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Cellular Compartmentation of Ehrlich Ascites Tumor Cells

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The digitonin method for the study of cellular compartmentation in mitochondrial and cytosolic fractions was applied to Ehrlich ascites tumor cells. The volume of mitochondrial and cytosolic water spaces are calculated to be 1.62 μl/30 $\times$ 10^6 cells and 12.7 μl/30 $\times$ 10^6 cells respectively, by the technique of ^3H_2O permeable and (^{14}C)-sucrose impermeable spaces. The validity of the methods was tested by the distribution of cytosolic (lactate dehydrogenase) and mitochondrial (citrate synthase and glutamate dehydrogenase) marker enzymes. As occurs in normal hepatic cells, an asymmetric distribution of ATP and ADP was observed. The ATP/ADP ratio in the cytosolic fraction was 7 times higher than in the mitochondrial fraction.

Key words: Cellular compartmentation, Ascites cells, Cytosolic volume, Mitochondria.

To determine the actual concentration of metabolites in the cytosolic and mitochondrial fractions, two methods, cellular permeabilization and cavitation technique, have been recently described for cellular compartmentation in isolated hepatocytes from rat liver (1, 16, 20, 21). In the most popular method, isolated liver cells are treated with digitonin in order to permeabilize the plasma membrane, and subsequently centrifuged through silicon oil to separate the two compartments. This method has also been used by LELYVELD and HOMMES (17) for fractionation of fetal rat liver cells. WIELAND *et al.* (3) have largely discussed the validity of the digitonin method for metabolite compartmentation in isolated hepatocytes. The method takes advantage of the fact that cytoplasmic membrane contains considerably more cholesterol than the mitochondrial membrane (4). In

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the present report a modification of the digitonin procedure is described for Ehrlich ascites tumor cells. Likewise, in order to know the metabolite concentration in the cytosolic and mitochondrial spaces, the water volumes of both compartments were accurately determined, by the technique of sucrose impermeable and water-permeable isotopic distribution.

Materials and Methods

Three strains of Ehrlich ascites tumors were used in this study: CBM strain from the «Centro de Biología Molecular», Madrid (Spain); Roma strain, supplied by Dr. Tommaso Galeotti, «Universitá Cattolica Sacro Cuore», Roma; INO strain, supplied by Dr. J. Coll, «Centro Ramón y Cajal», Madrid (Spain). The cells were maintained in two month old albino Swiss mice and harvested about 10 days after inoculation with 5×10^6 cells. The cells were withdrawn from the animals sacrificed by cervical dislocation and washed twice 0.15 M NaCl. Finally 60×10^6 cells/ml were suspended in 0.15 M NaCl, 6.15 mM KCl and 11 mM phosphate buffer pH 7.4.

Chemicals. Digitonin, MOPS (4 morpholinopropane sulfonic acid), dimethyl POPOP and PPO were obtained from Sigma; enzymes and coenzymes from Boehringer (Manheim, Germany). The silicon oils, AR 200 and AR 20, were provided by Wacker Chemie, G.m.b.H., München, (Germany). Tritiated water and (^{14}C)-sucrose were obtained from Amersham. Other chemicals and reagents were of the best grade available.

Assays. Adenine nucleotides were determined in the neutralized protein-free perchloric acid extracts using spectrophotometric enzymatic assays. ADP and AMP were determined by the method of JWOREK *et al.* (8), ATP as described by LAMPRECHT and TRAUTSCHOLD (11). Lactate dehydrogenase activity was determined according to BERGMEYER *et al.* (2); glutamate dehydrogenase by the method of KHANG *et al.* (9) and citrate synthase as described by SRERE (15).

Fractionation of tumor cells. Fractionation by digitonin method was performed at 0° C in 3 ml polypropylene centrifuge tubes loaded with 0.5 ml 1 M $HClO_4$ or 0.33 M sucrose, 1 ml silicon oil consisting in a mixture of types AR 200 and AR 20 (2:1 w/w). The upper layer consisted of 0.5 ml of a medium containing 0.25 M mannitol, 3 mM EDTA, 20 mM 4-morpholinopropane sulfonic acid pH 7, and digitonin. The values for whole cells were obtained by the same procedure, except the digitonin treatment. The values for cytosolic compartment were calculated by subtraction of values obtained for digition treated cells from those obtained for whole cells. Digitonin was purified by the method described by JANSKI and CORNELL (7). The use of a water soluble digitonin is critical for achieving a suitable preparation. Cell suspension (0.5 ml) was rapidly mixed with the upper layer by means of a Gilson pipetman P 1000 equipped with a bent disposable tip. After permeabilization of the cytoplasmic membrane, the cells were centrifuged for 90 s at 4,000 *g*. Because of lesser density of Ehrlich ascites tumor cells, the time of centrifugation has been elongated in comparison with the 30 s required in the conventional method for hepatocytes.

Determination of the volume of the mitochondrial and cytosolic water spaces. They were calculated by the method described by NICHOLLS (12) mod-

Table I. *Leakage of cytosolic and mitochondrial marker enzymes during the fractionation of Ehrlich ascites tumor cells (CBM strain).* The activity of whole cell was considered as 100 %.

Digitonin (mM)	Incubation time (s)	Lactate dehydrogenase (%)		Citrate synthase (%)		Glutamate dehydrogenase (%)	
		Cytosolic fraction	Mitochondrial fraction	Cytosolic fraction	Mitochondrial fraction	Cytosolic fraction	Mitochondrial fraction
0.50	5	95.3 ± 1.3	4.7 ± 1.3	3.5 ± 0.8	96.5 ± 0.8	1.8 ± 0.5	98.2 ± 0.5
0.50	10	96.0 ± 0.4	4.0 ± 0.4	3.3 ± 0.3	96.7 ± 0.3	2.9 ± 2.1	97.1 ± 2.1
0.50	15	95.7 ± 1.5	4.3 ± 1.5	4.4 ± 0.9	95.6 ± 0.9	4.8 ± 1.9	95.2 ± 1.9
0.75	5	96.4 ± 0.9	3.6 ± 0.9	5.1 ± 0.8	94.9 ± 0.8	3.0 ± 1.0	97.0 ± 1.0
0.75	10	96.0 ± 1.4	4.0 ± 1.4	6.4 ± 0.9	93.6 ± 0.9	5.4 ± 1.0	94.6 ± 1.0
0.75	15	97.1 ± 0.7	2.9 ± 0.7	7.0 ± 0.2	93.0 ± 0.2	7.1 ± 2.2	92.9 ± 2.2
1.00	5	97.7 ± 0.7	2.3 ± 0.7	8.3 ± 2.1	91.7 ± 2.1	5.2 ± 1.8	94.8 ± 1.8
1.00	10	98.0 ± 0.7	2.0 ± 0.7	14.1 ± 0.2	85.9 ± 0.2	10.0 ± 2.1	90.0 ± 2.1
1.00	15	96.9 ± 1.0	3.1 ± 1.0	18.1 ± 2.7	81.9 ± 2.7	15.7 ± 0.9	84.3 ± 0.9

ified as follows: 30 × 10^6 cells were incubated for 10 min in the upper layer of a polypropylene tube previously loaded with 0.5 ml of 5 % triton X-100, 0.33 M sucrose and 1 ml of silicon oil. The upper layer (0.5 ml) was labelled with 0.5 μCi of (^{14}C)-sucrose (10 mCi/mmol) and 2.5 μCi of ^3H_2O (180 mCi/mol). After the addition of 0.1 ml of 6 mM digitonin or buffer to the upper layer, the cells were centrifuged at 4,000 *g* for 90 s. Following centrifugation, 50 μl of the upper layer were added to 2.5 ml of scintillation liquid consisting of ethanol, toluene, and dioxane (1:1:1), dimethyl POPOP 0.1 g/l and PPO 5 g/l. The total volume of lower layer was added to 2.5 ml of the same scintillation liquid. Samples were counted in a Beckman LS-350 liquid scintillation counter. Quenching correction was calculated by the method of HENDLER (6). Total water space and the volume occupied by sucrose were obtained according to: space = 50 × n/N μl, where N is the CPM of isotope in the upper layer, and n the CPM of the isotope in the lower layer. The mitochondrial water space was calculated by subtraction of volume occupied by sucrose in permeabilized cells, from the total water cellular volume.

Results and Discussion

Table I shows the release of marker enzymes into the supernatant fraction during fractionation of Ehrlich ascites tumor cells in the CBM strain. Lactate dehydrogenase was used as cytosolic marker enzyme and citrate synthase and glutamate dehydrogenase as mitochondrial matrix marker enzymes. Since the mitochondrial membrane of the tumor has a larger cholesterol content than normal cells (18), the mitochondrial membrane of the tumor cells was made more permeable than the mitochondria of normal hepatic cells. As compared with the standard digitonin fraction procedure for hepatocytes, the time of exposure to digitonin was shortened by, at least, a factor of three (1). Moreover, at 2 mM digitonin used in hepatocyte fractionation, the mitochondrial membrane was made highly permeable, since almost one third of total citrate synthase was found in the cytosolic fraction. Similar results were found for Roma and INO strains.

Table II. *Subcellular distribution of adenine nucleotides in Ehrlich ascites cells (Roma strain).* Cells were preincubated to 37° under 95 % O_2 and 5 % CO_2 for 10 min, and treated with 1 mM digitonin for 5 s. Values are means of 4-5 replicate different experiments ± S.E.M.

	ATP (mM)	ADP (mM)	AMP (mM)	ATP/ ADP
Mitochondria	6.1 ± 0.5	8.0 ± 1.5	4.6 ± 1.1	0.76
Cytosol	3.7 ± 0.4	0.7 ± 0.2	0.4 ± 0.1	5.28

The volume of mitochondrial water space for INO cells was 1.62 ± 0.3 μl/30 × 10^6 cells; the total volume of the cellular water space was 14.3 ± 1.0 μl /30 × 10^6 cells. The volume of the mitochondrial water space for Ehrlich ascites cells was calculated to be about 11.3 % of the total volume of the cellular water space. These values are in very good agreement with those reported by WILLIAMSON (19) for hepatocytes, in which the mitochondrial water space was calculated to be about 9.5 % of the total water space.

The validity of the method was tested for the subcellular distribution of adenine nucleotides. Table II shows the concentration of adenine nucleotides in the mitochondrial and cytosolic fractions. About a third of the sum of adenine nucleotides was found in the mitochondrial fraction, as occurs in normal hepatic cells (13). Likewise,

ATP/ADP ratio in the cytosolic fraction was 7 times higher than that in the mitochondrial fraction, as is described for normal cells (14). The energy charge was 0.85 for cytosolic fraction and 0.54 for mitochondrial fraction; these values are very close to those found by AKERBOOM *et al.* (1) for hepatocytes, 0.91 for cytosolic fraction and 0.65 for mitochondrial fraction. The asymmetric distribution of ADP and ATP between the mitochondrial and extramitochondrial compartments suggest that in tumor cells the process of adenine nucleotides translocation may be similar to that described by KLINGENBERG *et al.* (5, 10) for normal cells.

The results presented here confirm the validity of the method, and suggest that permeabilization by digitonin could be a very interesting biochemical technique for the study of cytosolic and mitochondrial interrelation in tumors.

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Resumen

Se aplica el método de fraccionamiento celular con digitonina a células de tumor ascítico de Ehrlich. Los volúmenes de los espacios acuosos mitochondrial (1,62 μl/30 × 10^6 células) y citosólico (12,7 μl/30 × 10^6 células) han sido calculados por la técnica de ^3H_2O permeable y (^{14}C)-sacarosa no permeable a las membranas celulares. La validez del método se confirma por la distribución de enzimas marcadoras: lactato deshidrogenasa para la fracción citosólica, y citrato sintasa y glutamato deshidrogenasa para la fracción mitocondrial. Como ocurre en hepatocitos, se observa una distribución asimétrica de los nucleótidos ATP y ADP en citosol y mitocondria, siendo la razón ATP/ADP citosólica 7 veces mayor que la razón ATP/ADP mitocondrial.

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