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Isolation of Rat Adrenocortical Mitochondria

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Abstract

This report describes a relatively simple and reliable method for isolating adrenocortical mitochondria from rats in good, reasonably pure yield. These organelles, which heretofore have been unobtainable in isolated form from small laboratory animals, are now readily accessible. A high degree of mitochondrial purity is shown by the electron micrographs, as well as the structural integrity of each mitochondrion. That these organelles have retained their functional integrity is shown by their high respiratory control ratios. In general, the biochemical performance of these adrenal cortical mitochondria closely mirrors that of typical hepatic or cardiac mitochondria.

Keywords

Adrenal Cortex; Mitochondria; Isolation; Oxidative Phosphorylation; Rat

Introduction

Most biochemical data on adrenocortical mitochondria are based on organelles derived from domesticated animals, such as bovines [1-4], pigs [5], and sheep [6,7]. The use of these large animals almost always precludes endocrinological experimentation because of the required

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Author Contributions

Paola Solinas participated in the design and carried out the biochemical aspects of the experiment and was involved in the writing; Hisashi Fujioka performed the electron microscopy and participated in the writing; Bernard Tandler interpreted the electron micrographs and performed a major share of the writing; Charles L. Hoppel participated in the design of the experiment, interpreted the biochemical data, and was heavily involved in the writing.

volume of reactive agents and concomitant expenses involved in maintenance and clinical observation of such animals. Because of their small size, it would be desirable to use rats as a source of adrenocortical mitochondria, but isolation of these organelles from the rat adrenal cortex has proven an elusive goal due to the diminutive volume of the adrenal cortex in these animals. As a result, most biochemical studies of rat adrenocortical mitochondria have been based on tissue slices [8] or cultured cells [9], or cultured mouse adrenal tumor cells [10]. Although much has been learned from these preparations, the presence of *in situ* confounding factors in the former cannot easily be gainsaid. This report describes a relatively simple and reliable method for isolating rat adrenocortical mitochondria in good, reasonably pure yield. This method also has applicability to sundry, very small tissue specimens from which it is desired to isolate mitochondria.

Materials and Methods

Animal studies were approved by the Institutional Animal Care and Use Committees of Case Western Reserve University School of Medicine. Fischer 344 rats (6 month-old male) from the NIA colony at Harlan were used. They were housed in a temperature- and humidity controlled animal facility and had free access to food and water. On the day of the experiments, the rats were weighed (weight = 399 ± 21 g; mean ± SD; N=5) and killed by decapitation between 8 and 9 AM. After exsanguination, their adrenal glands were quickly dissected free of surrounding adipose tissue and excised.

Isolation of adrenocortical mitochondria

Each individual experiment made use of the paired adrenal glands from a single rat. In each case, the two extirpated glands were rinsed in ice-cold MSM medium (220 mM mannitol, 70 mM sucrose, 10 mM MOPS buffer, pH 7.4) (5 ml of MSM per gram of adrenal gland), then dried on filter paper and weighed (weight = 52 ± 9 mg; 13 ± 2 mg/100g body weight). The adrenal cortices were carefully separated from the medulla and minced with scissors. The comminuted tissue was homogenized with one stroke of a loose-fitting pestle using 10 ml MSM with 2 mM EDTA (1.0 ml 100 mM EDTA per 50 ml MSM per gram of adrenal cortex). The homogenate was centrifuged at 300 RPM for 10 minutes, then the supernatant was centrifuged at 6900 rpm for 10 min. The resultant pellet was washed twice by suspension in MSM and centrifuged at 6900 RPM for 10 min. The final pellet was resuspended in 0.1 ml of MSM per gram of adrenal cortex. Mitochondrial protein was measured using the Lowry assay [11]. Because the yield of isolated mitochondria was limited (15.8 ± 3.7 mg/ g wet weight), we employed high-resolution respirometry [12,13] (Oxygraph-2k from Oroboros, Innsbruck, Austria) using 2 ml of MIR05 (220mM sucrose, 60mM K-lactobionate, 0.5mM EGTA, 3mM $MgCl_2$, 20mM taurine, 10 mM KH_2PO_4, 20mM HEPES, pH7.1 and 1mg defatted BSA/ml) at 37°C. Datlab software (OROBOROS Instruments) was used for data acquisition and analysis. The measurement of integrated mitochondrial function (oxidative phosphorylation) was assessed with several substrates. The following substrates, uncoupler, and inhibitors were added (final concentration in the chamber): glutamate (10 mM), malate (5 mM), ADP (2.5 mM), cytochrome *c* (cyt. *c*, 10 μM), succinate (10 mM), dinitrophenol (DNP, 20 μM), rotenone (0.5 μM), antimycin A (2.5

μM), ascorbate (2 mM), tetramethylphenylenediamine (TMPD, 0.5 mM), and azide (15 mM).

Electron microscopy

Adrenocortical mitochondria isolated from 2 rats were subjected separately to electron microscopic examination. An aliquot of mitochondrial suspension was added to an equal volume of half-strength Karnovsky's fixative [14] and immediately spun down in a microfuge. The resultant pellet was transferred to fresh quarter-strength Karnovsky's fixative, where it remained for two hours. After a rinse in distilled water, the pellet was postfixed in ferrocyanide reduced osmium tetroxide [15] for two hours, then rinsed in distilled water. The pellets were soaked overnight in acidified 0.25% uranyl acetate [16]. Another rinse was followed by dehydration in ascending concentrations of ethanol, passage through propylene oxide, and embedment in Poly/Bed 812. Thin sections were stained with acidified methanolic uranyl acetate [16] followed by staining with Sato's triple lead stain as modified by Hanaichi et al. [17] and examined in a Zeiss CEM 902 electron microscope.

Results

In five separate experiments, individual Fischer 344 rats (6 mo old) were used to harvest mitochondria from the paired adrenal cortices to yield 15.8 ± 2.1 mg protein/g wet weight adrenal cortex (N=5). Morphological examination of the isolated adrenocortical mitochondria from two experiments using adult Fischer 344 rats revealed that these organelles were intact and relatively pure (Figure 1). There was an almost complete absence of contaminants. The scattered membranous segments probably were derived from smooth endoplasmic reticulum and plasma membranes. Aside from the fact that most of these organelles had assumed a globular shape, they were virtual simulacra of their *in situ* counterparts. Most retained their tubular cristae—the putative vesicles observed in many of the mitochondria actually are transversely-sectioned tubular cristae. Very few cristae had a lamelliform configuration.

To illustrate the physiological competence of the isolated mitochondria, we show the details of one experiment (Figure 2) out of the five, whereas Figure 3 combines the overall results of all experiments. In Figure 2, the addition of substrates, glutamate and L-malate (GM), leads to a boost in respiration (8.4 pmol/sec/mg), i.e., the leak state. When ADP (2.5mM) is added, there is a rise in rate to 33.6 pmoles/sec/mg. Thus, the stimulation by ADP results in a respiratory control ratio of ~ 4. The addition of cytochrome *c* produces a slight enhancement of the respiratory rate to 41.1, showing that the outer membrane is reasonably intact. A major increase in respiratory rate was elicited by the addition of succinate (GMSD). Only a 5% increase in rate followed the addition of an uncoupler (84.3). Rotenone, a selective inhibitor of complex I, decreased the rate to 67.9, showing that the respiratory rate is dependent solely on complex II (Figure 2). To determine the participation of complex III, antimycin A was added, resulting in a dramatic fall in respiration to 3.3 pmoles/sec/mg. To assess cytochrome *c* oxidase activity, tetramethylphenylenediamine and ascorbate were added, leading to an increase in the rate to 98.8. The complex IV inhibitor, azide, caused a steep fall in rate to 17.6. The data in Figure 3 show that the previous

illustration is quite typical; to make comparisons to other types of mitochondria feasible we have converted the units to nanoAtom oxygen / min / mg mitochondrial protein, which is the notation generally used. Additionally, the rate of oxygen consumption following addition of antimycin A, which is considered to be nonmitochondrial, has been subtracted from the original rates. A general characteristic of intact isolated mitochondria is the high level of coupling of respiration to ADP availability. For the acceptor control ratio, which is the rate of ADP-stimulated glutamate + malate oxidation (state 3) divided by the rate before ADP is added, a value of >4 is considered a mark of highly conserved mitochondrial function. Our mitochondria meet this criterion—the value of our preparations is 4.6 ± 1.2. The maximal capacity for substrate oxidation measured after an uncoupler is added to disassociate oxidation from phosphorylation is 71.9 ± 11.0 nAO/min/mg compared to oxidative phosphorylation of 67.9 ± 10.2. This finding shows that the adrenocortical mitochondrial oxidative capacity is only 5% greater than the oxidation coupled to phosphorylation.

Discussion

The method described here for isolating adrenocortical mitochondria from the rat is relatively simple, convenient, and reproducible. These organelles, which heretofore have been unobtainable in isolated form from a small laboratory animal such as the rat, are now readily accessible. Our electron micrographs show the high degree of mitochondrial purity obtained by our method, as well as the structural integrity of each mitochondrion. That these organelles have retained their functional integrity as well is shown by their high respiratory control ratios. In addition, the slight effect of added cytochrome c shows that the mitochondrial outer membrane remains reasonably intact. In general, the biochemical performance of these adrenal cortical mitochondria closely mirrors that of typical hepatic or cardiac mitochondria. Of course the mitochondrial preparations that are obtainable by our technique represent the entire population of the adrenocortex without regard to zonal distribution. So far, the only technique that permits examination of mitochondrial function in specific zones of the cortex is immunohistochemistry [18,19]. But immunohistochemistry is limited in the range of mitochondrial functions that can be tested. Using our technique could permit direct biochemical analyses and endocrine manipulation of adrenocortical mitochondria in a logistically feasible laboratory animal. By extension, it is now possible to examine the direct role of adrenocortical mitochondria in steroid biosynthesis in such test animals. Moreover, our method can be used to extricate mitochondria from very small tissue samples of whatever stripe.

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Highlights

A method for isolation of adrenocortical mitochondria from the adrenal gland of rats is described.

The purified isolated mitochondria show excellent morphological integrity.

The properties of oxidative phosphorylation are excellent.

The method increases the opportunity of direct analysis of adrenal mitochondria from small animals.

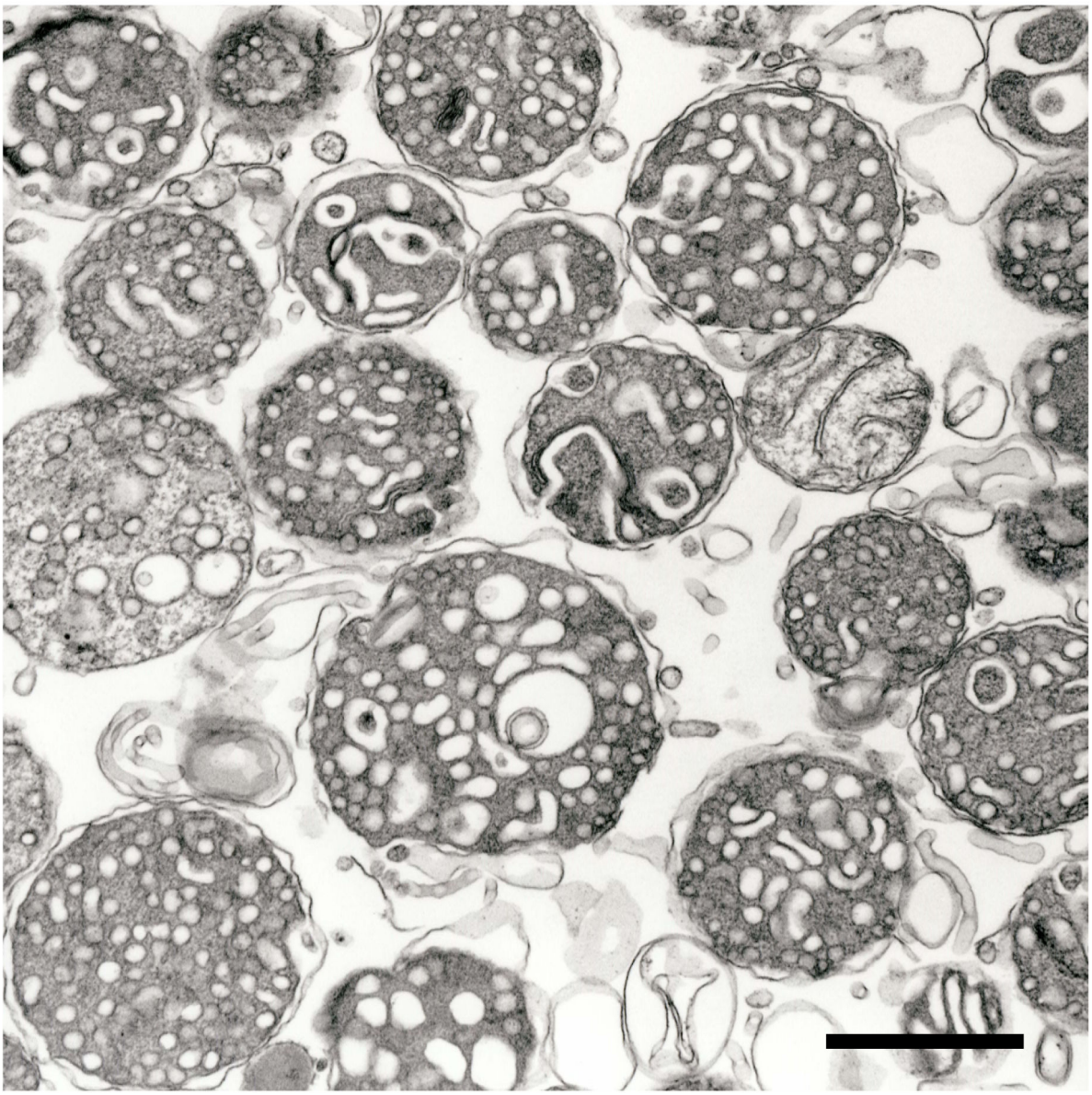

Figure 1.

An electron micrograph of isolated adrenocortical mitochondria. The mitochondria, which are globular, have retained their outer membranes. The cristae, which are predominantly finger-like, appear in transverse section as vesicles. There is a relatively low level of contamination of membranes of unknown origin. Scale bar = 1 μm

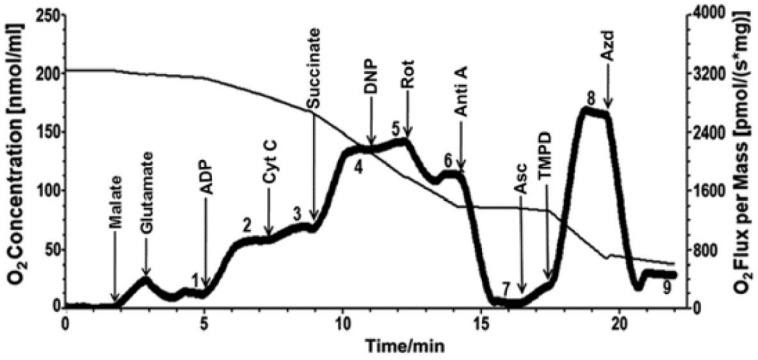

Figure 2.

An oxygraph trace of isolated adrenocortical mitochondrial respiration. This tracing from a single sample is representative of the sample (N=5) as a whole. The trace represents the oxygen consumption (right axis, bold line) and oxygen concentration (left axis, thin line) as a function of time. The arrows indicate addition of substrates, uncoupler, and inhibitors. The numbers represent the time when respiration was measured: (1) in the presence of glutamate +malate (GM) without ADP; (2) after addition of saturating ADP; (3) cytochrome *c* added to test integrity of the outer mitochondrial membrane, (4) succinate to measure respiration in the presence of GMS; (5) uncoupling with DNP; (6) after inhibition of complex I with rotenone [S(Rot)]; (7) residual oxygen consumption after inhibition of complex III with antimycin A; (8) complex IV respiration in the presence of ascorbate+TMPD (AsTm); (9) inhibition of complex IV with azide.

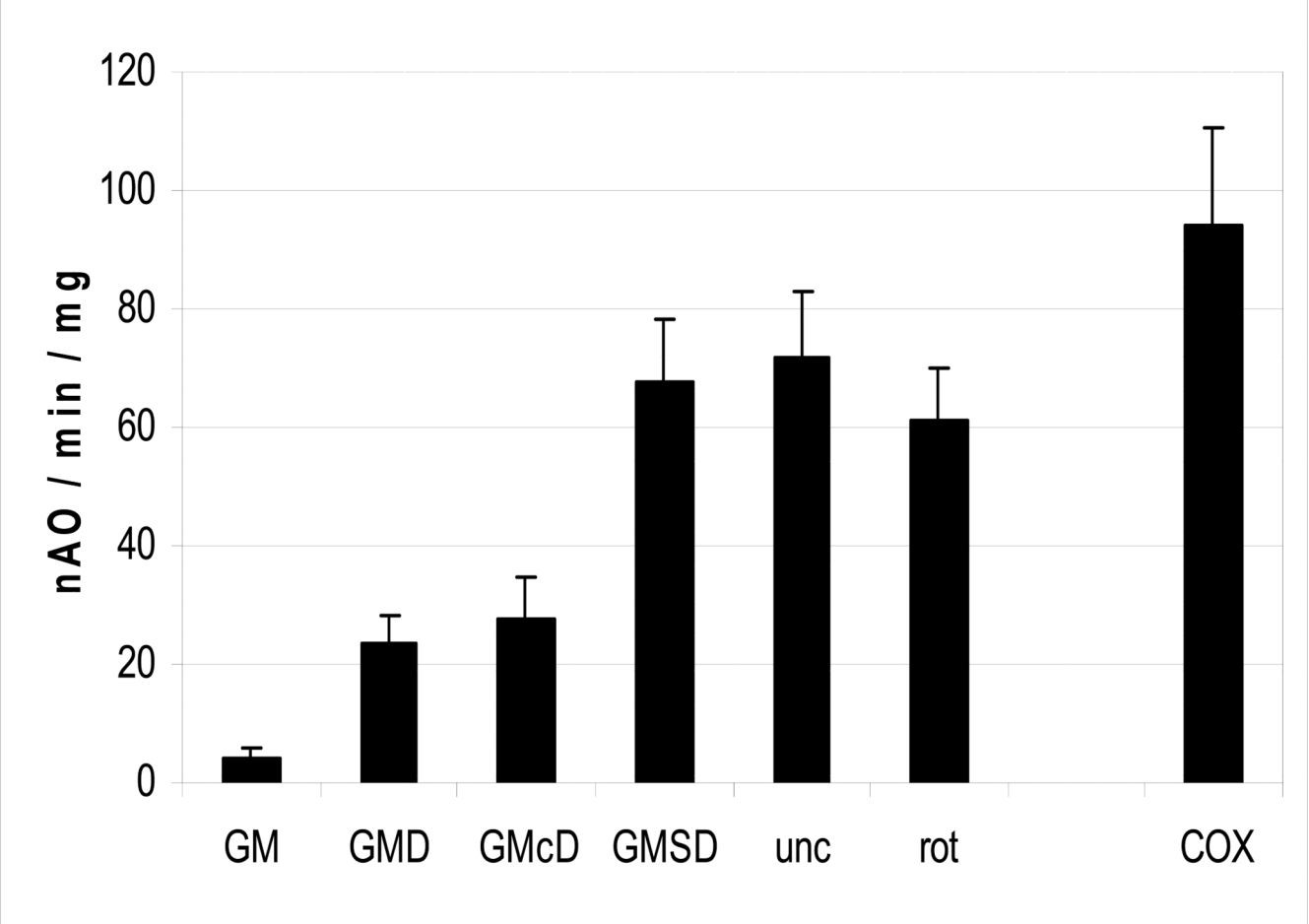

Figure 3.

A graph of the rates of respiration in rat adrenocortical mitochondria. The data are from five individual preparations. GM; 10 mM Glutamate + 2.5 mM L-Malate, GMD; 10 mM Glutamate + 2.5 mM L-Malate + 2.5 mM ADP, GMcD, 10 mM Glutamate + 2.5 mM L-Malate + 2.5 mM ADP + 10 μM cytochrome c, GMSD; 10 mM Glutamate + 2.5 mM L-Malate + 2.5 mM ADP + 10 μM cytochrome c + 10 mM Succinate, unc; 10 mM Glutamate + 2.5 mM L-Malate + 2.5 mM ADP + 10 μM cytochrome c + 10 mM Succinate + 20 μM Dinitrophenol, +rot; 10 mM Glutamate + 2.5 mM L-Malate + 2.5 mM ADP + 10 μM cytochrome c + 10 mM Succinate + 20 μM Dinitrophenol + 0.5 μM rotenone, COX; 15 mM azide inhibited oxidation of 2 mM ascorbate and 0.5 mM tetramethylphenylenediamine.