REVISTA ESPAÑOLA DE FISIOLOGIA, 31, 191-196. 1975

Convenient Procedures for Isolating the Mitochondrial Fraction Suitable for the Study of Mitochondrial Nucleic Acids from Normal and Neoplastic Cells

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(Received on 9 January, 1975)

D. GUERRITORE, A. FRIGOLA, E. PICCOLELLA and A. MONTALVO. *Convenient Procedures for Isolating the Mitochondrial Fraction Suitable for the Study of Mitochondrial Nucleic Acids from Normal and Neoplastic Cells.* Rev. esp. Fisiol., 31, 191-196. 1975.

A useful procedure for the large scale preparation of mitochondria from normal and neoplastic tissues is described using mannitol, sucrose, EGTA and bovine serum albumin. It overcomes satisfactorily the difficulties involved in the great lability of tumour cell mitochondria. No significant nuclear, cytoplasmic, lysosomal or bacterial contamination was present.

The study of the biogenesis of mitochondria attracts considerable interest. It has been reported that in neoplastic cells, mitochondria present differences, with respect to normal cells, in the properties of mitochondrial DNA (M-DNA) and the protein synthesis apparatus (6-8). However nothing is yet known about the significance of these changes in the neoplastic process. Therefore it seems important a comparative study, at a molecular level, between these systems in normal and neoplastic cells. Because of the interest of this approach, it seems necessary to dispose of a convenient procedure for the preparation of mitochondria capable of eliminating, as much as possible, any source of contaminants. With this aim, there has been developed in this paper a convenient procedure for large scale preparation of mitochondria from both normal and neoplastic tissues. It overcomes

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satisfactorily the difficulties involved in the great lability of tumour cell mitochondria and appears to be a very promising first step for further studies.

Materials and Methods

Preparation of mitochondria

Normal tissues. Rat liver: Two month old Wistar rats (Ar/IRE strain) fasted 12 hours before, were killed by stunning and decapitation. The livers were removed, minced and immediatly kept in the homogenization medium.

Mouse submaxillary gland: Adult male C3H/He mice (Ar/IRE strain) fasted 12 hours before, were killed by cervical dislocation. The subsequent operations were performed as described below.

Solid tumours. Submaxillary gland adenocarcinoma: Adult male C3H/He mice (Ar/IRE strain) bearing tumours and fasted 12 hours before, were killed by cervical dislocation 15 days after tumour transplantation. The tumours were excised from the surrounding tissues, minced and kept in the homogenization medium.

Plasma cell tumour: Adult male gold hamsters bearing a plasmocytoma, were killed by cervical dislocation 9-10 days after tumour transplantation. The subsequent operations were performed as described for submaxillary gland adenocarcinoma.

Ascites tumour cells. Tetraploid Lipschutz Ehrlich ascites tumour: Cells were obtained by harvesting the ascitic fluid from male swiss mice, killed by cervical dislocation 10 days after tumour transplantation. The ascitic fluid was removed by syringe and the cells were separated from the ascitic fluid by centrifugation at 900 $\times$ *g* for 5 minutes at 0-4° C.

A.H.130 Yoshida ascites hepatoma tumour: The cells were obtained by harvesting the ascitic fluids from male Wistar rats killed by cervical dislocation 6 days after tumour transplantation. The cells were collected as previously described for Ehrlich ascites tumour cells.

For both normal and neoplastic tissues, all steps were performed at 0-4° C using filter-sterilized solutions.

The minced tissues were kept in the medium containing 0.21 M mannitol, 0.07 M sucrose, 1 mM EGTA and 20 mM Tris buffer (pH 7.4) and homogenized in a Potter Elvehjem homogenizer in a ratio 1:10 (w/v). The homogenate was layered on 0.44 M sucrose and centrifuged for 10 minutes at 600 $\times$ *g*. The top layer was carefully taken off (avoiding the interface between the homogenization medium and 0.44 M sucrose) and centrifuged for 10 minutes at 12,000 $\times$ *g*. The resultant sediments were resuspended in 0.21 M mannitol, 0.07 M sucrose, 1 mM EGTA, 20 mM Tris buffer (pH 7.4) and 1 % bovine serum albumin (BSA), and washed twice. The final pellets were resuspended in the same medium to a final protein concentration of about 10-15 mg per ml.

For the ascites tumour cells the following procedure was carried out: the cells were washed twice with 0.21 M mannitol, 0.07 M sucrose and 1 mM EGTA. The packed tumour cells were then resuspended, in a volume of the same solution three times larger, with 0.1 M citric acid (400:1 v/v). The cells were homogenized in a tight Dounce homogenizer (10-20 strokes). The homogenate was centrifuged at 900 $\times$ *g* for 20 minutes. The top two thirds of the supernatant were collected and buffered to pH 7.4 with 1 M Tris. The resuspended sediments, in the same amount and medium as before, were again homogenized and centrifuged, and collection and neutralization was repeated as before. The supernatants of the second centrifugation were combined with those obtained from the first, and centrifuged at 5,000 $\times$ *g* for 20 minutes. Sediments were resuspended in 0.21 M mannitol,

0.07 M sucrose, 1 mM EGTA, 20 mM Tris buffer (pH 7.4) and 1 % BSA, and washed twice at 8,000 × *g* for 10 minutes, and finally, at 12,000 × *g* for 10 minutes. The final pellets were resuspended in the same medium to a final protein concentration of about 10-15 mg per ml.

Protein concentration was determinated as described by WADDELL (11) and modified by MURPHY (9).

Glucose-6-phosphatase activity, as control for microsomal contamination, was determinated as described by HARPER (4); acid phosphatase activity, as control for lysosomal contamination, was determinated according to GIANETTO (3). Bacterial contamination, was controlled by the surface viable count technique (1). Oxygen consumption, and phosphorylation of the mitochondrial suspension, were measured polarographycally in an Oxygraph (model KM of the Gilson Medical Electronics) equipped with a Clarck type, membrane coated, oxygen electrode (Yellow Spring Instruments), as described by ESTABROOK (2). The reaction mixture contained in final concentration 0.21 M mannitol, 0.07 M sucrose, 1 mM $MgCl_2$, 10 mM KCl, 1 mM EGTA, 10 mM phosphate buffer (pH 7.4), 1 % BSA and 2 mg of mitochondrial protein, to a final volume of 2.0 ml.

Results

Table I shows the oxygen consumption and phosphorylation of isolated mitochondria with various substrates.

The data for ADP/O ratios, respiratory control ratios and specific activity, clearly show that isolated mitochondria from all the studied sources, are tightly coupled, except those isolated from plasma cell tumour and Ehrlich ascites tumour cells. As can be seen, the best values were obtained using the succinate as substrate.

Table II shows the recovery of protein, glucose-6-phosphatase and acid phosphatase activities.

In all experiments performed, the mito-

Table I. *Oxygen consumption and phosphorylation of isolated mitochondria.*

Mitochondria	Substrates	ADP/O	R.C.R.	Specific* activity
Rat liver	Succinate	1.8	5.1	546
	Ketoglutarate	3.5	4.4	424
	Ketoglutarate + malate	3.2	3.9	512
	Pyruvate + malate	2.3	4.0	364
Mouse submaxillary gland	Succinate	1.7	5.0	386
	Ketoglutarate	3.3	3.6	321
Submaxillary gland adenocarcinoma	Succinate	1.9	4.8	232
	Ketoglutarate	3.7	4.1	225
	Pyruvate + malate	3.1	3.6	202
Plasma cell tumour	Succinate	1.7	2.5	262
	Ketoglutarate	2.6	2.3	115
Ehrlich ascites tumour cells	Succinate	1.8	3.3	200
	Ketoglutarate	2.6	2.5	128
Yoshida ascites tumour cells	Succinate	1.8	4.4	398
	Ketoglutarate	3.4	5.2	219
	Pyruvate + malate	2.8	3.3	155

* μmoles of PI esterified/min/mg of protein.

Table II. *Recovery of protein, glucose-6-phosphatase and acid phosphatase activities in the mitochondrial fraction.*

Mitochondria	Recovery % protein*	gluc-6-Pase**	acid Pase**
Rat liver	11.4	6.1	19.5
Mouse submaxillary gland	5.0	8.3	15.8
Submaxillary gland adenocarcinoma	6.6	7.1	24.6
Plasma cell tumour	5.3	6.4	17.3
Ehrlich ascites tumour cells	5.2	5.8	24.4
Yoshida ascites tumour cells	6.2	6.0	26.5

* Expressed as the percentage of protein amount present in the homogenate.
** Expressed as the percentage of enzymatic activity of the homogenate.

chondrial fractions were sufficiently germ free, since the bacterial contamination present was always lower than 1×10^3 viable bacterial cells per mg of mitochondrial protein, values below thoses indicated by KROON (5) for semisterile mitochondria.

Discussion

Large scale preparation of mitochondria from solid normal and neoplastic tissues and from ascites tumour cells followed in the present work, may be considered as a significant improvement on previously reported techniques, essentially because the described problem of the greater lability of tumour cell mitochondria has been solved employing media containing mannitol, EGTA and BSA. This choice of mannitol and EGTA rather than sucrose and EDTA, seems in fact to offer greater protection toward the permeability and oxydative functions of mitochondria. The presence in the medium of BSA is of great importance in order to avoid the swelling of the mitochondria (10, 12).

These media allow to obtain highly purified and tightly coupled mitochondria and it should also be emphasized that the procedures used are suitable for the isolation of phosphorylating mitochondria from a variety of normal and neoplastic tissues.

Acknowledgements

We are grateful to Prof. F. PALMIERI for critical reading of the manuscript. This work was supported by a grant from Consiglio Nazionale delle Ricerche, Italy.

Resumen

Se describe una técnica de gran utilidad para la preparación en gran escala de mitocondrias de tejidos normales y neoplásicos, empleando manitol, sacarosa, EGTA y albúmina sérica bovina. Esta técnica supera satisfactoriamente las dificultades debidas a la gran labilidad de las mitocondrias de células tumorales. Asimismo, las mitocondrias obtenidas no presentan contaminación nuclear, citoplasmática, lisosomal o bacteriana en cantidades significativas.

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