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Regulation of mitochondrial hexokinase in cultured HT 29 human cancer cells. An ultrastructural and biochemical study

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The involvement of the mitochondrial bound hexokinase in aerobic glycolysis was investigated in two subpopulations of the HT 29 human colon cancer cell line: a poorly differentiated one with high aerobic lactate production (referred as undifferentiated or standard cells), and an enterocyte-like differentiated one with lower lactate production (referred as differentiated or Glc$^-$ cells). After mild digitonin treatment, 85% of the total cellular hexokinase activity remained in the particulate fraction in both cell types. In both cases mitochondria appeared to be tightly coupled but the Glc$^-$ cells exhibited a significantly higher oxidation rate in the presence of glucose. Electron microscopy of freeze-fractured cells revealed the absence of contacts between the two limiting mitochondrial membranes in the highly glycolytic standard cells, whereas the contacts were present in the Glc$^-$ cells. Furthermore, we investigated the functional relationship between bound hexokinase (as hexokinase-porin complex) and the inner compartment of mitochondria isolated from standard and Glc$^-$ HT 29 cells. In contrast to the differentiated cells the hexokinase in undifferentiated standard cells was not functionally coupled to the oxidative phosphorylation. This suggests that the high rate of lactate formation in neoplastic cells is not caused by an increase of particulate hexokinase activity but rather by a disregulation of the hexokinase-porin complex caused by the absence of contact sites between the two mitochondrial membranes. In agreement with this interpretation, the hexokinase-porin complex could be completely removed by digitonin treatment in standard HT 29 cells, while this was not possible in mitochondria from Glc$^-$ cells.

Introduction

One of the most characteristic biochemical phenotype of cancer cells is their ability to sustain high aerobic rates of glycolysis [1] yielding high amounts of lactate The reason for this high aerobic lactate production of cancer cells is still not clearly understood It seems reasonable to assume a defect in the mitochondrial metabolism, but this has so far not been definitely established Singh et al [2] could correlate cellular transformation with increase in hexokinase activity in the particulate fraction the hexokinase activity was found to be risen more than 20-fold in some rapidly growing tumors, with approximately 70% of the activity being associated with the mitochondrial fraction [3] The particulate forms of hexokinase were found to be less sensitive to inhibition by glucose 6-phos-

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phate than are the soluble forms of the enzyme This has led Bustamente and Pedersen [4] to suggest that the resulting high levels of glucose 6-phosphate entering the glycolytic pathway may promote the formation of pyruvate concentrations that cannot be readily oxidized by the mitochondria However, the rate of glycolytic flux is not regulated by the glucose 6-phosphate levels but rather by the phosphorylation potential in the cytosol which controls the activity of phosphofructokinase and pyruvate kinase It has been observed in liver and brain [5,6] that the mitochondrial bound hexokinase preferentially uses intramitochondrial ATP, which consequently increases the rate of oxidative phosphorylation by direct ADP supply Based on these observations, it was hypothesized that it is not the free extramitochondrial ADP which regulates the rate of oxidative phosphorylation but rather glucose in liver [7,8] or creatine [9] in muscle, by exerting acceptor control via mitochondrial hexokinase or creatine kinase, respectively The unability of tumor cells to oxidize the pyruvate may result from a reduced number of mitochondria which is, however, not constantly observed [10] Alternatively, it may point to a defect in the functional coupling of the mitochondrial bound hexokinase to the inner compartment Hence, we suggest that mitochondrial hexokinase utilizes extramitochondrial ATP in neoplastic cells, implying that oxidative phosphorylation would not be supplied with ADP while the glycolytic flux would be greatly enhanced

The structural prerequisites of the functional coupling between hexokinase and oxidative phosphorylation are (1) the binding of the enzyme to the pore protein (porine) in the outer mitochondrial membrane [11,12] which has also been characterized in hepatoma mitochondria [13] and (2) the formation of contacts between the outer and inner limiting membranes [8] In the liver, these contacts have been shown to increase 4-fold in phosphorylating mitochondria (state 3) when compared to energized mitochondria (state 4) or freshly isolated mitochondria (state 1) [14] Furthermore, the increase of contacts in state 3 correlates to the degree of coupling between oxidative phosphorylation and electron transport The contacts are less numerous in loosely coupled mitochondria and are almost absent in uncoupled mitochondria [15]

In order to evaluate the importance of contacts between the two boundary membranes in physiological regulation of glycolysis and pyruvate oxidation, we compared two subpopulations of a neoplastic cell line which differ in aerobic lactate production

When grown in standard medium containing glucose, the HT 29 colon adenocarcinoma cells (standard cells) are known to be highly glycolytic and to accumulate high levels of glycogen [16,17] However, when these cells were adapted to grow in a glucose-free medium, they showed a reduced rate of lactate production and glucose consumption in the presence of readded glucose Concomitant with the change in metabolism, these cells displayed an enterocytic-like differentiation they are polarized in the cell layer and display apical and cystic brush border, tight junctions can be observed The morphological changes are accompanied by induction of specific enzymes as alkaline phosphatase and sucrase-isomaltase The enterocytic type of differentiation could be reversed after several passages when glucose was added [18]

Materials and Methods

Dulbecco's modified Eagle medium was obtained from Eurobio, Paris, France All other chemicals were purchased from Boehringer Mannheim and E Merck Darmstadt, F R G

Cell culture

The HT 29 cell-line derived from a human colon adenocarcinoma was established in permanent culture by J Fogh [19] The differentiated subpopulation of the HT 29 cell line was obtained from Dr A Zweibaum, Paris The cells were cultured in Dulbecco's modified Eagle medium supplemented with either 25 mM D-glucose and 10% fetal calf serum (undifferentiated cells) or without D-glucose (differentiated cells), plus 10% dialyzed fetal calf serum as described in Ref 18 Two days before analysis of the differentiated cells the medium was supplemented with 25 mM glucose

Digitonin treatment of the cells

The cells were harvested from the culture flasks by trypsination and were suspended in Krebs-Henseleit medium The suspension was adjusted to contain approx. 10 mg of protein per ml Aliquots of 0.5 ml were incubated at 30 °C for 30 s with concentrations of digitonin ranging between 10 and 200 μg/mg of protein and subsequently centrifuged for 30 s in a tabletop centrifuge. The supernatant was kept for analysis and the pellet was resuspended in Krebs-Henseleit medium

Isoenzyme electrophoresis

The structure bound hexokinase was extracted from the digitonin-treated cells by Triton X-100 This extract and the cytosolic fraction were run on agar gel electrophoresis as described by Allen et al [20] The isozymes were visualized by specific staining and the amount of the different isozymes was determined densitometrically

Assays

Hexokinase (EC 2 7 1 30) and lactate dehydrogenase (EC 1 1 1 27) were determined photometrically according to Bucher et al [21] The assays of adenylate kinase (EC 2 7 4 3) and succinate dehydrogenase (EC 1 3 99 19) were carried out as described recently [22] Glucose concentration was measured by incubating 20-μl aliquots for 30 min at 30 °C with a reaction mixture containing 0 15 M Tris (pH 7 6), 20 mM $MgCl_2$, 0 7 mM ATP, 0 5 mM NADP, 0 7 U/ml hexokinase and 0 6 U/ml glucose-6-phosphate dehydrogenase, the change in absorbance was read at 340 nm L-Lactate concentration was measured in 20-μl aliquots using lactate dehydrogenase and NAD

Protein was determined by the method of Lowry et al [23] using bovine serum albumin as standard

Isolation of mitochondria from HT 29 cells

HT 29 standard cells and Glc^- cells in the stationary phase were harvested with 0 25% trypsin in 0 13 M phosphate buffer (pH 7 2), Ca^{2+} and Mg^{2+} free, containing 0 53 mM EDTA, then suspended in culture medium to stop the trypsin action The cells were centrifuged at 500 × *g* for 3 min and resuspended in isolation medium containing 0 21 M mannitol, 0 07 M sucrose, 2 mM Hepes (pH 7 4) and 1 mM EGTA The suspension (10 ml) containing Glc^- cells was mixed with DEAE-cellulose (250 mg in 5 ml of isolation medium) according to Lawrence and Davies [24] and Nagarse in a final concentration of 15 μg/mg of total protein was added The suspension was stirred on ice for 7 min After incubation, the suspension was diluted 2-fold with isolation medium containing 1% bovine serum albumin, homogenized 15 times with a motor driven Teflon/glass homogenizer and subsequently centrifuged at 500 × *g* for 10 min The supernatant was centrifuged 10 min at 10000 × *g* The sediment was resuspended in isolation medium containing 1% bovine serum albumin and the mitochondria were sedimented by 10 min centrifugation at 14000 × *g* The final sediment was resuspended in isolation medium without EGTA containing 1% bovine serum albumin Mitochondria from standard HT 29 cells were isolated by the same procedure without addition of DEAE-cellulose

Respiratory measurements

Respiration was determined by a Clark type oxygen electrode at 23 °C according to Estabrook [25] Respiration of the HT 29 cell subpopulations was determined in culture medium in the presence of 25 mM glucose Respiration of mitochondria isolated from HT 29 cells and brain was measured in the respective mitochondrial isolation medium with 5 mM succinate as substrate

Treatment of isolated mitochondria with digitonin

Mitochondria isolated from HT 29 cells were suspended in the isolation medium so as to obtain a protein concentration of 10 mg/ml Aliquots of 0 1–0 2 ml of the suspension were incubated for 30 s at room temperature with concentrations of digitonin ranging from 0 1 to 1 0 mg/mg of protein The suspension was subsequently centrifuged for 1 min in a tabletop centrifuge The supernatant was removed and the sediment was resuspended in isolation medium

Fixation and embedding

The differentiated and undifferentiated cells were fixed in the culture dishes with 2 5%

glutaraldehyde in 0 1 M cacodylate buffer (pH 7 4) and postfixed with 2% osmium tetroxide The fixed samples were embedded in Spurr's epoxy resin, thin sectioned, post-stained with 'Locke's' uranyl acetate stain [26], and examined in a Zeiss 5M 10 electronmicroscope at 80 kV Mitochondrial area per cell area was determined with a MOP – AM2 picture analysing system (Kontron) on thin sections of the two HT 29 subpopulations

Freeze-fracture analysis

Differentiated and undifferentiated cells were grown on Thermanox (obtained from Lux Sci Corp USA) sheets under standard culture conditions described above Small pieces of the cell layer on Thermanox were subjected to rapid cryofixation with the sandwich-cryogen-jet-freezing method as described recently [27] The samples were broken in a Balzers 360M freeze-etch device at $-120\,^{\circ}C$ and 2 10^{-7} Torr, followed by Pt/C and C shadowing For electron microscopy, a Zeiss 5M 10 at 80 kV were used The morphological evaluations were performed using a Kontron MOP Am2 picture analysing system connected to a HP 9825 calculator The nomenclature of the exposed membranes follows that of Branton et al [28]

As a mean of quantifying the difference in fracture-plane deviations, the length of the edge where the fracture plane deflects was measured as related to the corresponding examined area In convex fractures, the edge of the exoplasmic face of the outer membrane was measured, whereas, in concave fractures, measurements were made of the exoplasmic face of the inner membrane These values (L) were expressed as length (μm) per unit of mitochondrial fractured membrane area The quantification was made in the areas where the curvature was low in order to avoid large distortions of the measured edgelines

Statistics

The statistical differences of the measured parameters between experimental and control groups were determined by the Mann-Whitney U-test and are shown as P % values

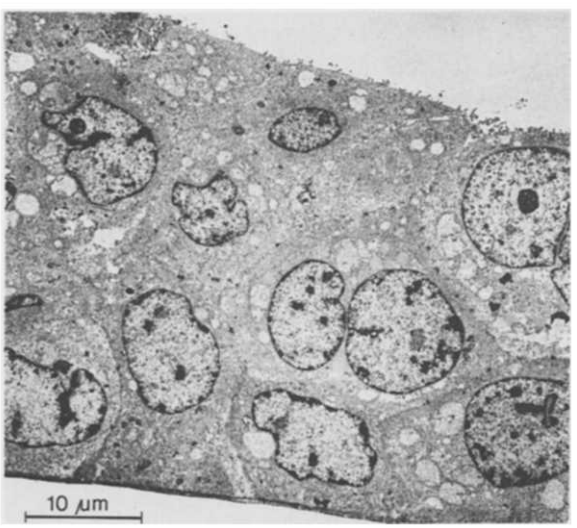

Fig 1 Thin section of undifferentiated HT 29 cells HT 29 tumor cells grown in the presence of 25 mM glucose were fixed in the culture dishes with 2 5% glutaraldehyde and 2% osmium followed by conventional embedding in Spurr's epoxy resin as described in Methods The substrate (lower margin) is covered by a multilayer of tightly packed undifferentiated not polarized cells The microvilli at the surface are not regularly orientated, tight junctions are not visible, but numerous desmosomes are present between the cells Bar = 10 μm

Results

Morphological characterization of the two HT 29 cell subpopulations

HT 29 cells cultured in a standard medium in the presence of 25 mM glucose grow as disorganized multilayers and display an unpolarized nucleus as well as cytoplasmic projections randomly dispersed at the cell periphery (Fig 1) Adaptation of the standard cells to glucose free medium changed the morphology significantly In contrast to the undifferentiated cells (Fig 1) the glucose-deprived (Glc^-) cells were covered with an apical brush border and formed a monolayer (Fig 2A) They were sometimes organized around small intercellular cysts (not shown) Tight junctions, considered as specific to polarized epithelial cell monolayers, were found between the cells (Fig 2B) Although both subpopulations of the adenocarcinoma were fixed under the same conditions, the mitochondria exhibited a very different structure they were swollen in the undifferentiated cells and condensed in the differentiated cells The swelling of the mitochondria was partially reduced when the undifferentiated cells were fixed in suspension after detachment from the substrate

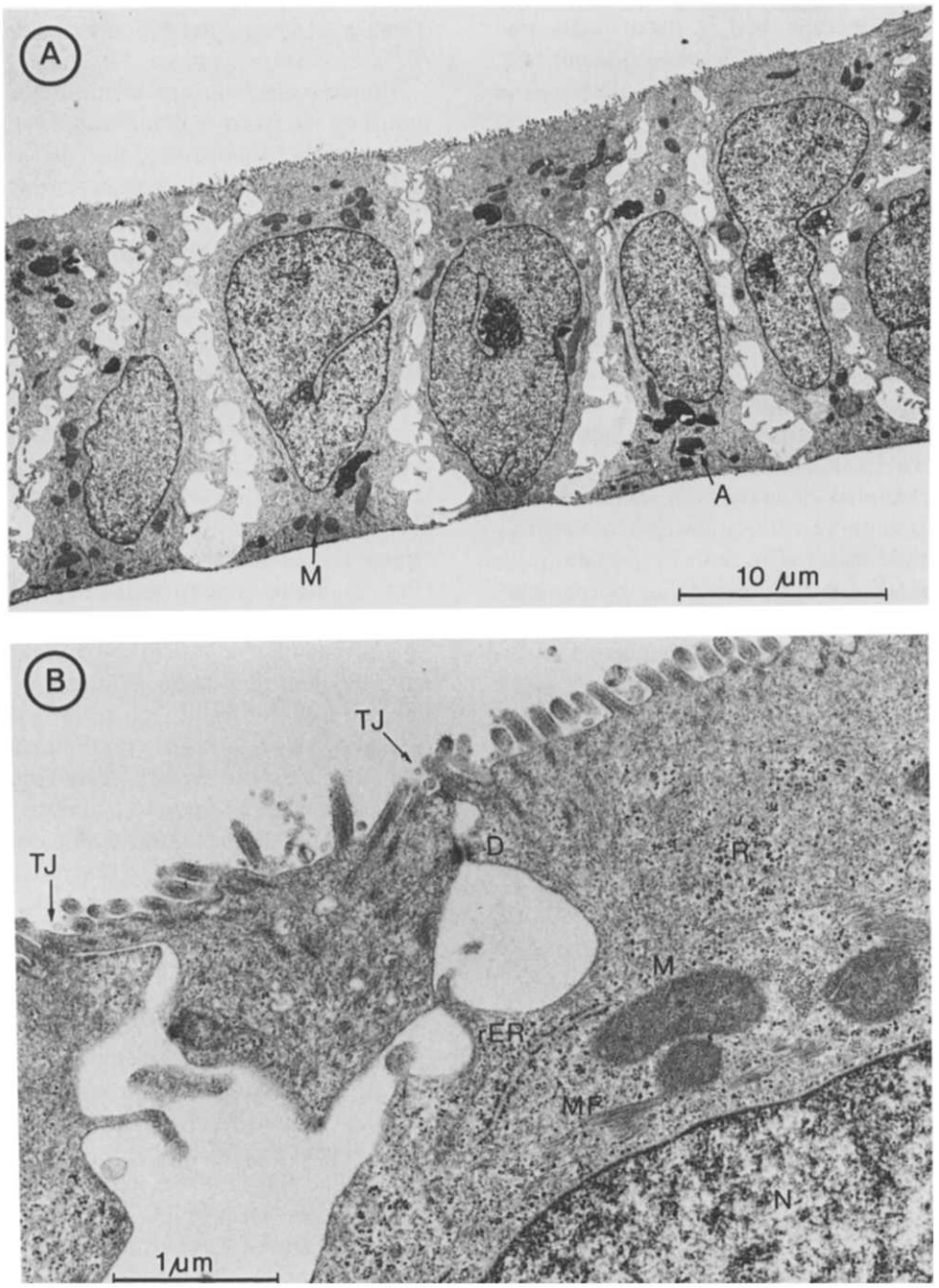

Fig 2 Thin section of differentiated HT 29 cells HT 29 cells adapted to glucose deprived medium were fixed on substrate and embedded as in Fig 1 (A) the cells forming a monolayer exhibit an apical brush border (upper margin) and are polarized Tight junctions are formed between the cells at the apical side (M = mitochondrion, A = autophagosome) Bar = 10 µm (B) detail micrograph from (A), TJ = tight junction, D = desmosome, rER = rough endoplasmic reticulum, R = ribosomes, M = mitochondrion, MF = microfilaments, N = nucleus Bar = 1 µm

(not shown), suggesting that the multilayer structure of the undifferentiated cells may affect the fixation process However, independent of which mode of fixation was chosen, the mitochondria in

the differentiated cells had a more condensed structure compared to the undifferentiated cells This points to the possibility that the difference in structure may be related to different functional states of the mitochondria in the two cell subpopulations

Metabolic characterization of the two HT 29 cell subpopulations

The glucose metabolism of the two cell types described above was analyzed two days after readdition of glucose to the differentiated cells Lactate production of the differentiated Glc^- cells was less than 50% of that observed in the undifferentiated cells (Table I) The lactate/glucose ratio of the undifferentiated standard cells was close to 2, suggesting that these cells utilize glucose mainly for energy production The ratio in the Glc^- cells was significantly lower However, as glucose may also enter other metabolic pathways, the calculation of the glycolytic ATP production from the amount of consumed glucose can result in an overestimation Also any pyruvate which is utilized in the oxidative metabolism reduces the production of lactate and thus leads to an underestimation of the glycolytic rate We used the lactate formation to estimate the glycolytic ATP production since Nakashima et al [29] assumed that the error of this calculation would be less than 8% (Table I)

TABLE I

GLUCOSE METABOLISM IN DIFFERENT TYPES OF HT 29 ADENOCARCINOMA CELLS

The glucose consumption and lactate production were measured in differentiated (adapted to glucose free medium) and undifferentiated HT 29 cells grown in the presence of glucose Analysis of the differentiated cells was performed two days after readdition of 25 mM glucose n = number of different experiments

	Undifferentiated cells (n = 24)	Differentiated cells (n = 21)
Glucose uptake (μmol/h per mg)	0 559 ± 0 095	0 300 ± 0 088
Lactate production (μmol/h per mg)	1 092 ± 0 21	0 420 ± 0 13
Lactate/glucose	1 94 ± 0 34	1 42 ± 0 24 (P% < 0 001)

Analysis of freeze fractured mitochondria in the two HT 29 cell subpopulations

Freeze-fractured mitochondria exhibit frequent jumps of the fracture plane between the inner and outer membranes provided that they are not chemically treated for fixation or cryoprotection. It has been postulated that these fracture-plane changes between the two boundary membranes represent intimate contacts between the two membranes, a phenomenon referred to as semifusion [30]. Therefore the frequency of fracture-plane jumps correlates to the frequency of contacts We have determined the frequency of fracture-changes as described in Methods by measuring the length of the edge where the fracture-plane deflects to the other layer In undifferentiated standard cells, mitochondria exhibiting fracture-plane deflections between the boundary membranes were very few (Fig 3), whereas they were frequent in the differentiated Glc^- cells, determined two days after re-addition of glucose (Fig 4) When exclusively the mitochondria showing fracture plane deflections were compared in both cell types, the frequency of deflections (representative for contact sites) was significantly lower in undifferentiated cells, whereas the mitochondria in differentiated cells exhibited values comparable to those observed in mitochondria of cultured hepatocytes [31] It should be noted that we detected only four mitochondria with fracture plane jumps within approx 12 10^4 freeze-fractured HT 29 standard cells, while we analyzed 21 detailed micrographs of mitochondria within 2 10^4 differentiated cells (Table II) However, the mitochondrial protein and the mitochondrial area per cell area determined in thin sections (Table IV) appeared to be similar in both cell populations Thus, one has to consider that in undifferentiated cells presumably a larger number of mitochondria exhibits smooth fracture faces because they are completely void of contacts In this case, it is difficult to distinguish between mitochondria and other cell organelles with similar size and particle distribution Therefore, the portion of the mitochondrial population without fracture-plane deflections could not be included into the quantification of contacts Consequently, the analysis overestimated the frequency of contacts in the mitochondria of undifferentiated HT 29 cells

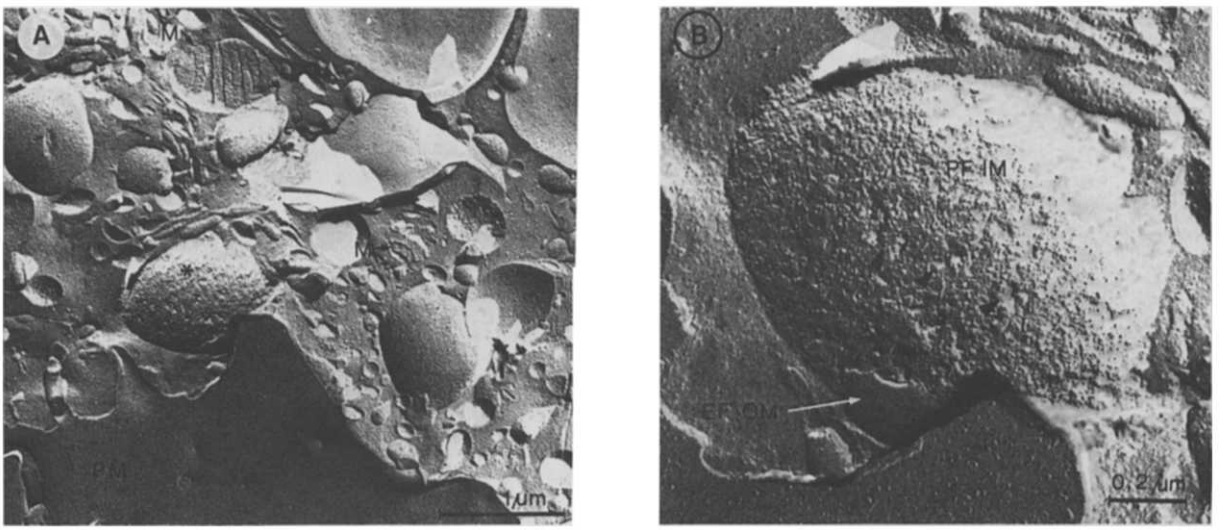

Fig 3 Freeze-fracture of undifferentiated HT 29 cells HT 29 cells were grown on Thermanox sheets They were fixed without chemical pretreatment on this substrate by the cryogen-jet method and freeze-fractured Mitochondria which exhibit fracture plane deflections between the two boundary membranes are very few (A) M = cross-fractured mitochondrion, * = mitochondrion with fracture plane deflection, PM = plasma membrane Bar = 1 µm (B) Higher magnification of the membrane fractured mitochondrion (*) from (A) Only one deflection between outer and inner membrane is seen (EF = exoplasmic face, PF = protoplasmic face, OM = outer membrane, IM = inner membrane) Arrow heads mark the entrances into pediculi cristae Bar = 0 2 µm

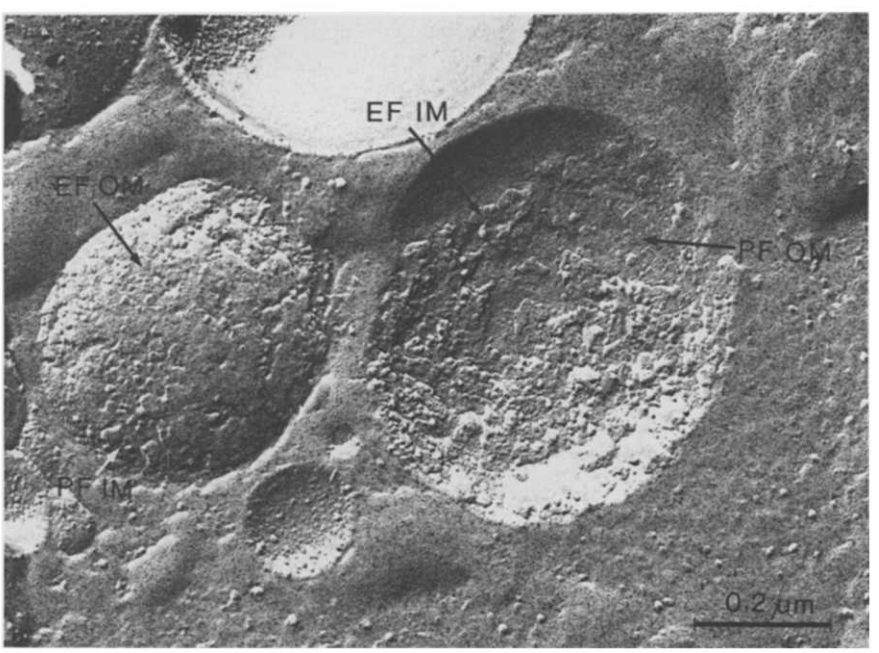

Fig 4 Freeze-fracture of differentiated HT 29 cells The sample of differentiated HT 29 cells was prepared as described in Fig 3 Mitochondria in this subpopulation show frequent fracture plane deflections between the two boundary membranes Nomenclature as in Fig 3 Bar = 0 2 µm

TABLE II

FREQUENCY OF CONTACT SITES IN MITOCHONDRIA OF FREEZE-FRACTURED UNDIFFERENTIATED AND DIFFERENTIATED HT 29 CELLS

HT 29 adenocarcinoma cells grown in the presence of glucose (undifferentiated) and cells of the same cell line adapted to glucose free medium (differentiated) were fixed by rapid freezing techniques and freeze-fractured The length of the fracture-plane edge (L) was examined in freeze-fractured mitochondria as described in Methods The determination was made two days after readdition of glucose to the differentiated cells The first column shows the approximate number of cells examined The second column presents the number of mitochondria with fracture plane deflections which were used to determine the length parameter (L) in column three Statistical differences of this measured parameter between the experimental groups are shown as P% value

	Number of cells examined	Number of mitochondria with patches	L ($\mu m/\mu m^2$)
HT 29 undifferentiated	12 10^4	4	4 8± 1 7
HT 29 differentiated	2 1 10^4	21	23 ±10 3 $P < 0$ 2%

Activity of hexokinase in the particulate fraction of the two HT 29 subpopulations

The differentiated and undifferentiated tumor cells were incubated for 30 s at 30 °C with increasing concentrations of digitonin After subsequent centrifugation the activity of lactate dehydrogenase, adenylate kinase, and hexokinase was determined in the pellet and the supernatant The activity of lactate dehydrogenase, which represents the soluble cytosolic fraction, was extracted from the sedimented cells According to these results, a concentration of 50 μg digitonin per mg of protein is sufficient to liberate 93% of the cytosolic fraction in both cell types (Figs 5 and 6) The main activity of the hexokinase was, however, not observed in the soluble fraction More than 80% of the total cellular activity of the enzyme appeared to be structure bound in both cell types The specific activity of the bound hexokinase in differentiated and undifferentiated cells was almost the same 45 or 40 mU/mg of structural protein (Figs 5 and 6)

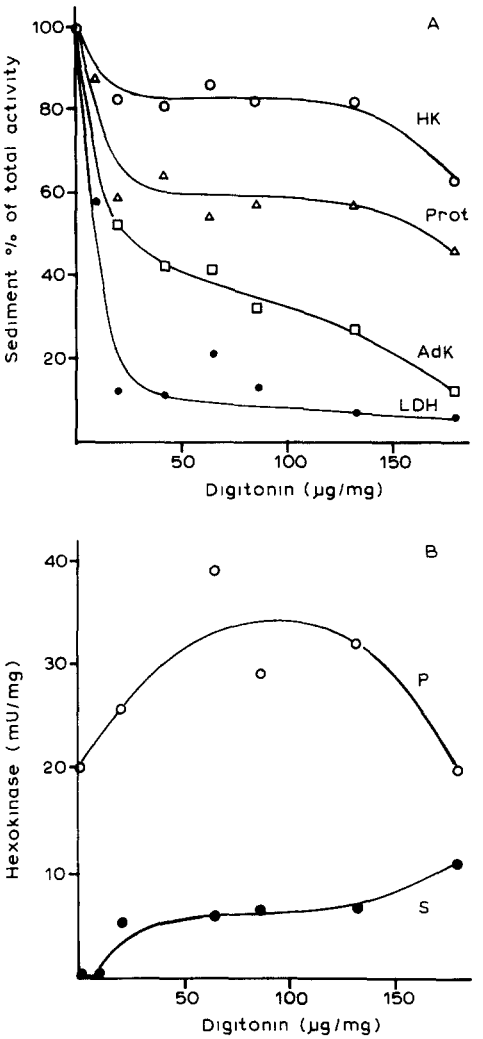

Fig 5 Extraction of cytosolic enzymes by treatment of undifferentiated HT29 cells with increasing concentrations of digitonin HT 29 cells grown with 25 mM glucose were suspended to a protein concentration of 10 mg/ml in 0 25 M sucrose, 25 mM Hepes (pH 7 5) and 5 mM $MgCl_2$ 1 ml aliquots of this suspension were incubated with differing concentrations of digitonin for 30 s at 30 °C Subsequently the suspension was centrifugated through a 30% sucrose layer for 30 s The resulting supernatant (S) and sediment (P) were analyzed for activity of adenylate kinase (AdK, □) hexokinase (HK, ○) and lactate dehydrogenase (LDH, ●) The activities of these enzymes in the different fractions are presented as percent of the total activity in A The specific activity of hexokinase in the different cellular fractions is shown in B

Analysis of the isozyme pattern of structure-bound hexokinase in undifferentiated cells

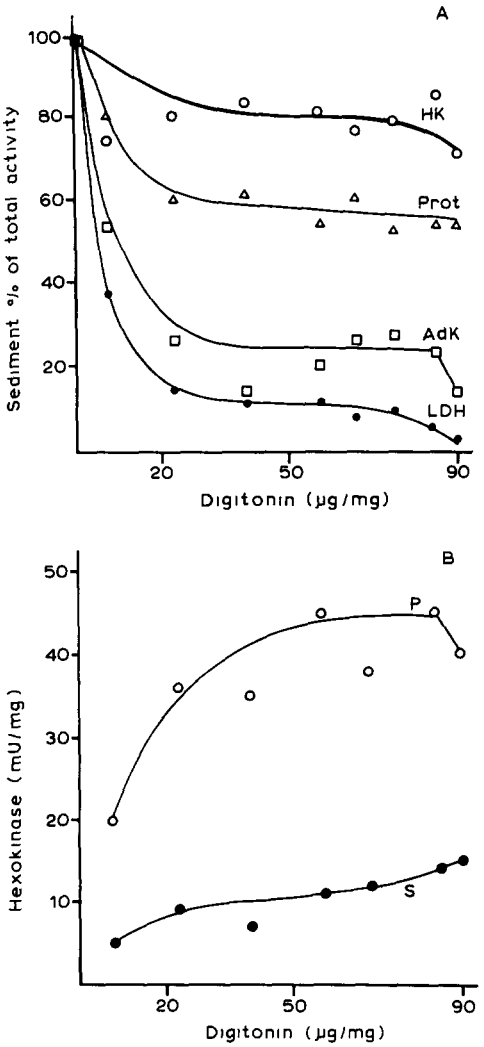

Fig 6 Extraction of cytosolic enzymes by treatment of differentiated HT29 cells with increasing concentrations of digitonin Tumor cells adapted to glucose deprived medium were analyzed 2 days after re-addition of glucose in the same way as described in Fig 5 Activities of adenylate kinase (AdK, □) and lactate dehydrogenase (LDH, ●), are shown in A as percent of total activity in the cells The specific activity of hexokinase in supernatant (S) and sediment (P) is presented in B

The isozyme analysis of the soluble and structure bound hexokinase activity showed that both fractions contained three isozymes According to the relative migration in the electrophoresis, 70% of the activity in the particulate fraction was represented by isozyme III, while isozymes II and III were prominent in the soluble fraction (Table III)

TABLE III

HEXOKINASE ISOENZYMES IN THE CYTOSOLIC AND PARTICULATE FRACTION OF UNDIFFERENTIATED HT 29 CELLS

Cytosolic and particulate fraction were separated by the digitonin technique (see Methods) The hexokinase remaining in the sedimented cells was desorbed by incubation with 0 5% Triton X-100 The concentration of the different isoenzymes was determined by densitometry after isoenzyme electrophoresis on agar gels The activity of the different isoenzymes is given as percent of the total stain intensity on the gel Mean of three experiments

Cellular fraction	Isoenzymes (%)		
	I	II	III
Soluble	19 6±0 68	43 2±3 2	33 58±2 2
Particulate	3 2±0 4	17 6±2 3	70 4 ±2 1

Volume of the mitochondrial fraction in different HT 29 cell subpopulations

The inability of the undifferentiated tumor cells to oxidize pyruvate could have been due to a reduced number of mitochondria Therefore, the specific activity of succinate dehydrogenase was measured in the cell homogenate and the isolated mitochondrial fraction Based on these data, we calculated that mitochondrial protein amounts 29% of the cellular protein in the standard cells and 21% in the Glc^- cells (Table IV) These values are comparable to what is found in liver cells

Furthermore, the area of mitochondria per cell area was determined by morphometric methods [32] in thin sections of 41 Glc^- cells and 24 standard cells as shown in Figs 1 and 2A The differentiated Glc^- cells were smaller compared to the undifferentiated cells and had a lower mitochondrial area per cell than the standard HT 29 cells (Table IV) The morphometric analysis agreed with the slightly higher amounts of mitochondrial protein in the undifferentiated cells, as calculated from the specific activity of succinate dehydrogenase

Oxidation rates in different HT 29 cell subpopulations

The respiration of the different cell subpopulations was determined with the oxygen electrode in culture medium containing 25 mM glucose The

TABLE IV

MITOCHONDRIAL PROTEIN AND AREA PER CELL IN HT 29 SUBPOPULATIONS

Mitochondria were isolated from HT 29 cells grown in the presence of glucose (undifferentiated) The cells were treated with Nagarse and homogenized in a teflon homogenizer in sucrose-mannitol isolation medium The activity of succinate dehydrogenase was determined in the homogenate and in the isolated mitochondria The amount of mitochondrial protein (expressed as percent in column 3 of the table) was calculated from the increase in specific activity in the mitochondrial fraction compared to the homogenate The mitochondrial area and cell area were determined by morphometry in electron micrographs from conventionally fixed, thin sectioned cells (Glc^- $n = 41$, standard $n = 24$) of the two HT 29 subpopulations

Cells	Succinate dehydrogenase (U/mg)		Mitochondrial protein (%)	Mean cell area (μm^2)	Mitochondrial area per cell area
	Homogenate	Mitochondria			
HT 29 undifferentiated	30 20 ± 2 50	102 85 ± 20 5	29	86 9 ± 30 9	0 08 ± 0 05
HT 29 differentiated	21 54 ± 2 38	103 95 ± 23 8	21	71 3 ± 35 7	0 0458 ± 0 021

respiration of both cell types in the completely uncoupled (by CCCP) state was comparable However, the oxidation rate of the differentiated Glc^- cells was 71% of that registrated in the uncoupled state, whereas the undifferentiated cells respired only 45% of the maximal rate The oxygen consumption in both cell types was due to coupled mitochondrial respiration since 90% of it was sensitive to oligomycin (Table V)

Characterization of mitochondria isolated from the two HT 29 subpopulations

Because disruption of HT 29 cancer cells needs treatment with protease and intense homogenization, the structure of the isolated mitochondria might become destroyed Therefore, the activity of characteristic soluble enzymes was determined by reference to the activity of the insoluble inner membrane enzyme succinate dehydrogenase in the cell homogenate and the isolated mitochondrial fraction glutamate dehydrogenase was chosen as representative for the matrix, adenylate kinase for the outer mitochondrial compartment, and hexokinase for the outer mitochondrial surface (Table VI) The relative activities of all three enzymes did not change in the isolated mitochondria when compared to the homogenate, suggesting that the mitochondria remained intact Moreover, the fact that the ratio of hexokinase activity per succinate dehydrogenase activity did not significantly decrease after isolation of the mitochondria confirms that almost all of the cellular activity of this enzyme is bound to the mitochondria

TABLE V

RESPIRATION OF DIFFERENT HT 29 SUBPOPULATIONS

Undifferentiated HT 29 cells (grown in the presence of glucose) and differentiated HT 29 cells (adapted to glucose free medium) were used in the post-confluent phase The differentiated cells were grown on glucose two days before the experiment The respiration was determined with a clark type oxygen electrode in 2 ml of Dulbecco's modified Eagle medium in the presence of 25 mM glucose The degree of coupling between mitochondrial oxidation and phosphorylation was determined by addition of 10 μg/ml oligomycin The maximal oxidation rate was determined by addition of 10 μM of CCCP

Cells	Respiration		
	acceptor controlled (% of uncoupled respiration)		uncoupled + CCCP (nmol O_2/min per mg)
	+ glucose	+ oligomycin	
HT 29 undifferentiated	44 9 ± 2 46	9 4 ± 0 71	9 77 ± 0 76
HT 29 differentiated	71 4 ± 4 21	3 66 ± 1 26	9 45 ± 0 40

TABLE VI

INTEGRITY OF MITOCHONDRIA ISOLATED FROM DIFFERENT SUBPOPULATIONS OF HT 29 CELLS

The integrity of isolated mitochondria was controlled by determination of soluble enzymes relative to membrane integrated succinate dehydrogenase (SDH) The following enzymes were determined in the cell homogenate and in the isolated mitochondrial fraction glutamate dehydrogenase (GLDH representing matrix enzymes), adenylate kinase (ADK representing enzymes of the outer mitochondrial compartment), hexokinase (HK representing enzymes located at the mitochondrial surface)

Cell fraction	GLDH/SDH	ADK/SDH	HK/SDH
HT 29 undifferentiated			
homogenate	5 28±0 26	12 49±1 06	1 55±0 33
mitochondria	5 53±0 51	12 52±1 32	1 68±0 05
HT 29 differentiated			
homogenate	11 14±0 4	17 51±2 9	1 00±0 13
mitochondria	15 16±1 8	16 54±2 5	0 88±0 17

TABLE VII

ACTIVITY OF BOUND HEXOKINASE IN ISOLATED MITOCHONDRIA FROM DIFFERENTIATED AND UNDIFFERENTIATED HT 29 CELLS

The activity of mitochondrial hexokinase was determined by a direct optical test system in mitochondrial isolation medium The assay system contained 4 mM $MgCl_2$, 4 mM phosphate, 5 mM NADP, 2 mM glucose, 0 5 U glucose-6-phosphate dehydrogenase and 5 μM rotenone ATP was provided either by addition of 2 mM ATP and 0 2 μM carboxy-atractyloside or by the oxidative phosphorylation from 10 mM succinate and 0 5 mM ADP

	Hexokinase activity (mU/mg)		
	intact mitochondria		lysed mitochondria (ATP, Triton X-100)
	ATP	ADP	
HT 29 differentiated	0 00	49 2±14 7	90 3±20 5
HT 29 undifferentiated	81 4±7 2	19 8± 3 6	102 2± 9 7

Activity of hexokinase in isolated mitochondria from the different HT 29 subpopulations

Mitochondria were isolated from undifferentiated and differentiated HT 29 cells Both types of mitochondria were well coupled and exhibited a P/O ratio of 1 6–1 8 and an acceptor control index of 6–8 with succinate as substrate The glucose phosphorylation by bound hexokinase was investigated using an optical test system in the mitochondrial isolation medium The ATP was provided either by direct addition and inhibition of the adenine nucleotide translocator (by carboxyatractyloside) or by oxidative phosphorylation from succinate, phosphate and ADP The initial hexokinase activity with external ATP in mitochondria from Glc^- cells was not measurable although the mitochondrial fractions of both HT 29 subpopulations had almost the same activity of the enzyme in the Triton X-100 lysate (Table VII) In the mitochondria of the Glc^+ cells hexokinase activity with external ATP was comparable to the activity determined in the Triton lysate When ATP was provided by the oxidative phosphorylation, glucose phosphorylation in the latter mitochondria decreased to 25% of the activity with external ATP, whereas a 2 4-times higher activity was observed in mitochondria from differentiated HT 29 cells This suggests that, although hexokinase is tightly bound to the mitochondrial surface of the undifferentiated cells, the enzyme does not preferentially use mitochondrial ATP

Desorption of structure bound hexokinase by digitonin in mitochondria from different subpopulations of HT 29 cells

It is known in liver mitochondria that digitonin detaches most of the outer membrane but leaves parts of it unaffected which are bound to the inner boundary membrane in the contact zones [33] Because hexokinase appears to be mainly bound to this part of the outer membrane in the contact sites [34], digitonin treatment cannot desorb bound hexokinase from mitochondria if contacts between the two boundary membranes are present [34] Therefore, the lack of contact sites in mitochondria of standard HT 29 cells could explain why hexokinase is found to be liberated by digitonin like adenylate kinase (Fig 7A) By contrast mitochondria from Glc^- cells, when treated in the same way, lost hexokinase at significantly higher digitonin concentrations than that required to liberate adenylate kinase suggesting that they contain contact sites (Fig 7B)

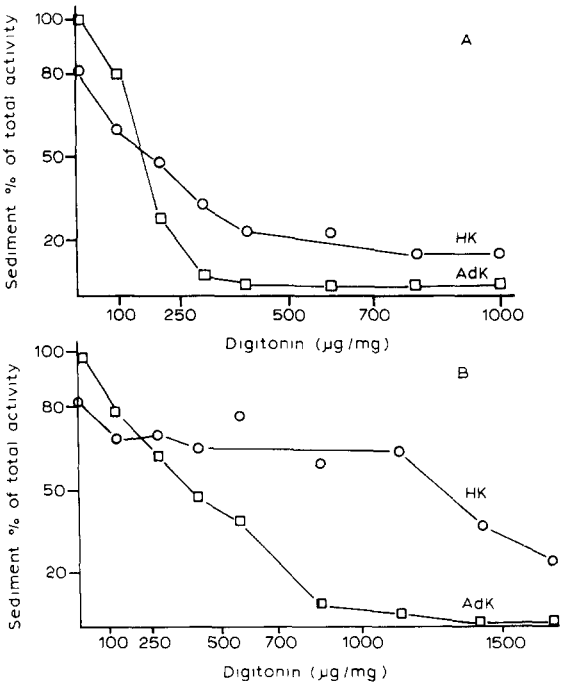

Fig 7 Effect of digitonin on the solubilization of bound hexokinase in isolated mitochondria from HT 29 cells Mitochondria isolated from undifferentiated (A) and differentiated HT 29 cells (B) suspended in isolation medium were incubated for 30 s with increasing concentrations of digitonin and subsequently centrifuged Activities of hexokinase (○) and adenylate kinase (□) were determined in the pellet and supernatant fraction

Discussion

In order to understand the mechanisms by which the mitochondrial metabolism in rapidly growing tumors is reduced, we compared two subpopulations of the HT 29 adenocarcinoma cell line which, as characterized by Zweibaum et al [18], vary in lactate production and morphological differentiation We calculated the relative contributions of glycolytic and oxidative metabolism to the cell ATP from the data in Tables I and V by assuming 1 mol ATP per mol produced lactate and 2 5 moles ATP per mol oxygen according to Nakashima et al [29] Based on this calculation the energy metabolism of both cell populations produced ATP with the same rate. However, the contribution of oxidative phosphorylation in the Glc^- cells was 83% and the glycolytic 17%, whereas in the Glc^+ cells both metabolic systems produced approximately half of the ATP (Table VIII) In both cell types more than 90% of the respiration was sensitive to oligomycin and was therefore, due to coupled oxidative phosphorylation This suggested a reduced ADP supply to the oxidative phosphorylation in the undifferentiated cells Alternatively, the undifferentiated cells may lack mitochondria This can be excluded, since maximal (uncoupled) oxidation rates (Table V) and concentrations of mitochondrial protein in the undifferentiated cells (Table IV) are comparable to what is found in the differentiated HT 29 cells and in isolated hepatocytes It has been reported that the elevated levels of mitochondria bound hexokinase are essential for the high rates of glycolysis in rapidly growing tumor cell lines [2–4] However, we found that the activity of mitochondrial hexokinase in the two subpopulations of tumor cells was approximately the same If the mitochondria bound enzyme has preferred access to the mitochondrially generated ATP as has been described for the liver [5] and brain [6], then, we are left to explain why the ADP supply was reduced in the undifferentiated HT 29 cells In view of these findings it appears that not the

TABLE VIII

RELATIVE CONTRIBUTION OF GLYCOLYSIS AND OXIDATIVE PHOSPHORYLATION TO CELL ATP PRODUCTION IN DIFFERENT SUBPOPULATIONS OF HT 29 CELLS

The rates of ATP production due to glycolysis were calculated from the lactate production in Table I, considering the formation of 1 mol ATP per mol lactate The production of ATP by the oxidative phosphorylation was calculated from the respiratory rates in Table V, assuming the formation of 2 5 mol ATP per mol of oxygen

Source of ATP	HT 29 cells	
	undifferentiated	differentiated
Glycolysis		
nmol/min per mg	18 9±3 4	7 0±2 1
% of total ATP	45 4	17 2
Oxidative phosphorylation		
nmol/min per mg	21 9±1 1	33 7±3 0
% of total ATP	54 7	82 8
Total ATP		
nmol/min/mg	40 1	40 8

amount but rather the regulation of the hexokinase-porin complex at the mitochondrial periphery in the undifferentiated cells is altered when compared to differentiated tumor and brain cells Indeed, in isolated mitochondria from the differentiated cells the initial activity rates of bound hexokinase with internally generated ATP were 50% of the activity observed in the Triton lysate and no activity was determined with externally added ATP The enzyme in mitochondria from undifferentiated HT 29 cells, however, appeared to use preferentially external ATP (Table VII) Similar results have been reported with hepatoma mitochondria by Nelson et al [35] This apparent absence of a functional coupling between hexokinase and oxidative phosphorylation in mitochondria from undifferentiated neoplastic cells points to a lack of a channeling mechanism for ATP in these mitochondria We have postulated that the topological relationship between hexokinase bound to the outer membrane pore and the inner membrane transport systems is mediated by the formation of contacts between the two boundary membranes [8] In agreement with this postulate, the frequency of mitochondrial contact sites was significantly reduced in freeze-fractured samples of undifferentiated cells, while contact sites were present in differentiated HT 29 cells (Table II)

A structural difference of the mitochondria in the two cell subpopulations became also evident in conventional fixed and embedded thin-sectioned samples The swelling of the mitochondria in undifferentiated cells may result from a different behaviour during fixation, because these cells grew as a multilayer However, it may also be explained by the reduced mitochondrial ADP supply in the undifferentiated cells compared to the differentiated one As observed by Hackenbrock et al [36], in ascites tumor cells, such swollen mitochondria represent orthodox (non-phosphorylating mitochondria) which could be ultrastructurally transformed into condensed mitochondria by artificial (addition of 2-deoxyglucose) increase of the intracellular ADP level

The presence of contact sites can be demonstrated by electron microscopy and also by treating the mitochondria with digitonin, because they are responsible for the incomplete removal of outer membrane by this reagent [33] Proteins located in the outer membrane in the area of contacts remain attached during this treatment [37] As observed by electron microscopy [38] and confirmed by binding to the isolated contacts [34] hexokinase adsorbs preferentially to the pore in the contacts In agreement to this finding, the activity of this enzyme becomes only partially desorbed by digitonin from mitochondria of rabbit heart [39], rat kidney [40], liver [34] and brain [41] However, in mitochondria from Novikoff ascites tumor [42] and the undifferentiated HT 29 cells (Fig 7A) hexokinase is desorbed by digitonin just like adenylate kinase, again suggesting the absence of contacts

The absence of contact sites in mitochondria of undifferentiated tumor cells may explain the lack of functional coupling of hexokinase However, this observation cannot account for the elevated level of mitochondrial hexokinase described in many tumor cells [2,3] Together, these data suggest that the binding of hexokinase in undifferentiated HT 29 cells is regulated independently from the functional coupling of the enzyme, although the contact sites in differentiated cells can be considered as preferred binding sites

The content of the pore-forming protein which is required for hexokinase binding is not significantly elevated in mitochondria from neoplastic cells [13] and, therefore, cannot account for the increased hexokinase binding [43] The same situation is found in brain where, like in neoplastic cells, most of the hexokinase is mitochondrial In the case of brain a binding property specific for isozyme I was proposed, but the observed amount of this isozyme in the mitochondrial fraction of standard HT 29 cells was low Thus the high affinity of tumor mitochondria for hexokinase remains unexplained However, the reduced rates of oxidative phosphorylation may be due to a lack of efficient transfer of phosphate acceptor in the absence of contact sites Although the distribution of other peripheral kinases in mitochondria of HT 29 cells has not yet been studied, one has to consider that the functional coupling of such kinases, like creatine kinase [44], may also be effected by the reduction of contact sites Creatine kinase has been recently located in the contact sites of brain mitochondria (unpublished results)

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