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High aerobic glycolysis of rat hepatoma cells in culture: Role of mitochondrial hexokinase

(L-lactic acid/D-glucose/D-galactose/liver/neoplasia)

ERNESTO BUSTAMANTE* AND PETER L. PEDERSEN

Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

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ABSTRACT A tumorigenic anchorage-dependent cell line (H-91) was established in culture from an azo-dye-induced rat ascites hepatoma. When grown in a glucose-containing medium the cells exhibit high rates of lactic acid production characteristic of rapidly growing tumor cells. However, when glucose is replaced with galactose the cells grow equally well but exhibit only moderately elevated rates of lactic acid production. The molecular basis for this observation cannot be attributed to differences in permeability because initial rates of glucose and galactose entry into hepatoma cells are identical. Rather, the activity of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) is found to be high in hepatoma cells, about 20-fold higher than that of control and regenerating rat liver. Moreover, tumor hexokinase activity is not inhibited by low concentrations (<0.6 mM) of the reaction product glucose 6-phosphate. Additionally, 50% of the hexokinase activity of hepatoma cells is found associated with the mitochondrial fraction. This fraction is 3-fold enriched in hexokinase activity relative to the homogenate and 4-fold enriched relative to the nuclear and postmitochondrial fractions. Tumor mitochondrial hexokinase appears to be coupled directly to oxidative phosphorylation, because addition of glucose to respiring hepatoma mitochondria (after a burst of ATP synthesis) results in stimulation of respiration. In contrast, glucose has no effect on the respiration of mitochondria from control and regenerating liver. These results suggest that the high glycolytic capacity of H-91 hepatoma cells is due, at least in part, to an elevated form of hexokinase concentrated in the mitochondrial fraction of the cell.

The most characteristic biochemical phenotype of cancer cells is their propensity for high aerobic rates of glycolysis (1-4). Rapidly growing tumor cells, in particular those growing in ascitic form, are known to exhibit markedly elevated rates of lactic acid production when compared with normal cells (1-4). Cells from slowly growing tumors also have elevated rates of aerobic glycolysis (4), although the elevation is not nearly as dramatic.

The reason for the high aerobic lactic acid production of rapidly growing cancer cells has puzzled biochemists for more than 50 years, and as of this date a satisfactory explanation acceptable to most workers in the field of cancer research has not been forthcoming. Possible explanations for the very "high glycolysis" of rapidly growing cancer cells usually involve mitochondria, which are assumed to be either defective in some capacity or less effective than glycolytic enzymes in competing for common intermediates (2, 5).

To more definitely establish the relationship(s) between mitochondria and glycolysis in rapidly growing cancer cells, the studies described in this paper have focused on differences in the catabolism of glucose and galactose by a hepatoma cell line growing in culture. Because such cancer cells grow equally well on both carbohydrate sources but produce large amounts

of lactic acid only when grown on glucose, the hepatoma culture system seemed ideal for exploring the molecular events responsible for the "high glycolysis" of cancer cells.

METHODS

Cell Line and Tissue Culture. The cell line H-91 was established in this laboratory from the azo-dye-induced rat ascites hepatoma AS-30D (6). Rats bearing the AS-30D ascites tumor cells at passage 300 were generously provided by A. C. Griffin of the M. D. Anderson Hospital and Tumor Institute. The ascites cells were cultured under conditions described below. In order to select for a population of cells capable of anchoring, floater cells were removed daily for 2 months. The anchorage-dependent cells were then cloned by serial dilution, and the cell line H-91 was grown from a clone consisting of fewer than 200 cells.

H-91 cells were routinely grown at 37° in closed Falcon flasks made of sulfonated polystyrene using L-15 Leibovitz medium (7) supplemented with 5% (vol/vol) fetal bovine serum (heat inactivated) at pH 7.4 and under a 100% air atmosphere. This growth medium contains 4 mM galactose and a negligible amount of glucose. The cells were transferred weekly from one into five flasks using isotonic 0.25% (wt/vol) trypsin as a detaching agent. It is important to note that H-91 cells retain their original tumorigenicity and rapid tumor cell growth in rats.

Cell Fractionation. H-91 cells were removed from flasks with trypsin, and suspended in H-medium (8) at a density of about 5×10^7 cells per ml. The cell suspension was homogenized by sonication for 15 sec with the small probe of a Bronwill sonicator at 20% output. All subsequent operations were performed at 4°. The cell homogenate, which typically consisted of 90% disrupted cells, was centrifuged at $770 \times g$ for 5 min. The resulting sediment was suspended in H-medium and constitutes the nuclear (N) fraction. The supernatant was centrifuged at $7900 \times g$ for 10 min. The resulting sediment was suspended in H-medium and constitutes the mitochondrial (M) fraction. The resulting supernatant constitutes the postmitochondrial (S) fraction. Regenerating rat liver was obtained surgically (9). Mitochondria were prepared from control and regenerating rat liver (10).

Transport Assays. Hexose transport was measured at 37° and pH 7.4 in a 0.1-ml system containing 140 mM NaCl, 2.7 mM KCl, 10 mM KP_i , and 10^6 to 4×10^6 cells. The process was started by adding 5 mM hexose containing 0.1 μ Ci of [^{14}C]-hexose. To correct for nonspecific trapping and adsorption, 5 mM unlabeled hexose containing 0.1 μ Ci of [^{14}C]sucrose was substituted for the radioactive hexose in control experiments.

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Abbreviations: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; H-medium, 200 mM mannitol/70 mM sucrose/2 mM Hepes/defatted bovine serum albumin at 0.5 g/liter, pH 7.4.

* On leave from the Universidad Peruana Cayetano Heredia, Lima, Perú.

Table 1. Glycolytic activity of H-91 cells in glucose- and galactose-containing media

Hexose in growth medium	Initial concentration, mM	Cell number $\times 10^{-6}$		Hexose utilized, $\mu\text{mol}/24 \text{ hr}$	Lactic acid formed, $\mu\text{mol}/24 \text{ hr}$	% hexose utilized converted to lactic acid
		Initial	After 24 hr			
Glucose	5	5.0	10.1	11.2	20.8	92
Galactose	5	5.0	9.7	4.0	7.2	90

See *Methods* and legend to Fig. 1 for details of growth conditions and biochemical assays. Volume per flask was 8 ml.

Aliquots (0.09 ml) of the incubation medium were transferred onto 0.1 ml of an oil mixture (2:1, dibutyl phthalate/dinonyl phthalate) in a 0.45-ml conical plastic tube and centrifuged at maximum speed in a Beckman 152 Microfuge for 20 sec. The end of the incubation period was taken as the time the Microfuge was started. The minimum incubation time in our hands was 9 sec. The tips of the tubes (containing the packed cells) were sliced and placed in vials containing 1% (wt/vol) sodium dodecyl sulfate and 0.1 M NaOH and incubated overnight at 55°. Radioactivity was estimated by liquid scintillation counting.

Biochemical Determinations. Respiration rates of isolated mitochondria were determined polarographically with a Clark oxygen electrode (11) in a closed 3-ml system containing 220 mM mannitol, 70 mM sucrose, 2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, 2.4 mM K_2P_i buffer, 0.5 mM sodium EDTA, 1.8 mM MgCl_2 , 5 mM succinate, and defatted bovine serum albumin (0.17 g/liter), at pH 7.4 and 22°. Mitochondria, ADP, glucose, Glc-6-P, oligomycin, and 2,4-dinitrophenol were added at concentrations indicated in Fig. 6.

Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) activity was determined by following the formation of NADPH at 340 nm in a 1-ml system containing 32 mM Hepes buffer, 12 mM MgCl_2 , 6.5 mM ATP, 0.9 mM NADP^+ , 1 unit Glc-6-P dehydrogenase, and 0.5 mM D-glucose, at pH 7.6 and 22°. In experiments where the effect of Glc-6-P was tested, hexokinase activity was measured at 340 nm in a 1-ml coupled reaction system containing 40 mM Tris/ HCO_3 buffer, 10 mM MgCl_2 , 5 mM KCN, 0.2 mM NADH, 0.64 mM phosphoenolpyruvate, 2 mM ATP, 55 units pyruvate kinase, 0.5 mM glucose, and 6 units lactate dehydrogenase, at pH 7.9 and 22°. In some experiments where the coupled reaction system was used, it was necessary to block endogenous oxidation of NADH with additions of oligomycin or excess KCN before glucose was added.

L-Lactic acid was determined by measuring at 340 nm the concentration of NADH formed after 30 min in a 2.4-ml system containing 42 mM hydrazine sulfate, 170 mM glycine, 0.4 mM EDTA, 0.85 mM NAD^+ , and 23 units lactate dehydrogenase, at pH 9.5 and 22°.

Protein concentration was determined by the method of Lowry *et al.* (12).

Tissue culture reagents were purchased from Microbiological Associates. Glucose, galactose, and lactate dehydrogenase (type VI) were from Sigma; Glc-6-P dehydrogenase was from Boehringer Mannheim; and ATP, ADP, NAD^+ , NADH, and NADP^+ were obtained from P-L Biochemicals. [^{14}C]Glucose, [^{14}C]galactose, and [^{14}C]sucrose were purchased from New England Nuclear. 3-O-methyl[^{14}C]glucose was a generous gift from Saul Roseman of this University.

RESULTS

Results presented in Fig. 1A show that during the first 2 days in culture H-91 hepatoma cells grow as well in the presence of growth medium supplemented with 20 mM galactose as in

medium supplemented with 20 mM glucose. This observation is consistent with earlier findings (13) in other cell lines. The amounts of lactic acid produced during the growth period are depicted in Fig. 1B. Significantly, cells grown in glucose-supplemented medium produce at least 2- to 3-fold more lactic acid at all time points than cells grown in galactose-containing medium. The initial rates of lactic acid production measured in a separate experiment were found to be 750 nmol/hr-mg when cells were grown in glucose-supplemented medium and 250 nmol/hr-mg when cells were grown in galactose-supplemented medium. The latter value is only moderately elevated relative to literature values (14) for lactic acid production in liver slices.

As indicated in Table 1, a reduction of the initial hexose concentration in the growth medium from 20 mM to 5 mM yields similar results. H-91 cells double in about 24 hr when grown on media supplemented with either 5 mM glucose or 5 mM galactose. During this time period about 3 times more glucose is utilized than galactose. Moreover, about 3 times more lactic acid is formed when glucose is present in the growth medium than when galactose is present. In both cases about 90% of the hexose utilized is converted to lactic acid (assuming 1 mol of hexose = 2 mol of lactic acid).

The simplest explanation that can account for the enhanced lactic acid production by H-91 cells growing in glucose relative to galactose, is that glucose might be transported into the cells more rapidly. However, rapid transport measurements (9 sec

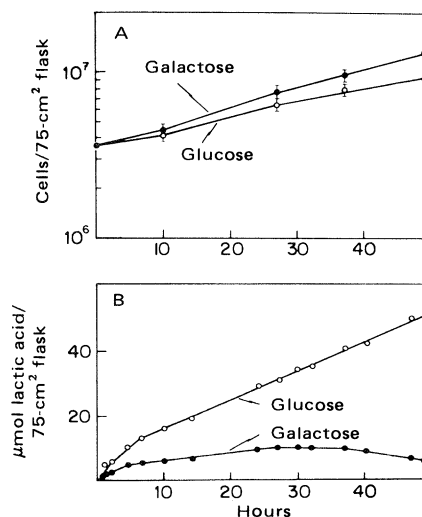


FIG. 1. Kinetics of growth (A) and lactic acid production (B) of H-91 hepatoma cells in culture. The cells were seeded in duplicate per experimental point with 3×10^6 cells per flask (plating efficiency = 85%) and incubated for 24 hr. Mean doubling time is 24 hr. At day zero fresh L-15 growth medium was provided and supplemented with 20 mM glucose or 20 mM galactose as shown in the figure. Two flasks were processed at the time points indicated. The extracellular fluid was collected and cleared of cell debris. The supernatants were assayed for lactic acid. The anchored cells remaining in the flask were trypsinized, washed in saline, counted with a hemocytometer, and assayed for protein.

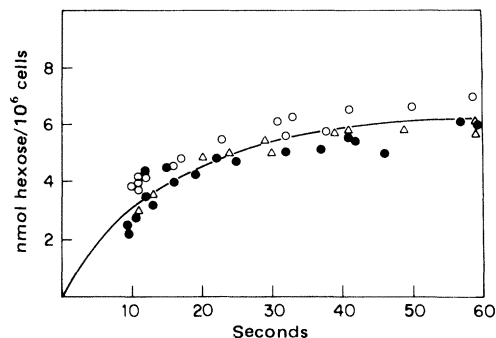


FIG. 2. Hexose transport by intact H-91 hepatoma cells. Cells growing exponentially in galactose-containing medium were trypsinized off the flasks, washed five times in saline, and suspended in saline at densities ranging from 10^7 to 4×10^7 cells per ml. Transport of [14 C]galactose (\bullet), [14 C]glucose (\circ), and 3-O-methyl[14 C]glucose (Δ) was measured. Final hexose concentration was 5 mM in all cases. A blank value for sucrose, to which cells are impermeable, (about 0.7 nmol/ 10^6 cells) was subtracted from all points in order to correct for nonspecific trapping.

to 1 min) carried out at 5 mM hexose show that glucose and galactose enter H-91 cells at the same rate (Fig. 2). Transport of hexose during this time period is not complicated by metabolism because 3-O-methylglucose, a nonmetabolizable sugar, is found to enter H-91 cells at the same initial rate as glucose and galactose. (When measurements are made over longer periods of time, glucose "uptake" is found to be greater than galactose "uptake," a finding that, as will be emphasized below, most likely reflects enhanced phosphorylation of glucose relative to galactose.) The transport measurements summarized in Fig. 2 are consistent with those of Hatanaka (15), who found that glucose and galactose are transported in a similar manner in fibroblasts transformed by mouse sarcoma virus.

An alternative explanation for the enhanced rates of lactic acid production by H-91 cells growing in glucose relative to galactose focuses on the enzymatic steps involved in converting these two sugars to the common glycolytic intermediate Glc-6-P. As shown in Fig. 3, glucose, upon entering most cells, is converted directly to Glc-6-P by hexokinase (or by glucokinase in liver), whereas conversion of galactose to Glc-6-P involves several enzymatic steps (Leloir pathway) which bypass the hexokinase reaction. It seemed likely, therefore, that hexokinase was either elevated in activity and/or unregulated in H-91 cells.

As noted in Fig. 4, the hexokinase activity of H-91 cells is markedly elevated (about 20-fold) with respect to activities found in both control and regenerating rat liver. This elevated hexokinase activity is not inhibited by Glc-6-P concentrations up to 0.6 mM. Glc-6-P (<0.6 mM) is known to inhibit the activity of brain hexokinase (16, 17), a finding that we have confirmed under our assay conditions using tissue homogenates.

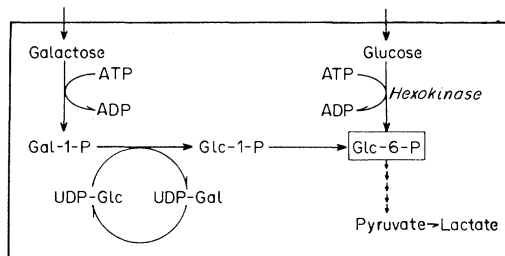


FIG. 3. Pathway of extracellular galactose and glucose leading to intracellular glucose 6-phosphate. Note that galactose enters the glycolytic mainstream bypassing the hexokinase step via the Leloir pathway.

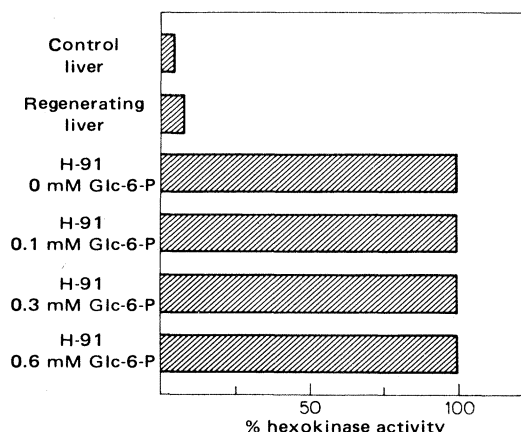


FIG. 4. Hexokinase specific activity in homogenates from control rat liver, regenerating rat liver, and H-91 hepatoma cells. 100% = 124 nmol of glucose/min-mg protein. The glucose concentration in the assay for hexokinase activity in H-91 cells was 0.5 mM, while that for control and regenerating liver was 13 mM. The activity of the liver tissue homogenates in the presence of 0.5 mM glucose was negligible. Therefore, it is possible that the enzyme activity measured in control and regenerating rat liver was actually exerted also by the high- K_m enzyme glucokinase.

With this information in hand, it became of interest to ascertain whether the hexokinase of tumor cells was associated with the mitochondrial fraction, both because there are reports in the literature of bound hexokinase of particulate nature (18-24), and because alterations in the interaction between mitochondrial and glycolytic reactions have been considered for many years to be a major factor responsible for high aerobic lactic acid production in tumor cells (1-5). Fig. 5A shows that the bulk of the hexokinase activity of H-91 cells is shared in about equal proportion by the mitochondrial and postmitochondrial fractions. The activity found in the nuclear fraction correlates well with the percent unbroken cells present in H-91 homogenates and, consequently, can be most likely ascribed to unbroken cells sedimenting in the nuclear fraction. Fig. 5B shows the same experimental data presented in the form of a de Duve plot (25). This figure emphasizes that the specific activity of the mitochondrial fraction is 3-fold higher than that of the homogenate, and 4-fold higher than the activities of the nuclear and postmitochondrial fractions. These results indicate that although the hexokinase activity of H-91 cells is equally distributed among the mitochondrial and postmitochondrial fractions, the former is 4-fold more enriched in hexokinase activity than the latter.

Subcellular fractions were assayed also for 5'-nucleotidase, a plasma membrane marker, and glucose-6-phosphatase, a microsomal marker. Less than 20% of the total cell activity of these enzymes was found in the mitochondrial fraction.

Finally, it became of interest to ascertain whether the hexokinase of H-91 cells is coupled in some way to the oxidative phosphorylation system of mitochondria. Fig. 6 shows that after a burst of ATP synthesis, glucose significantly stimulates the respiration of coupled hepatoma mitochondria, a result that should be obtained only if the hexokinase reaction (glucose + ATP \rightleftharpoons Glc-6-P + ADP) occurs at the expense of mitochondrially synthesized ATP. Significantly, this glucose-stimulated respiration is inhibited by oligomycin, and in turn enhanced by the uncoupler 2,4-dinitrophenol to a maximal rate indicating coupling of the hexokinase reaction with the oxidative phosphorylation system. The glucose-stimulated respiration of H-91 mitochondria is not subject to inhibition by up to 0.6 mM Glc-6-P, in analogy to the lack of inhibition of hexokinase activity in H-91 homogenates.

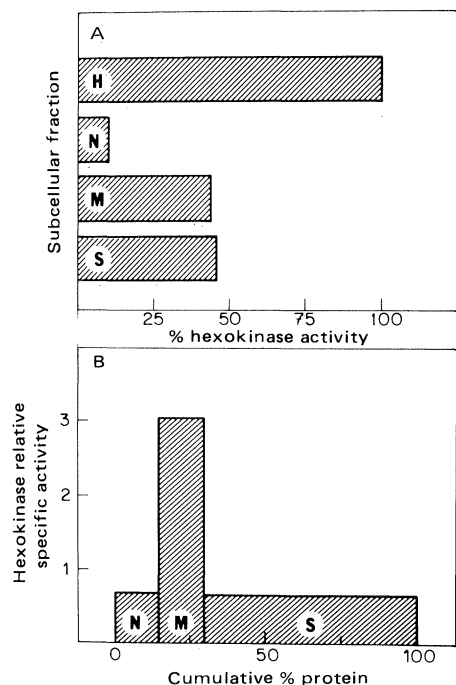


FIG. 5. Subcellular distribution of hexokinase activity in H-91 hepatoma cells. The cells were fractionated by the differential centrifugation scheme described in *Methods*. The subcellular fractions were assayed for hexokinase activity and protein concentration. (A) Distribution of total hexokinase activity. (B) Distribution of relative specific activity. Total hexokinase activity in homogenate = 2.1 μmol of glucose per min. Total protein in homogenate = 18.4 mg. Hexokinase recovery was 95%. Protein recovery was 96%. H = homogenate; N = nuclear fraction; M = mitochondrial fraction; S = postmitochondrial fraction.

Fig. 6 also shows that glucose is unable to stimulate the respiration of mitochondria from control and regenerating rat liver. However, when tumor mitochondria (in amounts which would not detectably increase oxygen consumption via their own respiration) are added to respiring normal mitochondria, glucose is now able to stimulate respiration, as shown in Fig. 6 A and C. These results emphasize that high levels of hexokinase activity bound to the mitochondria of H-91 hepatoma cells are characteristic of the neoplastic state and not a property of the tissue of origin or of rapidly dividing normal liver cells.

DISCUSSION

These results show that hepatoma cells (H-91) grown in galactose as the sole hexose in the medium produce only moderately elevated levels of lactic acid relative to rat liver. This is in agreement with the observation of Eagle *et al.* (13) in other cell lines. However, when glucose is included in the growth medium, markedly elevated levels of lactic acid are produced. The high aerobic glycolytic rate exhibited by H-91 cells growing in the presence of added glucose seems to be due, at least in part, to a form of hexokinase concentrated in the mitochondrial fraction which is not readily inhibited by Glc-6-P. This conclusion is based upon known differences in the catabolism of glucose and galactose, and substantiated by the following six lines of information reported here: (i) Glucose and galactose are transported into H-91 cells with equal facility. (ii) The hexokinase activity of H-91 cells is elevated 20-fold over liver and regenerating liver. (iii) At least 50% of the cellular hexokinase is associated with the mitochondrial fraction. (iv) The mitochondrial hexokinase is 4-fold higher in specific activity than other subcellular fractions. (v) Both the homogenate and mitochondrial hexokinase activities of fresh preparations

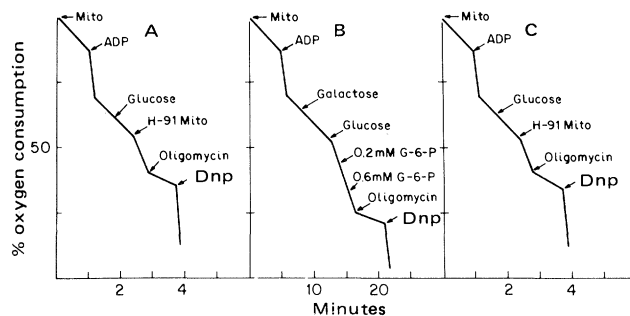


FIG. 6. Typical polarographic traces of mitochondria. (A) Control liver. Indicated additions were: 2.5 mg of control liver mitochondria, 0.25 μmol of ADP, 0.5 μmol of glucose, 0.1 mg of H-91 mitochondria, 1 μg of oligomycin, 0.1 μmol of 2,4-dinitrophenol (Dnp). (B) H-91 hepatoma. Indicated additions were: 1.2 mg of H-91 mitochondria, 0.25 μmol of ADP, 0.5 μmol of galactose, 0.5 μmol of glucose, 1 μg of oligomycin, and 0.1 μmol of Dnp. (C) Regenerating liver. Indicated additions were: 2.5 mg of regenerating liver mitochondria, 0.25 μmol of ADP, 0.5 μmol of glucose, 0.1 mg of H-91 mitochondria, 1 μg of oligomycin, and 0.1 μmol of Dnp. In all cases, total oxygen dissolved in 3 ml = 1.44 μatoms of oxygen at 22°. G-6-P, glucose 6-phosphate.

are not inhibited by up to 0.6 mM Glc-6-P. (vi) Mitochondrial hexokinase is able to phosphorylate glucose at the expense of mitochondrially synthesized ATP.

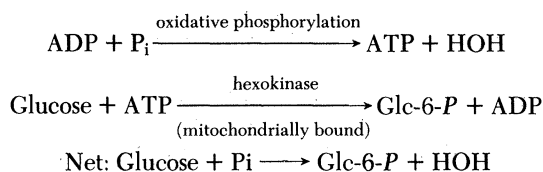
Mitochondrially bound hexokinase has been studied previously by Rose and his colleagues (21, 26) and by other investigators in ascites tumor cells (Ehrlich and sarcoma 37), and in brain and heart (for a review see ref. 16). Mitochondrial hexokinase has been studied also in the rat uterus (22) and shown to increase in activity in response to estrogens, which stimulate glucose utilization by this tissue. The enzyme is localized in the outer mitochondrial compartment and can be solubilized under specified conditions by Glc-6-P and ATP (21). Although the mitochondrial enzyme in brain and some other tissues has been reported to be inhibited by Glc-6-P, there is much controversy surrounding the time and ion dependence of this inhibition and its physiological significance (16, 27). There does seem to be general agreement, however, that particulate forms of hexokinase are less sensitive to inhibition by Glc-6-P than are soluble forms of the enzyme (16, 28-30). Consistent with this view, and in experiments to be reported elsewhere, we find that the hexokinase activity of fresh homogenates of H-91 cells is not markedly inhibited until >1 mM Glc-6-P is added to the assay. However, when the homogenate is frozen and thawed several times to release the particulate enzyme, the hexokinase activity is inhibited $>50\%$ by only 0.2 mM Glc-6-P.

Hexokinase activity [low- K_m enzyme(s)] can be detected in liver mitochondria provided a radioactive assay procedure is employed (23). We should emphasize, however, that the specific activity of the liver mitochondrial enzyme (~ 1 nmol/min-mg in our hands) is relatively insignificant compared to the hexokinase specific activity of H-91 mitochondria (~ 300 nmol/min-mg). In fact, there is very little hexokinase activity in liver homogenates (specific activity = 4-5 nmol/min-mg) relative to H-91 hepatoma cells (specific activity = 124 nmol/min-mg). Thus, transformation of liver hepatocytes to give H-91 cells results in markedly enhanced activity levels of hexokinase, much of which becomes mitochondrially bound.

The finding that the hexokinase activity of H-91 cells is concentrated in the mitochondrial fraction is of considerable interest because this type of interaction provides a direct physical link between the mitochondrial oxidative phosphorylation system and the cytosolic glycolytic pathway. This finding, together with the fact that hexokinase activity of H-91 cells is markedly elevated, may help explain the very high

aerobic glycolysis of this cell line. Thus, the mitochondrial location of high activity levels of hexokinase may allow for the rapid and efficient formation of large amounts of Glc-6-P and ADP. The resulting high levels of these reaction products upon entering the glycolytic pathway may promote the formation of amounts of pyruvate that cannot be oxidized readily by the mitochondria.

The inability of the tumor mitochondrial population to oxidize excessive amounts of pyruvate may be compounded. Thus, the mitochondrial content of H-91 cells (our unpublished observations), similar to other rapidly growing tumor cells (31), is reduced by 50% or more relative to liver tissue. Moreover, ATP synthesis coupled to high levels of hexokinase activity in tumor mitochondria may lower the concentration of P_i in the vicinity of the site of oxidative phosphorylation (32) as shown below:



Racker and his colleagues (33-35) have recently indicated in a rather "absolute tone" that high glycolysis of rapidly growing cancer cells results because of aberrant ATPase activities within such cells. It is suggested that these aberrant ATPases may vary from one cancer cell to the other. Support for this hypothesis is derived from studies of the effect of known ATPase inhibitors on lactic acid production by intact cells. However, inhibitor studies have not been done on control (normal cells), nor has it been established to what extent alleged ATPase inhibitors affect other enzymatic activities in the tumor lines examined. With respect to the views of Racker and his colleagues (33-35) it should be pointed out that mitochondrial hexokinase functions as a partial ATPase in converting ATP to ADP. Moreover, quercetin, one of the ATPase inhibitors used in the studies of these investigators, has recently been shown to markedly inhibit mitochondrially bound hexokinase of Ehrlich ascites cells (24), a finding that we have recently confirmed with the mitochondrial enzyme of H-91 cells. Thus, it is not unreasonable to suggest that the so-called aberrant ATPase-like activities in tumor cells may largely reflect elevated levels of particulate hexokinases.

Finally, it is important to note that earlier studies by Horecker and his colleagues (36, 37) have shown that the appearance of hexokinase activity in an unidentified particulate fraction correlates with the transformation function in chick embryo cells infected with a Rous sarcoma virus mutant. Moreover, Hatanaka (15) has shown that viral transformation of mouse fibroblasts also results in an elevation of hexokinase activity. These results are supported by additional findings summarized in this report which show that markedly elevated levels of mitochondrially bound hexokinase are a characteristic of the hepatoma cell line studied and not a normal property of control and regenerating rat liver. It seems clear then that the expression of maximal hexokinase activity and possibly a particulate binding factor are repressed in some normal cells and become manifest upon transformation.

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