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ADENINE NUCLEOTIDE TRANSPORT IN HEPATOMA MITOCHONDRIA

CHARACTERIZATION OF FACTORS INFLUENCING THE KINETICS OF ADP AND ATP UPTAKE

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Initial velocity measurements of [^{3}H]ADP and [^{3}H]ATP uptake have been made with mitochondria isolated from Morris hepatomas of differing growth rates, and factors known to influence the rates of nucleotide exchange have been examined in an effort to determine whether the elevated rates of aerobic glycolysis in these tumors can be attributed to altered carrier activity. These studies included the determination of the apparent kinetic constants for nucleotide uptake as a function of the mitochondrial energy state and the dependence of transport rates on temperature. Also included in these studies were measurements of the mitochondrial levels of endogenous inhibitors, divalent cations and internal adenine nucleotides. Results obtained showed that with mitochondria isolated from the various tumor lines, the apparent kinetic constants for nucleotide uptake are different from those of control rat or regenerating liver mitochondria; the apparent V_{max} values for both ADP and ATP uptake are significantly lower. Furthermore, under conditions of a high-energy state, the K_m and V_{max} values for ATP uptake are greater than the K_m and V_{max} value for ADP uptake but that under uncoupled conditions, the opposite is observed. Comparison of the levels of mitochondrial Ca^{2+}, Mg^{2+}, long-chain acyl-CoA ester and adenine nucleotide from the various mitochondria showed that important differences exist between liver and hepatoma mitochondria in the levels of Ca^{2+}, long-chain acyl-CoA ester and AMP. Mitochondrial Ca^{2+} levels are elevated 3–5-fold in all tumor lines, and for Morris 7777 hepatoma (a rapidly growing tumor) by a remarkable 70-fold; whereas the levels of acyl-CoA ester and AMP are significantly lower in the more rapidly growing tumors. Arrhenius plots for nucleotide uptake in mitochondria from liver and hepatoma are characterized as being biphasic, having similar activation energies above and below the break point temperature (28–38 and 6–16 kcal/mol, respectively). However, the transition temperature for mitochondria from the various hepatomas is uniformly 4–5°C lower than mitochondria from control liver. The latter difference may reflect a variation in membrane composition, most probably lipid components. It is concluded that the presence of elevated levels of Ca^{2+} and lower levels of AMP in hepatoma mitochondria and difference of membrane compositions may play an important role in limiting adenine nucleotide transport activity in vivo and that the impaired carrier activity may contribute to higher rates of aerobic glycolysis observed in these tumors.

Introduction

Recently, much effort has been devoted towards defining the physiological role of mitochondrial adenine nucleotide transport and

Abbreviations CCCP, carbonyl cyanide *m*-chlorophenylhydrazone, Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

the factors which can influence the net exchange thereby possibly controlling cellular energy metabolism. It is now known that there are at least four important parameters which can influence rates of nucleotide exchange· (a) the mitochondrial energy state [1,2]; (b) the levels of endogenous inhibitors (e.g., long-chain acyl-CoA esters) [3,4]; (c) the size of the internal exchangeable adenine nucleotide pool [1]; and (d) the chemical activity of the internal pool [5,6]. In addition, it is also known that the nucleotide carrier catalyzes the one-four-one exchange of extramitochondrial ADP and ATP, respectively, for intramitochondrial ADP and ATP [7,8] and is exclusively responsible for the exchange of adenine nucleotides across the inner mitochondrial membranes The latter observation is particularly important, since a shift in metabolic competition in favor of glycolysis could occur if the rate at which cytosolic ADP was made available to mitochondria for phosphorylation was reduced to the extent that the rate of mitochondrial ATP synthesis was limited

The observation that many different tumor cells exhibit elevated rates of aerobic glycolysis raises the possibility that this phenomenon may be contributed to by an alteration in the rates of nucleotide exchange. In the present study we have examined this possibility by measuring transport rates in a series of hepatomas whose growth rates correlate with rates of aerobic glycolysis [9–11]. Previously, we have shown that the rate of ADP uptake in hepatoma mitochondria is inversely correlated to the hepatoma growth rate and the tumor size [12]. Aiming to understand the characteristics of the nucleotide transport in these tumor mitochondria, we paid attention to the factors that are known to influence their rates; these include energetic states of the mitochondrial membrane, levels of mitochondrial divalent cations, temperature dependence and internal mitochondrial nucleotide levels. Some parts of the preliminary results have been presented [13,14].

Materials and Methods

Materials

Coenzyme A, NAD, ATP, ADP, NADH, NADP, acetyl phosphate, carboxyatractyloside, oligomycin, phospho*enol*pyruvate, CCCP, bovine serum albumin (fraction V), pyruvate kinase (rabbit liver), yeast hexokinase, glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*), lactate dehydrogenase (rabbit muscle), myokinase, citrate synthetase (pigeon muscle) and malate dehydrogenase (bovine heart) were obtained from Sigma Chemical Co, St. Louis, MO. [2,8-^{3}H]ADP (26.4 Ci/mmol) and [2-^{3}H]ATP (16 Ci/mmol) were purchased from New England Nuclear and Amersham, respectively. Male Sprague-Dawley rats (250–350 g) were from Taconic Farms, Germantown, NY. Male Buffalo strain rats weighing 140–150 g were obtained from Simonsen Laboratory, Gilroy, CA, and shipped to Dr. H.P Morris, Howard University, Washington, DC. where Morris 7777, 7800, 7794-A and 16 cell lines were transplanted into the hind legs and the animals subsequently transferred to our laboratory. The hepatoma cell lines chosen were a fast-growing, poorly differentiated tumor (hepatoma 7777, growth rate of 12–20 days), two well differentiated tumors with intermediate growth rates (hepatoma 7800, 3–5 weeks, and 7794 A, 4–6 weeks) and a slow-growing, highly differentiated tumor (hepatoma 16, 4–6 months). All other reagents used were of highest purity grade commercially available

Methods

Adenine nucleotide transport assay. Mitochondrial uptake of radioactive ATP and ADP was monitored using the carboxyatractyloside stop method [15,16] as follows. In a final volume of 0.95 ml, 0.5–1.0 mg of mitochondria was preincubated for 5–10 min at 2°C in a medium containing 116 mM KCl, 21 mM Tris-HCl (pH 7.4) at 2°C, 1.05 mM EDTA, 13 mM sucrose, 0 53 mM Hepes and 2.0 μg oligomycin. The assay was started by adding graded amounts (50 μl) of radioactive ADP or ATP into reaction tubes under constant vortex-mixing. In these experiments, radioactive nucleotide was added by using a series of Hamilton syringes contained in an apparatus designed to allow simultaneous addition of substrate or inhibitor. After 12 s the reaction was stopped by injecting 50 μl of 200 μM carboxyatractyloside (using another series of syringes) and the mitochondria were centrifuged at 12000 × g for 4 min in a Beckman microfuge. The supernate was aspirated

and the mitochondrial pellet washed with 1 ml of KCl medium (110 mM KCl, 20 mM Tris-HCl (pH 7.4), 1.0 mM EDTA) containing 10 μM carboxyatractyloside and centrifuged as before. The pellet was dissolved in 0.2 ml of 2% SDS, transferred to a scintillation vial containing 3.0 ml of counting fluid and counted by liquid scintillation. Corrections for nonspecific counts trapped by mitochondria in the adherent fluid and sucrose-permeable space were made by subtracting values obtained in which carboxyatractyloside was added before the labeled nucleotide. Under these conditions, the first-order rate constant for nucleotide uptake remained unchanged during the first 15–20 s of the assay. The reproducibility of duplicate sets of assays was $\pm 5\%$ S.E. All other additions to the assay medium are described in the figure legends.

The apparent kinetic constants were calculated from linear portions of the curve by the direct linear-plot method of Eisenthal and Cornish-Bowden [17].

Determination of long-chain acyl-CoA esters. Long-chain acyl-CoA esters were extracted from 10–15 mg of mitochondria by adding 2 parts of 5% (w/v) perchloric acid to 3 parts of mitochondrial suspension. The denatured protein and precipitated acyl-CoA esters were pelleted by centrifugation and the supernatant was saved for subsequent adenine nucleotide analysis. The precipitated esters were dissolved in 0.5 ml of 20 mM Tris-HCl (pH 7.4), and hydrolyzed by adjusting the pH to 12.5–13.0 with 1.0 M KOH. After a 20 min incubation at room temperature, the solution was neutralized with 5% perchloric acid and the CoA levels were determined by the cycling catalytic assay procedure of Michal and Bergmeyer [18].

Determination of mitochondrial adenine nucleotides Mitochondrial AMP, ADP and ATP levels were measured by enzymatic analysis of neutralized perchloric acid extracts obtained from 10–15 mg of mitochondria AMP and ADP levels in the neutralized extracts were measured by the method of Jaworet et al. [19] while ATP levels were measured by the procedure of Lamprecht and Trauschold [20]

Isolation of mitochondria Rat liver mitochondria were isolated from male Sprague-Dawley rats by the method of Johnson and Lardy [21] in 0.25 M sucrose, 10 mM Hepes, 1 mM EDTA, pH 7.4. Hepatoma mitochondria were isolated using the procedure of Kaschnitz et al. [22] from tumors weighing less than 2 g for rat hepatomas in the presence of 1% serum albumin. The choice of using this size of tumor was to obtain mitochondrial preparations of good respiratory control ratio and appeared to have intact double membranes as examined by electron microscope [23]. Preparations with respiratory control ratio values of lower than 2.5 (using succinate as substrate as in Ref. 24) were not used for the present studies. Regenerating livers were obtained from rats 48 h after partial hepatectomies, involving removal of about 70% of the liver (left and median lobes). The procedure of Higgins and Anderson [25] was followed The endogenous contents of Mg^{2+} and Ca^{2+} in mitochondria were measured by atomic absorption using samples dissolved in 3 ml of 2% SDS. Standard curves of 0–2 ppm Mg^{2+} and 0–10 ppm Ca^{2+} were run for each set of determinations in the presence of 0.25 M sucrose and 0.5% SDS for internal corrections Other detailed procedures are described in the figure legends

Results

Effect of the mitochondrial energy state on kinetics of ADP and ATP uptake

Experimental results shown in Tables I and II characterize the effects of the mitochondrial energy state on the apparent kinetic constants of ADP and ATP uptake. These values were obtained from measurements of transport activity in mitochondria isolated from small tumors in the presence of albumin and assayed with and without added respiratory substrate, and uncoupling agent. The basic design of these experiments involves determining the initial velocity for nucleotide uptake under the indicated experimental conditions. Assays performed in the absence of added substrate or uncoupling agent are referred to as a 'low-energy state' or control for comparison with assays performed in the presence of an uncoupling agent or oxidizable substrate, the latter is referred to as a 'high energy state'

ADP uptake. Results in Table I show that under

TABLE I

APPARENT KINETIC CONSTANTS OF ADP UPTAKE IN RAT LIVER AND MORRIS HEPATOMA MITOCHONDRIA EFFECTS OF MITOCHONDRIAL ENERGY STATE

Mitochondria were isolated from liver and tumors weighing 0 5–2 0 g in a sucrose medium, as described in Materials and Methods, in the presence of 1% albumin Initial rates of ADP uptake were measured as described in the text Final concentrations of labeled ADP added to the assay medium were 1, 2 5, 5, 10 and 20 μM for assays measured in the absence of uncoupler and 2 5, 5, 10, 20 and 40 μM for assays measured in the presence of uncoupler Assays performed under conditions of a high-energy state were made by including 5 mM succinate in the preincubation medium For each determination, assays were run in duplicate The apparent V_{max} and K_m values were calculated from linear portions of double-reciprocal plots containing a minimum of four data points at higher substrate concentrations as described before The V_{max} values and K_m are means ±S E expressed in nmol/mg per min and in μM, respectively The numbers in parentheses are numbers of determinations

Mitochondrial source	Low-energy state		+2 μM CCCP		High-energy state	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Regenerating liver	6 6±1 1 (3)	14 2±4 3 (3)	4 9±0 8 (3)	8 6±2 2 (3)	3 0±0 4 (2)	6 7±1 0 (2)
Rat liver	5 5±0 6 (4)	8 0±1 9 (4)	4 3±1 0 (8)	11 6±3 0 (8)	1 9±0 3 (3)	5 8±1 2 (3)
Hepatoma 16	2 1±0 2 (5)	5 7±0 7 (5)	2 7±0 6 (4)	10 3±2 7 (4)	1 6±0 3 (2)	2 7±0 7 (2)
Hepatoma 7800	3 2±0 6 (4)	4 2±0 8 (4)	3 8±1 0 (4)	10 3±3 0 (4)	2 2±0 5 (2)	2 0±0.3 (2)
Hepatoma 7794A	2 5±0 5 (3)	6 9±2 8 (3)	3 9±0 2 (3)	9 2±1 2 (3)	1 9±0 2 (3)	4 2±1 0 (3)
Hepatoma 7777	1 8±0 8 (3)	2 6±0 7 (3)	2 2±0 4 (8)	5 7±1 2 (8)	2 1±0 3 (3)	2 2±0 3 (3)

conditions of a low-energy state (control), transport activity in mitochondria isolated from the different tumor lines is quite similar and has only about 50% the activity of control or regenerating liver The apparent K_m values also tended to be lower in hepatoma mitochondria with the lowest values seen in the more rapidly growing tumors. Including 1 mg of serum albumin in the assay medium increased the V_{max} value in liver and tumor by about 10–15% (results not shown) while having little effect of the apparent K_m value, so that transport activity in tumor still remained only less than 50% of normal. This effect of albumin may involved retarding a leakage of endogenous nucleotides from mitochondria as well as removing endogenous inhibitors. Lower transport activity in hepatoma mitochondria was also observed under uncoupled conditions In addition, similar to con-

TABLE II

APPARENT KINETIC CONSTANTS OF ATP UPTAKE IN RAT LIVER AND MORRIS HEPATOMA MITOCHONDRIA EFFECTS OF MITOCHONDRIAL ENERGY STATE

Mitochondria were isolated from liver and hepatomas weighing 0 5–2 0 g and assayed as described in Materials and Methods Final concentrations of labeled ATP added to the assay medium were 5, 10, 20, 40 and 80 μM for assays measured in the absence of uncoupler and 2 5, 5, 10, 20, 40 μM for those in the presence of uncoupler and 30, 50, 90, 180 and 350 μM for assays measured in the presence of succinate The apparent V_{max} and K_m values were calculated as described in Table I

Mitochondrial source	Low-energy state		+2 μM CCCP		High-energy state	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Regenerating liver	4 1±0 5 (3)	58 5± 5 7 (3)	2 5±0 2 (3)	19 5±7 8 (3)	6 4±0 2 (3)	104 6±12 2 (3)
Rat liver	4 3±0 3 (6)	49 5±11 6 (6)	2 2±0 3 (4)	12 5±2 6 (4)	5 9±0 8 (4)	115 0±10 5 (4)
Hepatoma 16	2 5±0 8 (2)	42 0± 8 2 (2)	1 8±0 2 (2)	9 5±1 5 (2)	3 6±0 3 (2)	95 2± 8 5 (2)
Hepatoma 7800	2 0±0 5 (3)	25 5±11 5 (3)	1 7±0 2 (3)	12 1±2 2 (3)	3 0 (1)	123 0 (1)
Hepatoma 7794A	1 9±0 4 (2)	35 0±10 1 (2)	1 7±0 2 (2)	12 3±1 8 (2)	2 6 (1)	165 0 (1)
Hepatoma 7777	1 6±0 6 (2)	45 0± 0 9 (2)	1 6±0 3 (4)	5 8±0 8 (4)	4 0±0 2 (2)	105 5±20 2 (2)

trol liver, uncoupling of tumor mitochondria had a marked effect on the K_m value for ADP uptake, increasing it almost 2-fold. This effect of uncoupling agent is in agreement with previous results of Vignais et al. [2,26] who suggested that it may involve energy-dependent conformational changes in the carrier. The observation that higher transport rates are achieved by including albumin in the isolation medium compared to only adding it to the assay medium indicates that agents are present in homogenates of tumors which act to lower transport activity and that albumin can reverse their effects.

A comparison of the kinetic constants for mitochondria isolated in the presence of albumin and assayed with uncoupler versus with added respiratory substrate (succinate) shows that a transition to a high-energy state lowers both the K_m and V_{max} values for ADP uptake. In addition, a comparison of liver and hepatoma mitochondria under these conditions shows that the V_{max} and K_m values are lower in the tumor mitochondria.

ATP uptake. The values for the apparent kinetic constants obtained from measurements of ATP uptake under the same conditions as ADP uptake are shown in Table II. Overall the same general pattern observed for ADP uptake was observed for ATP uptake, i.e., both V_{max} and K_m values were lower in tumor mitochondria than normal liver mitochondria and that there was no apparent correlation between values of the kinetic constants and tumor growth rate. Specifically, it was observed that similar to results obtained for ADP transport, the rates of ATP uptake measured under conditions of a lower energy state are lower in tumor mitochondria than control liver and that this difference could not be corrected by including albumin in the assay medium. Results in Table II indicate that under conditions of both a high-energy and uncoupled state, the differences between the values of the kinetic constants for normal and tumor mitochondria were less than the difference when this ATP uptake was under conditions of a low-energy state.

In summary, a transition from a high-energy state to an uncoupled state results in an increase in both the K_m and V_{max} values for ADP uptake whereas the opposite effect is true for ATP uptake. A comparison of the kinetic constants of ADP and ATP uptake under a high-energy state shows that for both normal and hepatoma mitochondria $K_m(ADP) << K_m(ATP)$ and $V_{max}(ADP) < V_{max}(ATP)$ In the uncoupled state the reverse was, in general, observed, i.e, $V_{max}(ADP) \geq V_{max}(ATP)$. These observations are significant, since they demonstrate that the mitochondrial energy state in mitochondria from hepatoma, as well as from liver, is an important factor in influencing rates of nucleotide exchange.

Adenine nucleotide levels in hepatoma mitochondria

The levels of adenine nucleotides in mitochondria isolated from small tumors are shown in Table III The most notable difference between normal and tumor mitochondria is the low levels of AMP in hepatomas 7777 and 7800 In the case

TABLE III

MITOCHONDRIAL ADENINE NUCLEOTIDE LEVELS IN SMALL TUMORS

Mitochondrial AMP, ADP and ATP levels were measured by enzymatic analysis, as described in Materials and Methods, of neutralized perchloric acid extracts obtained from 10–15 mg mitochondria (low-energy state) isolated in the presence of 1% albumin from liver and tumors weighing 0 5–2 0 g Data shown are the mean values ±S E Values are expressed in nmol/mg protein E, exchangeable pool, T, total nucleotide pool

Tumor line	AMP	ADP	ATP	E	T
MH 7777	1 9±0 2 (12) [a]	2 5±0 3 (13)	1 7±0 2 (13)	4 3±0 4 (13)	6 3±0 5 (12) [a]
MH 7800	2 3±0 6 (3) [a]	5 3±1 1 (3)	1 0±0 3 (3)	6 3±1 4 (3)	8 6±0 8 (3)
MH 7794A	3 0 (1)	3 2 (1)	3 2 (1)	6 4 (1)	9 4 (1)
MH 16	7 7±1 2 (3)	3 1±1 0 (3)	1 2±0 4 (3)	4 3±0 9 (3)	12 0±2 0 (3)
Rat liver	5 8±0 6 (3)	3 2±0 3 (3)	1 3±0 3 (3)	4 5±0 4 (3)	10 3±0 5 (3)

[a] Significantly different from rat liver at $P \leq 0\ 02$

TABLE IV

Mg^{2+}, Ca^{2+} AND LONG-CHAIN ACYL-CoA ESTER LEVELS IN SMALL TUMORS

Mitochondrial Mg^{2+}, Ca^{2+} and long-chain acyl-CoA ester levels were measured by atomic absorption and enzymatic analysis, respectively, as described in Materials and Methods In the case of Mg^{2+} and Ca^{2+} determinations, mitochondria (low-energy state) used in these studies were isolated in the presence of 1% albumin For acyl-CoA analysis, albumin was omitted from the isolation medium Data shown are the mean values ± S E Numbers in parentheses represent the number of determinations BW 7756 is a mouse tumor in C57LJ mice obtained from Jackson Laboratory, ME, it is included for comparison with rat hepatomas Values are expressed in nmol/mg protein

Tumor line	Mg^{2+}	Ca^{2+}	Acyl-CoA
BW 7756	19 7±1 8 (3)	23 4 ± 4 4 (3)	0 14±0 02 (3)
MH 7777	20 8±1 2 (11)	322 ±32 (10)	0 27±0 04 (5)
MH 7800	27 9±0 9 (2)	15 4 ± 1 8 (2)	0 35±0 05 (2)
MH 7794A	25 5 (1)	14 6 (1)	0 45 (1)
Rat liver	25 1±2 2 (4)	4 66± 0 73 (4)	0 72±0 05 (2)

of the 7777 tumor, the AMP levels are reduced to the extent that the total pool size is significantly lower than control. Interestingly though, the exchangeable pool size was preserved, seemingly at the expense of AMP. Eboli et al. [27] have observed a similar situation in mitochondria isolated from Morris hepatoma 3924-A, another rapidly growing tumor. The authors found although the levels of ADP + ATP are similar in tumor compared to liver, AMP levels were less than 20% of normal. These findings may be significant, since they indicate that much of the potential reserve capacity for higher transport activity in tumor mitochondria is absent. That is, tumor mitochondria with seemingly normal transport rates but which have low AMP levels will be unable to increase appreciably their exchangeable pool size (by conversion of AMP to ADP or ATP) and therefore could not meet increased metabolic demand if the occasion arose At present, it is not certain whether this condition represents a general feature of all rapidly growing Morris hepatomas although the finding that the total pool size in hepatoma 7800 was also lower than in control liver, again due to low levels of AMP, would seem to support this suggestion.

The important parameter with regards to transport activity is the levels of ADP + ATP The results presented in Table III demonstrate that for all tumor lines these levels differ only slightly from those in control liver. It is important to bear in mind that the nucleotide levels reported here were determined from mitochondria isolated with added albumin. In the absence of added albumin, leakage of nucleotides from tumor mitochondria can and does occur. When transport rates measured under conditions of a low-energy state were determined with mitochondria isolated in the absence of added albumin, lower rates were observed [12,14]. This fact may well be due to a lower level of nucleotides in these mitochondria.

Mg^{2+}, Ca^{2+} and long-chain acyl-CoA levels in hepatoma mitochondria

The levels of divalent cations and long-chain acyl-CoA esters are shown in Table IV. In all tumor lines Mg^{2+} levels were essentially identical with those in control liver. In marked contrast to these findings, Ca^{2+} levels in tumor mitochondria were significantly higher than those in control liver and for hepatoma 7777, by a remarkable 70-fold. These levels of Ca^{2+} agree well with the report by LaNoue et al. [28], although Ca^{2+} levels in the present study were 3-fold higher for the 7777 tumor. The accumulation of Ca^{2+} by mitochondria can lead to inhibition of nucleotide exchange owing to a reduction in the levels of free nucleotides by chelation with Ca^{2+} [27].

Results in Table IV also show that the levels of long-chain acyl-CoA are lower in tumor mitochondria with the lowest values seen in the more rapidly growing tumors. These observations are consistent with previous reports by Halperin et al. [29] and Sul et al. [30] who showed that acyl-

CoA ester levels are reduced in tumor tissue, as well as in the mitochondrial fraction [30], and may indicate that the extent to which these agents can influence transport rates in vivo is impaired.

Temperature dependence of ADP and ATP transport in hepatoma mitochondria

The Arrhenius plots in Fig. 1 illustrate the temperature dependence of ADP and ATP uptake in the mitochondria from various hepatomas and control liver. In these studies efforts were made to stabilize mitochondria against the damaging effects of elevated temperature by including 200 μM dibucaine in the assay medium to inhibit phospholipase activity [31] As can be seen in the figure, in all cases a break in the plot was observed for both ADP and ATP uptake. For hepatoma mitochondria the break-point temperature averaged 9.6°C, varying between 8 1 and 10.2°C compared to a range of 13 5–14 0°C for liver mitochondria. These findings are in contrast to the recent report by Sul et al. [30] who observed no

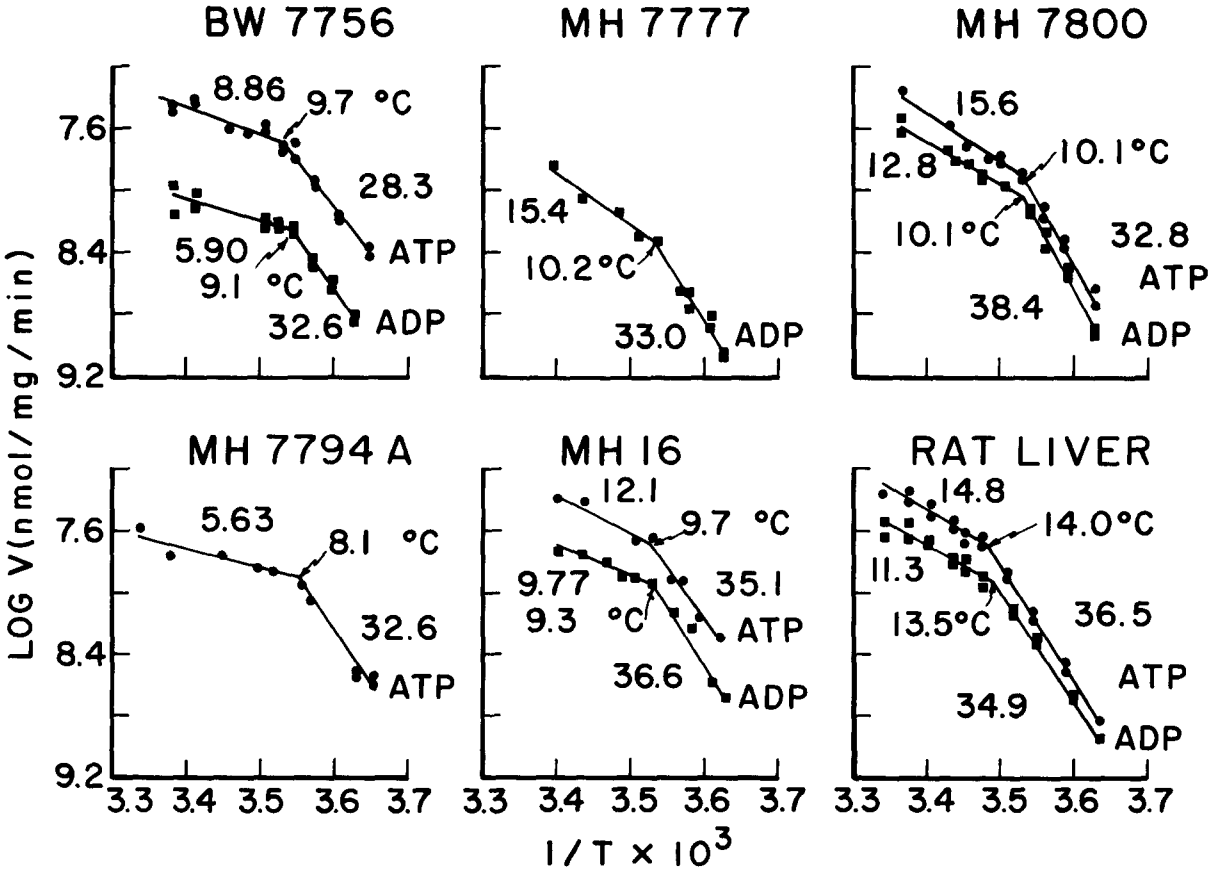

Fig 1 Arrhenius plots for ADP and ATP uptake in mitochondria isolated from liver and small hepatomas Velocity of ADP and ATP uptake was measured in a KCL medium, as described in Materials and Methods, in the presence of 2 μg oligomycin, 5 mM succinate and 200 μM dibucaine (added to stabilize mitochondria at higher temperatures) After a 5 min preincubation at the indicated temperature, the reaction was started by adding, in a final concentration, 20 μM [^{3}H]ADP or 350 μM [^{3}H]ATP (spec act $\geq$ 3000 cpm/nmol) At 0°C the reaction was allowed to run for 22 s and assay times were decreased by 4 s for each 3°C rise in temperatures until the reaction time was reduced to 2 s at which point the same reaction time was used for higher temperatures Arabic numerals shown in the figure without units indicate the activation energies in kcal/mol The break-point temperature (in °C) and the best-fit line by two-line linear regression are also shown Mitochondria used in these studies were isolated in a medium containing 1% albumin BW 7756 is a mouse tumor in C57LJ mice obtained from Jackson Laboratory, ME, it is included for comparison with rat hepatomas

break in the Arrhenius plot for ATP uptake in mitochondria from Morris hepatoma 5123C. The reasons for the apparent discrepancy in results are not immediately clear but the fact that their measurements were performed at times far removed from initial velocity may be significant. In a previous report, Klingenberg [32] indicated that the break-point temperature for nucleotide uptake in liver mitochondria was 18°C Recently, this figure has been revised downward to 14°C [33] in good agreement with the results reported here Also shown in the figure is that the activation energies for ADP and ATP uptake are similar in normal and tumor mitochondria, varying between 6 and 16, and 28 and 38 kcal/mol, respectively, above and below the break point. These values correspond well with previously reported values for liver mitochondria [32].

Normally, a discontinuity in the Arrhenius plot involving membrane-bound enzymes is suggestive of a phase transition of the membrane lipids Since the break-point temperature in the various tumor mitochondria was uniformly 4–5°C lower than in liver mitochondria, these findings may be indicative of an altered membrane lipid composition In recent years, several groups have examined this issue and have shown that the mitochondrial lipid composition in Morris hepatomas differs substantially from that of liver mitochondria [34–36]. This was evidenced by variations in the fatty acid composition (high oleic acid levels [34,35]), acyl specificity in the group-1 position of phospholipids (a large proportion of unsaturated instead of saturated fatty acids was found to be present [34]) and the relative proportion as well as total amount of the phospholipids [34–36]. Cholesterol was also shown to be elevated in tumor mitochondria [37,38]. Thus, it seems reasonable to suggest that the lower break-point temperatures in tumor mitochondria may result from a difference in the composition of membrane lipids and in particular from the higher proportion of unsaturated fatty acids.

Discussion

Effects of the mitochondrial energy state on the kinetics of nucleotide uptake

In this report we have examined the effects of the mitochondrial energy state and other parameters on the kinetics of ADP and ATP uptake in an effort to determine whether the higher rates of aerobic glycolysis in tumor may result from altered carrier activity. In these studies it was shown that for all tumor lines the effect of energization or deenergization of mitochondria on transport rates was similar qualitatively to that in control liver for the uptake of both ADP and ATP and that the values for the apparent V_{max} in tumor, to a large extent, were lower than those in liver. Specifically, it was observed that with respect to an uncoupled state, energization of mitochondria increased the V_{max} and, especially, the K_m values for ATP uptake while for ADP uptake the reverse was true. The observation that the V_{max} value for ATP uptake was increased is a new finding and may be of physiological significance with respect to the competition between cytosolic ADP and ATP for entrance into the mitochondria when it is considered that under the same experimental conditions the V_{max} value for ADP uptake was 2–3-fold lower. This finding is important, since it indicates that the presence of saturating cytosolic levels of ADP would not necessarily guarantee V_{max}, as has been assumed [39], due to competition by free cytosolic ATP whose levels are of the order of its K_m value [40]. Thus, when considering the degree of competition between the mitochondrial and glycolytic enzymes for phosphorylation of cytosolic ADP, the cytosolic ATP levels may be important, since as their levels increase ATP could act as a feedback inhibitor of mitochondrial ATP synthesis by preventing the uptake of ADP into mitochondria

Earlier results in our laboratory [4] showed that albumin activated transport rates in mitochondria by increasing the V_{max} for nucleotide uptake and that this effect was accompanied by a removal endogenous long-chain acyl-CoA esters [41]. Since several groups have provided good evidence that these agents are competitive inhibitors for nucleotide exchange [42–44], a higher level of acyl-CoA esters was expected for the observed low rates of nucleotide transport. However, in contrast, the present study showed that long-chain acyl-CoA level was lower in tumor than in control rat liver mitochondria. This observation may be related to a 10-fold lower fatty acid-binding protein content in Morris hepatoma [45]

Temperature dependence of ADP and ATP transport and the levels of mitochondrial adenine nucleotides and divalent cations in hepatoma mitochondria

The present study showed that tumor mitochondria exhibit a break point in the Arrhenius plot for the uptake of both ADP and ATP with a transition temperature in tumor uniformly 4–5°C lower than in liver, in contrast to the report by Sul et al. [30], who observed no break point for ATP uptake in mitochondria from Morris hepatoma 5123 C. As indicated earlier, the reasons for the apparent discrepancy in results are not immediately clear but the fact that their measurements were performed at times far removed from initial velocity may be significant. Furthermore, in the present study efforts were made to stabilize mitochondria against the damaging effects of elevated temperature by including 200 μM dibucaine in the assay medium to inhibit phospholipase activity [31]. It was also shown that the activation energies above and below the transition point were similar in normal and tumor mitochondria, varying between 6 and 16 and 28 and 38 kcal/mol, respectively.

The observed differences in the levels of adenine nucleotides and Ca^{2+} in hepatoma mitochondria may be sufficient to explain the low nucleotide transport activity in the mitochondria. In our view, the high Ca^{2+} level in tumor mitochondria reported here and by others [27,28] may induce compartmentation of nucleotides due to its higher affinity for ATP than ADP [46]. One compartment, consisting primarily of ATP, would only slowly undergo exchange whereas the other compartment, composed primarily of ADP, would undergo more rapid exchange, since it known that the nucleotide carrier will only transport free nucleotides and not metal complexes [47–49].

The observations that high levels of Ca^{2+} in mitochondria as the cause of low nucleotide uptake and preferentially lower rates of ATP efflux [27,50] may have important consequences with regard to the ability of these mitochondria to synthesize extramitochondrial ATP. The newly synthesized ATP molecule, selectively complexed with Ca^{2+} in the matrix, would not be available for transport and effectively could result in futile cycling of ADP It would follow, therefore, that since the oxidative phosphorylation of cytosolic ADP is critically dependent on the rates of ATP export, the presence of high levels of matrix Ca^{2+} in tumor mitochondria, in situ, could interfere with this reaction and thus augment rates of aerobic glycolysis.

A second factor which may be important in determining whether transport rates in tumor mitochondria affect rates of aerobic glycolysis deals with the concept that the 'potential reserve capacity' of tumor mitochondria to exhibit higher rates of transport may be reduced due to the low level of AMP found in the more rapidly growing tumors (hepatomas 7777, 3924-A [27] and perhaps 7800). This concept refers to the evidence that the rates of nucleotide exchange in mitochondria are largely dependent on the size of the internal exchangeable pool [51]. Thus, metabolic states which arise and lead to a variation in the pool size, through interconversion with intramitochondrial AMP, would likely influence transport rates Consequently, tumor mitochondria which have reduced levels of AMP will be unable to increase appreciably the size of their exchangeable pool and the ability to increase transport rates will be limited The potential impact this condition could have on rates of aerobic glycolysis would depend on the degree to which nucleotide transport, as a function of the exchangeable pool size, limits the overall rate of ATP synthesis. This issue is a matter of controversy and additional studies are needed to determine whether transport limits this reaction.

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