

## Energy Metabolism of Tumor Cells

REQUIREMENT FOR A FORM OF HEXOKINASE WITH A PROPENSITY FOR MITOCHONDRIAL BINDING\*

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Rat liver cytoplasm (postnuclear supernatant) has a low aerobic glycolytic rate in the presence of added glucose, ATP, ADP,  $P_i$ , and  $NAD^+$ , whereas cytoplasm from Ehrlich ascites tumor cells exhibit a high aerobic glycolytic rate which is typical of rapidly proliferating tumor cells. Tumor mitochondria, unlike liver mitochondria, contain bound hexokinase which constitutes about 70% of the total cellular hexokinase activity.

The high aerobic glycolytic rate of Ehrlich tumor cytoplasm is reduced markedly if the mitochondria are removed and can be restored almost completely upon addition of the hexokinase-containing tumor mitochondria to tumor cytosol (postmitochondrial supernatant). Addition of tumor mitochondria to liver cytosol can enhance its glycolytic rate to levels approaching those of tumor cytoplasm, whereas added liver mitochondria are without effect on the already low glycolytic rate of liver cytosol. Addition of tumor mitochondria to tumor cytosol increases its glycolytic rate to the level of tumor cytoplasm, as mentioned above, but liver mitochondria added to tumor cytosol actually depress its glycolytic rate to the level of liver cytosol.

The stimulatory effect of tumor mitochondria on liver cytosol can be ascribed to its associated hexokinase activity since hexokinase specifically removed from mitochondria of tumor cells can also enhance the glycolytic rate of liver cytosol. The depressing effect of added liver mitochondria on tumor cytosol glycolysis suggests that liver mitochondria can compete more effectively than tumor mitochondria for a common intermediate and/or cofactor.

Examination of 12 different tumor cell lines revealed that only those which reached maximum size in 1 month or less, and which have elevated glycolytic activities, had detectable mitochondrially associated hexokinase activity. The studies reported here describe resolution and reconstitution of tumor cytoplasm, supplementation of cytosol with intact mitochondria or mitochondrial hexokinase, and a survey of mitochondrial hexokinase content in various tumors, and provide strong evidence for the view (Bustamante, E., and

Pedersen, P. L. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 3735-3739) that a form of hexokinase with a propensity for mitochondrial binding plays a key role in the high aerobic glycolysis of cancer cells.

One of the prominent properties characteristic of rapidly growing tumor cells is their capacity to sustain high rates of glycolysis under aerobic conditions (1-5). This seems to be a general property of highly malignant tumors independent of their carcinogenic origin. Moreover, high aerobic glycolysis is now known to be one of the few immediate biochemical changes which correlate with the transformation function in cells infected with tumor viruses temperature sensitive for transformation (6). Despite the many years that have elapsed since the original description of such behavior (1), much remains to be elucidated as to the pertinent biochemical changes a cell undergoes upon transformation.

In previous reports (5, 7) we have shown that rapidly growing hepatoma cells grown in culture can exhibit both a high glycolytic state or a low glycolytic state depending on the hexose present in the medium as the sole carbohydrate source. We showed that H-91 hepatoma cells have a low glycolytic rate when grown on galactose, and exhibit a high aerobic glycolytic rate only when grown on a hexose which can be a substrate for hexokinase, such as glucose (5). The observation suggested to us that the high glycolytic capacity of these cancer cells might be due either to an enhanced rate of glucose transport relative to galactose transport or to an unique elevated form of hexokinase. We showed that a form of hexokinase bound to hepatoma mitochondria is, at least in part, responsible for such high rates of glycolysis (5). In a subsequent report we described the kinetic properties of hexokinase bound to hepatoma mitochondria and defined conditions for removing the enzyme from the mitochondrial membrane (8).

Experiments described in this communication were undertaken to evaluate directly the requirement of rapidly growing Ehrlich ascites tumor cells for mitochondrial hexokinase in supporting a high aerobic glycolytic rate. The following questions were addressed in this study. (a) Can the high glycolytic rate characteristic of Ehrlich ascites cell cytoplasm be reduced when the mitochondria (with their associated hexokinase) are removed? (b) Will readdition of tumor mitochondria result in a reconstitution of the original glycolytic activity? (c) What is the differential effect on the normally low glycolytic rate of liver cytosol of hexokinase-containing (*i.e.* tumor) mitochondria and of hexokinase-free (*i.e.* liver) mitochondria? (d) What is the differential effect on the normally high glycolytic rate of tumor cytosol of added tumor mitochondria compared to

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added liver mitochondria? (e) Can addition of hexokinase specifically solubilized from tumor mitochondria enhance equally well the glycolytic rate of liver cytosol? (f) is mitochondrial hexokinase associated only with tumor cell lines reported to have an elevated glycolysis and rapid growth rate? Answers to all of these questions are provided below.

#### EXPERIMENTAL PROCEDURES

##### Materials

The NU strain of Ehrlich ascites tumor cells was generously provided by Dr. A. L. Lehninger of this University, and was propagated by weekly intraperitoneal injection into male Swiss albino mice. Morris hepatomas were a generous gift of Dr. H. P. Morris of Howard University College of Medicine (Washington, D. C.).

Hepes,<sup>1</sup> NAD<sup>+</sup>, BSA, glucose, a standard lactic acid solution, glucose-6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*, whose cofactor can be either NAD<sup>+</sup> or NADP<sup>+</sup>), and lactic dehydrogenase (type XVIII) were purchased from Sigma Chemical Co. Sucrose was from Baker. ATP and ADP were from P-L Biochemicals. Nagarse was purchased from Enzyme Development Corp., New York. Reagents for protein determination were purchased from Bio-Rad Laboratories. All other reagents used were analytical grade.

##### Methods

**Cell Fractionation**—The homogenization medium used throughout was 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, and 0.1% BSA, pH 7.4. Mitochondria from rat liver and mitochondria from Morris hepatomas were prepared according to the high yield procedure of Bustamante *et al.* (9). The mitochondrial fractions prepared as described exhibited ADP-stimulated respiration with succinate as substrate and were judged on this basis to be functionally intact (9). Rat liver cytosol was prepared by centrifuging the first postmitochondrial fraction (9) at 48,000 rpm in the Beckman type 65 rotor (150,000 × *g*, average) for 45 min at 4 °C. The supernatant was the liver cytosol fraction. Ehrlich tumor mitochondria were prepared according to the procedure described by Pedersen *et al.* (10) which involves the use of the protease Nagarse. This mitochondrial fraction was found to have an acceptor control ratio of about 6 with succinate as respiratory substrate.

In order to prepare Ehrlich tumor cytoplasm and cytosol, cells (10 ml, 10<sup>8</sup> cells/ml) were homogenized by sonic oscillation at 4 °C with the large probe of a Bronwill Biosonik sonic oscillation apparatus for a total of 30 s (in three 10-s bursts) at 80% output. The homogenate was centrifuged at 3,000 rpm for 5 min at 4 °C in a Sorvall rotor to sediment nuclei and cell debris. The postnuclear supernatant or cytoplasmic fraction was centrifuged at 10,000 × *g* for 10 min at 4 °C in the Sorvall rotor. The resulting supernatant was centrifuged at 48,000 rpm in the Beckman type 65 rotor for 45 min at 4 °C. The final supernatant was the Ehrlich tumor cytosol fraction.

**Determination of Glycolytic Activity**—Glycolysis was determined at 37 °C by measuring the amount of lactic acid produced after 30 min in a final volume of 0.3 ml containing 4 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM KP<sub>i</sub>, 4 mM ATP, 4 mM ADP, 2.8 mM NAD<sup>+</sup>, and 4 mM glucose, pH 7.4. The reaction was started by the addition of 0.07 ml of a mixture of the above to 0.23 ml of the subcellular fraction to be assayed. The reaction was stopped by the addition of 0.7 ml of 5% (w/v) trichloroacetic acid. The tubes were chilled for about 10 min and centrifuged at 10,000 rpm in a Sorvall rotor for 10 min at 4 °C. The supernatants were collected and taken to about pH 9 by the addition of 0.06 ml of 3.5 N NaOH. Aliquots of 0.05 to 0.2 ml were taken to measure lactic acid. Lactic acid in such samples was determined spectrophotometrically by measuring the amount of NADH formed at 340 nm in a 1-ml system containing 42 mM hydrazine sulfate, 170 mM glycyl glycine, 0.4 mM EDTA, 0.85 mM NAD<sup>+</sup>, and 20 units of lactic dehydrogenase, at pH 9.5 and 25 °C. The assay mixture usually reached end point for NADH formation at about 30 min. The absorbance readings were calibrated against a standard curve made with a 4.4 mM lactic acid standard.

**Determination of Hexokinase Activity**—Hexokinase (EC 2.7.1.1) was determined spectrophotometrically at pH 7.7 and 25 °C by following the formation of NADH at 340 nm in a 1-ml system containing 24 mM Hepes, 12.5 mM MgCl<sub>2</sub>, 2.5 mM NAD<sup>+</sup>, 5 mM glucose, 6 mM ATP, and 0.4 unit of glucose-6-phosphate dehydrogenase.

<sup>1</sup> The abbreviations used are: Hepes, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; BSA, defatted bovine serum albumin.

**Solubilization of Mitochondrial Hexokinase**—Hexokinase was specifically solubilized from tumor mitochondria by the procedure of Bustamante and Pedersen (5). Fresh tumor mitochondria were incubated for 10 min at 25 °C in a medium containing 3 mM glucose 6-phosphate in 250 mM sucrose and 30 mM Hepes, pH 7.4. The suspension was centrifuged at 10,000 × *g* for 10 min at 4 °C. The resulting supernatant was dialyzed against 2,000 volumes of 250 mM sucrose and 30 mM Hepes, pH 7.4, at 4 °C for at least 8 h. The dialysate is referred to as "solubilized mitochondrial hexokinase."

**Determination of Protein**—Protein was determined by the method of Bradford (11) as formulated by Bio-Rad Laboratories, using a 1.4 mg/ml of  $\gamma$ -globulin solution as protein standard.

#### RESULTS

**Loss of Glycolytic Capacity in Ehrlich Ascites Cytoplasm upon Removal of the Mitochondrial Fraction**—Fig. 1 shows that cytoplasm (postnuclear supernatant) from Ehrlich ascites tumor cells has a high aerobic glycolytic rate in the presence of added glucose, ATP, ADP, P<sub>i</sub>, and NAD<sup>+</sup>. This is typical of rapidly growing tumor cell lines (1–5). It is significant to note (Fig. 1) that removal of the particulate fraction from tumor cytoplasm results in a markedly decreased rate of aerobic glycolysis. This is an indication that an important constituent of the glycolytic system has been removed upon centrifugation. Table I shows that hexokinase, the first enzyme in the glycolytic pathway, is localized to a large extent (about 70%) in the mitochondria of Ehrlich ascites tumor cells. This is in agreement with the subcellular localization of this enzyme in several other cell types examined to date including other strains of Ehrlich ascites cells (5, 12–15). Thus, removal of mitochondria from tumor cytoplasm in the experiment depicted in Fig. 1 results in a cytosolic fraction poor in hexokinase activity with a markedly decreased rate of aerobic glycolysis.

**Reconstitution of Original Glycolytic Activity of Tumor Cytosol upon Addition of Tumor Mitochondria**—Table II shows that when isolated tumor mitochondria are added back to tumor cytosol, the original glycolytic capacity of tumor cytoplasm is restored. Table II shows also that such reconstitution of the original lactic acid production rate is also observed when expressed on a specific activity basis. The mitochondrial fraction does not catalyze glycolysis (data not shown). Therefore, restoration of the original glycolytic rate

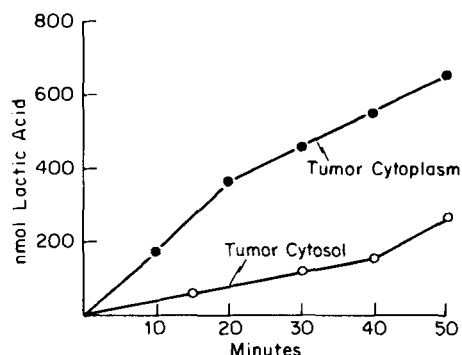


FIG. 1. Kinetics of lactic acid production by Ehrlich tumor cytoplasm and cytosol. A cytoplasmic fraction was prepared from Ehrlich tumor cells as described under "Methods." An aliquot of such fractions was centrifuged at 10,000 × *g* for 10 min at 4 °C in a Sorvall rotor. The supernatant was subsequently centrifuged at 48,000 rpm for 45 min at 4 °C in a Beckman type 65 rotor. The resulting supernatant was the cytosol fraction. Aliquots (equal volumes) of the cytoplasm (0.92 mg of protein) and of its cytosol (0.64 mg of protein) were assayed at 37 °C for glycolytic activity during the indicated time periods as described under "Methods." Results shown represent the average of duplicate determinations. The term "tumor cytoplasm" (●—●) refers to the postnuclear supernatant of the homogenate. The term "tumor cytosol" (○—○) refers to the soluble fraction of cytoplasm free of mitochondria and microsomes.

TABLE I

*Subcellular distribution of hexokinase in Ehrlich tumor cytoplasm*

A cytoplasmic fraction was prepared from Ehrlich ascites tumor cells as described under "Methods" and centrifuged at 10,000  $\times$  g for 10 min at 4 °C in a Sorvall rotor. The resulting sediment is referred to in this table as "mitochondria." The supernatant was subsequently centrifuged at 48,000 rpm for 45 min at 4 °C in the Beckman type 65 rotor. The resulting sediment is referred to in this table as "microsomes." The final supernatant is referred to in this table as "cytosol." Results shown are normalized and represent a typical experiment with a recovery of 92%. Hexokinase activity and protein determinations were carried out as described under "Methods."

	Hexokinase total activity	Protein	Hexokinase specific activity
	nmol glucose/min	mg	nmol glucose/min $\times$ mg
Cytoplasm	3660	43	85
Mitochondria	2730	10	273
Microsomes	30	2	15
Cytosol	900	31	29

when tumor cytoplasm is reconstituted should be a reflection of some mitochondrial factor. For reasons indicated above, the obvious candidate was hexokinase.

Fig. 2, in agreement with the results presented in Table II, shows upon addition of increasing amounts of tumor mitochondria, the originally high glycolytic rate of tumor cytoplasm can be restored. About 0.4 mg of tumor mitochondria per mg of cytosol is required to attain maximal reconstitution (Fig. 2). In order to test whether the mitochondrial constituent essential for high aerobic glycolysis was hexokinase bound to tumor mitochondria, it was important to carry out a control experiment with liver mitochondria, as they contain little or no detectable hexokinase activity (hepatocytes contain essentially no hexokinase, not to be confused with glucokinase EC 2.7.1.2 (see Refs. 16–18)).<sup>2</sup> Fig. 2 shows that, in contrast to the case where tumor mitochondria are added, liver mitochondria added to glycolyzing tumor cytosol actually cause a decrease in its rate of lactic acid production. Significantly, liver mitochondria reduce the glycolytic rate of tumor cytosol to levels approaching those of liver cytosol.

**Elevation of Glycolytic Activity in Liver Cytosol by the Addition of Tumor Mitochondria**—To test whether mitochondrial hexokinase of Ehrlich ascites tumor cells is required for elevated glycolytic activity, Ehrlich tumor mitochondria were added to the cytosol of a cell type that is normally characterized by a low glycolytic rate. Whole liver has a very low glycolytic rate, and has a specific hexokinase activity (low  $K_m^{\text{Glu}}$ , EC 2.7.1.1) of about 3 milliunits/mg<sup>3</sup> of protein (about 30 times lower than ascites tumor cells) which is mostly localized in the cytosolic fraction (5). Fig. 3 shows that rat liver cytosol has a low glycolytic rate under aerobic conditions. Significantly, its glycolytic rate is enhanced severalfold when increasing amounts of tumor mitochondria are added to the assay system. In contrast, liver mitochondria are without effect on the low glycolytic rate of liver cytosol. Although the tumor mitochondria-enhanced glycolytic rate of liver cytosol is not as high as that of tumor cytoplasm, such a markedly

<sup>2</sup> In this article we refer throughout to hexokinase (EC 2.7.1.1). This activity is detected in assays containing only 5 mM glucose. It should not be confused with glucokinase (EC 2.7.1.2), which is detectable at much higher concentrations of glucose (100 mM). Hexokinase is a characteristic activity of rapidly growing tumors and is present at very low activity levels in liver, where glucokinase is the predominant glucose phosphorylating enzyme. Liver mitochondria have a hexokinase activity level of less than 1 milliunit/mg (19).

<sup>3</sup> One milliunit of hexokinase activity is defined as the amount of enzyme that will catalyze the phosphorylation of 1 nmol of glucose in 1 min at 25 °C and pH 7.7.

increased glycolytic rate clearly indicates that under our assay conditions a limiting factor for liver cytosol to sustain high rates of aerobic glycolysis is a constituent present in tumor mitochondria but absent from liver mitochondria.

TABLE II

*Reconstitution of tumor high aerobic glycolysis*

Cytoplasmic and cytosolic fractions were prepared from Ehrlich ascites tumor cells as described in the legend to Fig. 1. Mitochondria were prepared from Ehrlich cells as described under "Methods," and had a hexokinase specific activity of 318 milliunits/mg of protein. An aliquot containing 0.46 mg (146 milliunits) was added to the cytosolic fraction as shown below. Results presented are averages of duplicate determinations.

	Total glycolytic activity	Protein	Glycolytic specific activity
	nmol lactate/30 min	mg	nmol lactate/30 min $\times$ mg protein
Cytoplasm	398	0.92	433
Cytosol	164	0.64	256
Cytosol + mitochondria	483	1.10	439

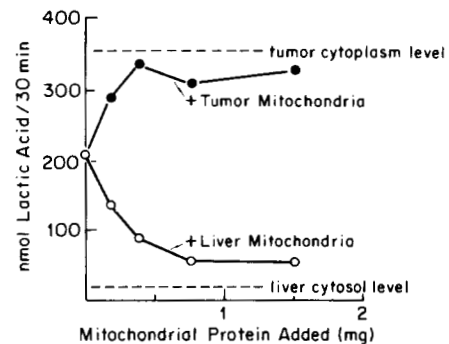


FIG. 2. Effect of tumor and liver mitochondria on the glycolytic rate of tumor cytosol. Ehrlich tumor cytosol, Ehrlich tumor mitochondria, and rat liver mitochondria were prepared as described under "Methods." Aliquots of fresh tumor cytosol (0.8 mg of protein) were incubated with indicated amounts of fresh tumor (●—●) or liver (○—○) mitochondria and assayed for glycolytic activity at 37 °C as described under "Methods." Hexokinase activity of tumor mitochondria was 318 milliunits/mg of protein. There was no detectable hexokinase activity in the liver mitochondrial fraction under identical assay conditions. Results shown are averages of duplicate determinations.

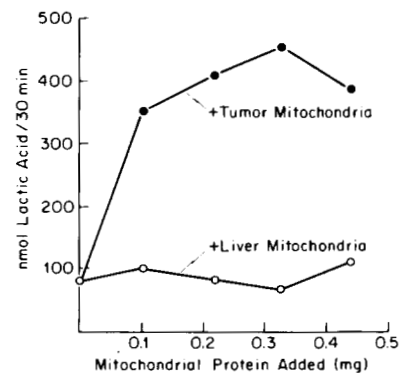


FIG. 3. Effect of tumor and liver mitochondria on the glycolytic rate of liver cytosol. Liver cytosol, Ehrlich tumor mitochondria, and rat liver mitochondria were prepared as described under "Methods." Aliquots of fresh liver cytosol (3.6 mg of protein) were incubated with indicated amounts of fresh tumor (●—●) or liver (○—○) mitochondria and assayed for glycolytic activity at 37 °C as described under "Methods." Results shown are averages of duplicate determinations. Hexokinase activity of tumor mitochondria was 318 milliunits/mg of protein. There was no detectable hexokinase activity in the liver mitochondrial preparation under identical assay conditions.

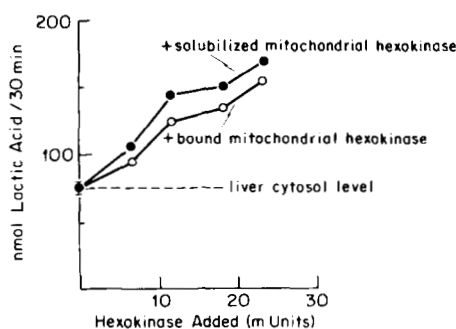


FIG. 4. Stimulation of the glycolytic rate of liver cytosol by mitochondrially bound and solubilized tumor mitochondrial hexokinase. Liver cytosol and tumor mitochondria were prepared as described under "Methods." Tumor mitochondria were washed and suspended in 250 mM sucrose and 30 mM Hepes, pH 7.4, and kept frozen until used. Such fraction is referred to as "bound mitochondrial hexokinase" (hexokinase specific activity = 273 nmol of glucose/min  $\times$  mg of protein). Solubilized tumor mitochondrial hexokinase was prepared as described under "Methods." The hexokinase specific activity of such preparation was 1050 nmol of glucose/min  $\times$  mg of protein). 2.5 mg of liver cytosol were incubated with the indicated amounts of either bound mitochondrial hexokinase (O—O) or solubilized mitochondrial hexokinase (●—●), and aerobic glycolysis was assayed at 37 °C as described under "Methods." Results shown are averages of duplicate determinations.

TABLE III

Correlation between growth rate and mitochondrial hexokinase activity

Mitochondrial fractions were prepared from homogenates of the tumors listed below as described under "Methods." Growth rate is defined as the time that usually elapses between transfers or that is needed for a solid tumor to reach 1.5 cm in diameter. Listed growth rates correspond to those recorded by Dr. Morris' laboratory in 1976 (42). Liver growth rate was estimated as the typical *in vivo* doubling time for hepatocytes. Mitochondrial hexokinase specific activity was determined as described under "Methods." MH stands for Morris hepatoma (all solid tumors). Values listed in this table are averages of at least duplicate determinations.

	Growth rate	Mitochondrial hexokinase specific activity
	months	nmol glucose/min $\times$ mg protein
Liver	12.0	<3
MH 7794A	4.5	<3
MH 4123C	2.5	<3
MH 44	2.0	<3
MH 8995	1.7	<3
MH 66	1.5	<3
MH 9121F	1.0	7
MH 9A	1.0	19
MH 3924A	1.0	55
L 1210 ascites tumor cells	0.2	180
Novikoff hepatoma (solid)	0.2	280
H-91 hepatoma cells (culture)	0.2	330
Ehrlich ascites tumor cells	0.2	380

**Elevation of Glycolytic Activity in Liver Cytosol by the Addition of Mitochondrial Hexokinase**—Fig. 4 shows that the stimulatory effect of tumor mitochondria on the glycolytic rate of liver cytosol can be mimicked on an activity basis by a preparation of hexokinase specifically solubilized from tumor mitochondria. (The hexokinase used in the reconstitution experiment was released from mitochondria in soluble form with 3 mM glucose 6-phosphate as described under "Methods.") This strongly suggests that the constituent present in tumor mitochondria (but absent from liver mitochondria) which is responsible for the enhancement of aerobic glycolysis is a form of hexokinase which has a propensity for binding to mitochondria.

**Correlation of Growth Rate and Glycolytic Capacity of Several Tumors with Mitochondrial Hexokinase Activity**—Table III lists 13 tissues, including 12 tumor types, arranged according to their growth rate (expressed in terms of months that either elapse between transplantations or that are needed for a solid tumor to reach 1.5 cm in diameter). As shown in Table III, only those tumors with a growth rate equal to or faster than 1 month have detectable mitochondrially associated hexokinase activity. It is a well known fact that the more dedifferentiated a tumor is (the more rapidly it grows), the higher its capacity to carry out high aerobic glycolysis (20–22). It is significant to note that only the tumor cell lines reported to have a capacity for high aerobic glycolysis (20–22) have detectable mitochondrially associated hexokinase activity, while the slow growing tumors that are known to have a near normal liver capacity (20–22) for aerobic glycolysis do not have detectable mitochondrial hexokinase activity.

## DISCUSSION

Results presented in this report provide six lines of evidence which indicate that a form of hexokinase with a propensity for mitochondrial binding plays a key role in the high aerobic glycolysis typical of rapidly growing tumor cells. (a) Tumor mitochondria contain (associated to their external membrane; see Refs. 14, 23) about 70% of the total cellular hexokinase activity, whereas liver mitochondria have no detectable hexokinase activity. (b) Removal of mitochondria from tumor cytoplasm results in a markedly diminished glycolytic capacity. (c) Readdition of tumor mitochondria to mitochondria-depleted cytoplasm (cytosol) restores its original glycolytic capacity. (d) Hexokinase-containing mitochondria (tumor) but not hexokinase-free mitochondria (liver) can enhance markedly the aerobic glycolytic rate of liver cytosol. (e) The enhancing effect of tumor mitochondria on liver cytosol glycolysis can be accounted for in terms of their associated hexokinase activity. (f) Of 12 different tumor lines tested, only those which have a rapid growth rate, and which are reported to be highly glycolytic (20–22), have detectable hexokinase activity in their mitochondrial fractions.

Significantly, several workers have shown that the mitochondrial fraction of tumors can enhance the glycolytic rate of cytosolic fractions (20, 24–26) although the factor(s) responsible was never identified. In recent years several indirect lines of evidence from other laboratories have accumulated which indicate that a particulate form of hexokinase is at least partially responsible for the capacity of certain animal tissues to sustain high aerobic glycolysis. Singh *et al.* (6) reported that an increased level of hexokinase correlates with the transformation function using Rous sarcoma virus mutants temperature sensitive for transformation. In a more recent report these same workers have shown that the transformation of chick embryo fibroblasts by Rous sarcoma virus enhances the proportion of hexokinase that is bound to a particulate fraction (27). In addition, Ballatori and Cohen (28) have reported that the high aerobic glycolytic rate of renal papillary tissue can be strongly correlated with an increased mitochondrial hexokinase specific activity relative to the cortical and medullary regions of the kidney (which exhibit a low glycolytic rate). Finally, it seems important to note that the correlation between tumor growth rate and mitochondrial hexokinase activity reported in this communication (see Table III) agrees with the molecular correlation concept proposed by Weber (21, 22).

Racker and his colleagues (29–34) have emphasized in recent years that the glycolytic rates of most cells are determined by the rates at which these cells are able to regenerate ADP and P<sub>i</sub> from ATP. Although they state that "uncoupled"

acceleration of the mitochondrial ATPase, in some tumors, and of the plasma membrane  $\text{Na}^+, \text{K}^+$ -ATPase in other tumors may be primarily responsible for their elevated glycolytic rates, there have been little data presented to strongly support this view. In fact, it has been shown that mitochondria isolated from most tumor cells carry out coupled ATP synthesis almost as well as normal mitochondria (for a detailed review see Ref. 3). Thus, they operate primarily in the direction of ATP synthesis not hydrolysis. The original work on the  $\text{Na}^+, \text{K}^+$ -ATPase showed that the inhibitors ouabain (29) and quercetin (32) inhibit both the activity of this enzyme and glycolysis in tumor cells (32), but no control studies were carried out to ascertain quercetin this inhibitor also inhibits the  $\text{Na}^+, \text{K}^+$ -ATPase and glycolysis of normal cells. More recently Graziani (35) and Podhacer *et al.* (36) have shown that quercetin also inhibits the mitochondrial hexokinase activity of Ehrlich ascites cells.

The recent finding of Racker and his colleagues (33, 34) that a protein kinase of Ehrlich ascites cells phosphorylates the  $\text{Na}^+, \text{K}^+$ -ATPase and evidently makes it less coupled is of obvious interest, but the question of how specific this and other protein kinases are in tumor cells in the *in vivo* situation remains unknown. Moreover, futile cycling of ATP by an uncoupled cellular ATPase would be predicted to deplete the cancer cell of ATP essential for cell growth and division.

Hexokinase provides the carbon,  $\text{P}_i$ , and ADP "gate" for the glycolytic system. Operationally, hexokinase is a "coupled" ATPase in the sense that it feeds mitochondrial phosphate directly into the glycolytic system and provides the cofactor ADP for later steps (5). It is not difficult, therefore, to envision that a coupled hexokinase in elevated and unregulated form (and not an uncoupled  $\text{Na}^+, \text{K}^+$ , or mitochondrial ATPase) may be the "ATPase" essential for high glycolytic rates in rapidly growing cancer cells.

Experimental data reported in this communication argue against the view that only an uncoupled  $\text{Na}^+, \text{K}^+$ -ATPase (providing excess ADP and  $\text{P}_i$ ) is primarily responsible for high glycolytic rates. Thus, it is shown that liver cytosol is not able to carry out high aerobic glycolysis in the presence of added ADP,  $\text{P}_i$ , and ATP unless it is also provided with mitochondrial hexokinase. Moreover, in the presence of added ADP,  $\text{P}_i$ , and ATP, tumor cytoplasm depleted of hexokinase-containing mitochondria has a markedly lower rate of aerobic glycolysis than the system reconstituted with mitochondrial hexokinase. It seems clear, therefore, that addition or subtraction of hexokinase-containing mitochondria in glycolyzing systems can increase or decrease their glycolytic rate despite the high and continuous availability of ADP,  $\text{P}_i$ , and ATP in the assay media. Thus, "even in the midst of plenty," glycolytic systems do not carry out high aerobic glycolysis unless hexokinase is present in elevated amounts.

It is of interest to note that the only enzyme required by the cytosolic fraction to convert this fraction into a highly glycolytic state is the presence of a hexokinase with a propensity for mitochondrial binding. This observation certainly does not rule out a role for other key regulatory enzymes of the glycolytic pathway (*e.g.* phosphofructokinase and pyruvate kinase). Indeed, we believe they probably become limiting at the point where glycolytic activity of liver cytosol supplemented with mitochondrial hexokinase becomes maximal but still does not reach the glycolytic level corresponding to tumor cytoplasm (see Fig. 3). Nevertheless, these results strongly point out that the first limiting enzyme in high aerobic glycolysis is mitochondrially associated hexokinase.

Current studies in this laboratory are directed at better understanding two other aspects of data presented in this communication. First, it is not clear why tumor mitochondria

cannot accommodate (by transport and/or oxidation) the excess pyruvate formed in cancer cells. Rather it is converted to lactic acid. Is this due to a defective or limiting pyruvate transport or oxidation system in tumor mitochondria as suggested by Greenhouse and Lehninger (37, 38) or to the "M" form of lactic dehydrogenase in tumor cells which might compete more effectively for the pyruvate (39, 40)? Secondly, it is not completely clear why hexokinase is preferentially localized bound to the mitochondrial fractions in highly glycolytic tumors. This question takes on added interest since we were able to show that either the free or bound forms of the enzyme can enhance the glycolytic activity of the liver cytosolic fraction. Perhaps the binding of mitochondrial hexokinase to its recently discovered receptor protein (41) makes it more resistant to substrate or product inhibition under intense enzyme activity during high aerobic glycolysis *in vivo*.

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