

# OXIDATIVE PHOSPHORYLATION IN RAT ORAL MUCOSAL MITOCHONDRIA

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Oral mucosal mitochondria were isolated and characterized morphologically by electron microscopy. Polarographic measurements were made of respiration and oxidative phosphorylation in the mitochondrial preparations. ADP:O ratios approaching or slightly exceeding the theoretical maxima and stabilized respiratory control ratios were achieved with malate + glutamate, succinate and ascorbate-N,N,N',N' tetramethyl-p-phenylenediamine (TMPD) as substrates. Inhibition by rotenone, antimycin A, azide, and cyanide established the classical electron transport chain as the major pathway of mitochondrial respiration. Respiration of the oral mucosal mitochondria was stimulated by DNP in the presence of succinate. DNP-stimulated respiration exceeded that observed in the presence of ADP plus Pi and increasing the concentration of DNP progressively inhibited respiration.

Oxidative phosphorylation (ATP synthesis) and electron transport in oral mucosa or skin mitochondria have not been extensively investigated. Those studies which have been conducted have yielded variable and generally low levels of respiration both in the presence and absence of substrate [1-4]. Recent studies of mitochondria isolated from human fibroblast cell cultures suggest that these mitochondria oxidatively phosphorylate ADP to ATP in the presence of Pi and appropriate substrates [5]. However, quantitative assessment of mitochondrial ATP synthesis in oral mucosa and skin has been hindered by the extreme difficulty of homogenizing these tissues without damaging mitochondrial integrity and uncoupling oxidative phosphorylation.

The role played by oxidative phosphorylation in oral mucosal metabolism would aid in our understanding of the gingival responses to local injury and periodontal disease. For

example, it has been reported that collagen synthesis and remodeling during periodontal tissue repair has been significantly altered by diabetes [6], scurvy [7], and protein deficiencies [8]. The events affecting collagen synthesis and remodeling under these conditions may also reflect changes at the oxidative phosphorylation level especially since ATP is required for many synthetic processes associated with collagen synthesis.

In this paper we report procedures for the isolation of morphologically intact mitochondria from rat oral mucosa which consistently displayed good rates of respiration, respiratory control, and ADP:O ratios approaching theoretical yields with appropriate substrates. In addition, electron transport pathways utilized for respiration by oral mucosa were examined and defined through the use of classical inhibitors and uncouplers of respiration and oxidative phosphorylation in relation to energy conservation.

## METHODS AND MATERIALS

### Reagents

Antimycin A (type III), rotenone, ethyleneglycol-bis[B-amino ethyl ether]N,N' tetraacetic acid (EGTA), BSA-Fraction V (fatty acid free) bovine serum albumin, D + mannitol, dihydronicotinamide adenine dinucleotide phosphate (reduced) (NADPH) (type I), *p*-nitrophenyl-phosphate, adenosine 5' monophosphoric acid (5'AMP) (type V), ribonuclease (RNAase) (type XII-A), cytochrome c (type III), and the sodium salts of adenosine 5' diphosphate (ADP) (grade 1), glutamate, ascorbate, malate, and urate were obtained from Sigma. Hepes, and sodium succinate were obtained from Calbiochem, sucrose (density gradient-ribonuclease free) from Schwartz-Mann; crystalline albumin was obtained from Pentex; 2,4 dinitrophenol (DNP), and sodium azide were obtained from Fisher. TMPD was obtained from Eastman Kodak Co., and was recrystallized before it was used.

### Electron Microscope Reagents

Sodium cacodylate and uranyl acetate were obtained from J. T. Baker; glutaraldehyde and paraformaldehyde were obtained from Fisher, and osmium tetroxide and Epon 812 were obtained from Polysciences.

## METHODS

### Tissue Preparation-Isolation of Mitochondria

Sherman strain (60 gm) weanling rats (12 animals/group) were sacrificed by decapitation. Palatal gingiva was quickly removed from the left and right lingual maxillary molar regions and placed in cold buffer. Approximately 10 mg of wet weight tissue was obtained per animal utilized in these studies. We did not attempt to separate the epithelial from the dermal layer by mechanical, chemical, or enzymatic means because of the difficulty in obtaining either a clean mechanical separation of the tissue layers or viable mitochondria after using these dissociation methods [9].

Palatal intact gingiva was first scissor minced, then homogenized in a Polytron PT-10 homogenizer (Brinkman) at setting 4.5 for 4-5 sec in cold buffer. The buffer (MSE-Hepes 1% BSA) contained: 0.2 M mannitol, 0.07 M sucrose, 1 mM EGTA, 0.05 M Hepes pH 7.4, and 1% BSA Fraction V (fatty acid free). Oral mucosal homogenates were centrifuged at 600 g-10 min to remove cell debris and nuclei. The 600 g supernatant was centrifuged at 6,400 g-25 min in rotor 40.2 (Spinco L2-65B centrifuge) and the 6400 g-25 min sediment was

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### Abbreviations:

ADP: adenosine 5' diphosphate

ADP:O: The ratio of ADP removed from the media to the amount of oxygen consumed.

5'AMP: adenosine 5' monophosphoric acid

BSA-Fraction V: (fatty acid free) bovine serum albumin

DNP: 2-4 dinitrophenol

EGTA: ethyleneglycol-bis[B-amino ethyl ether] N,N' tetraacetic acid

EM: electron microscope

Hepes: N-2 hydroxyethylpiperazine N'-2 ethanesulfonic acid

KCN: potassium cyanide

NADPH: dihydronicotinamide adenine dinucleotide phosphate (reduced)

RCl: respiratory control index. The ratio of the respiratory rate (State 3) in the presence of ADP to the rate (State 4) following the expenditure of ADP.

RNAase: ribonuclease

TMPD: N,N,N',N' tetramethyl-p-phenylenediamine

resuspended in cold buffer. The resuspended crude mitochondrial pellet was centrifuged at 1,500 g-10 min. (MSE LR-4 centrifuge) to remove extraneous nonmitochondrial proteins. Centrifugation above 600 g in the initial separation step tends to trap the mitochondria in the sediment and subsequent washing of the pellet does not release the mitochondria from the sediment. The 1500 g supernatant was centrifuged at 6400 g-25 min and the crude mitochondrial preparation (0.15 mg protein) was either fixed for EM evaluation or suspended in buffer and analyzed for oxidative phosphorylation. The yield of mitochondrial protein in our preparations was 1.3  $\mu$ g per mg wet weight tissue.

#### Histologic Observations

Palatal gingiva consisted of a stratified squamous epithelium which is keratinized on its surface. The lamina propria consisted of dense connective tissue which was not highly vascular. The lamina propria contained only few elastic fibers which for the most part were confined to the walls of blood vessels. This is typical of palatal gingiva and mucosal observations [10].

#### Respiration and Oxidative Phosphorylation

Oxygen uptake was measured polarographically with a Clark oxygen electrode (Yellow Spring Inst. Co. Biological Oxygen Monitor (Model 52)) at 25°C in a total volume of 1 ml. All reaction media contained MSE-Hepes-1% BSA Buffer pH 7.4, and 5 mM  $K_2HPO_4$ . Substrates (10 mM succinate or 10 mM malate + 10 mM glutamate) and 50 nanomoles ADP were added where indicated in Fig 1. Succinate utilization was determined in the presence of 1  $\mu$ g of rotenone. Ascorbate-TMPD medium contained: 1  $\mu$ g antimycin A, 10 mM ascorbate, 0.2 mM TMPD, and 25 nanomoles of ADP were added to stimulate state 3 respiration. The ADP:O ratio and respiratory control index were calculated from the electrode traces as described by Estabrook [11], and the oxygen content of the medium was calibrated by the method of Tyler and Gonze [12].

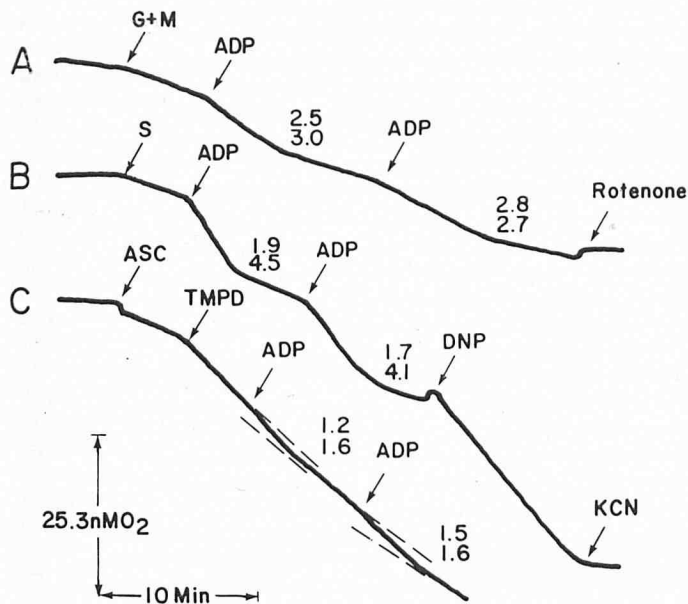


FIG 1. Polarographic tracings of rat oral mitochondrial respiration. Reaction medium is MSE-Hepes-1% BSA + 5 mM  $K_2HPO_4$ . Tracing A, 10 mM each L-glutamate and DL-malate (G+M) were added to the reaction medium containing mitochondria. ADP (46 nm) was added to induce state 3 respiration. Respiration was inhibited by the addition of 1  $\mu$ g rotenone. Tracing B, 10 mM succinate (S) was added to the reaction medium containing mitochondria and 1  $\mu$ g rotenone. ADP (47 nm) was added to initiate state III respiration. The addition of  $3 \times 10^{-4}$  M DNP uncoupled oxidative phosphorylation and  $3 \times 10^{-4}$  M KCN completely inhibited respiration. Tracing C, 10 mM ascorbate and 0.2 mM TMPD were added to the reaction medium containing mitochondria and 1  $\mu$ g antimycin A. ADP (25 nanomoles) was added to initiate state 3 respiration. Mitochondrial protein A = 0.122 mg, B = 0.153 mg, and C = 0.140 mg. Total volume was 1.0 ml in all reactions. The numbers to the right of the tracings represent the ADP:O ratio (upper) and the respiratory control index (lower).

In each experiment, oral mucosal mitochondria were run through 4 to 5 cycles of transition between state 3 and state 4 respiration. ADP:O ratios and respiratory control indexes (RCIs) were determined for each cycle. Average ADP:O ratios and RCIs reported herein are averages obtained from 3-4 separate experiments per substrate.

#### Cytochrome Oxidase

Cytochrome oxidase (E.C. 1.9.3.1) activity was analyzed by following the oxidation of ferrocytochrome c at  $E_{550nm}$  at 25°C as previously described [13] in a Gilford Model 2400 spectrophotometer. Specific activities are expressed as nm cytochrome c oxidized per mg protein per minute  $\pm$  standard error.

#### NADPH Cytochrome c Reductase

NADPH cytochrome c reductase (E.C. 1.6.2.4) activity was determined by following the reduction of ferricytochrome c at  $E_{550nm}$  at 25°C spectrophotometrically. The assay media contained 23 nm cytochrome c, 10  $\mu$ M of phosphate buffer pH 7.75, 30  $\mu$ M KCN, and 28  $\mu$ M of NADPH [14].

#### Acid Phosphatase

Acid phosphatase (E.C. 3.1.3.2) activity was determined by the method of Andersch and Szczypinski [15] using *p*-nitrophenylphosphate as a substrate. Experimental tubes were incubated at 37°C for 60 min and read spectrophotometrically at  $E_{410nm}$ .

#### Uricase

Uricase (E.C. 1.7.3.3) activity was determined spectrophotometrically by following the change in absorption  $E_{292nm}$  at 37°C for 10 min in the presence of sodium urate and appropriate buffer as described by Reid [16].

#### 5'-Nucleotidase

5'-Nucleotidase (E.C. 3.1.3.5) activity was determined by analyzing the amount of Pi cleaved (Fiske and Subarow [17]) after incubation of the homogenate or crude mitochondrial sample with 5'AMP, and appropriate buffer for 60 min at 37°C as described by Reid [16].

#### Protein

Protein was determined by the Lowry Folin phenol reagent method [18] using crystalline bovine albumin as a standard.

#### EM Preparation of Mitochondria

Mitochondrial pellets were fixed in cacodylate-buffered paraformaldehyde-glutaraldehyde fixative, stained with osmium tetroxide and uranyl acetate by the method of Hay and Revel [19], and embedded in Epon 812 [20]. Mitochondrial pellets were cut on an LKB-Huxley microtome, collected on formvar coated grids, and viewed on an RCA EMU-3H electron microscope.

## RESULTS

#### EM and Biochemical Characterization of the Mitochondrial Pellet

The crude mitochondrial pellet (Fig 2) contained intact condensed mitochondria which ranged in diameter from 0.3-0.5  $\mu$ . Typical cristae associated with the inner membranes were seen in these mitochondrial preparations. The crude pellet also contained plasma membranes, lysosomes, and polyribosomes as contaminants.

Cytochrome oxidase recoveries in the 6,400 g crude mitochondrial pellet ranged from 60-65% of beginning activity (i.e., total homogenate) and 55% after the 1,500 g centrifugation step. However, the mitochondria were purified 10-fold from the total homogenate as determined by the increase in the specific activity of cytochrome oxidase from  $97.0 \pm 8.5$  SE in the total homogenate to  $934.9 \pm 67.5$  SE for the mitochondrial fraction.

The degree of contamination of our crude mitochondrial pellet by microsomes, lysosomes, plasma membranes and peroxisomes was determined by analyzing the distribution of marker enzyme activities associated with these organelles

## Respiration of Oral Mucosal Mitochondria

Substrate	Oxygen-Consumption uMO <sub>2</sub> /hr/mg protein		Respiratory Control Ratio	ADP/O
	State 4	State 3		
n = 20 Malate + Glutamate	0.14 ± 0.01	0.36 ± 0.04	2.5 ± 0.1	3.2 ± 0.1
n = 14 Succinate	0.39 ± 0.04	1.45 ± 0.08	4.0 ± 0.2	1.6 ± 0.1
n = 16 Ascorbate + TMPD	0.57 ± 0.04	0.89 ± 0.06	1.4 ± 0.1	1.4 ± 0.1

Measurements of oxygen consumption and calculation of respiratory control indices and ADP:O ratios are described under Materials and Methods. Substrates and ADP were added as described under Methods. n = number of state 3 to state 4 transition cycles analyzed.

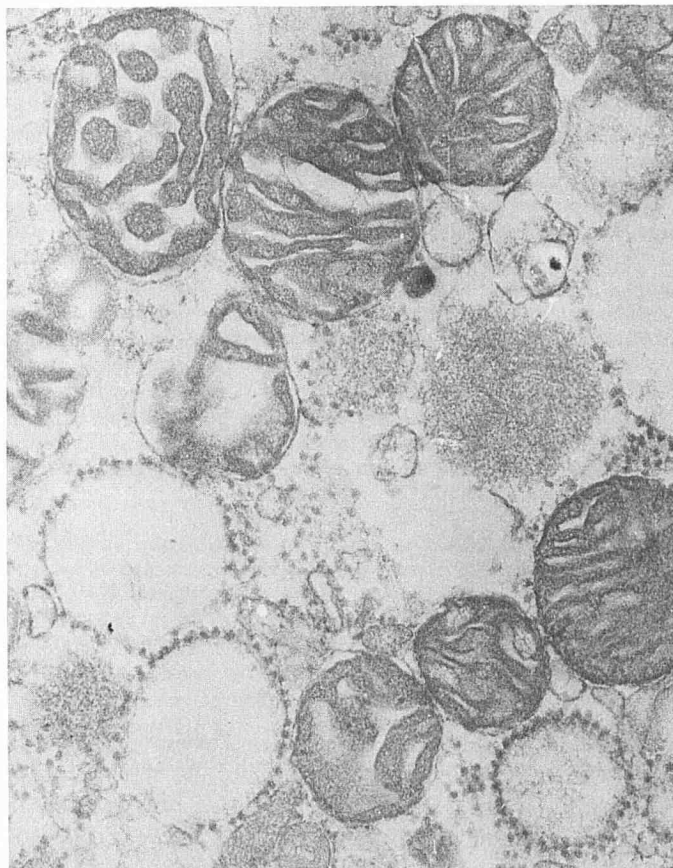


FIG 2. Electron micrograph of isolated mitochondrial fraction from rat oral mucosa ( $\times 48,725$ ).

and membranes. NADPH cytochrome c reductase (microsomal), acid phosphatase (lysosomal), 5' nucleotidase (plasma membrane) and uricase (peroxisome) activities were determined in the total homogenate and crude mitochondrial pellet. The data revealed that our mitochondrial preparation was devoid of peroxisomal marker activity. However, our preparation was contaminated by 10.2, 12.9, and 12.7% respectively of microsomal, lysosomal and plasma membrane enzyme marker activities.

#### Purification of Mitochondria from Pellets

Attempts to further purify our mitochondrial preparation by the use of ribonuclease, or discontinuous sucrose density gradients have met with failure. Ribonuclease (160 U/ml) was added to the homogenizing medium and maintained at that concentration for 55 minutes during the preparation of the mitochondrial pellet. The mitochondrial pellet was washed 2 $\times$  to remove extraneous substrates and RNAase. The mitochondria in the presence of succinate  $\pm$  ADP had poor respiration (QO<sub>2</sub>). This may be due to RNAase (basic protein) inhibition of mitochondrial cytochrome oxidase [21].

Crude mitochondrial pellets placed on discontinuous sucrose

gradients, were found to concentrate (32% of initial cytochrome oxidase activity) in a broad band between densities of 1.13 and 1.31. Subsequent analysis of the mitochondrial fraction (EM) revealed the presence of swollen mitochondria, polyribosomes, and membranal contaminants similar to those found in initial preparations.

#### Respiration

Oxygen consumption (state 4) in the presence of malate + glutamate, succinate and ascorbate-TMPD are summarized in the Table. When ADP was added in the presence of these substrates, respiration was stimulated with a transition from state 4 to state 3. Respiration rates (state 4 and 3) were significantly greater in heart mitochondria than in oral mucosal mitochondria. Typical polarographic traces of oral mucosal mitochondrial respiration in the presence of malate + glutamate, succinate and ascorbate-TMPD are seen in Fig 1. ADP:O ratios approaching the theoretical values were obtained in the presence of malate + glutamate and succinate (Fig 1, Table). ADP:O ratios with ascorbate-TMPD were greater than the theoretical value (Table, Fig 1), and the addition of 1  $\mu$ g of rotenone or 1  $\mu$ g of antimycin A did not alter the ratio. The RCI values obtained in the presence of malate + glutamate, succinate and ascorbate-TMPD are summarized in the Table. Respiratory control was demonstrated to be operative at all three sites of energy conservation.

#### Inhibitors and Uncouplers of Oxidative Phosphorylation

Rotenone ( $2.5 \times 10^{-6}$ M) inhibited respiration by mitochondria utilizing malate + glutamate as substrates (Fig 1) and antimycin A ( $1.9 \times 10^{-6}$ M) was found to act similarly in the presence of succinate. The addition of cyanide ( $1 \times 10^{-4}$ M) and azide ( $5 \times 10^{-4}$ M) completely inhibited respiration and oxidative phosphorylation in mitochondria utilizing either substrate.

DNP ( $3 \times 10^{-4}$ M) uncoupled oxidative phosphorylation and produced maximal stimulation of respiration by mitochondria utilizing succinate (Fig 2). The addition of increasing concentrations of DNP (0.6–3.0 mM) progressively inhibited respiration. Fifty percent inhibition of the maximum stimulated rate of respiration occurred at  $1.5 \times 10^{-3}$ M DNP.

#### DISCUSSION

Early studies of oxidative metabolism of skin and oral mucosal mitochondrial preparations indicated generally poor performance in terms of (1) respiration in the presence and absence of substrates, (2) ability to oxidatively phosphorylate, and (3) morphological integrity at the EM level [1–4]. Recent studies of isolated pig epidermis mitochondria revealed that mitochondrial respiration was stimulated by substrates, however, the mitochondria were uncoupled in terms of oxidative phosphorylation [4]. Only 7 percent of the cytochrome oxidase activity was recovered by these investigators in the heavy and light mitochondrial fractions prior to discontinuous sucrose gradient analysis indicating poor recovery. Our recoveries in non-separated intact palatal gingiva were approximately 8-fold higher than those reported for mechanically separated pig epidermis mitochondria [4]. Attempts to purify our preparation using discontinuous sucrose gradients were unsuccessful.

ful. After gradient centrifugation our mitochondria appeared swollen and significant amounts of nonmitochondrial contaminants were still present at the ultrastructural level. On the other hand, pig epidermis heavy mitochondrial fractions after gradient separation were reported to be intact, condensed, and free of major contaminants [4]. Our inability to remove nonmitochondrial contaminants by this method may be due to possible interactions among albumin, glycoproteins, and mucopolysaccharides present in the isolating medium, or released by homogenization, which cause mitochondria to form coacervates with these substances.

Our oxidative phosphorylation data represent the contribution made by both epithelial and dermal mitochondria. The question arises as to which compartment contributed the bulk of the activity. Recent evidence obtained in our laboratory suggests that after elastase dissociation of the epithelial from the dermal compartment, that the bulk of the oxidative metabolic activity resides in the dermal layer. Analysis of the percent distribution of the recombined enzymatic activities after elastase dissociation of the oral mucosal compartments revealed that  $69.8 \pm 2.5$  and  $79.8 \pm 1.4\%$  respectively of the cytochrome oxidase and succinic cytochrome c reductase (mitochondrial marker) activities were associated with the dermal compartment. The dissociated epithelial compartment contained  $30.3 \pm 2.6$  and  $20.2 \pm 1.4\%$  respectively of the cytochrome oxidase and succinic cytochrome c reductase activities. Isolated mitochondria prepared from the elastase dissociated compartments were found to be altered at the ultrastructural and biochemical levels and could no longer oxidatively phosphorylate ADP to ATP [9].

As a result of the data obtained in the present experiments, electron transport pathways in oral mucosal mitochondria have been fully characterized for the first time. Inhibition of respiration and oxidative phosphorylation by rotenone, antimycin A, cyanide, and azide were of the classical types, coinciding with those reported for heart mitochondria, submitochondrial particles, and isolated enzyme systems [22,23].

The rate of oxygen consumption by mitochondria depends on the presence of various metabolites. Mitochondrial steady-states are classified by relating the rate of respiration to the concentration of the rate limiting components in the system. For example in state 4, substrate is present in excess, but because the ADP content of the system is low, respiration is slow. On the other hand respiration is rapid in state 3, because substrate and ADP are present [24]. The ratio of moles of ADP added to gram atoms of oxygen consumed during state 3 respiration is equal to the ADP:O ratio. The dependence of respiratory rate on ADP concentration is called respiratory control (RCI) or more specifically acceptor control and it is defined as the ratio of state 3/state 4 respiration rates. Marked deviations from the theoretical ADP:O ratios for a particular substrate or increasing RCIs after each state 3 stimulation (ADP) are indicative of poorly coupled or uncoupled mitochondria.

The coupling of respiration to energy conservation and transfer at the 3 characteristic respiratory sites were confirmed by the ADP:O ratios observed with malate + glutamate, succinate, and ascorbate-TMPD, and our values were similar to those reported for human cultured fibroblast [5], and human placental mitochondria [25,26]. Our ascorbate-TMPD ratios exceeded 1.0, however, the addition of rotenone or antimycin A did not lower the ratios as reported for liver mitochondria [27]. DNP uncoupled oxidative phosphorylation and with increasing concentration inhibited respiration in our preparation, an effect similar to that reported for liver [28, 29].

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