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BIOFLAVONOID REGULATION OF ATPase AND HEXOKINASE ACTIVITY IN EHRLICH ASCITES CELL MITOCHONDRIA

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SUMMARY

(1) The mitochondrial ATPase (EC 3.6.1.3) Ehrlich ascites cell mitochondria, was inhibited by D-glucose under physiological concentrations of ATP. The generation of ADP by the mitochondrial bound hexokinase, seems to be the reason for the D-glucose inhibitory effect. Reversal of the inhibitory effect of ADP on Ehrlich ascites cell mitochondria ATPase by an ATP-regenerating system was achieved.

(2) Dissociation of mitochondrial bound hexokinase from the mitochondria eliminated the inhibitory effect of D-glucose. Rebinding of the hexokinase to the mitochondria regenerated the D-glucose inhibitory effect on Ehrlich ascites cell mitochondria ATPase.

(3) Bioflavonoids such as quercetin inhibit the mitochondrial hexokinase activity, but do not change the mitochondrial ATPase activity of isolated Ehrlich ascites tumor cell mitochondria.

(4) The inhibitory effect of bioflavonoids on mitochondrial bound hexokinase activity is shown to be dissociable from the ascites tumor cell mitochondria and seems to be associated with regulatory rather than catalitic sites of the enzyme.

INTRODUCTION

The ATPase (EC. 3.61.) activity of different pump systems was shown to be inhibited by bioflavonoids [1–4]. In malignant cells, it was suggested that bioflavonoids inhibit aerobic glycolysis by inhibition of their excessive ATPase activity [2]. Quercetin (3,3′,4′,5,7-pentahydroxyflavone) was shown to inhibit the ATPase activity of beef heart submitochondrial particles without interfering with the oxidative phosphorylation reaction [1]. Furthermore, this compound was shown to inhibit the Ca^{2+} ATPase activity of trypsin treated chloroplast coupling factor-1 (CF_1) [3]. Thus, the quercetin binding sites are possibly located on the large subunits of

Abbreviation: DHMF, 4′,5,7-hydroxy-3,6-methoxyflavone.

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the CF_1. Recently, a model was proposed for the relative location of quercetin sites on the largest CF_1 subunits [5].

The location of the mitochondrial hexokinase on the outer mitochondrial membrane in Ehrlich ascites tumor cells [6] and the mitochondrial ATPase in the inner membrane [7] provides the possibility to determine the site of action of inhibitors, such as quercetin, which are associated with the control mechanism of energy metabolism. Recently it has been suggested that the equilibrium between soluble and mitochondrial bound hexokinase may be controlled by change of metabolite levels such as ATP and glucose 6-phosphate [6, 8, 9]. The association of hexokinase with mitochondria as well as soluble fraction of cells may have biological importance in sugar metabolism and energy turnover. The question which arises in this work is to find out whether the association, dissociation or inhibition of hexokinase of the mitochondria controls enzymatic activities which are integrated in these particles.

MATERIALS AND METHODS

Preparations

Ehrlich ascites tumor cells were maintained by inoculation in mice and harvested after 7–10 days of growth as described [10]. Cells from 10–12 mice were suspended at 4° C in 100 ml 50 mM sodium *N*-tris (hydroxy methyl) methylglycine buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl and 2 mM $MgCl_2$. After 10 min centrifugation at 800 × *g*, the packed tumor cells (approximately 5 ml) were mixed with 1 ml 0.1 M Tris · HCl buffer, pH 7.4, containing 0.25 M mannitol and 2 mM $MgCl_2$. The mixture was poured into a pre-cooled mortar and ground in the presence of 3 g of washed glass beads, as described [11]. A typical separation by differential centrifugation of the homogenate, and the ATPase and hexokinase activities in each fraction are represented in Table I. After grinding, cell debris and glass beads were discarded by centrifugation at 800 × *g* for 5 min. The supernatant which is referred to as 'Homogenate' was centrifuged at 12 000 × *g* for 10 min. The supernatant obtained after this centrifugation is referred to as 'microsomes and soluble

TABLE I

DISTRIBUTION OF ATPase AND HEXOKINASE ACTIVITIES IN EHRLICH ASCITES CELL HOMOGENATE

Fractions from Ehrlich ascites cells were prepared as described in Materials and Methods. 1 unit represents the degradation or formation of 1 μmol substrate per min at 30 °C.

Fraction	Total protein (mg)	ATPase activity		Hexokinase activity	
		μmol/ mg per min	units	μmol/ mg per min	units
Homogenate	1146	0.038	43.6	0.058	66.5
Mitochondria	45.6	0.270	12.3	0.620	28.2
Mannitol supernatant	31.9	0.185	5.9	0.210	6.7
Microsomes and soluble enzymes	915	0.026	23.8	0.032	29.3

enzymes'. The pellet after the 12 000 × *g* centrifugation was mixed with 1 ml 0.25 M mannitol and centrifuged again at 12 000 × *g* for 10 min. The supernatant of this centrifugation was referred to as 'mannitol supernatant' and the pellet as 'mitochondria'.

Solubilized mitochondrial hexokinase was prepared according to a previous procedure [6].

Soluble hexokinase was obtained by homogenization of Ehrlich ascites tumor cells. One volume of packed cells was suspended in four volumes of distilled water at 4 °C. The homogenization was carried on with a Dounce homogenizer. The homogenate was centrifuged for 30 min at 30 000 × *g*. The pellet, which contains broken cells, nuclei and mitochondria was discarded. The supernatant was used for determination of soluble hexokinase activity.

Assays

Hexokinase activity was assayed by coupling the phosphorylation of D-glucose by ATP to the glucose-6-phosphate dehydrogenase reaction [12]. A sample of particles or soluble enzyme was added to a cuvette in a final volume of 1 ml 50 mM potassium tricine buffer, pH 7.6, containing 2 mM $MgCl_2$ 100 mM KCl, 5 mM NaCl, 10 mM glucose, 0.4 mM $NADP^+$ and 0.5 units glucose-6-phosphate dehydrogenase. The rate of the enzyme activity was determined with the Gilford spectrophotometer maintained at 30° C. The reaction was incubated for 10 min with and without inhibitors and started by the addition of sodium ATP to a final concentration of 2 mM.

The mitochondrial ATPase activity was assayed by addition of the particles in a final volume of 1 ml, containing potassium 50 mM tricine buffer, pH 7.6, 2 mM $MgCl_2$, 100 mM KCl and 5 mM NaCl (tricine/potassium buffer). ATP regenerating system which consists of 5 mM phosphoenol pyruvate and 2 units of pyruvate kinase [13] was added to the reaction mixture as indicated in the legends of the tables and the figures. The reaction was started after 10 min preincubation at 30 °C by the addition of 2 μmol ATP and stopped after 10 min by addition of 50 μl 40 % trichloroacetic acid. After centrifugation, the supernatant was assayed for P_i as described [14]. The ATPase activity at low concentration of mitochondrial protein was determined by the release of $^{32}P_i$ from [γ-^{32}P]ATP as described [15]. This assay was performed under the same conditions of the previous determinations of ATPase activity except that the reaction was started by addition of 2 mM labeled ATP. The specific radioactivity in each assay was 0.003 μCi/mM. Protein was determined according to the method of Lowry et al. [16].

Chemicals

The chemicals used were all of analytical reagent grade. Adenine nucleotides, D-hexoses, glucose 6-phosphate, quercetin, phosphoenol pyruvate, pyruvate kinase, yeast hexokinase, $NADP^+$, tricine and bovine serum albumin were obtained from Sigma (St. Louis) Corp. Rutamycin was generously supplied by Dr. G. E. Mallet of Eli Lilly. 4',5,7-hydroxy-3,6-methoxyflavone (DHMF) was generously supplied by Dr. E. Wollenweber.

RESULTS AND DISCUSSION

Interrelationship between ATPase and hexokinase activities in Ehrlich ascites cell mitochondria

Highly purified hexokinase from yeast was suggested to contain ATPase activity [17]. Furthermore, the ATPase activity of hexokinase was considered as a possible candidate for production of ADP and P_i in tumor cells [18]. Since the solubilized mitochondrial hexokinase does not contain significant ATPase activity (Table II), the possibility that only the bound hexokinase may contain ATPase activity was tested in the experiment represented in Table III. It is shown in this table that atractyloside a compound which was shown to inhibit the translocation of nucleotides through the

TABLE II

RESOLUTION AND RECONSTITUTION OF D-GLUCOSE SENSITIVITY TO EHRLICH ASCITES CELL MITOCHONDRIAL ATPase

Freshly prepared mitochondria, or the same material treated with 1 M NaCl and washed with 0.25 M mannitol as described [6], served these experiments for reconstitution of D-glucose sensitivity, 0.55 units of solubilized mitochondrial hexokinase were added to 50 μg mitochondria in a final volume of 0.3 ml, which contains 20 μmol triethanolamine/chloride pH 7.4 and 2 μmol $MgCl_2$. The mixture was incubated for 10 min at 30 °C and the particles were recovered by centrifugation. Number of experiments, 5. Mean values ±S.D. are given.

	ATPase activity (μmol/mg per min)	
	Control	+10 mM D-glucose
Mitochondria	0.28±0.02	0.13±0.02
Treated mitochondria	0.30±0.03	0.28±0.02
Solubilized mitochondrial hexokinase	0.03±0.005	0.03±0.005
Treated mitochondria plus solubilized mitochondrial hexokinase	0.31±0.04	0.17±0.02

TABLE III

THE EFFECT OF RUTAMYCIN, AZIDE AND ATRACTYLOSIDE ON EHRLICH ASCITES CELL MITOCHONDRIAL ATPase IN THE PRESENCE OF D-GLUCOSE

Mitochondria, 50 μg/ml were preincubated in tricine/potassium buffer for 10 min at 30 °C in the presence or the absence of the compounds as indicated. The reaction was started by addition of 2 μmol ATP. The liberation of P_i from ATP was determined after 10 min incubation. Number of experiments, 4. Mean values ±S.D. are given.

Additions	ATPase activity (μmol/mg per min)	
	Control	+10 mM D-glucose
No additions	0.28±0.03	0.120±0.02
+8 μg/ml rutamycin	0.08±0.02	0.030±0.004
$+10^{-3}$M azide	0.06±0.01	0.025±0.004
$+5 \cdot 10^{-5}$M atractyloside	0.12±0.02	0.050±0.006

mitochondrial membranes [19], does not prevent the D-glucose inhibitory effect. If atractyloside acts to prevent the transport of ATP to the inner compartment of the mitochondria, then at least 60 % of the ATPase activity tested in these experiments is located in the inner side of the mitochondria. Furthermore, 70–80 % from the ATPase activity in mitochondria isolated from Ehrlich ascites cells is sensitive to mitochondrial ATPase inhibitors such as rutamycin and azide. This ATPase activity is further inhibited by the addition of 10 mM D-glucose. These observations may suggest a different location of ATPase activity and the D-glucose inhibitory effect on ATPase activity in these mitochondria.

The kinetics of dissociation and association of mitochondrial bound hexokinase from Ehrlich ascites cell mitochondria were already studied [6]. When the mitochondrial bound hexokinase was dissociated from the mitochondria by the same method (Table II), abolition of the D-glucose inhibitory effect on ATPase activity was observed. Regeneration of the D-glucose inhibitory effect was obtained almost completely by rebinding of the solubilized mitochondrial hexokinase to the treated mitochondria. This observation further indicates that mitochondrial bound hexokinase, which is probably located on the outer mitochondrial membrane, may regulate the mitochondrial ATPase activity.

In previous studies [6], the bound hexokinase of ascites tumor cell mitochondria was proposed to be external to the ATP transport barrier. Therefore the possibility that the D-glucose inhibitory effect of mitochondrial ATPase is due to product inhibition of ADP formed by extensive mitochondrial hexokinase activity was tested in the experiment represented in Table IV. At concentration of 10 mM, the D-hexoses: glucose, fructose, mannose and glucose amine, all substrates for hexokinase, inhibit the ATPase activity of isolated mitochondria. On the other hand D-galactose which is a poor substrate for hexokinase was found to be ineffective. By using the ATP regenerating system which consists of 5 mM phosphoenol pyruvate and 2.0 units of pyruvate kinase, the inhibitory effect of the different hexoses was abolished almost completely and stimulation of the ATPase activity in comparison with the control was observed.

TABLE IV

EHRLICH ASCITES TUMOR CELLS ATPase ACTIVITY IN ISOLATED MITOCHONDRIA. THE EFFECT OF DIFFERENT D-HEXOSES AND ATP REGENERATING SYSTEM

Mitochondria from Ehrlich ascites cells, 50 μg/ml, were preincubated for 10 min at 30 °C in the presence of 10 mM D-glucose, D-fructose, D-mannose, D-galactose and D-glucosamine. The reaction was started by addition of 2 μmol ATP. Inorganic phosphate was determined after 10 min incubation. The data represented are the mean of 4 experiments. Mean values ±S.D. are given.

	ATPase activity (μmol/mg per min)	
	Control	+Regenerating system
No additions	0.23±0.02	0.33±0.03
+10 mM D-glucose	0.08±0.01	0.33±0.02
+10 mM D-fructose	0.07±0.01	0.33±0.04
+10 mM D-mannose	0.07±0.01	0.31±0.03
+10 mM D-galactose	0.23±0.02	0.34±0.04
+10 mM D-glucosamine	0.11±0.01	0.33±0.03

TABLE V

THE EFFECT OF D-GLUCOSE ON $^{32}P_i$ PRODUCTION AT DIFFERENT PROTEIN CONCENTRATIONS OF EHRLICH ASCITES CELL MITOCHONDRIA

The formation of $^{32}P_i$ was determined as described in Materials and Methods. Number of experiments, 4. Mean values ±S.D. are given.

	$^{32}P_i$ formation (nmol per 10 min)	
	Control	+10 mM D-glucose
10 μg/ml protein	35± 4	16±2
20 μg/ml protein	60± 5	25±3
30 μg/ml protein	85±10	40±5
50 μg/ml protein	140±15	65±5

In order to find out whether the decrease in ATP concentration or the generation of ADP is the main reason for the shown inhibitory effect, the mitochondrial ATPase activity in presence of 10 mM D-glucose was tested at different concentrations of mitochondrial protein. As indicated in Table V similar inhibition of ATPase activity was achieved at different concentrations of mitochondrial protein. Thus, the inhibitory effect of ADP on ATPase activity [13] rather than the drop of ATP level seems to be the main reason for the D-glucose inhibitory effect on the Ehrlich ascites cell mitochondria ATPase.

Regulation of mitochondrial ATPase and hexokinase activities by bioflavonoids

The distinct location of the hexokinase and ATPase in Ehrlich ascites cell

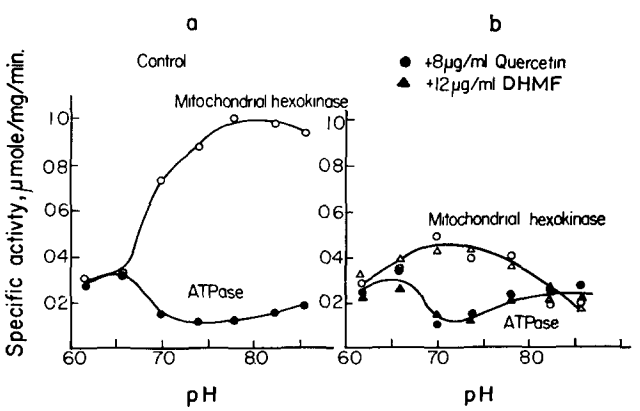

Fig. 1. ATPase and hexokinase activities in Ehrlich ascites cell mitochondria. (a) The pH dependence of Ehrlich ascites cell mitochondrial ATPase and hexokinase activities. For determination of ATPase activities, 50 μg/ml were used as described in Table I. Hexokinase was tested on 20 μg/ml samples of isolated Ehrlich ascites cell mitochondria. (b) The effect of quercetin and DHMF on ATPase and hexokinase activities at different pH ranges. Ehrlich ascites cells mitochondria were preincubated for 10 min at 30 °C in the presence of 8 μg/ml quercetin or 12 μg/ml DHMF. The data represented are the mean of 3 experiments with S.D. of 8–15 %.

mitochondria permits the definition of the sites involved with the regulation of the ATP metabolism by bioflavonoids. The high lactate formation in malignant cells was considered to be associated with high ATPase activity and low pH levels [18]. Since quercetin was shown to inhibit various ATPase systems [1–4], it was interesting to find whether variation of the proton concentration in the medium influences the bioflavonoids effect on the ATPase and hexokinase activity in Ehrlich ascites cell mitochondria. Therefore, the pH dependence of the quercetin inhibitory effect on Ehrlich ascites cell mitochondria ATPase and mitochondrial bound hexokinase activities was studied (Fig. 1). It is clear from Fig. 1a, that along with the pH increase there is elevation of the mitochondrial bound hexokinase activity whereas the Ehrlich ascites cell mitochondria ATPase activity slightly decreases.

Furthermore, it is shown in Fig. 1b that both bioflavonoids, quercetin and DHMF decrease the pH-dependent elevation of mitochondrial hexokinase activity. On the other hand, the Ehrlich ascites cell mitochondria ATPase activity is unaffected by both bioflavonoids in all the pH ranges tested. Thus, in these mitochondria, the pH variation influences the bioflavonoid inhibitory effect on enzymatic activity which is associated with the outer and not with the inner mitochondrial membrane.

As already proposed in Tables IV and V, high generation of ADP is the reason for the D-glucose inhibitory effect on Ehrlich ascites cell mitochondria ATPase. Is it possible that quercetin prevents the ADP inhibitory effect on Ehrlich ascites cell mitochondria ATPase? The experiment presented in Table VI shows that at a concentration of 1 mM ADP, 60 % of the ATPase activity is inhibited. Quercetin, at a concentration of 12 μg/ml does not prevent the ADP inhibitory effect. This observation further indicates that the bioflavonoid effect on these mitochondria is associated with the inhibition of the hexokinase activity rather than with the abolition of the ADP inhibitory effect.

The effect of quercetin on dissociation of mitochondrial bound hexokinase

Glucose 6-phosphate was found to be a potent inhibitor of rat brain hexokinase activity [20]. In several reports it was proposed that the binding and dissociation of

TABLE VI

THE EFFECT OF ADP AND QUERCETIN ON MITOCHONDRIAL ATPase IN EHRLICH ASCITES CELLS MITOCHONDRIA

ATPase activity of Ehrlich ascites cell mitochondria was determined in the presence and absence of 1 mM ADP as described in Table I.

	ATPase activity (μmol/mg per min)	
	Expt. I	Expt. II
Control	0.25	0.27
+1 mM ADP	0.11	0.13
+12 μg/ml quercetin	0.24	0.25
+ADP+quercetin	0.11	0.12

TABLE VII

THE EFFECT OF D-GLUCOSE, GLUCOSE 6-PHOSPHATE, QUERCETIN AND DHMF ON EHRLICH ASCITES CELL MITOCHONDRIA ATPase

Ehrlich ascites cell mitochondrial ATPase activity was determined after preincubation of 10 min at 30 °C with 1 mM glucose 6-phosphate, 8 μg/ml quercetin or 12 μg/ml DHMF in the presence or absence of 10 mM D-glucose. The data represented are the mean of 4 experiments. Mean values ±S.D. are given.

	ATPase activity (μmol/mg per min)	
	Control	+10 mM D-glucose
No additions	0.29±0.03	0.12±0.01
+1 mM glucose 6-phosphate	0.30±0.04	0.28±0.03
+8 μg/ml quercetin	0.30±0.03	0.29±0.02
+12 μg/ml DHMF	0.31±0.04	0.30±0.03

mitochondrial hexokinase is regulated by glucose 6-phosphate, ATP, P_i and bivalent cations [6, 8, 9].

In the experiment represented in Table VII it is shown that 1 mM glucose 6-phosphate, 8 μg/ml quercetin or 12 μg/ml DHMF abolish the D-glucose inhibitory effect of the mitochondrial ATPase activity. Is the abolition of the D-glucose inhibitory effect on the mitochondrial ATPase by the bioflavonoids due to resolution of the hexokinase from the mitochondria? As shown in Table VIII, after 10 min preincubation at 30 °C, glucose 6-phosphate and ATP cause a release of the hexokinase activity from the Ehrlich ascites cell mitochondria as reported [6, 8]. On the other hand, a slight, if any, dissociation of hexokinase from these mitochondria was found after preincubation with 12 μl/ml quercetin. Furthermore, the D-glucose inhibitory effect

TABLE VIII

ATPase AND HEXOKINASE ACTIVITIES IN ATP, GLUCOSE 6-PHOSPHATE AND QUERCETIN TREATED EHRLICH ASCITES CELL MITOCHONDRIA

Ehrlich ascites cell mitochondria, 50 μg protein per ml, were incubated in 0.25 M mannitol for 10 min at 30 °C. When indicated in Table V, mM ATP, 1 mM glucose 6-phosphate or 12 μg/ml quercetin were added in the preincubation medium. After centrifugation for 10 min at 10 000 × *g*, ATPase and hexokinase activities were measured. Bovine serum albumin at concentration of 3 mg/ml was added to the reaction mixtures to avoid inhibitory effects of bound quercetin [21]. Number of experiments, 4. Mean values ±S.D. are given.

	ATPase activity (μmol/mg per min)		Hexokinase activity (μmol/mg per min)
	Control	+10 mM D-glucose	
Mitochondria	0.31±0.03	0.15±0.02	0.58±0.04
ATP-treated mitochondria	0.32±0.02	0.30±0.03	0.17±0.02
Glucose 6-phosphate-treated mitochondria	0.30±0.02	0.31±0.03	0.18±0.02
Quercetin-treated mitochondria	0.33±0.03	0.17±0.02	0.54±0.03

TABLE IX

THE EFFECT OF BIOFLAVONOIDS ON PARTICULATE AND SOLUBLE HEXOKINASE ACTIVITY

Mitochondria and fraction which contains microsomes and soluble enzymes were obtained as described in Materials and Methods. Solubilised hexokinase was obtained from Ehrlich ascites cell mitochondria as described [6]. Number of experiments, 4. Mean values ±S.D. are given.

	Hexokinase activity (μmol/mg per min)		
	Control	+8 μg/ml quercetin	+8 μg/ml DHMF
Mitochondria	0.65±0.05	0.20±0.02	0.30±0.03
Soluble hexokinase	0.05±0.01	0.05±0.01	0.05±0.01
Solubilized mitochondrial hexokinase	0.55±0.1	0.21±0.02	0.25±0.03
Yeast hexokinase	108 ±5	102 ±4	105 ±5

of the ATPase activity was diminished by preincubation with glucose 6-phosphate and ATP, but it persists after the quercetin preincubation treatment. These observations may indicate that the inhibitory effect of quercetin on the mitochondrial bound hexokinase does not involve dissociation of the hexokinase from the mitochondria.

Does the inhibition of the hexokinase by quercetin depend on the location of its enzymatic activity? In the experiment represented in Table IX, it is shown that both mitochondrial bound hexokinase and solubilized mitochondrial hexokinase are inhibited by both bioflavonoids, whereas, both compounds do not inhibit soluble hexokinase activity obtained from Ehrlich ascites tumor cells or yeast. Thus, the bioflavonoid inhibitory effect of Ehrlich ascites cell mitochondria may be associated with a regulatory but not catalytic sites of the hexokinase activity. The observations that 12 μg/ml quercetin changes both V and K_m values for ATP and D-glucose (Table X), further indicate that quercetin affects mitochondrial bound hexokinase activity on regulatory rather than catalitic sites.

The inhibitory effect of quercetin on beef heart mitochondrial ATPase [1] and on Ehrlich ascites cell mitochondrial hexokinase on one hand, but its lack of

TABLE X

V AND K_m VALUES FOR ATP AND D-GLUCOSE IN MITOCHONDRIAL BOUND HEXOKINASE

Ehrlich ascites cell mitochondria were incubated at different concentrations of D-glucose or ATP at 30 °C. The hexokinase activity was determined as described in Materials and Methods.

	K_m/(mM)		V (μmol/mg per min)	
	Control	+12 μg/ml quercetin	Control	+12 μg/ml quercetin
D-Glucose	0.27	0.82	0.09	0.05
ATP	0.42	0.72	0.05	0.03

effect on Ehrlich ascites cell mitochondrial ATPase, may indicate the existence of dissociable bioflavonoid regulatory site for both enzymatic activities in Ehrlich ascites cell mitochondria.

The inhibition of growth and proliferation of tumor cells by bioflavonoids *in vitro* was suggested to be due to the regulation of ATPase systems [21]. The observations in this study indicate, that other membrane bound enzymes, such as hexokinase, which are inhibited by bioflavonoids, may be taken into consideration for the inhibitory effect of these compounds on tumor cells growth. Furthermore, bioflavonoids may regulate growth of cells by direct inhibition of ATPase systems as proposed [1-3, 21] or indirectly as presented in this work.

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