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Adenine Nucleotide Transport in Hepatoma Mitochondria and Its Correlation with Hepatoma Growth Rates and Tumor Size[1]

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ABSTRACT

Initial rates of [^{3}H]adenosine diphosphate and [^{3}H]adenosine triphosphate uptake were measured in mitochondria isolated from normal rat liver, regenerating liver, mouse hepatoma BW7756, and four Morris hepatomas (7777, 7800, 7794A, and 16) of varying degrees of malignancy. Results obtained demonstrate that (*a*) the apparent K_m and V_{max} values for adenosine diphosphate and adenosine triphosphate uptake are significantly lower in hepatoma compared to normal or regenerating liver mitochondria, (*b*) the V_{max} values for adenosine diphosphate uptake correlate with tumor growth rate, and (*c*) the K_m values for adenosine triphosphate in both hepatoma and normal mitochondria are lowered in the presence of added uncoupling agents; however, the extent of decrease is much less in fast-growing tumors than in slow-growing tumors and normal tissues.

Studies examining the causes of reduced transport rates in hepatoma mitochondria showed that they are independent of the mitochondrial energy state and associated with substantially lower levels of the total and exchangeable adenine nucleotides. Additional studies revealed that transport rates are also dependent on the size of the tumor from which the mitochondria are isolated. Mitochondria isolated from small tumors (<2 g) had higher transport rates as well as higher levels of exchangeable and total adenine nucleotides than those isolated from larger tumors (4 to 6 g). Endogenous inhibitor levels also varied as a function of tumor size; free fatty acid levels increased, whereas acyl coenzyme A levels declined in mitochondria isolated from larger tumors. These results seem to indicate that, during the progression of tumor growth, mitochondria are experiencing cellular environmental changes that will affect overall tumor cell metabolism.

INTRODUCTION

Since the original observation by Warburg (47) that many tumor cells have high rates of aerobic glycolysis, various theories have been proposed claiming this lesion to be of mitochondrial or cytosolic origin. These include defective respiratory enzymes (47), impaired hydrogen shuttle systems (5), and reduced cellular mitochondrial content (39), all of which result in a reduced mitochondrial oxidative capacity and partially uncoupled ion pumps which fuel glycolysis by producing excessive amounts of rate-limiting substrates (40). It is now clear, however, that hydrogen (9, 15) and electron transport (29) in tumor mitochondria is either not defective or the defects appear to be unrelated to lactate production, and it is uncertain to what extent lowered mitochondrial protein contents or uncoupled ion pumps have on rates of glycolysis.

One area of research that may be particularly relevant to this problem and has received little attention is the role that the mitochondrial adenine nucleotide carrier may have in influencing rates of aerobic glycolysis. The mitochondrial adenine nucleotide carrier catalyzes a one-for-one exchange of intramitochondrial ADP and ATP for extramitochondrial ADP and ATP (26, 38). Because ADP is the primary phosphate acceptor of oxidative phosphorylation, the rate at which cytosolic ADP is made available to the F_1-ATPase enzyme for phosphorylation may control the overall rate of ATP synthesis. Various investigators have examined this point and have indicated that nucleotide transport may indeed limit rates of oxidative phosphorylation, although the extent to which this occurs *in vivo* is unclear (10, 24, 27, 30). Regardless of whether activity of the nucleotide carrier limits the rate of ATP synthesis or not in normal cells, it is important to consider the consequences that reduced rates of nucleotide transport would have on cellular energy metabolism under pathological states (*e.g.*, neoplasia).

Control of aerobic glycolysis can be viewed to arise from a dynamic competition for cytosolic NADH, ADP, and P_i by mitochondrial and glycolytic enzymes. Thus, competition for these substrates could be shifted in favor of glycolysis if rates of ADP transport were sufficiently reduced to the extent that mitochondrial oxidation capacity were impaired.

In the present study, rates of ADP and ATP uptake were measured in mitochondria isolated from normal and regenerating liver and a series of Morris hepatomas the growth rates of which correlate with rates of aerobic glycolysis (higher rates in the more rapidly growing tumors). Transport rates were measured under different energy states, and factors known to influence carrier activity were monitored. Results obtained demonstrate that transport rates in hepatoma mitochondria are significantly lower than those in control liver and correlate with tumor growth rate. These rates, however, are also dependent on the size of the tumor from which the mitochondria were isolated. Some preliminary results were reported in abstract form (1, 3).

MATERIALS AND METHODS

Materials

CoA, NAD, ATP, ADP, NADH, NADP, acetylphosphate, carboxyatractyloside, oligomycin, palmitic acid, phosphoenolpyruvate, *m*-chlorocarbonylcyanide phenylhydrazone, 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, Boston, Mass., and Amersham/Searle Corp., Arlington Heights, Ill., respectively. Male Sprague-Dawley rats (250 to 350 g) were from Taconic Farm, Germantown, N. Y. Female C57L/J mice and the hepatoma BW7756 cell line were from The Jackson Laboratory, Bar Harbor, Maine. Male Buffalo rats

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weighing 140 to 150 g were obtained from Simonsen Laboratories, Gilroy, Calif., and shipped to Dr. H. P. Morris, Howard University, Washington, D. C., where the Morris 7777, 7800, 7794A, and 16 cell lines were transplanted into the hind legs; the animals were subsequently transferred to our laboratory. 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 (4, 46) as follows. In a final volume of 0.95 ml, 0.5 to 1.0 mg of mitochondria was preincubated for 5 to 10 min at 2° in a medium containing 115 mM KCl, 21 mM Tris-HCl (pH 7.4) at 2°, 1.05 mM EDTA, 13 mM sucrose, 0.53 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 2.0 μg of oligomycin. The assay was started by adding graded amounts (50 μl) of radioactive ADP or ATP into reaction tubes under constant vortexing. 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 sec, the reaction was stopped by injecting 50 μl of 200 μM carboxyatractyloside (using another series of syringes), and the mitochondria were centrifuged at 12,000 × g for 4 min in a Beckman microfuge. The supernatant was aspirated, and the mitochondrial pellet was washed with 1 ml of KCl medium [110 mM KCl, 20 mM Tris-HCl (pH 7.4), and 1.0 mM EDTA] containing 10 μM carboxyatractyloside and centrifuged as before. The pellet was dissolved in 0.2 ml of 2% sodium dodecyl sulfate, 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 to 20 sec of the assay. The reproducibility of duplicate sets of assays was ±5% (S.E.). All other additions in the assay medium are described in the table legends.

The apparent kinetic constants were calculated from linear portions of the curve by the direct linear plot method of Eisenthal and Cornish-Bowden (12).

Free Fatty Acid Determination. The endogenous free fatty acid levels in mitochondria were determined as described by Nixon and Chan (34), except for the inclusion of a silicic acid washing step to remove phospholipids which may contribute to the developed color. This was accomplished by adding 0.25 g of silicic acid to the acid-washed organic phase, vortexing the tubes for 3 min, and separating the 2 phases by centrifugation. The supernatant was pipeted off and analyzed as described before (34) without further modification.

Determination of Long-Chain Acyl-CoA Esters. Long-chain acyl-CoA esters were extracted from 10 to 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 to 13.0 with 1.0 N 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 (33).

Determination of Mitochondrial Adenine Nucleotides. Mitochondrial AMP, ADP, and ATP levels were measured by enzymatic analysis of neutralized perchloric acid extracts obtained from 10 to 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 Trautschold (28).

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 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 1 mM EDTA (pH 7.4). Hepatoma mitochondria were isolated using the same procedure as for rat liver from tumors weighing 4 to 6 g for rat hepatomas and 1.5 to 2.5 g for the BW7756 mouse hepatomas in the presence and absence of 1% serum albumin. Mitochondria used in studies examining the effects of tumor size on transport activity were isolated in a sucrose medium to which 1% albumin was or was not included as indicated. The intactness of the tumor mitochondrial preparations was judged by measuring the RCR[2] (14) and by electron microscopic examination (41). Hepatoma mitochondria prepared with sucrose medium exhibited respiratory control with RCR values between 2.2 and 3.5 (using succinate as substrate, as in Ref. 8). Preparations with albumin added in the homogenization medium showed improved RCR (range, 2.5 to 4.0) when additional albumin was included in the oxygen electrode chamber during the RCR measurement, in agreement with previous reports (23, 41). Of particular interest was our observation that, in the presence of albumin, the rate of State 3 respiration was promptly and sharply decreased back to State 4 respiration after the added ADP was exhausted, while without albumin, the transition from State 3 to State 4 respiration was slow and gradual. Nevertheless, the calculated ADP/O ratios both with and without (by extrapolation in the latter case because of the curved tracings between State 3 and State 4 respirations) added albumin were the same and not different from values obtained with control rat liver mitochondria. Furthermore, the addition of albumin had no effect on the appearance of the apparently intact double membrane of the mitochondrial preparations as examined by electron microscope (41). Regenerating livers were obtained from rats 48 hr after partial hepatectomies, involving removal of about 70% of the liver (left and medium lobes). The procedure of Higgins and Anderson (17) was followed.

Data Analysis. Statistical analysis was performed using a 2-tailed Students' *t* test.

RESULTS

An important point addressed by the present study is whether adenine nucleotide transport rates in hepatoma mitochondria are altered in a manner which may contribute to the higher rates of aerobic glycolysis seen in many tumor cell lines. To examine this, mitochondria were isolated from a series of Morris hepatomas the growth rates of which correlate with rates of aerobic glycolysis (6, 43, 48). The hepatoma cell lines chosen were a fast-growing, poorly differentiated tumor (Hepatoma 7777, growth rate of 12 to 20 days), 2 well-differentiated tumors with intermediate growth rates (Hepatomas 7800, 3 to 5 weeks; Hepatoma 7794A, 4 to 6 weeks) and a slow-growing, highly differentiated tumor (Hepatoma 16, 4 to 6 months). For comparative purposes, the fast-growing, poorly differentiated mouse Hepatoma BW7756 (2 to 4 weeks) was also included in these studies.

Apparent Kinetic Constants of ADP and ATP Uptake in Hepatoma Mitochondria

ADP Uptake. Tables 1 and 2 summarize the apparent kinetic constants obtained from experiments measuring initial rates of ADP and ATP uptake in mitochondria isolated from control liver and the various hepatomas. The apparent V_{max} and K_m (Table 1) values for ADP uptake are significantly lower in hepatoma mitochondria compared to control liver; in the absence of uncoupler, the V_{max} values correlate with tumor growth rate (*i.e.*, fast < intermediate < slow < normal).

Several factors are known to influence the rates of nucleotide exchange, including the levels of endogenous inhibitors (7, 31), the mitochondrial energy state (4, 35, 36, 42, 46), and the size

[2] The abbreviation used is: RCR, respiratory control ratio.

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Table 1

Apparent kinetic constants of ADP uptake in control rat liver and hepatoma mitochondria: effects of serum albumin and uncouplers

Initial rates of ADP uptake were measured as described in "Materials and Methods." Where indicated, 1 mg of serum albumin, 2 μM *m*-chlorocarbonylcyanide phenylhydrazone, or 20 μM dinitrophenol (uncoupler) was included in the preincubation mix. The reaction was started by adding 50 μl of labeled nucleotide (ADP, 1 to 20 μM final concentration; specific activity, ≥3000 cpm/nmol) and stopped after 12 sec by adding 50 μl of 200 μM carboxyatractyloside. 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 4 data points at higher substrate concentrations by the direct linear plot method (12). V_{max} values for hepatoma mitochondria with no addition are significantly lower than those for control liver, at $p = 0.04$ or less.

Mitochondrial source (growth rate)	No addition		+ serum albumin		+ uncoupler	
	V_{max} (nmol/mg/min)	K_m (μM)	V_{max} (nmol/mg/min)	K_m (μM)	V_{max} (nmol/mg/min)	K_m (μM)
Rat liver	3.94 ± 1.4[a] (7)[b]	8.2 ± 2.1 (7)	5.46 ± 0.6 (4)	8.0 ± 1.9 (4)	4.32 ± 1.0 (8)	11.6 ± 3.0 (8)
Regenerating liver	4.85 ± 1.6 (4)	9.4 ± 3.4 (4)	6.55 ± 1.1 (3)	14.2 ± 4.3 (3)	4.93 ± 0.8 (3)	8.6 ± 2.2 (3)
Hepatoma 16 (4–6 mos.)	2.22 ± 0.2 (5)	3.49 ± 1.9 (5)	3.21 ± 0.42 (3)	4.01 ± 0.71 (3)	2.79 ± 0.52 (4)	4.1 ± 1.9 (4)
Hepatoma BW7756 (mouse) (2–4 wk)	2.01 ± 0.27 (4)	1.51 ± 0.27 (4)	3.43 ± 0.42 (3)	3.27 ± 0.88 (3)	2.08 ± 0.02 (2)	1.42 ± 0.08 (2)
Hepatoma 7800 (3–5 wk)	1.56 ± 0.52 (5)	3.01 ± 0.61 (5)	2.06 ± 0.62 (5)	2.33 ± 0.64 (5)	2.35 ± 0.64 (3)	6.59 ± 3.2 (3)
Hepatoma 7794A (4–6 wk)	0.73 ± 0.28 (4)	3.26 ± 1.9 (4)	1.25 ± 0.48 (4)	3.56 ± 1.6 (4)	0.87 ± 0.31 (4)	5.3 ± 4.2 (4)
Hepatoma 7777 (12–20 days)	0.42 ± 0.07 (4)	4.88 ± 0.93 (4)	0.85 ± 0.31 (4)	7.37 ± 2.2 (4)	0.37 ± 0.09 (4)	5.85 ± 1.79 (4)

[a] Mean ± S.E.

[b] Numbers in parentheses, number of determinations.

Table 2

Apparent kinetic constants of ATP uptake in control rat liver and hepatoma mitochondria: effects of serum albumin and uncouplers

Initial rates of ATP uptake (ATP, 1 to 100 μM final concentration; specific activity, ≥2000 cpm/nmol) and calculation of kinetic constants were determined as described in the legend to Table 1.

Mitochondrial source	No addition		+ serum albumin		+ uncoupler	
	V_{max} (nmol/mg/min)	K_m (μM)	V_{max} (nmol/mg/min)	K_m (μM)	V_{max} (nmol/mg/min)	K_m (μM)
Rat liver	3.16 ± 1.25[a] (5)[b]	42.3 ± 10.3 (5)	4.25 ± 0.6 (5)	49.2 ± 15.9 (5)	2.2 ± 0.6 (4)	10.5 ± 2 (4)
Regenerating liver	4.53 ± 1.7 (4)	64.2 ± 11.6 (4)	5.89 ± 1.02 (3)	75 ± 13.2 (3)	2.51 ± 0.35 (3)	11.7 ± 3.1 (3)
Hepatoma 16	1.75 ± 0.66 (4)	43.6 ± 6.7 (4)	2.35 ± 0.5 (3)	35 ± 8.9 (3)	1.82 ± 0.19 (3)	10.7 ± 0.76 (3)
Hepatoma BW7756 (mouse)	0.77 ± 0.14 (3)	4.27 ± 0.7 (3)	1.02 ± 0.45 (2)	4.45 ± 1.5 (2)	0.61 ± 0.2 (2)	1.56 ± 0.44 (2)
Hepatoma 7800	0.72 ± 0.33 (3)	10.1 ± 1.4 (3)	1.43 ± 0.63 (3)	26.9 ± 8.9 (3)	0.56 ± 0.15 (3)	3.86 ± 2.6 (3)
Hepatoma 7794A	0.55 ± 0.23 (4)	18.1 ± 6.5 (4)	1.23 ± 0.38 (3)	25.7 ± 4.5 (3)	0.80 ± 0.27 (3)	8.6 ± 1.4 (3)
Hepatoma 7777	0.44 ± 0.3 (4)	11.4 ± 8.0 (4)	0.64 ± 0.23 (3)	9.46 ± 3.8 (3)	0.96 ± 0.3 (3)	5.7 ± 2.6 (3)

[a] Mean ± S.E.

[b] Numbers in parentheses, number of determinations.

of the internal ADP-plus-ATP pool (37). Lower rates of nucleotide transport in hepatoma mitochondria might, therefore, be due to one or more of these factors. To evaluate this, the effects of adding serum albumin (to lower endogenous inhibitor levels) and dinitrophenol or *m*-chlorocarbonylcyanide phenylhydrazone (to uncouple mitochondria) to the assay medium were studied. Albumin was used for these studies since we had shown previously that it is capable of activating transport activity in mitochondria by the removal of long-chain acyl-CoA esters (7).

Results shown in Table 1 demonstrate that addition of 1 mg of serum albumin to the assay medium increased rates of ADP uptake in both hepatoma and control liver mitochondria while having a minimal effect on the apparent K_m. The increase in the V_{max} value varied between 25 and 65% for the various tumor lines, compared to a 37% activation in control liver mitochondria. Assays performed in the presence of titrating amounts of albumin (0.05 to 5.0 mg/mg of mitochondria) showed that activation was approximately half-maximal with 0.25 mg albumin and nearly maximal at 1 mg (results not shown). The limited activation of ADP uptake by albumin suggests that, although endogenous inhibitors may lower rates of nucleotide transport, their effects on tumor mitochondria are not sufficient to account for low transport activity.

Low rates of ADP uptake were also observed in hepatoma mitochondria assayed in the presence of an uncoupling agent. As seen in Table 1, uncoupling of mitochondria by addition of 2 μM *m*-chlorocarbonylcyanide phenylhydrazone or 20 μM dinitrophenol slightly increases the K_m and V_{max} values for ADP uptake in control liver and, in general, hepatoma mitochondria (an exception was seen in Hepatoma BW7756, a mouse tumor), although transport rates remained low in tumor mitochondria. This opposite effect of uncoupler on ADP (in contrast to effects on ATP; see below) is consistent with results reported previously (4, 46).

ATP Uptake. Table 2 summarizes the apparent kinetic constants of ATP uptake determined under conditions identical to those for ADP. Similar to the results for ADP uptake, both the apparent V_{max} and K_m values for ATP uptake in hepatoma mitochondria are significantly lower than those for control liver. The effect of serum albumin on rates of ATP transport is also similar to its effect on ADP transport. Including 1 mg of serum albumin in the assay medium increased the apparent V_{max} value for ATP uptake while having little effect on the apparent K_m.

In normal mitochondria, uncoupling agents are known to activate ATP transport by increasing the affinity of the carrier for ATP (36, 42). Results in Table 2 show that uncoupling agents affect ATP uptake in hepatoma mitochondria in a similar manner by lowering the apparent K_m to <10 μM. Comparing the results in Tables 1 and 2 shows that, although the effects of uncoupling agents and serum albumin on the apparent kinetic constants for

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Table 3

Mitochondrial adenine nucleotide levels

Mitochondrial AMP, ADP, and ATP levels were measured by enzymatic analysis of neutralized perchloric acid extracts obtained from 10 to 15 mg of mitochondria, as described in "Materials and Methods." Unless otherwise indicated, values for hepatoma mitochondria are significantly lower than those for control liver, at $p = 0.05$ or less.

	nmol/mg of protein				
Tumor line	AMP	ADP	ATP	E[a]	T
BW 7756	2.6 ± 0.2[b] (6)[c]	1.5 ± 0.3 (7)	0.9 ± 0.2 (7)[d]	2.2 ± 0.3 (7)	4.7 ± 0.6 (6)
MH 7777	1.4 ± 0.1 (15)	1.2 ± 0.1 (17)	0.5 ± 0.1 (14)	2.1 ± 0.4 (14)	2.9 ± 0.3 (13)
MH 7800	1.5 ± 0.9 (2)	2.4 ± 0.1 (2)[e]	1.5 ± 0.1 (2)[d]	3.9 ± 0.3 (2)[d]	5.4 ± 0.6 (2)
MH 7794A	0.5 (1)	0.7 (1)	1.0 (1)	1.8 (1)	2.2 (1)
MH 16	2.9 ± 0.5 (2)	1.4 ± 0.3 (2)	0.7 ± 0.2 (2)[d]	2.2 ± 0.1 (2)	5.0 ± 0.6 (2)
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] E, exchangeable pool (ADP + ATP); T, total pool (AMP + ADP + ATP).
[b] Mean ± S.E.
[c] Numbers in parentheses, number of determinations.
[d] Values not different from normal.
[e] $p = 0.15$.

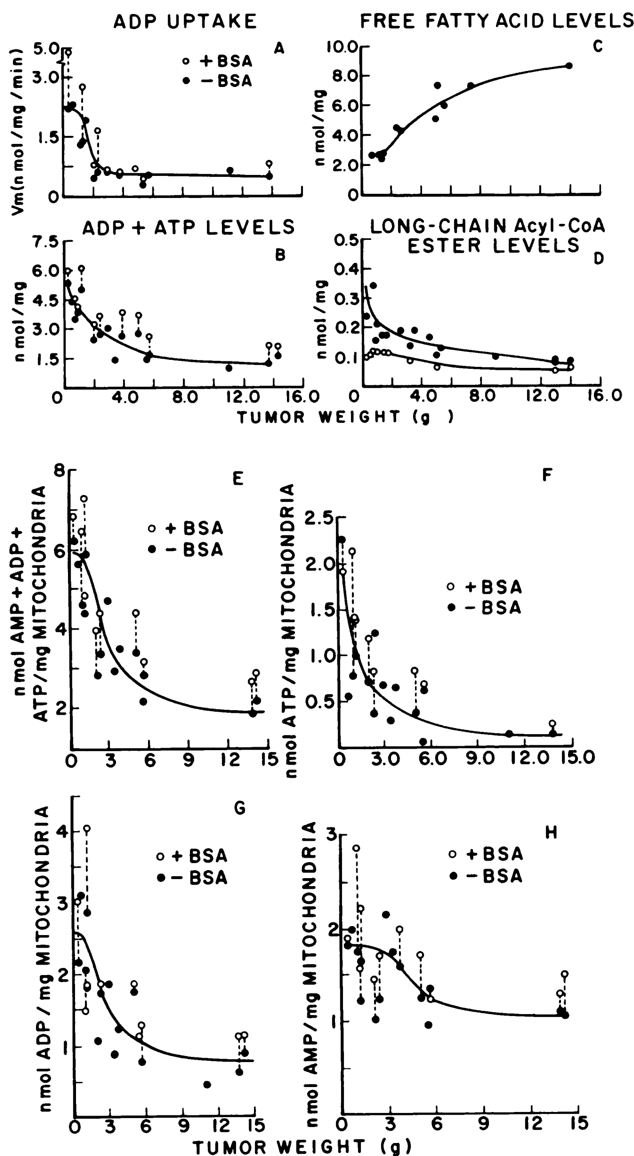

Chart 1. Effects of tumor size on Morris Hepatoma 7777 mitochondria. Morris Hepatoma 7777 mitochondria were isolated in a sucrose medium with and without added albumin (*BSA*; 1%), as described in "Materials and Methods," and the parameters indicated were monitored as a function of tumor size. Prior to homogenization, tumor tissue was weighed, and necrotic and hemorrhagic areas were dissected free. In *A*, initial rates of ADP uptake were measured as described in the legend to Table 1 in the presence of 20 μM dinitrophenol or 2 μM *m*-chlorocarbonylcyanide phenylhydrazone. Intramitochondrial ATP, ADP, and AMP levels (*F* to *H*), free fatty acid levels (*C*), and long-chain acyl-CoA ester levels (*D*) were measured as described in "Materials and Methods."

ADP and ATP uptake are qualitatively similar (but quantitatively different) in normal and tumor mitochondria, the values of these constants remain lower in hepatoma compared to normal mitochondria under all assay conditions tested. These results indicate that low rates of nucleotide uptake in hepatoma mitochondria are probably not accountable by the effects of endogenous inhibitors or the mitochondrial energy state.

Internal Adenine Nucleotide Levels in Hepatoma and Liver Mitochondria

The possibility that low rates of nucleotide transport in hepatoma mitochondria resulted from low levels of internal exchangeable nucleotides was examined by quantitating their levels in neutralized perchloric acid extracts of mitochondria.

The levels of AMP, ADP, and ATP in control liver and the various hepatoma mitochondria are shown in Table 3. Indeed, in nearly every case, the size of the internal exchangeable (ADP + ATP) and total (AMP + ADP + ATP) adenylate pool is significantly lower in hepatoma mitochondria compared to control liver. These results strongly indicate that low rates of nucleotide uptake in hepatoma mitochondria are partially due to lower internal adenine nucleotide levels.

Effects of Tumor Size on Hepatoma 7777 Mitochondria

Insight as to the significance of low levels of adenine nucleotides in hepatoma mitochondria became apparent when it was discovered that their levels and corresponding rates of transport were highly dependent on the size of the tumor from which the mitochondria are isolated. Data shown in Chart 1 are the results of a study in which rates of ADP uptake, internal adenine nucleotide levels, and levels of endogenous inhibitors were monitored in mitochondria isolated from Hepatoma 7777 of various sizes. Results presented show that when mitochondria were isolated from tumors much smaller (<2 g) than those used in the above studies (4 to 6 g), transport rates (Chart 1*A*) and exchangeable (Chart 1*B*) and total (Chart 1*E*) nucleotide pool size were significantly higher. Results in Chart 1, *F*, *G*, and *H*, show that mitochondrial ATP levels decline more rapidly than do ADP levels and that AMP levels are most stable in mitochondria isolated

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from larger tumors. Endogenous inhibitor levels also varied as a function of tumor size. Free fatty acid levels were higher (Chart 1*C*), whereas long-chain acyl-CoA ester levels (Chart 1*D*) were lower in mitochondria isolated from larger tumors.

Previously, we had shown that the presence of high levels of free fatty acids in tumor mitochondrial preparations may damage mitochondrial membranes and inhibit uncoupler-activated ATPase activity and ATP transport (2). It was also shown that including serum albumin in the isolation medium lowered fatty acid levels and increased rates of ATP transport and uncoupler-stimulated ATPase activity. Results in Chart 1 show that when Hepatoma 7777 mitochondria are isolated in the presence of 1% albumin, higher transport rates were also observed, as well as an increase in the size of the exchangeable and total nucleotide pool and lower acyl-CoA ester levels. This effect of albumin on nucleotide and inhibitor levels indicates that, in addition to removing acyl-CoA esters, albumin may protect mitochondria from membrane-damaging agents present in tissue homogenates and thus prevent the loss of exchangeable nucleotides. This explanation may largely account for the observed increase in rates of nucleotide uptake in hepatoma mitochondria, seen in Tables 1 and 2, when albumin was added to the assay medium. The mitochondria used in these studies were isolated from tumors weighing 4 to 6 g and, as seen in Chart 1, such mitochondria contain high levels of free fatty acids and low levels of internal nucleotides (*cf.* Table 3). Thus, it would seem that the higher transport activity observed when albumin was added to the assay medium is partially due to the removal of long-chain free fatty acids from mitochondria, which can induce loss of internal nucleotides. The ability of free fatty acids to induce loss of internal nucleotides may explain the earlier observation by Harris *et al.* (16), who reported that oleic acid acts as a noncompetitive inhibitor for nucleotide uptake.

The protective effect that albumin has on mitochondria is limited. Results in Chart 1 show that the presence of 1% albumin in the isolation medium is not sufficient to prevent the loss of transport activity in mitochondria isolated from tumors >2 g and suggests either that mitochondria are altered prior to isolation (*i.e.*, *in vivo*) or that this level of albumin is not sufficient to prevent the accumulation of membrane-damaging agents by mitochondria. Evidence in support of the former suggestion is that free fatty acid levels in mitochondria isolated from large BW7756 tumors (3 g) were reduced from 8 to 10 nmol/mg to undetectable levels when 1% albumin was included in the isolation medium. Thus, this amount of albumin is sufficient to prevent the accumulation of these agents by mitochondria.

DISCUSSION

Importance of Tumor Size as a Factor Influencing Rates of Nucleotide Transport. An initial survey of the kinetics of ADP and ATP uptake in hepatoma mitochondria revealed that transport activity (V_{max}) in all mitochondria studied was significantly lower than in control or regenerating liver and that reduced rates of transport are likely a result of a lower content of endogenous adenine nucleotides. In contrast to our initial expectation, the K_m were also lower than that of normal rat liver mitochondria. However, this simultaneous decrease in both V_{max} and K_m is not unique; in many enzyme systems, a low K_m is not a necessarily important component of enzymatic catalysis (*e.g.*, see Ref 32; for reviews, see Ref. 20). In fact, it has been argued that it is catalytically advantageous to bind substrates weakly, insofar as the K_m is not higher than substrate concentration. This is true in the case of normal rat liver mitochondria, since the K_m are in μM range, while adenine nucleotides in the cell are at mM concentrations. A lower K_m in tumor mitochondria may indeed represent a higher binding energy with a consequent lower transport rate, as reflected by lower V_{max} values obtained experimentally in the present study. More importantly, the V_{max} for ADP uptake correlates inversely with the tumor growth rate (Table 1). Additional studies showed, however, that these results, as well as the levels of long-chain free fatty acids and their acyl-CoA ester derivatives, vary with tumor size. Transport rates, internal adenine nucleotide levels, and levels of long-chain acyl-CoA esters are lower and free fatty acid levels are higher in mitochondria isolated from larger tumors. The latter finding is considered especially significant, since the occurrence of hypoxic cells in solid tumors and particularly large tumors has been well documented (22, 25). Thus, the results presented showing a progressive decline in transport activity and associated events in Hepatoma 7777 mitochondria are interpreted to indicate the development of more hypoxic cells in the center of larger tumors.

Tumors of these sizes were chosen since they are typically used by investigators who study not only mitochondrial function (29) but also various other cellular activities in Morris hepatomas (18). In retrospect, the results reported by LaNoue *et al.* (29), demonstrating lower rates of glutamate and malate transport in the same Morris hepatoma lines used here, are not unexpected. These authors reported that mitochondria were harvested when tumors had achieved a diameter of 2 to 3 cm (4 to 8 g). It seems reasonable to suggest that if mitochondria isolated from tumors of this size are sufficiently depleted of adenine nucleotides, then the levels of other small metabolites (*e.g.*, aspartate and 2-oxoglutarate) would likewise be reduced. This effect would lower transport rates, since these and some other anion transports in mitochondria also operate by an obligatory one-for-one exchange mechanism.

Evidence in the literature which supports the finding that tumor size can influence the activity of the resultant mitochondria comes from earlier studies by Emmelot and Bos (13). They reported, in 1961, that mitochondria isolated from small, "early" tumors had significantly higher rates of uncoupler-activated ATPase activity and rates of fatty acid oxidation than mitochondria isolated from large, "old" tumors. It was also shown that when the floating lipid fraction, which is obtained from "old" transplants during the isolation of mitochondria after the first high-speed spin, was added to liver mitochondria, uncoupler-activated ATPase activity was inhibited, while ATPase activity in the presence of Mg^{2+} was increased, in contrast to lipid from young transplants. Analysis of the lipid fraction from "old" transplants showed that it contained high levels of free fatty acids. When similar studies were carried out with other tumor lines, the authors observed a close parallel between the mitochondria-damaging effects of lipids contained in the hepatomas and the activities of mitochondria isolated from the corresponding hepatomas. It is well documented that solid tumors have deficient vascular beds (44) and often contain large populations of hypoxic cells (22, 25). As tumors grow larger, ischemic regions develop, and some necrosis is evident. Most investigators have reported the presence of necrotic regions in tumors weighing 4 to 6 g and have attempted to circumvent this problem by carefully dissecting these areas free prior to tissue homogenization. In addition,

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serum albumin is usually included in the isolation medium to bind lipid compounds which may damage mitochondrial membranes. In this study, we have also attempted to dissect free necrotic regions prior to tissue homogenization. However, as seen in Chart 1, even when mitochondria were isolated from "necrotic-free" 7777 tumors in the presence of 1% albumin, these efforts did not prevent the loss of transport activity and the levels of internal adenine nucleotides. The results shown indicate that the protective effect of albumin is limited and may also indicate the characteristics of hepatoma mitochondria *in vivo*. Other evidence which may be viewed as consistent with these findings is the report by Kaschnitz *et al.* (23), who observed that the acceptor:control ratios in mitochondria isolated from rapidly growing tumors weighing 2 g (Hepatoma 3683) and 1.5 to 8.0 g (Hepatoma 3924A) were significantly lower than those from tumors weighing 0.5 to 0.7 g (Hepatoma 7777), even though mitochondria were isolated in the presence of 2% albumin.

The occurrence of hypoxic cells in solid tumors may be considered to arise simply from the lack of development of a sufficiently organized vascular net to keep pace with the expanding tumor mass. In this view, it may well be expected that, for a given size tumor, ischemic-like effects would be more pronounced in rapidly growing than in slowly growing tumors. Evidence that such effects occur are results reported by Weber *et al.* (49), who showed that whole-tissue adenine nucleotide levels are significantly lower in a fast-growing tumor (Hepatoma 3924A) and a tumor with an intermediate growth rate (Hepatoma 5123D) compared to a slow-growing tumor (9816A) and control liver. Vary *et al.* (45) have examined the relationship between adenine nucleotide metabolism and ischemia and concluded that the reduction of whole-tissue adenine nucleotide levels is an excellent marker for the onset of irreversible ischemic tissue damage. Other evidence for this effect is the results from the present study, which show that transport rates are lower and more severely depressed in mitochondria isolated from a large, fast-growing tumor (Hepatoma 7777) than from a large, slow-growing tumor (Hepatoma 16). Thus it appears that ischemic-like effects are more prominent in larger, rapidly growing tumors than in smaller, slow-growing tumors.

The implication that ischemic effects may vary with tumor size and growth rate may indicate that rates of aerobic glycolysis would be similarly affected. Dinescu-Romalo and Mihai (11) have shown that uncoupling agents and inhibitors of adenine nucleotide and electron transport increase rates of aerobic glycolysis in rat liver slices at levels which are not cytotoxic. Since long-chain free fatty acids can act both as uncoupling agents and as inhibitors of adenine nucleotide transport, their accumulation in tumor mitochondria as a consequence of hypoxia may well influence rates of aerobic glycolysis *in vivo*. Higher rates of aerobic glycolysis would be expected in larger, rapidly growing tumors than in smaller, slower-growing tumors. As was described under "Introduction," rates of aerobic glycolysis have been shown to correlate with tumor growth rate in Morris hepatomas, with higher rates occurring in more rapidly growing tumors (6, 43, 48). A key issue, therefore, is the size of the tumors used in these studies. The report by Burk *et al.* (6) simply states that tumors were used "when (they) had reached sufficient size." Weber *et al.* (49) are somewhat more precise and report that tumors are harvested when they are approximately 1.5 cm in diameter. Since the report mentioned above by Weber *et al.* (49) on adenine nucleotide levels in Morris hepatomas used tumors of this size and since the loss of tissue adenine nucleotides has been shown to be a good indicator of tissue ischemia, their results may indicate that ischemic effects occur in more rapidly growing tumors which have reached 1.5 cm in diameter and thus contribute to the elevated rates of aerobic glycolysis observed.

To summarize, the results presented indicate that the adenine nucleotide transport activity in all tumor mitochondria studied was significantly lower than in control or regenerating rat liver mitochondria. Furthermore, the V_{max} for ADP uptake correlates inversely with the tumor growth rate. In addition, ischemic-like effect may occur in tumors of large size and, as discussed above, may also be more pronounced in rapidly growing tumors than in slowly growing tumors. This last conclusion is consistent with the findings of Emmelot and Bos (13), who also reported that tumor size may influence mitochondrial function. However, it should be pointed out that the present study is the first to demonstrate that the observed decline in mitochondrial nucleotide transport activity as a function of tumor size is probably a result of tissue hypoxia.

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