min/mg. The K_m (from the y intercept, i.e. K_m/V) is 74.6 μM for the polytron preparation. Similarly, the V derived for the intermyofibrillar mitochondria is 1750 nmol/min/mg and the K_m for Ca^{2+} is 189 μM. The presence of a low K_m process for the intermyofibrillar mitochondria at Ca^{2+} concentrations ranging from 0.93 to 9.0 μM is suggested by a significant deviation from linearity in the low Ca^{2+} portion of the kinetic plot. After five reiterative analyses [16], the V of this portion of the slope was determined to be 11.5 nmol/min/mg with a K_m of 3.7 μM. However, it should be pointed out that at such low Ca^{2+} concentrations, an error of 0.5 nmol Ca^{2+} in the $[Ca^{2+}]/v$ term could lead to a similar slope deviation so that the current methods cannot ascertain the presence of such a component.

As we did for the Ca^{2+} uptake experiments in Fig. 2, it was important to assure that Nagarse treatment per se was not responsible for the higher K_m observed for the intermyofibrillar mitochondria. Therefore, we investigated the effect of direct Nagarse treatment (25 μg/mg protein) on the polytron (subsarcolemmal) mitochondria as shown in the inset to Fig. 4. Nagarse did not alter the kinetic parameters for Ca^{2+} uptake in the subsarcolemmal mitochondria (K_m = 78 μM, V = 619 nmol/min/mg). To examine the possibility that tissue cytosolic enzymes contribute to a possible modification of the polytron mitochondria, Nagarse (5 mg/g wet weight) was added directly to the 500 × g supernatant fraction following polytron homogenization. Polytron mitochondria subsequently isolated from this fraction demonstrated near theoretical Ca^{2+} uptake rates from 26.7 to 66.7 μM Ca^{2+} added (succinate-supported). In addition, state 3 respiration was also in the range reported for the control subsarcolemmal mitochondrial isolation. However, when the 500 × g supernatant fraction was allowed to incubate with Nagarse for 5 min at 4°C, rates of Ca^{2+} uptake were depressed from 25 to 50%, with Ca^{2+} release observed at 80 nmol Ca^{2+} added per mg mitochondria. These results suggest that mild treatment of the polytron mitochondrial supernatant with Nagarse does not effect the Ca^{2+} uptake kinetics observed. However, a more prolonged exposure with Nagarse may have significant deleterious effects on the capacity of the polytron mitochondria for Ca^{2+} accumulation.

Other experiments on rat heart mitochondria (data not shown) yielded results similar to those seen with the canine heart mitochondrial preparations. For the intermyofibrillar and subsarcolemmal mitochondria from rat heart, the kinetic constants were respectively: V = 833 nmol/mg/mg, K_m = 102 μM and V = 455 nmol/min/mg, K_m = 50 μM. Again the subsarcolemmal mitochondrial values were not affected by Nagarse treatment.

Since it has been reported previously that heart mitochondrial Ca^{2+} uptake is not limited by respiratory chain activity [17], we examined Ca^{2+} uptake by the polytron and Nagarse mitochondria prepared from dog heart in the presence of 1 mM MgATP as the energy source for Ca^{2+} uptake. At a final total Ca^{2+} concentration of 66 μM (tetramethylmurexide as indicator dye), ATP-dependent Ca^{2+} uptake by the polytron mitochondria was significantly lower than the Nagarse mitochondria. Thus, although conditions for assay of calcium uptake were markedly different (i.e., presence of magnesium and ATP both of which alter mitochondrial calcium uptake characteristics) the differences between mitochondrial populations persist. Therefore, the differences in Ca^{2+} uptake between the two preparations is not a simple function of respiratory differences.

We also considered the possibility that Ca^{2+} recycling, i.e. an efflux component, may limit Ca^{2+} uptake in the Polytron preparation. A cycle of efflux and reuptake would be detected as a prolonged stimulation of oxygen consumption following the initial calcium uptake burst. Therefore, oxygen consumption rates by the two mitochondrial preparations were compared before, during and following Ca^{2+} uptake (80 nmol Ca^{2+}/mg mitochondria) in a succinate-supported uptake medium. Ca^{2+} uptake by the polytron and Nagarse mitochondria was accompanied by augmentation of oxygen consumption rates of 151 and 181 natoms/min/mg, respectively, again indicating that respiratory

activity is not rate-limiting (compare to ADP-stimulated rates) under Ca^{2+} uptake conditions. The subsequent respiratory rates following Ca^{2+} uptake (presumably due to calcium cycling) were 16% (Nagarse mitochondria) and 11% (Polytron mitochondria) higher than the rate of oxygen consumption prior to Ca^{2+} addition. Therefore, our results cannot be explained by increased calcium cycling.

Discussion

The existence of two different types of mitochondria in skeletal muscle has been proposed. Müller [18] has suggested that the subsarcolemmal mitochondria and intermyofibrillar mitochondria have different functions (reflecting their comparmentation in the cell) which can be distinguished both morphologically and biochemically. A successful biochemical isolation of these mitochondrial types should reflect the morphological differences observed.

Hülsmann et al. [19] reported isolation of two types of mitochondria from skeletal muscle following loose and tight homogenization with a Potter-Elvehjem homogenizer. Using a combination of polytron homogenization and Nagarse treatment, their findings were subsequently extended to cardiac muscle [20]. Palmer et al. [7] have characterized the cellular origin and biochemical properties of the polytron and Nagarse-prepared mitochondria from rat heart. From their electron microscopic evidence, it was suggested that mild polytron homogenization releases mitochondria from below the sarcolemmal membrane, whereas Nagarse specifically isolates mitochondria associated with the myofibrils.

The biochemical characteristics of muscle mitochondria prepared using the polytron and Nagarse procedures appear to correlate well with information obtained at the ultrastructural level. In agreement with morphometric studies on triiodothyronine-treated rats where the volume density of subsarcolemmal mitochondria increases approximately 2-fold over that of the myofibrillar mitochondria [21], a doubling in the yield of polytron-isolated mitochondria from hyperthyroid rat heart was observed [20]. We have demonstrated increases and decreases in state 3 respiration of skeletal muscle subsarcolemmal mitochondria in response to endurance training and disuse, respectively [22]. These data support morphological evidence of adaptation of subsarcolemmal mitochondria to alterations in the levels of contractile activity [23].

Our mitochondrial preparations from both rat and canine heart isolated according to the method of Palmer et al. [7], demonstrate structural and functional integrity with respiration and enzymatic activities as well as cytochrome content in the ranges reported [7]. Rat heart mitochondria isolated using Nagarse contain 50% more cytochrome aa_3 than the polytron mitochondria, although cytochrome oxidase activities were not significantly different between the two preparations. These results support the contention that rates of electron transfer are related to activation of the respiratory carriers [15] and demonstrate that the degree of purity of the isolated mitochondrial fractions is similar with respect to cytochrome components.

Rates of respiration-supported Ca^{2+} uptake for both mitochondrial preparations were determined over a wide range of Ca^{2+} concentrations. The V of Ca^{2+}

uptake for the Nagarse preparation was 3-fold greater compared to the polytron-prepared mitochondria (1750 versus 577 nmol/min/mg). Although the K_m values for Ca^{2+} uptake are three times (polytron mitochondrial) to an order of magnitude higher (Nagarse mitochondria) than values for heart mitochondria reported using other techniques of measurement [24,25], the K_m values reported there are similar to those observed using murexide as the metallochrome indicator dye [26]. The close linear correlation between the initial velocities of Ca^{2+} uptake as a function of Ca^{2+} concentration irrespective of the dye employed, as well as the derived Hill coefficient, suggests that only one process is responsible for Ca^{2+} accumulation in both mitochondrial types. The deviation from linearity at low Ca^{2+} concentrations using the Woolf-Hanes plot may be attributed to a small degree of uncertainty in free Ca^{2+} concentration that would effect the kinetic plot more significantly in the low Ca^{2+} range than at high Ca^{2+} concentrations.

The differences in Ca^{2+} uptake velocities between the subsarcolemmal and intermyofibrillar mitochondria could reflect differences in the membrane potential, concentrations of a Ca^{2+} channel protein [27,28], or the mitochondrial Ca^{2+} uniport mechanism [17]. A deleterious effect of Nagarse on mitochondrial Ca^{2+} uptake has been shown by others [26]. In contrast with these latter studies, our milder Nagarse preparation had higher rates of Ca^{2+} accumulation as well as higher oxygen consumption and fatty acid oxidation. It is still possible that the apparent lower affinity of these mitochondria for Ca^{2+} could be due to partial proteolysis of a Ca^{2+} binding site. The lack of effect of Nagarse on Ca^{2+} kinetic parameters of the polytron-prepared subsarcolemmal mitochondria, i.e., no significant increases in either V or K_m for Ca^{2+}, or in first-order rates of uptake would tend to argue against this explanation and suggests that the differences observed derive from biological differences between the two mitochondrial populations.

As yet, the significance of the differences observed by ourselves and others between the two classes of mitochondria found in cardiac [7,20] and skeletal [19,22] muscle is uncertain. The presence of a population of mitochondria with greater respiratory capacity and Ca^{2+} uptake capacity in the myofibrillar space where ATP consumption is most prominent is intriguing. In addition, while respiration-supported Ca^{2+} transport may not be of the order of affinity to be important in beat-to-beat control of excitation-contraction coupling, it may provide an efficient 'Ca^{2+} buffer' mechanism within the muscle to prevent Ca^{2+} overload syndromes as described in ischemia [29] and in catecholamine-induced myopathy [30]. In regard to the former situation, our recent data [31] describing the sensitivity of Ca^{2+} transport in these two mitochondrial populations to palmitoyl-CoA and palmitoyl-carnitine (which are markedly augmented during ischemia [32]) suggest that this physiologic function may be impaired early in the ischemic process.

Further suggestion of the physiological relevance of this compartmentation of mitochondria is provided in skeletal muscle where subsarcolemmal mitochondria were found to respond preferentially to endurance training and to disuse atrophy [23].

Thus, studies on Ca^{2+} uptake using two different preparations of muscle mitochondria may be significant not only for procedural considerations, but

also because of the proposed cellular origin of these mitochondrial types. The ability of mitochondria to act as a Ca^{2+} buffer mechanism in contractile tissue could depend on the relative rates of Ca^{2+} accumulation, and on differences in cellular compartmentation as well.

Acknowledgement

This material was developed by the Section of Cardiovascular Sciences (Myocardial Biology) of the National Heart and Blood Vessel Research and Demonstration Center, Baylor College and Medicine, a grant supported project of the National Heart, Lung and Blood Institute, National Institutes of Health, Grant No. HL 17269 and Grant HL 23,161 and HL 13870. Charlotte A. Tate was a post-doctoral trainee of HL 07282, the Cardiovascular Sciences Training Grant. Paul E. Wolkowicz and Alice Chu are graduate fellows in the Department of Biochemistry, Baylor College of Medicine.

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