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Respiratory Characteristics of Mitochondria Isolated from Squamous Epithelium of the Hamster Cheek Pouch[1] (39888)

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Introduction. The vast majority of cancers in man is epithelial in origin, with the epithelial lining of the gastrointestinal tract being particularly susceptible (1). Epidemiological evidence indicating that both dietary excesses and deficiencies play a role in the etiology of gastrointestinal tract cancer in man has been recently reviewed by Wynder *et al.* (2). To date, little, if any, information is available on the effect of dietary alterations on the metabolism of cells of the upper gastrointestinal tract. Thus, the availability of model systems for the investigation of early metabolic events associated with neoplasia, and/or the predisposing factors which are associated with the increased risk of neoplasia, are highly desirable.

The cheek pouch of the Syrian golden hamster provides such a model system for the study of the etiology of oral cavity cancer. The pouches are bilateral evaginations of the oral cavity mucosa. Histologically, the epithelial layer is from three to seven cells thick and is characterized as slightly keratinized, stratified, squamous epithelium, supported by fibrous connective tissue. In contrast to skin, the epithelium is relatively homogeneous in that it does not contain significant amounts of other cellular elements (i.e., sebaceous glands or hair follicles).

It is the purpose of this communication to describe the preparation of epithelial sheets from the cheek pouch of the Syrian golden hamster and to demonstrate their utility as a starting material for subcellular fractionation of squamous epithelial cells.

Materials and methods. Chemicals. Adenosine 5′-diphosphate (grade IV); adenosine 5′-triphosphate, disodium salt; collagenase (Type I); cytochrome *c* (Type III); 2,4-dinitrophenol; L-glutamic acid; Hepes (*N*-2-hydroxyethyl piperazine-*N*¹-2 ethane sulfonic acid); L-malic acid; nicotinamide; oligomycin; phospho(enol)pyruvate, trisodium salt; pyruvate kinase (Type II); and Tricine (*N-tris*-[hydroxymethyl]-methyl glycine) were obtained from Sigma Chemical Co., St. Louis, Missouri. Sucrose, special enzyme grade, was obtained from Schwarz/Mann, Orangeburg, New York.

After adjustment to appropriate pH, all aqueous solutions were filtered through 0.45-μm HAWP Millipore filters (Millipore Corp., Bedford, Mass.).

Preparation of epithelial sheets. Three to five adult male Syrian golden hamsters obtained from Sprague-Dawley, Madison, Wisconsin, were sacrificed by CO_2 anesthesia. Cheek pouches were everted, excised, and placed in ice-cold 0.8% NaCl, 0.04% KCl, 1 m*M* Tricine, pH 7.4 (buffer A), and a single longitudinal cut was made. Pouches were washed with several changes of buffer A and then pinned to a paraffin board with the epithelial surface down.

Following removal of muscle and excess areolar connective tissue, the pouches were rinsed with buffer A and repositioned on the paraffin board with the epithelial surface up. Approximately 0.2 ml of 0.5% (w/v) collagenase solution (dissolved in buffer A) was injected between the cheek pouch and the paraffin board. Trapped air bubbles were gently expressed and care was taken to ensure that none of the collagenase solution came in direct contact with the epithelial surface. The preparation was then placed in a closed humidified incubation chamber and incubated at 37° for 20 min. After incubation, the preparation was removed and the epithelial layer was separated as a single intact sheet by gentle scraping with a dulled surface. Isolated epithelial sheets were suspended in buffer A and centrifuged at 480*g*

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for 10 min at 4° in a Sorvall RC2-B centrifuge. The resultant cell pellet was finely minced and suspended in 0.5 vol of 300 m*M* sucrose, 0.5 m*M* EDTA, 5 m*M* Tricine, pH 7.4 (buffer B), per gram wet weight or original tissue and homogenized manually in a Tenbroeck tissue grinder.

Isolation of squamous epithelial mitochondria. The cell homogenate was passed through four layers of cheesecloth and then centrifuged at 480*g* for 10 min. The resultant supernatant was centrifuged at 9800*g* for 10 min, the supernatant was discarded, and the pellet was suspended in an equivalent volume of buffer B and centrifuged as before. The pellet was suspended in a minimal volume of buffer B (usually 1–2 ml).

Isolation of hamster liver mitochondria. Hamster livers were homogenized in 8 vol of buffer B per gram wet weight and isolated by differential centrifugation as previously described for rat liver (3).

Measurement of oxygen consumption. The rate of oxygen consumption was determined with either a Gilson oxygraph equipped with a Clark-type oxygen electrode (Model K-ICC, Gilson Medical Electronics, Middleton, Wis.) or a Rank oxygen electrode systen (Rank Bros., Cambridge, England). Analysis of identical preparations on both instruments indicated less than a 5% difference in the rates of oxygen consumption. The Rank oxygen electrode system was preferred for analysis of squamous epithelial mitochondria since the sensitivity of the measurements can be increased by reducing the assay volume to as little as 0.3 ml. Mitochondrial respiratory activity was determined as described by Estabrook (4). The basic assay medium consisted of 225 m*M* sucrose, 10 m*M* potassium phosphate, 5 m*M* $MgCl_2$, 20 m*M* KCl, 20 m*M* Hepes, pH 7.4. Temperature of the reaction vessel was maintained at 30°. Other additions are as described.

Mitochondrial cytochrome analysis was performed as described by Williams (5) and recorded on a Cary Model 118 C scanning spectrophotometer. Cytochrome content was calculated as described by Vanneste (6).

Enzyme assays. Mitochondrial fractions were analyzed for rotenone-insensitive NADH cytochrome *c* reductase, succinate cytochrome *c* reductase, and ATPase, as described by Fleisher and Fleisher (7). Inorganic phosphate was measured colorimetrically (8). Cytochrome oxidase activity was determined spectrophotometrically as described by Wharton and Tzagoloff (9). Protein was determined by the method of Lowry *et al.* (10). All results are expressed as means ± standard deviation. The data were analyzed by Student's *t* test.

Results. Homogenization of the epithelial sheets obtained following collagenase treatment yields 10.9 ± 1.8 mg of protein/g wet weight of original cheek pouch. Of this, only 4%, or 0.41 ± 0.06 mg, was recovered in the mitochondrial fraction. Analysis of the various fractions for succinate cytochrome *c* reductase activity indicated that 25 to 35% of the total activity could be recovered in the mitochondrial fraction.

Comparison of the respiratory activity of mitochondria isolated from hamster liver and from squamous epithelium is presented in Table 1. The rates of state 4 respiration in the presence of succinate are similar for both preparations. Addition of ADP results in a marked stimulation of respiration in both mitochondrial preparations. The addition of the uncoupler, dinitrophenol, to mitochondria in state 4 results in a greater stimulation of respiration in liver than in squamous epithelial mitochondria. State 4 rates of respiration, in the presence of the NAD-linked substrates glutamate and malate, were similar for both preparations and were approximately one-half of those rates observed in the presence of the succinate. Both the ADP- and dinitrophenol-stimulated rates of respiration were lower in the squamous epithelial preparation. The disparity between liver and squamous epithelium mitochondria, with respect to NAD-linked respiratory capacity, probably results from the presence of a potent NADase in the latter preparation. Omission of nicotinamide from the reaction mixture resulted in complete abolishment of both ADP and dinitrophenol stimulation. Analysis of both mitochondrial preparations for cytochrome content is presented in Table II.

Table III compares the specific activities of several mitochondrial marker enzymes.

TABLE I. RESPIRATORY CAPACITY OF LIVER AND SQUAMOUS EPITHELIAL MITOCHONDRIA.[a]

	Nanoatoms of O/min/mg of protein			
	State 4	State 3	+ 100 μ *M* DNP	RCR[b]
(A) Succinate supported				
Liver	28.1 ± 3.4	107.7 ± 11.5	146.0 ± 18.1	3.83
Squamous epithelium	32.2 ± 2.7	81.2 ± 7.1	96.4 ± 8.4	2.45
(B) Glutamate-malate supported				
Liver	15.8 ± 2.6	79.2 ± 5.5	105.2 ± 6.2	5.00
Squamous epithelium	15.4 ± 2.7	44.7 ± 9.1	55.1 ± 9.1	2.90

[a] The basic reaction mixture contained 10 m*M* succinate (part A) or 10 m*M* malate, 5 m*M* glutamate, 20 m*M* nicotinamide (part B). Reactions were initiated by the addition of either 1–2 mg of liver mitochondria or 0.3–0.6 mg of squamous epithelial mitochondria. State 3 respiration was initiated by the rapid injection of 125 μ*M* ADP. After return to state 4 respiration, the uncoupled rate of respiration was initiated by the injection of dinitrophenol dissolved in ethanol. The values presented are expressed as means ± SD of four separate mitochondrial preparations.

[b] RCR = Respiratory control ratio.

TABLE II. CYTOCHROME CONTENT OF LIVER AND SQUAMOUS EPITHELIAL MITOCHONDRIA.

Cytochrome type	Concentration (nmoles/mg of protein)	
	Liver	Squamous epithelium
b	0.090 ± 0.007 (5)	0.082 ± 0.009 (3)
c_1	0.060 ± 0.011 (5)	0.111 ± 0.010 (3)
c	0.134 ± 0.010 (5)	0.127 ± 0.021 (3)
$a + a_3$	0.116 ± 0.013 (5)	0.264 ± 0.013 (3)
	Concentration ratio	
b	0.78	0.30
c_1	0.52	0.42
c	1.16	0.48
$a + a_3$	1.00	1.00

Rotenone-insensitive NADH cytochrome *c* reductase activity, found in both microsomal and outer mitochondrial membranes, is increased in squamous epithelial mitochondria. In contrast, the activity of succinate cytochrome *c* reductase, an inner mitochondrial membrane marker, is approximately one-half that associated with liver mitochondria. Activation of latent enzyme activity by freeze-thawing failed to decrease the differences in activity. Sonication or inclusion of 0.3% cholate in the assay mixture gave results similar to those obtained by freeze-thawing (McCoy, G. D., unpublished results). The activity of cytochrome oxidase in squamous epithelium mitochondria is approximately two-thirds that obtained for liver mitochondria.

Examination of mitochondrial ATPase activity revealed the most striking differences between squamous epithelial and liver mitochondria. Endogenous rates of ATP hydrolysis were markedly lower in squamous epithelial mitochondria and, in contrast to liver mitochondria, dinitrophenol stimulation of ATPase activity was not observed.

Discussion. Previous investigations have described isolation of mitochondria from skin epithelium (11, 12). Although these preparations demonstrated substrate-supported respiration, as well as the presence of mitochondrial marker enzymes, they appear to have been damaged during isolation, since no ADP stimulation of respiration was observed. The procedure described in this study yields an active mitochondrial preparation capable of coupled respiration in quantities suitable for biochemical studies.

The concentration ratio obtained for hamster liver mitochondrial cytochromes is in agreement with previously reported values for rat and mouse liver (13). In contrast, the ratios of $b/a + a_3$ and $c/a + a_3$ are depressed in squamous epithelial mitochondria. At the present time, it is not clear whether the differences in cytochrome $a + a_3$ content in Table II and cytochrome oxidase activity in Table III are due to interference with the spectral analysis by some unidentified absorbing compound or to the presence of a cytochrome oxidase molecule of lower catalytic capacity in squamous epithelial mitochondria. The apparent disparity between cytochrome oxidase activities is of potential interest in terms of the structure and function of the electron transport system in squamous epithelial mitochondria.

The most striking difference observed be-

TABLE III. ENZYME-SPECIFIC ACTIVITIES OF MITOCHONDRIA FROM LIVER AND SQUAMOUS EPITHELIUM.[a]

	Liver	Squamous epithelium
NADH cytochrome *c* reductase[b]	0.221 ± 0.008 (5)	0.286 ± 0.030* (5)
Succinate cytochrome *c* reductase		
Fresh	0.044 ± 0.015 (6)	0.027 ± 0.006** (6)
Freeze-thawed	0.132 ± 0.022 (6)	0.068 ± 0.018[c] (6)
Cytochrome oxidase	0.520 ± 0.051 (4)	0.372 ± 0.038* (4)
ATPase[c]		
Control	0.263 ± 0.040 (4)	0.136 ± 0.024* (6)
+100 μM DNP	0.515 ± 0.042 (4)	0.128 ± 0.011* (6)

[a] All enzyme assays were performed at 30°. All values are expressed as micromoles per minute per milligram of protein ± SD. The numbers in parentheses indicate the number of separate mitochondrial preparations.

[b] Assays performed in the presence of 0.1 μg/ml of rotenone.

[c] Addition of 3 μg/ml of oligomycin results in 88% inhibition of liver ATPase and 86% inhibition of squamous epithelial ATPase.

* Significant difference from liver, $P < 0.01$.

** Significant difference from liver, $P < 0.05$.

tween squamous epithelial and liver mitochondria is the absence of stimulation of mitochondrial ATPase by dinitrophenol under conditions which stimulate liver mitochondrial ATPase. The inability of dinitrophenol to stimulate mitochondrial ATPase has been observed previously for mitochondria isolated from a wide variety of hepatocellular carcinomas (14–16), and it has been shown that the loss of dinitrophenol sensitivity is not associated with gross alteration in the composition of the mitochondrial ATPase (17). The lack of stimulation of mitochondrial ATPase we observe does not appear to be due to the isolation of damaged mitochondria, since our preparations show good respiratory control, dinitrophenol-sensitive respiration, and activation of succinate cytochrome *c* reductase by procedures which are known to disrupt membrane integrity.

Our finding that mitochondria isolated from normal noncancerous tissue and tumor mitochondria share similar properties requires further experimentation before any definitive conclusions can be drawn as to its functional significance. The extent to which this phenomenon exists at the next-higher level of organization, the intact cell, is currently under investigation.

We believe that the studies reported here demonstrate the feasibility of undertaking biochemical investigation of the metabolic consequences of dietary alterations in specific target tissues, and that investigations of this type will lead to a better understanding of the etiology of diet-related cancers in man.

Summary. A procedure is described for the preparation of sheets of squamous epithelium from the cheek pouch of the Syrian golden hamster. The use of these sheets for the isolation of a mitochondrial fraction capable of coupled respiration is demonstrated. These mitochondria were compared to hamster liver mitochondria with respect to respiratory capacity, enzymatic activity, and cytochrome content. Squamous epithelial mitochondria exhibited increased rotenone-insensitive NADH cytochrome *c* reductase activity, while the activities of succinate cytochrome *c* reductase, oligomycin-sensitive ATPase, and cytochrome oxidase decreased. The ratios of cytochromes $c/a + a_3$ and $b/a + a_3$ were lower in squamous epithelial mitochondria.

The uncoupler, dinitrophenol (DNP), stimulated oxygen consumption of both mitochondrial preparations. In contrast to liver mitochondria, the ATPase of squamous epithelial mitochondria was not stimulated by DNP.

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