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INSTITUTO DE FISIOLOGIA CELULAR

CONTROL DE LA DUPLICACION CELULAR POR MODULACION DE LA FOSFORILACION OXIDATIVA EN LA LINEA TUMORAL AS - 30D

T E S I S

Que para obtener el grado de

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A mi amado esposo Pedro A. con infinito amor y admiración

A Samy, lo más valioso de mi vida

A mi mamá y hermanas con todo mi cariño

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RESUMEN

Las células tumorales de rápido crecimiento adquieren características metabólicas diferentes a las de los tejidos de los cuales derivan. Entre estas características destacan la acelerada actividad glucolítica aún en presencia de concentraciones saturantes de oxígeno y la baja dependencia del metabolismo oxidativo. Estas observaciones han hecho suponer que energéticamente la célula tumoral depende primordialmente del catabolismo de la glucosa. Sin embargo, en la línea tumoral ascítica AS-30D, derivada de hígado, la glucolisis no es la única vía activa; otras vías metabólicas relacionadas con el metabolismo oxidativo, como la degradación de cuerpos cetónicos (acetoacetato, β-hidroxibutirato) y el catabolismo de glutamato también se encuentran muy activas. En esta tesis, nosotros hemos aportado una serie de evidencias que indican que AS-30D, a diferencia de otras líneas tumorales de rápido crecimiento, tiene un metabolismo de tipo oxidativo y no glucolítico, i.e., mantienen una actividad de fosforilación oxidativa (FO) similar a la registrada en hepatocitos: la velocidad respiratoria es muy alta (100 ngA O/min/10^7cels, 95 % sensible a oligomicina) y en el líquido de ascitis (fracción libre de células) se registra una alta concentración de sustratos de la FO como glutamina (4 mM) y oxígeno (50 µM) mientras que la glucosa se encuentra en baja concentración (23 µM). Desde el punto de vista energético, la FO es la vía que suministra la mayor cantidad de ATP (99%), indicando que puede mantener la duplicación celular, la biosíntesis de proteínas y ácidos nucleicos. En este contexto, la NADH oxido-reductasa (sitio I de la cadena respiratoria) y el bloque de enzimas consumidoras de ATP son los puntos de mayor control de la FO.

La adición de glucosa (5 mM) o fructosa (10 mM) a células AS-30D en suspensión promueve un abatimiento parcial en la respiración celular acoplada a la síntesis de ATP (54 y 34%, respectivamente), fenómeno llamado *Efecto Crabtree*. En la literatura existe discrepancia sobre la naturaleza del factor responsable de esta inhibición. Nuestros estudios con células y mitocondrias aisladas de AS-30D demostraron que existe más de un factor responsable, i.e., el efecto Crabtree es el resultado de un control multisitio. Entre estos factores de

control se encuentran la disponibilidad de fosfato y la variación en el pH. Después de la adición de glucosa, se promueve una disminución abrupta en ambos lo que da como consecuencia la inhibición de algunas enzimas de la FO. La adición de un desacoplante clásico estimuló la respiración celular previamente inhibida por glucosa, lo que demuestra que el sistema fosforilante de la FO es el que se encuentra afectado. Este efecto es interesante en un tumor oxidativo ya que es posible utilizar a la glucosa como un modulador de la vía que aporta primordialmente el ATP tumoral.

Este trabajo demuestra que *in situ*, la línea tumoral AS-30D depende del metabolismo mitocondrial. Por lo cual, hemos hipotetizado que el abatimiento de la FO (ya sea modificando la fuente de carbono externa ó inhibiendo directamente a la vía con inhibidores o fármacos selectivos) disminuirá la proliferación tumoral. En cultivos primarios de AS-30D, las células mantienen un crecimiento diferencial dependiendo de la fuente de carbono externa: solo son capaces de proliferar en condiciones donde el metabolismo oxidativo prevalece (en presencia de glutamina o glutamato), mientras que en condiciones glucolíticas no se promueve su crecimiento, aunque se mantienen viables (80%). La mayor densidad celular se alcanza en presencia de ambos sustratos ya que determinamos que con glutamina el aporte de ATP es mayor que con glucosa, sin embargo la glucosa provee de otros intermediarios (fosforibosil pirofosfato) que la glutamina es incapaz de suministrar. Los inhibidores y fármacos que afectan la FO (oligomicina, rotenona, rodamina 123 y 6G, nimesulide, nabumetona, clofazimida, y baicaleina) promovieron un abatimiento total en el crecimiento tumoral mientras que los inhibidores glucolíticos (iodoacetato, gosipol) disminuyeron solo el 30%. Los fármacos que mostraron mayor índice de toxicidad fueron los compuestos de la familia de las rodaminas (123 y 6G); sus valores de IC_{50} oscilaron en el intervalo de 1-2 µM.

Los resultados de esta tesis sugieren que una estrategia bioquímica para reducir la tasa proliferativa de células tumorales oxidativas es la modulación de la FO.

SUMMARY

The fast-growth tumor cells acquire different metabolic characteristics from those of the tissues which originated them. Among those characteristics the ones that stand out are the accelerated glycolytic activity even in the presence of saturating oxygen concentration and the lesser dependence on the oxidative metabolism. These observations have led to assume that, energetically, the tumor cell depends mainly on glucose catabolism. However, in AS-30D, an ascitic tumor line derived from the liver, the glycolysis is not the only active pathway; other metabolic ways related to the oxidative metabolism, such as the ketone bodies degradation (acetoacetate, β-hydroxibutyrate) and the glutamate catabolism, are also very active. In this thesis, we show a series of evidences indicating that AS-30D cells, in contrast with several other fast-growth tumor lines, have an oxidative type of metabolism and not a glycolytic one, *i.e.*, they maintain an oxidative phosphorylating activity (OP) similar to that registered in hepatocytes: The respiratory rate is very high (100 ngA O/ min/ 10^7cells, 95% oligomycin-sensitive) and the ascites liquid (free-cell fraction) registers a high concentration of oxidative phosphorylation substrates such as glutamine (4 mM) and oxygen (50 μM) while glucose is found in a low concentration (23 μM). From the energetic point of view, the OP is the pathway that provides the largest amount of ATP (99%), to sustain cell duplication, and protein and nucleic acid biosynthesis. In this context, the NADH oxido-reductase (site I of the respiratory chain) and the enzyme block for ATP utilization were the points of larger control of the OP.

The addition of glucose (5 mM) or fructose (10 mM) to an AS-30D suspension promotes a partial depression in the cellular respiration coupled to the ATP synthesis (54% and 34%, respectively), a phenomenon named Crabtree effect. There is a discrepancy in the literature, over the nature of the responsible factor for this inhibition. Our studies with cells and mitochondria isolated from AS-30D demonstrated that there is more than one controlling factor, *i.e* , the Crabtree effect is the result of a multisite control. Phosphate availability and pH variation are some of these factors. After glucose addition, an abrupt decrease in both factors is promoted causing the inhibition of several enzymes of the OP system. The addition of a classical uncoupler stimulated the

cellular respiration previously inhibited by glucose, which demonstrates that the phosphorylative system is the target of the Crabtree effect. This of particular interest in an oxidative tumor since it opens the possibility of using glucose as a modulator of the pathway that provides most of the ATP in these tumor cells.

This work shows that *in situ*, the tumor line AS-30D depends on mitochondrial metabolism. Thus, we hipothesized that the depression of the OP (either by modifying the carbon external source or by the direct inhibition of the pathway with inhibitors or selective drugs) would diminish tumoral proliferation. In AS-30D primary culture, the cells grow differentially depending on the external carbon source: they maintain their proliferation only under conditions where the oxidative metabolism prevails (in the presence of glutamine or glutamate) but not under glycolytic conditions, although they remain viable (80%). The highest cellular density is reached in the presence of both substrates since we determined that with glutamine the ATP supply is larger than with glucose; however, glucose provides other intermediary (phosphoribosyl pyrophosphate) which glutamine is incapable of producing. The inhibitors and drugs that affect the OP (oligomycin, rotenone, rhodamines 123 and 6G, nimesulide, nabumetone, clofazimide, and baicalein) promoted a total depression in the tumoral growth while the glycolytic inhibitors (iodoacetate, gossipol) diminished the growth by 30%. The drugs that showed greater toxicity index were the compounds of the rhodamine family (123 and 6G); their IC_{50} values were in the range of 1-2 μM.

The results of this thesis suggest that a biochemical strategy to reduce the proliferation of tumoral oxidative cells would be the modulation of the OP.

INDICE

CAPITULO 1

ASPECTOS GENERALES DEL METABOLISMO TUMORAL

1.1 Metabolismo intermediario de las células tumorales de rápido crecimiento

La mayor parte de los tumores experimentales, sean sólidos [transplantados usualmente en la pierna posterior de rata (sarcomas, carcinomas)] o líquidos [transplantados en la cavidad intraperitoneal de rata o ratón (ascíticos)], pueden clasificarse en tres categorías (Tabla 1) [1]:

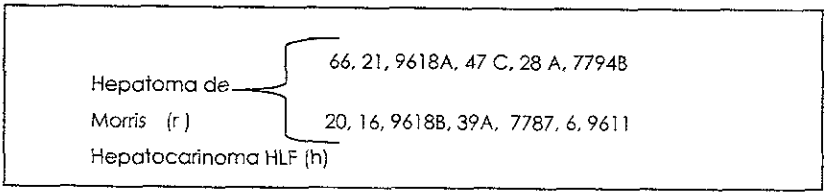
I) Tumores de lento crecimiento (altamente diferenciados)
II) Tumores de crecimiento intermedio (bien diferenciados)
III) Tumores de rápido crecimiento (pobremente diferenciados).

Algunas de las líneas tumorales mostradas en la Tabla 1, presentan una segunda clasificación numérica y alfabética, *i.e.*, de una línea establecida se originan otras líneas con características fenotípicas diferentes a la original.

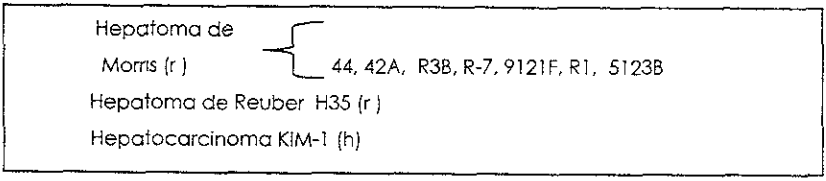
De los tres grupos, los tumores de rápido crecimiento son los que presentan las diferencias bioquímicas, histológicas, y cariotípicas más marcadas en comparación con su tejido de origen [1].

Entre las diferencias bioquímicas más relevantes del metabolismo tumoral, particularmente en las líneas de rápido crecimiento, destacan la acelerada glucólisis aún en presencia de concentraciones saturantes de O_2. Aunque se ha propuesto que esta elevada actividad glucolítica sostiene los niveles de ATP requeridos por la célula para duplicarse, algunos tumores muestran una clara dependencia del metabolismo oxidativo. A continuación se presenta un artículo de revisión donde se exponen las características bioquímicas sobresalientes en las líneas tumorales de rápido crecimiento, particularmente la línea ascítica de AS-30D utilizada en este estudio, denotando los cambios relevantes en algunas vías metabólicas y la importancia de la fosforilación oxidativa en el suministro de ATP celular

(1) Tumores de lento crecimiento



(2) Tumores de mediano crecimiento



(3) Tumores de rápido crecimiento

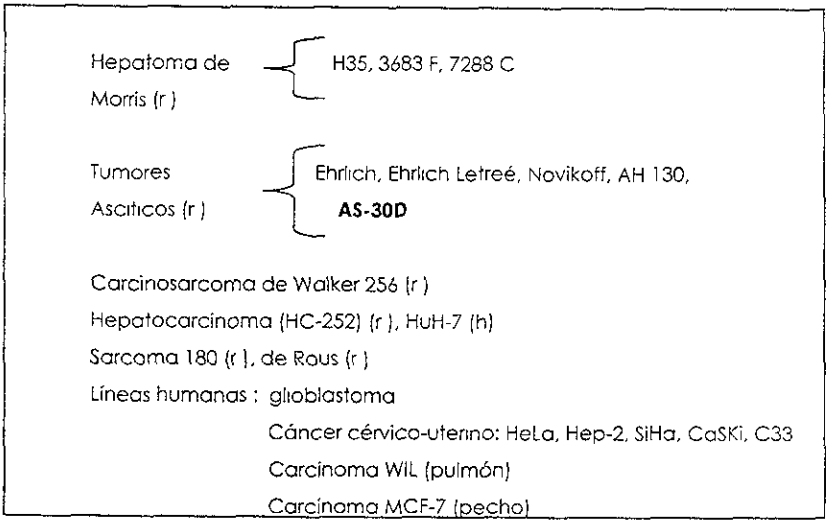


Tabla 1 **Clasificación temporal de las diferentes líneas tumorales que se desarrollan en roedores (r) y humanos (h).** Los tumores de mediano y lento crecimiento se desarrollan en meses y años, respectivamente, mientras que los de rápido crecimiento se desarrollan en semanas (sólidos) o días (asciticos) Modificado de Pedersen, 1978

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Review Article

Intermediary Metabolism of Fast-Growth Tumor Cells[1]

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Abstract

Tumor cells show several modifications in their metabolism in comparison with normal cells. In particular, tumor cells show an accelerated glycolysis and a low O_2 dependence, which are metabolic modifications involved in the resistance of many tumor cell lines to radiation. Thus, a strategy to enhance the radiosensitivity could be the transformation of the glycolytic metabolism of tumor cells into an oxidative type of metabolism, i.e., to induce the ATP supply to depend solely on oxidative phosphorylation. Therefore, this review emphasizes the relevance of oxidative phosphorylation on tumor cells regarding (a) its contribution to ATP supply for cell duplication during the proliferative phase, and (b) the possible therapeutic implications of having oxidative rather than glycolytic tumor cells. (*Arch Med Res* 1998; 29:1)

KEY WORDS: Tumor cells; Intermediary metabolism, Glycolysis; Oxidative phosphorylation, Metabolic Control Theory.

Introduction

Tumor cell lines show differentiation states which range from less dedifferentiated cells with similar metabolic characteristics to normal cells (low glycolysis and growth rates), for example Morris 66, Morris 47C, and Morris 7794 A hepatomas (1), to largely dedifferentiated cells with a definitive different metabolism from normal cells (fast growth and glycolysis rates). As an example of the latter, there exist ascites cellular lines, such as AS-30D, Ehrlich, hyperdiploid chain Ehrlich Lettré, and Walker-256 carcinosarcoma. The main characteristic of fast-growth tumor cells (FGTC) is their high glycolytic rate, which results in an increase in lactic acid concentration, even in the presence of saturating O_2 concentrations (see Figure 1). In addition, in FGTC the lactic acid may be formed from glutamine through the formation of malate (from glutamine) in the mitochondrial matrix, and the conversion of malate to pyruvate catalyzed by a cytosolic malic enzyme (1,2). A high aerobic glycolysis is also found in other proliferative non-tumoral cell types.

It appears that in all tumoral cell types studied, in addition to glycolysis, glutaminolysis (Ehrlich, Ehrlich Lettré), nucleic acid synthesis, lipid synthesis (Novikoff hepatoma, AS-30D, Ehrlich), cholesterol synthesis (Morris 3924A, AS-30D) and ketone bodies usage (AS-30D) are also stimulated. However, the metabolic behavior is not the same in all tumor types. In Ehrlich and Ehrlich Lettré, the Krebs cycle enzyme activities are low, as is pyruvate oxidation. On the contrary, in AS-30D hepatoma cells, the Krebs cycle enzyme activities and pyruvate oxidation are higher than in normal hepatocytes (3).

Thus, the control of some metabolic pathways in tumor cells varies from line to line. The principal differ-

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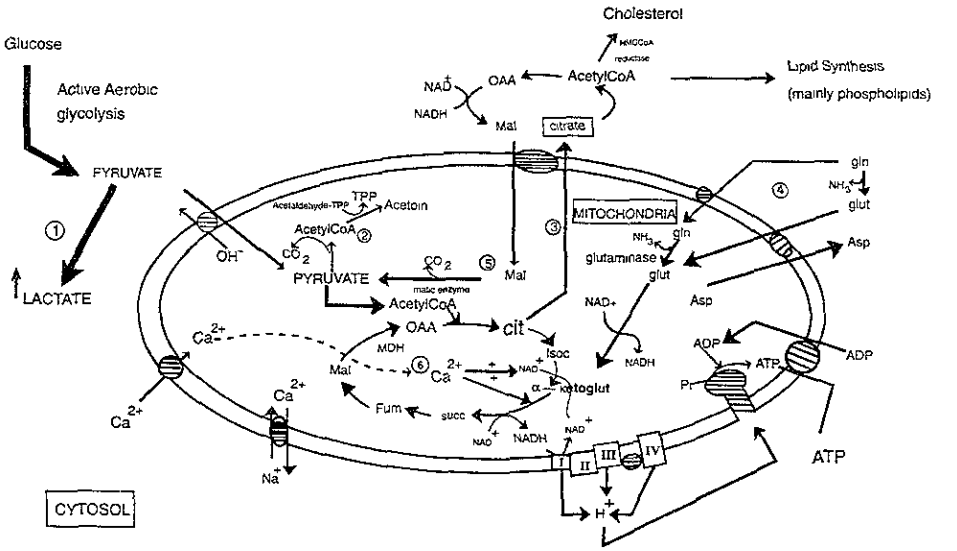


Figure 1 Principal metabolic derivations found in fast growth tumor cells 1, about 90% of pyruvate formed from glucose is reduced to lactate, and the rest is oxidated by tumoral PDH 2, mitochondrial pyruvate is decarboxylated to acetoin; which is transformed to other products through ATP-depending reactions. 3, citrate is released from mitochondria to feed the cholesterol and triglyceride pathways. 4, glutamine feeds the tumoral Krebs cycle. 5, cytosolic malate is oxidized to pyruvate and CO_2 by NAD(P) -depending malic enzyme 6, isocitrate and α-ketoglutarate dehydrogenases are stimulated by Ca^{2+}, which produces an increment in the matrix NAD(P) H to drive ATP synthesis 7, the cytosolic acetyl CoA is substrate for cholesterol and lipid biosynthetic pathways.

ences and similarities between tumor cells and normal cells are presented in Table 1.

In some ascites tumor cells (Ehrlich, Ehrlich Lettré), but not all (AS-30D), only a low percentage (1.5%) of the total consumed glucose is transformed into acetyl CoA by the pyruvate dehydrogenase complex, whereas 0.7% is used in the pentose phosphate pathway, 2% for lipid synthesis and the rest (93%) is accumulated as lactate (4).

It seems that the glycolytic high rate in FGTC is a metabolic strategy for living in hypoxic states The hypotheses proposed to explain this event are the following:

A) There exist isoenzymatic pattern changes in two of the three enzymes that control the glycolytic pathway in normal conditions: hexokinase (HK) and phosphofructo-1-kinase (PFK-1) (2) Tumoral HK is present in greater concentration and activity than those observed in normal hepatocytes (1 vs. 124 nmol/min/mg prot in liver and tumor cells H-91, respectively) (5) It has been reported that PFK-1 exerts much of the flux control of the glycolytic pathway in both FGTC and normal cells Argilés and López-Soriano (6) reported that the elevated activity of PFK-1 found in Walker-256 carcinosarcoma is due to the rise in lactic acid levels that increases the cytosolic proton concentration (pH diminishes), which, in turn, is a positive modulator of this enzyme. This pH effect on PFK-1 may explain the low O_2 sensitivity of glycolysis in tumor cells; in other words, a negligible Pasteur effect should prevail in tumor cells (it should not have glycolysis inhibition by oxygen), but this has not been evaluated (6)

Interestingly, overexpression of PFK-1 in *Saccharomyces cerevisiae* increased glycolytic flux under aerobic conditions without modifying glycolysis in anaerobic conditions (7). Thus, similar to tumor cells, the Pasteur effect was abolished in these cells. The increased glycolytic flux was accompanied by a decrease in the oxidative phosphorylation flux. An inverted pH gradient across the plasma membrane has been described in some tumor lines, including different hepatoma types (8). Despite the enhanced production of lactic acid, and in contrast with the behavior found in Walker-256 carcinosarcoma, the intracellular pH in most tumor cells is always constant (pHi = 7.1) In fact, it has been found that the intracellular pH is slightly more basic than the

Table 1
Intermediary Metabolism of Normal Proliferating Cells and Tumor Cells

Metabolic feature	Normal proliferating	Tumoral cells	Reference
Lactate formation	↓		1,2,5,7
Pasteur effect	↓	↓↓	1,5
Glutaminolysis	↑	↑↑	19,20
Pyruvate Oxidation	↑	a)Ehrlich and Lettré ↓↓ b)AS-30D	12,13,15 3,31
Acetoacetate and β-OHbutyrate Oxidation	↑	↑↑ ↓↓	16,17 16,17
Krebs Cycle enzyme activities	↑	a)Ehrlich and Lettré ↓↓ b)AS-30D ↑↑	12,13 3,24
Calcium sensitive-dehydrogenases enzymes	n r.	a)Ehrlich and Lettré n r. b) AS-30D	24
Malic enzyme activity	↑	↑↑	23
Lipogenesis	↑	↑↑	2,18
Cholesterogenesis	0	↑↑	1,4

Note: The different fast-growth tumor cell lines and quiescent cellular lines (in G_1 phase) were compared with mature normal cells using the following symbols: ↑, two-three fold increase; ↑↑, fourfold increase, ↓, decrease; 0, unchanged; n.r , not reported.

extracellular pH (pHe = 6.8), which implies an active simultaneous release of lactate and H^+ to the extracellular space (8)

B) Boxer and Devlin (9) proposed that glycolysis is accelerated in neoplastic cells because of lack of activity in the transference systems of cytosolic reductor equivalents to mitochondria (known as aspartate/malate and glycerol 3-P shuttles). This hypothesis proposes that since most of pyruvate is transformed into lactic acid, with a concomitant cytosolic NADH oxidation, the transference systems are limited by the cytosolic low NADH availability. However, Grivell et al (10) demonstrated that in Ehrlich cells, the rate of transference of reducing equivalents from the cytosol to the mitochondrial matrix is similar to that found in normal hepatic cells, depending on the substrate used (0.67 μmol NADH transported/mg prot/min) Therefore, the transference of reducing equivalents seems not to be the main event involved in the induction of the high rate of glycolysis in tumor cells

C) A third hypothesis proposes that the complex of the pyruvate dehydrogenase (PDH), which catalyzes the oxidation of pyruvate into acetyl CoA, is competitively inhibited by acetoin, an unusual 4-carbon metabolite that results from the pyruvate non-oxidative decarboxylation (11) [Ki = 41μM acetoin vs. Km = 16.5 μM pyruvate] (data reported for AS-30D hepatoma cells). In addition, cytosolic pyruvate is transformed into lactate by an LDH isoenzyme that has greater activity in tumor cells than in normal cells (50% more activity). Acetoin has been evaluated in Ehrlich and AS-30D hepatoma cells (11,12).

Formation of Acetoin in Tumor Cells

The formation of acetoin (Figures 1 and 2) in tumor mitochondria occurs in two steps (12,13):

A) Pyruvate enters into mitochondria in exchange for OH^- (14) and is decarboxylated by tumoral PDH to form acetyl CoA. Next, an activated acetaldehyde (hydroxyethyl-thiamine-pyrophosphate-enzyme complex) reacts with acetyl CoA to produce diacetyl, an acetoin immediate precursor. In this last reaction, coenzyme A and thiamine pyrophosphate are released. In contrast to normal mitochondria, tumor mitochondria are not able to oxidize the aldehydes, most likely because the aldehyde dehydrogenase (AlDH) is located only in

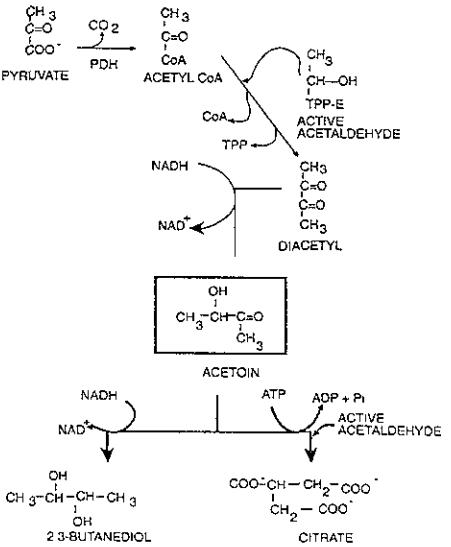


Figure 2 Schematic representation of acetoin formation from pyruvate by tumor mitochondria Abbreviations. PDH, pyruvate-dehydrogenase complex; HE-TPP-E, hydroxyethyl-thiamine-pyrophosphate-enzyme complex Modified from (12)

the cytosol in tumor cells. Tumoral AIDH only oxidizes aromatic aldehydes, and uses NAD(P$^+$) as cofactor, while normal AIDH oxidizes aliphatic and aromatic aldehydes and uses only NAD$^+$ as cofactor (13) The AIDH reaction does not require ATP, although TTP$^+$ and Mg^{2+} are essential for activity.

B) Formed diacetyl is reduced to acetoin, using NADH. The acetoin formed in tumor mitochondria is rapidly degraded in an ATP-dependent reaction to citrate and two non-identified products. Formed citrate probably derives from condensation of acetoin and other active acetaldehyde. In liver, in pathological conditions such as alcoholism, acetoin can be reduced to 2,3-butanediol.

Another important characteristic of tumor cells is that the PDH complex is activated by physiological concentrations of AMP (from 0.5 to 1 mM), which does not occur in normal tissues This effect is probably involved in the formation of acetoin, since AMP activates the oxidative decarboxylation of pyruvate to acetoin, and increases its concentration in the cytosol (15). In such a situation, where tumoral PDH is partially inhibited by acetoin and stimulated by AMP, mitochondrial pyruvate may be transformed either into acetyl CoA or into acetoin: acetoin may return to the Krebs cycle through the generation of acetate and then, acetyl CoA

"Truncated" Krebs Cycle in Tumor Mitochondria

The citrate formed from acetyl CoA and oxaloacetate in Ehrlich cells (13) does not continue its oxidation rapidly, because the aconitase and isocitrate dehydrogenase have low activities; therefore, citrate is preferentially released from mitochondria (see Figure 1). The tricarboxylate carrier is faster in tumor mitochondria (four fold) than in normal mitochondria (4). Since citrate is actively released from mitochondria in Ehrlich and Ehrlich Lettré cells, the Krebs cycle cannot be fully achieved; for this reason, it has been called the "truncated Krebs cycle". In the cytosol, the citrate is used for sterol synthesis, mainly cholesterol (Figure 1)

Kelleher et al (16) found that, in contrast to normal hepatocytes, ketone bodies are highly metabolized by AS-30D hepatoma cells. It appears that mitochondrial succinyl-CoA-acetyl-transferase, the enzyme that catalyzes the first step in acetoacetate oxidation to acetoacetyl CoA, shows a 40-fold increase in activity in tumor cells (8 vs 334 nmol/min/mg prot in liver and AS-30D mitochondria, respectively) These authors (16) suggested that the elevated consumption of acetoacetate is due to the high demand of lipids required by tumor cells (Figure 3) Moreover, when acetoacetate at physiological concentrations (2 mM) was added to tumor cells, the lipid synthesis and CO_2 production increased in the presence of glucose (5 mM), indicating that acetoacetate is an important fuel metabolite during glycolysis

β-hydroxybutyrate is not heavily used by AS-30D hepatoma cells in comparison to acetoacetate, probably because these cells have a very low β-hydroxybutyrate dehydrogenase activity, as previously reported for other tumor cell lines (17).

Briscoe et al. (16) have questioned the Parlo and Coleman hypothesis (4) of the truncated "Krebs cycle", at least for AS-30D cells: The CO_2 produced from acetoacetate, acetate and pyruvate (these last two measured in Kelleher's laboratory) indicates a substantial flux of citrate through the Krebs cycle toward malate (see Figure 3). Briscoe's observations support Dietzen's data (3) that, in AS-30D hepatoma cells, the Krebs cycle is not truncated, since acetyl group units are sufficient to provide the carbons necessary for oxidative metabolism during lipogenesis.

Another outstanding characteristic of tumor cells is their high sterol synthesis rate, mainly that of cholesterol. The explanation for the rise in cholesterol concentration is the lack of control of the ß-hydroxymethyl glutaryl-CoA reductase (4). This enzyme that transforms hydroxymethyl-glutaryl-CoA to mevalonate does not respond to the negative feedback mechanism that exists in normal tissue; i.e., it is not allosterically inhibited by its product: cholesterol. Parlo and Coleman (4) proposed

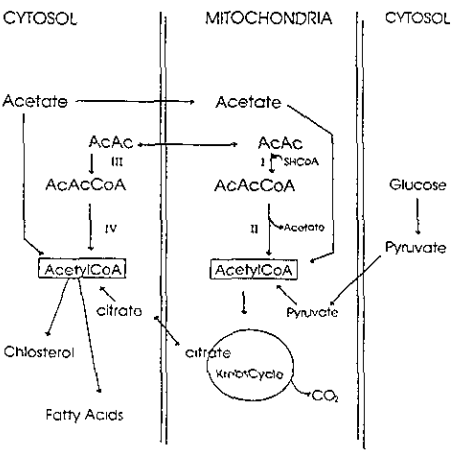


Figure 3. Pathway of acetoacetate (AcAC) in AS-30D) hepatoma cells. Acetoacetate carbons may contribute to lipid synthesis via 1) a mitochondrial route involving 3-oxyacid thiolase (I) and acetoacetylCoA and cytosolic thiolase (IV). AcetylCoA is produced from acetate and pyruvate by a mitochondria acetate thiokinase and PDH, respectively. A specific carrier for acetoacetate has not been identified, most likely this metabolite travels through the membrane in the protonated (acidic) from

that this effect resides at the genetic level, where the cholesterol-sensitive, ß-hydroxymethylglutaryl-CoA-reductase *locus* is expressed in a modified, cholesterol-insensitive form. The cholesterol synthesis in tumor cell membranes, including the mitochondrial membranes, induces important physiological changes; one of these changes in intact mitochondria is the diminution of the passive inner membrane H^+ permeability that may be related to drastic changes in membrane potential-dependent metabolism of tumor cells (13).

In Morris 3924-A hepatoma and Ehrlich mitochondria, the pyruvate carbon skeletons are preferentially used for cholesterol synthesis (Figure 1), rather than energy production (oxidation to CO_2) (18). Parlo and Coleman (4), and Dietzen and Davis (18), using Ehrlich and AS-30D cells, respectively, reported that the mitochondrial membrane cholesterol content is four toten fold more than that observed in normal mitochondria (from 1.9 cholesterol µg/mg protein in normal cells - 8 cholesterol µg/mg protein in hepatoma cells); 87% cholesterol is found in the external, and the rest, in the internal mitochondrial membrane. Parlo and Coleman reasoned that these data support the "truncated Krebs cycle" hypothesis proposed for Ehrlich cells, where an increment in mitochondrial membrane cholesterol induces kinetic alterations of the tricarboxylate carrier (cholesterol increases the carrier activity, which restricts the utilization of citrate by aconitase, and favors the release of citrate to the cytosol) Once in the cytosol, the ATP-citrate lyase transforms citrate into acetyl CoA, which is the building block for cholesterol synthesis (18).

To compensate for the low availability of matrix citrate in Ehrlich and Ehrlich Lettré hepatoma cells, alternative pathways to complete this "truncated cycle Krebs" are used. In this regard, the glutaminolytic pathway is the most important, apparently due to a greater activity of glutaminase and glutamyltransferase in tumor mitochondria (19,20).

Glutamine is the main metabolite fuel in Ehrlich and Ehrlich Lettré hepatoma cells, even in the presence of saturating concentrations of glucose. Mitochondrial glutamine uptake is faster in tumor than in normal cells (52 nmol/min vs.15 nmol/min, respectively) (20). In tumor and normal mitochondria, glutamine is deaminated to form glutamate by a phosphate-dependent glutaminase (21) An increase in the cytosolic-free calcium concentration ($[Ca^{2+}]c$) stimulates the glutaminase activity in hepatocytes (22), although it is not known whether Ca^{2+} modulates the tumor enzyme Glutamate may enter the Krebs cycle by transamination (glutamate/oxaloacetate transaminase) and dehydrogenation (glutamate dehydrogenase) to form α-ketoglutarate (Figure 1) Thus, in this case, glutamate and glutamine are the main sources of mitochondrial malate, which in turn is transformed into oxaloacetate by a NAD(P⁺)-dependent malate dehydrogenase (Figure 1)(23). On the other hand, the exogenous malate is preferentially transformed into pyruvate and CO_2 by the mitochondrial NAD(P⁺)-dependent malic enzyme in FGTC. This last enzyme is probably bound to the matrix surface of the inner membrane in association with the malate transport system (13,23). The pyruvate formed by the malic enzyme: (a) can be transformed either into acetoin and acetyl CoA; (b) may be reduced to lactate by lactate dehydrogenase, or (c) may compete with glycolytic pyruvate for binding (and oxidation) by the AMP-dependent pyruvate dehydrogenase. Under this scheme, the prevailing source of citrate should be exogenous malate (23).

The presence of a "truncated Krebs cycle" in Ehrlich, Ehlich Lettré and Morris 7777 ascites hepatoma cells implies that, in these cells, a mitochondrial NADH pool of sufficient magnitude to drive oxidative phosphorylation (<5 % NADH (24)) might not be generated. However, in contrast to these tumor cells (4), the Krebs cycle in AS-30D hepatoma mitochondria is not truncated (4,12,13), and therefore, it contains the complete enzymatic battery without any apparent abnormality (3).

The activities of the Krebs cycle enzymes in AS-30D hepatoma mitochondria are fourfold greater than activities found in normal mitochondria in the absence (3) or in the presence of Ca^{2+} (25). Therefore, the oxidative block in the pathway does not appear to be the rate-limiting step of oxidative phosphorylation in this type of tumor mitochondria.

Tumor cells require a high ATP supply to support the accelerated formation of new molecules (mainly proteins and nucleic acids) for rapid growth and dedifferentiation. The question that arises is whether this high ATP demand can be solely supported by the glycolytic pathway. Muller et al. (26) calculated that only 13% of consumed ATP was produced by glycolysis in Ehrlich cells, cultured in an aminoacid-conditioning medium These authors (26) proposed that the rest of the cell ATP which was consumed was produced by oxidative phosphorylation Therefore, a hypothesis proposed by our group is that, in fast-growth ascites tumor cells, the ATP supply is provided by both glycolysis and oxidative phosphorylation and, consequently, the contribution of each pathway has to be evaluated for the different tumor cell lines.

In this respect, it has been determined for Ehrlich ascites tumor cells that the transition to a resting state from a proliferating growth state induced a reduction in total ATP generation, which correlated with a diminution in oxidative phosphorylation (approx 50%), whereas glycolysis remained constant (27). But when tumor cells were growing, oxidative phosphorylation increased in parallel with some highly ATP-consuming processes such as protein turnover, RNA synthesis and the activities of the Na^+/K^+ and Ca^{2+} ATPases. The enhancement of the cell ATP-consuming processes is very likely one of

the signals (i.e., elevation of cytosolic ADP and Pi) that activate glycolysis and oxidative phosphorylation (28).

Clinical Implications of Manipulation of Energy Metabolism in Tumor Cells

Since it is conceivable that energy metabolism (and cell duplication) in many tumor cells depends on both pathways, glycolysis and oxidative phosphorylation, it is not surprising that attempts to use tumor energy metabolism as a target for antineoplastic therapy have failed. However, when simultaneous inhibition of both pathways was assayed, a significant reduction of cell growth (50%) in Walker-256 carcinosarcoma was obtained (29). Diminution of glycolysis was achieved by reducing gluconeogenesis in the host (rat) with 3-mercaptopicolinate, a competitive inhibitor of phosphoenol pyruvate carboxykinase. Oxidative phosphorylation was specifically blocked with rhodamine 6G. A separate treatment of tumor cells with 3-mercaptopicolinate or rhodamine 6G did not alter the growth rate (29).

A specific inhibition of glycolysis of several tumor cell lines also seemed to be attained by gossypol, a polyphenolic bisnaphthalene aldehyde, through the inhibition of NAD- and NADP-dependent enzymes (30). In this last study (30), it was confirmed that the sole inhibition of oxidative phosphorylation by rhodamine 123 did not affect the growth rate of tumor cells. However, the rhodamine-resistant cells were strongly depressed by gossypol. This observation places gossypol as an attractive potential antineoplastic drug which should be experimentally tested in other tumor lines. However, no new reports pursuing gossypol effect have appeared since 1990, suggesting a failure to reach positive results

Another promising antineoplastic drug is clofazimine, commonly used for the prevention of leprosy (31). This drug showed a cytotoxic effect on a chemoresistant human carcinoma cell line, reducing the tumor size by one-third by inducing an increase in the mitochondrial membrane H^+ permeability (31), the increase in H^+ permeability induces collapse of the H^+ electrochemical gradient and hence, inhibition of oxidative phosphorylation

Role of Ca^{2+} and Mg^{2+} in Tumor Cell Intermediary Metabolism

Recently, Wolf et al. (32) demonstrated the existence of a plasma membrane Mg^{2+}/Na^+ antiport in Ehrlich cells, which was very similar to that found in erythrocytes. It seems that this carrier is activated by cAMP (approximately 50 μM) through a phosphorylation process

It has been proposed that there is an increment in the mitochondrial matrix free Ca^{2+} concentration ($[Ca^{2+}]m$) in tumor cells as a result of an elevation of cytosolic Ca^{2+}, which occurs in response to an external stimulus (hormones or growth factors) (33-35). Ca^{2+} is taken up in isolated tumor mitochondria by an electrophoretic uniporter similar to that found in normal mitochondria. This uniporter is sensitive to ruthenium red and lanthanum. On the other hand, mitochondrial Ca^{2+} efflux is catalyzed by an Na^+/Ca^{2+} exchange, which is also found in brain and heart mitochondria (36).

It has been determined that ATP in micromolar concentrations (100 μM) produces an increment in $[Ca^{2+}]c$ through the interaction with P_2 purinergic receptors (34,35). ATP is a powerful agonist of this receptor, which is associated with the generation of inositol trisphosphate (34); IP_3 stimulates the release of Ca^{2+} from the endoplasmic reticulum (fourfold over the basal). A lesser release of Ca^{2+} is observed after a second addition of ATP. The authors (34) proposed that such an effect is due to a refilling of intracellular Ca^{2+} pools through an ATP-sensitive mechanism.

In AS-30D tumor mitochondria, the elevation of $[Ca^{2+}]m$ stimulates at least two of the three enzymes of the Krebs cycle that are reported to be Ca^{2+}-sensitive in normal mitochondria: the isocitrate dehydrogenase and the α-ketoglutarate dehydrogenase (Figure 1). The effect of Ca^{2+} on these enzymes in tumor and normal mitochondria is the same: it decreases the Km for their corresponding substrates. Stimulation of these dehydrogenases, and hence flux through the Krebs cycle, may activate oxidative phosphorylation (25).

Crabtree Effect in Tumor Cells

It has also been found that Ca^{2+} and Mg^{2+} participate in a characteristic reaction of tumor cells called Crabtree effect or glucose and deoxyglucose respiratory inhibition, considered as the opposite of the Pasteur effect.

Evtodienko et al. (37) demonstrated that in the presence of physiological concentrations of glucose, $[Ca^{2+}]c$ increased from a basal of 170 nM up to 300 nM. However, a careful analysis of their results reveals that such an increment may not be significant, since the magnitude of these $[Ca^{2+}]c$ fluctuations is commonly found in normal and tumor cells Notwithstanding, the authors proposed several hypotheses: (A) the existence of a glucose receptor (similar to that found in pancreatic β-cells) that initiates the cascade reactions that lead to the release of Ca^{2+} from the endoplasmic reticulum and then, to an elevation of $[Ca^{2+}]c$ However, these authors (37) did not show the calibration curves of fluorescence obtained from cells loaded with the calcium fluorescent probe, which is important for assessing a good signal calibration. They also used a Kd value for the Ca^{2+}-indicator complex obtained in solution, but they did not attempt to obtain the Kd value for the intracellularly-trapped indicator. Experience from our laboratory indi-

cates that if such precautions are not taken, a misinterpretation of experimental fluorescence data is highly possible (38) (B) A second hypothesis by Evtodienko et al. (37) was that glucose alters Ca^{2+} homeostasis. When glucose (or deoxyglucose) enters the glycolytic pathway, it is rapidly phosphorylated by intracellular hexokinases. The effect of this phosphorylation is the fast acidification of the cytoplasm, which results from the appearance of an additional dissociable H^+ when glucose or deoxyglucose is phosphorylated by ATP. Glucose induced an elevation in $[Ca^{2+}]c$ up to 300 nM, a concentration that has been reported (39) to inhibit mitochondrial synthesis and hydrolysis of ATP in normal cells, and to affect the activity of the ATP/ADP carrier through either of the following mechanisms:

i) Formation of an ATP-Ca^{2+} complex that competes with ATP-Mg^{2+} for specific binding sites (40), or

ii) Ca^{2+} promotes association of the ATP synthase inhibitory subunit to the enzyme; since there is more ATPase inhibitory protein in tumor mitochondria than in normal mitochondria, an enhanced sensitivity of the ATP synthase for Ca^{2+} should be expected (41).

Mg^{2+} is also involved in the Crabtree effect, since it has been observed that the addition of glucose to Ehrlich tumor cells induces a massive release of intracellular Mg^{2+}. In consequence, there is a diminution in the internal Mg^{2+} concentration and hence, a respiratory inhibition, because Mg^{2+} is a cofactor of several mitochondrial reactions (32).

Effect of Oxygen and pH Gradients in Tumor Development

Solid tumors are hypoxic, limited in nutrients and exposed to a highly acidic environment (due to elevated glycolysis) that leads to the generation of microenvironmental changes (42). Hypoxia and acidic pH have been identified as factors involved in resistance to radiotherapy and chemotherapy in many tumor cells (43). Apparently, high sensitivity to radiation in tumor cells is associated with an oxidative type of metabolism. Görlach and Acker (44) have found that it is possible to change from a glycolytic metabolism to an oxidative metabolism, varying O_2 and pH gradients which, interestingly, enhances the sensitivity of tumor cells to radiotherapy. These authors (44) have used, as an experimental model of poor tissue vascularization, two colon human tumor cell lines (HT29 and U118MG) These tumor cells are cultured as tridimensional multicellular spheroids that, in contrast with tumor cells cultured in monolayer, reveal a greater sensitivity to radiation and chemotherapy Tumor cells organized as spheroids generate both O_2 and pH gradients.

These authors (44) found that the two colon tumor cell lines showed very specific metabolic differences, despite the fact of having originated from the same type of tumor cell. HT29 cells generated a radial oxygen gradient with a partial pressure of 107 mm Hg on the periphery and 6 mmHg in the center, while U118MG cells generated a smaller O_2 gradient of 47 mmHg in the center and 78 mmHg on the surface (see Figure 4).

Regarding the pH gradient, it was found that in HT29 cells, the surface pH was more basic (pH 7.3) than the center (pH 7.07), while in U118MG cells, the center of the nodule was more acidic (pH 6.93) than the periphery Moreover, these authors found that in the U118MG line, the greater lactate production correlated with a high lactate dehydrogenase activity (800 U/g wet weight) and with a lower oxidation of pyruvate (60 nmol pyruvate/min x mg protein).

These observations indicated that the tumor line U118MG was metabolically more glycolytic, whereas the HT29 line was more oxidative (see panel A, Figure 4). Interestingly, when both tumor lines were exposed to oxamate, significant metabolic changes were apparent. Oxamate is a structural analog of pyruvate and competitively inhibits lactate dehydrogenase, although it may also inhibit mitochondrial pyruvate transport. In the presence of oxamate, lactate dehydrogenase activity decreased drastically in U118MG cells (50%, down to 400 U/g wet weight); although there was not an evident change in the oxygen gradient, a drastic change in the pH gradient was apparent (see panel B of Figure 4). Thus, the addition of oxamate induced a change from a glycolytic to an oxidative type of metabolism in this tumor cell line. To the contrary, in HT29 cells, which are predominantly oxidative, the presence of oxamate induced an inhibition of pyruvate oxidation (pyruvate oxidation decreased from 100 nmol to 50 nmol of oxidated pyruvate/min x mg protein), which makes these cells more dependent on glycolysis than on oxidative phosphorylation.

These findings may have clinical applications, since radiobiological studies indicate that O_2 influences tumor cell radiosensitivity. In line with the last statement, a study of patients with cervical cancers showed that hypoxic tumors were significantly larger than normoxic tumors: patients with hypoxic tumors had worse survival probabilities and a lower response to radiotherapy than patients with normoxic tumors (45). Consequently, the conversion of glycolytic cells into oxidative cells may enhance the deleterious effects of radiation and chemotherapy on previously resistant tumor cells.

This interesting proposal should be evaluated, taking into consideration some recent and relevant findings about tumor energy metabolism For instance, nitric oxide reversibly inhibits respiratory chain activity, leading to a collapse of the mitochondrial H^+ gradient and ATP synthesis in hepatoma AH130 cells and mitochondria (46); this nitric oxide effect lasts longer at low $[O_2]$, around 25 μM, than at high $[O_2]$ (>200 μM) A large number of macrophages can be found in the ascites fluid

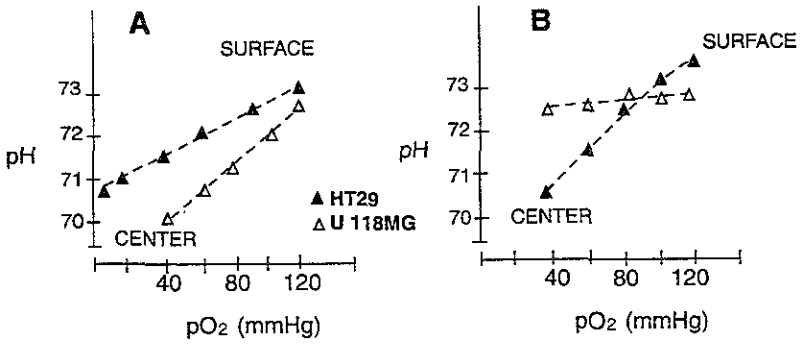


Figure 4. Relationship between O_2 and pH gradients in HT29 and U118MG multi-spheroids in normal conditions (A) and exposed to 40 mM oxamate (B). Modified from (44).

and since macrophages may release fairly large amounts of nitric oxide, it can be predicted that the *in vivo* rate of oxidative phosphorylation in ascites tumor cells is a balance between the [nitric oxide], the $[O_2]$ and the supply of oxidizable substrates· at high $[O_2]$, tumor cells are able to achieve a high rate of oxidative phosphorylation, whereas the opposite can be expected at low $[O_2]$.

Control of Oxidative Phosphorylation in AS-30D Cells and Isolated Mitochondria

Analysis of control of oxidative phosphorylation in isolated liver mitochondria and other non-tumoral mitochondrial types have revealed that control of this pathway is distributed among several steps (28). The main control points are the ATP/ADP carrier, the cytochrome oxidase, the dicarboxylate carrier, the ATP synthase and the b-c_1 cytochrome complex, which show flux-control coefficients (Ci) (28,47) in the range of 0.2-0.5, i.e., each of these steps controls the flux-pathway 20 - 50%, depending on the particular conditions of incubation.

Our group found that in mitochondria isolated from AS-30D cells, the control of oxidative phosphorylation was mainly shared by the ATP/ADP carrier (Ci = 0.70) and the ATP synthase (Ci = 0.25) (48) It was proposed that the high control coefficient calculated for the ATP/ADP carrier indicated that this step exerted a severe limitation of flux Flux limitation of oxidative phosphorylation could be due to carrier inhibition by high levels of endogenous Ca^{2+}, which are consistently found in isolated tumor mitochondria (49)

We have recently found that the control of oxidative phosphorylation in AS-30D hepatoma cells (*in situ* mitochondria) was not exerted by the two enzymes reported for isolated mitochondria (48), but by the NADH dehydrogenase or Site I of the respiratory chain

It should be mentioned that the calculation of Ci was carried out using different equations. In our work by López-Gómez et al. (48), it was considered that the inhibitors of oxidative phosphorylation were irreversible, that is, once the enzyme-inhibitor complex was formed, there were no ways to dissociate them. Therefore, the Ci value was calculated from the equation proposed by Wanders et al. (47) for irreversible inhibitors·

$$Ci = \frac{-Imax}{Fo}\left[\frac{(dF)}{(dI)}\right] I = 0,$$

where Imax is the minimal inhibitor concentration required to reach maximal inhibition, Fo is the pathway flux in the non-inhibited system, and dF/dI is the change of flux induced by small inhibitor concentrations when [I] tends to zero. Calculation of Ci from this equation is based only on the initial points of the curve, where it is feasible to extrapolate to zero inhibition (Figure 5). It should be noted that this analysis depends heavily on inspection of such initial points to carry out lineal regression that permits extrapolation toward the origin In other words, in this calculation of Ci a strict criterion does not exist to determine which points of the curve should be considered.

At present, it is known that most oxidative phosphorylation inhibitors bind in a pseudo-irreversible manner to their respective enzyme (non-competitive, tightly bound inhibition); this means that the inhibitor remains strongly attached to the enzyme, but is able to dissociate (50).

Accordingly, Gellerich et al. (51) proposed an equation that takes into account the Kd (dissociation constant) of the enzyme-inhibitor complex for non-competitive, tightly-bound inhibition·

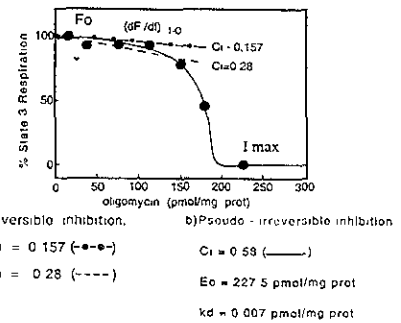
$$J=\left[\frac{n(Jo-Ji)^2 \quad x \quad E^n}{Ci \times Jo \times Eo^n + [(n-Ci) \times Jo - (n \times Ji)] \times E^n}\right]+Ji$$

$$E^n + (Kd + I - Eo) \times E - Kd \times Eo = 0,$$

where Jo and Ji are the flux rates in the non-inhibited (E = Eo) and completely inhibited (E = 0) systems, respectively, Kd is the dissociation constant of the inhibitor (I)-enzyme complex, and n is an empiric exponent that establishes the relationship of the substrate concentration with the reaction catalyzed by E (with n = 1 the reaction is a linear function of the substrate concentration: [S]<Km).

Analysis of our recent titration curves with isolated hepatoma mitochondria using the equation for irreversible inhibitors showed that the Ci of the ATP synthase was approximately 0.3, very similar to values previously reported by our group (48). However, when analysis of the same experimental data was made using the equation for pseudo-irreversible inhibitors, the estimated Ci was approximately 0.1, a value much closer to that obtained with intact cells (see Figure 5).

It should also be borne in mind that the determination of Ci using the equation for irreversible inhibitors requires from the researcher the following: (a) the inspection of the initial titration points that may be linearly extrapolated to the no-inhibition point, and (b) the decision concerning how many points to include in such a linear regression analysis. This is better illustrated in Figure 5, where two slopes with a high correlation coefficient can be calculated depending on the number of experimental points considered. In contrast, the use of the equation for pseudo-irreversible inhibitors involves non-linear regression analysis and, hence, the curve fitting covers the entire range of experimental points (solid line in Figure 5). Therefore, this last control analysis is also more accurate.



a) Irreversible inhibition.
Ci = 0 157 (-•-•-)
Ci = 0 28 (------)

b)Pseudo - irreversible inhibition
Ci = 0 59 (——)
Eo = 227 5 pmol/mg prot
kd = 0 007 pmol/mg prot

Figure 5. Graphic representation of the estimation of flux control coefficients using irreversible inhibitors (dashed lines) or pseudo-irreversible inhibitors (solid line). The solid line is the best-fitting to the Gellerich et al. (51) equation of the experimental points (to estimate Ci using these equation, a non-linear regression analysis is required; this was performed by using the commercially available computer program GraphPad Inplot4). The dashed line represents the Ci value calculated using the first four (-•-•-) or the first five points of the curve (- - -)

We have found that, in AS-30D hepatoma cells, in spite of using saturating concentrations of specific mitochondrial inhibitors of the respiratory chain or the ATP synthase, the rate of cellular respiration cannot be fully inhibited, reaching a similar value of 85 - 90% inhibition. This observation indicates the existence of non-mitochondrial oxygen consumption reactions, which amount to about 10 - 15% total cell O_2 uptake. Therefore, oxidative phosphorylation is the main O_2-consuming reaction in AS-30D hepatoma cells, although this question should be further examined in other tumor cell lines, in which the non-mitochondrial, O_2-consuming reactions could have a more significant contribution.

To elucidate the predominant metabolic pathways used by hepatoma cells for the generation of ATP, we have determined the concentration of relevant metabolites under various conditions. Table 2 compares data of metabolites determined in this laboratory with those reported for Walker-256 carcinoma (6). As expected for tumor cells, glucose was rapidly consumed in both cell lines as well as glutamate and glycogen (data calculated only for AS-30D cells). Lactate was actively accumulated, whereas ATP, pyruvate and glutamine remained without change during the evaluated period. We found that, in opposition to what was reported in (16), β-hydroxybutyrate and acetoacetate were also consumed in AS-30D cells. In the first case, this is a surprising observation, because a low activity of tumoral β-hydroxybutyrate dehydrogenase in AS-30D cells has been reported (17), acetoacetate utilization may be due to a fast formation of acetyl CoA for lipid and cholesterol synthesis. Glutamate utilization indicates that it is a more usable metabolite to provide carbon skeletons that feed the Krebs cycle than, for example, pyruvate, which was not adequately consumed. It was also evident that AS-30D cells have an active glucogenolysis.

On the other hand, we have found a relatively high oxygen concentration in the free-cell hepatoma liquid, this observation indicates that a limitation for O_2 in the respiratory chain (the apparent Km of cytochrome c oxidase for O_2 is less than 1 μM (52) is unlikely. That is, we may consider AS-30D cells to be of the oxidative type.

Some authors have suggested (53) that oxidative phosphorylation in tumor mitochondria is affected at the cytochrome oxidase and succinate dehydrogenase levels. Studies of the a, b, c and c1 cytochromes revealed

that their activities were decreased and therefore, that they worked in a deficient manner compared to normal liver mitochondria (49). However, these findings should not be generalized, since they have only been observed in Morris hepatoma mitochondria.

Another factor, in addition to a lower cytochrome activity, that has also been postulated as a possible cause of tumor cell dependence on glycolysis as the energy-producing pathway, is the small amount of mitochondria in tumor cells (1,53). However, this situation again depends on the type of tumor lines studied; for instance, in Walker tumor cells, the quantity of mitochondria oscillates between 20 and 40% of that of normal cells. In Ehrlich and possibly AS-30D, the content is equal to the normal. Moreover, the activity of some tumor mitochondrial dehydrogenases was high (3,24). Therefore, the assumption that the low content of mitochondria and/or deficiency in some enzyme activities is the reason for the low activity of oxidative phosphorylation is, at least, questionable.

Perspectives

This review emphasizes the importance of oxidative phosphorylation for ATP supply in cells that for many decades have been considered exclusively glycolytic

Table 2
Variation of Metabolites in AS-30D Hepatoma and Walker-256 Carcinosarcoma Cells (6)

	AS-30D (nmol/10^7cells)		Walker-256 carcinosarcoma
Metabolite	Freshly-Obtained cells	40-min incubation at 37°C in Ringer medium	μmol/g wet weight
Glucose	3.2	1.1	9.23-4.67
Glycogen	μg/10^7cells 2.3	μg/10^7cells 0.7	not reported
Glutamine	0.32	0.24	6.32-5.23
Glutamate	2.4	11.5	not reported
Lactate	1.3	3.0	0.372-0.4
Pyruvate	0.2	0.2	2.29-3.8
β-OHbutyrate	0.3	0.15	0.1-0.095
Acetoacetate	0.21	0.03	0.062-0.071
ATP	1.1	1	2.0-2.0

The data shown regarding AS-30D hepatoma cells represent the mean of five different preparations assayed. Data are from the authors' laboratory.

We are currently carrying out the first experiments that will allow, using the metabolic control theory, to elucidate which enzymes control the flux rate of the pathway. Once the enzymes are identified, the pathway could be manipulated using specific inhibitors. We are assuming that cellular duplication depends on ATP supply that arises from glycolysis as much as from oxidative phosphorylation. Therefore, the extent of tumor growth diminution induced by sole inhibition of oxidative phosphorylation should depend on the degree of participation of oxidative metabolism.

Since the Pasteur effect is negligible in tumor cells and PFK-1 is involved in such a response, it would be interesting to measure the effect of the different, relevant modulators (citrate, pH, P_i, adenine nucleotides) of PFK-1 in the isolated enzyme. These results may help to predict enzyme behavior under physiological conditions and to establish the role of PFK-1 in the radiotherapy resistance of some tumor cell lines.

The Ci values determined for tumor cells are not entirely of physiological relevance, since the isolation of cells removes extracellular metabolites which are not replenished during the experiments. Thus, considering the data of Table 2, the Ci values should also be determined in the presence of, at least, the main oxidizable substrates of tumor cells, glutamine and glutamate, which are present in high concentration in the cell-free ascites liquid and which are actively consumed by AS-30D cells (see Table 2).

Assuming that Ca^{2+} plays a central role in the mechanism of the Crabtree effect, it appears that the determination of $[Ca^{2+}]c$ and $[Ca^{2+}]m$ are required, as well as the measurement of the total contents of the cell and mitochondrial Ca^{2+}, under the different additions that trigger the Crabtree effect. Along the same lines, our data showed that the concentration of glucose in the cell-free ascites liquid was lower than 50 μM, while the serum glucose was above 2 - 4 mM. Therefore, the Crabtree effect in AS-30D cells is very likely of a small magnitude (<10% inhibition of cell respiration).

Studies using tumor cell cultures should allow to readily manipulate the glycolytic metabolism inducing transformations toward different degrees of oxidative metabolism. Thus, according to the Görlach and Acker hypothesis (44), this experimental approach could enhance the sensitivity of tumor cells to radiotherapy.

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eferences

1. Pedersen PL. Tumor mitochondria and the bioenergetics of cancer cells. Prog Exp Tumor Res 1978, 22:190
2. Eigenbrodt E, Fister P, Reinacher M. New perspectives on carbohydrate metabolism in tumor cells. In. Regulation of Carbohydrate Metabolism Peimer R, editor Boca Raton, FL: CRC Press: 1984. 6(4): p. 141.
3. Dietzen DJ, Davis J. Oxidation of pyruvate, malate, citrate, and cytosolic reducing equivalents by AS-30D hepatoma mitochondria. Arch Biochem Biophys 1993, 305 91
4. Parlo RA, Coleman PS. Continuous pyruvate carbon flux to newly synthesized cholesterol and the suppressed evolution of *pyruvate-generated CO_2 in tumors: further evidence for a persistent truncated Krebs cycle in hepatomas.* Biochim Biophys Acta 1986; 886:169
5. Bustamante E, Pedersen PL High aerobic glycolisis of rat hepatoma cells in culture: Role of mitochondrial hexokinase Proc Natl Acad Sci 1977; 74:3735.
6. Argilés JM, López-Soriano FJ. The energy state of tumor-bearing rats. J Biol Chem 1991, 266:2978
7. Davis SE, Brindle KM Effects of overexpression of phosphofructokinase on glycolysis in the yeast *Saccaromyces cerevisiae.* Biochemistry 1992, 31.4729
8. Stubbs M, Rodrigues L, Howe FA, Wang J, Jeong K, Veech RL, Griffiths JR. Metabolic consequences of a reversed pH gradient in rat tumors. Cancer Res 1994, 54 4011
9. Boxer GE, Devlin TM Pathways of intracellular hydrogen *transport. Science 1961; 134.1495*
10. Grivell AR, Korpelainen EI, Williams CJ, Berry MN. Substrate-dependent utilization of the glycerol 3-phosphate or malate/aspartate redox shuttles by Ehrlich ascites cells Biochem J 1995, 310.665
11. Baggeto LG, Lehninger AL. Formation and utilization of acetoin, an unusual product of pyruvate metabolism by Ehrlich and AS-30D tumor mitochondria J Biol Chem 1987, 262 9535
12. Baggeto LG, Testa-Parussim R Role of acetoin on the regulation of intermediate metabolism of Ehrlich ascites tumor mitochondria Its co- ibution to membrane cholesterol enrichment modifying passive proton permeability. Arch Biochem Biophys 1990, 283.241
13. Baggeto LG. Deviant energetic metabolism of glycolytic cancer cells Biochimie 1992, 74:959.
14. *LaNoue K, Schoolwerth A. Metabolite* transport in mitochondria Annu Rev Biochem 1979, 48.871.
15. Lazo A, Sols A Pyruvate dehydrogenase complex of ascites tumour. Biochem J 1980, 190 705.
16. Briscoe DA, Fiskum G, Holleran AL, Kelleher JK Acetoacetate metabolism in AS-30D hepatoma cells Mol Cell Biochem 1994, 136.131.
17. Ohe K, Morris HP, Weinhouse S D-β-hydroxybutyrate dehydrogenase activity in liver and tumor Cancer Res 1967, 27 1360.
18. Dietzen DJ, Davis EJ Excess membrane cholesterol is not responsible for metabolic and bioenergetic changes in AS-30D hepatoma mitochondria Arch Biochem Biophys 1994, 309 341
19. Ding JL, Smith GD, Peters TJ. Subcellular localization and isolation of γ-glutamyltransferase from rat hepatoma cells Biochim Biophys Acta 1981, 661 191
20. *Molina M, Segura JA, Aledo JC, Medina MA, Núnez de Castro* I, Márquez J Glutamine transport by vesicles isolated from tumour-cell mitochondrial inner membrane Biochem J 1995; 308:629
21. *Lazo PA. Amino acids and glucose utilization by different* metabolic pathways in Ascites-Tumour Cells. Eur J Biochem 1981, 117.19
22. Corvera S, García-Sainz JA. Hormonal stimulation of mitochondrial glutaminase Biochem J 1983, 210 957.
23. Moreadith RA, Lehninger AL. Purification, kinetic behavior, and regulation of NAD(P)$^+$ malic enzyme of tumor mitochondria J Biol Chem 1984, 259:6222.
24. Moreno-Sánchez R, Hogue BA, Hansford R Influence of NAD-linked dehydrogenase activity on flux through oxidative phosphorylation. Biochem J 1990; 268:421
25. *Murphy AN, Kelleher JK, Fiskum G. Calcium sensitive Isocitrate* and 2-oxoglutarate dehydrogenase activies in rat liver and AS-30D hepatoma mitochondria Biochem Biophys Res Commun 1988, 157:1218.
26. Müller M, Siems W, Buttgereit F, Dumdey R, Rapoport SM Quantification of ATP-producing and consuming processes of Ehrlich ascites tumour cells Eur J Biochem 1986, 161:701.
27. Schmidt H, Siems W, Muller M, Dumdey R, Rapoport SM ATP-producing and consuming processes of Ehrlich mouse ascites tumor cells in proliferating and resting phases Exp Cell Res 1991, 194:122
28. Moreno-Sánchez R, Torres-Márquez ME. Control of oxidative phosphorylation in mitochondria, cells and tissues Int J Biochem 1991, 23.1163
29. Fearon KCH, Plumb JA, Burns HJG, Calman KC Reduction of *the growth rate of the Walker 256 tumor in rats by rhodamine 6G* together with hypoglycemia Cancer Res 1987; 47:3684.
30. Jaroszewski JW, Kaplan O, Cohen JS Action of Gossypol and Rhodamine 123 on wild type and multidrug-resistant MCF-7 human breast cancer cells: ^{31}P nuclear magnetic resonance and toxicity studies. Cancer Res 1990, 50 6936.
31. Sri-Pathmanathan RM, Plumb JA, Fearon KCH Clofazimine alters the energy metabolism and inhibits the growth rate of a human lung-cancer cell line *in vitro* and *in vivo* Int J Cancer 1994, 56:900.
32. Wolf F, Di Francesco A, Cittadini A. Characterization of magnesium efflux from Erlich ascites tumor cells Arch Biochem Biophys 1994, 308 335
33. Dubyak GR, De Young MB Intracellular Ca^{2+} mobilization activated by extracellular ATP in Ehrlich ascites tumor cells J *Biol Chem 1985, 260.10653*
34. Torres-Márquez ME, Mejía S, Moreno-Sánchez R Modulation of the ATP induced $[Ca^{2+}]c$ increase in AS-30D hepatoma cells. Int J Biochem 1993; 25 1109.
35. Torres-Márquez ME, Moreno-Sánchez R Measurement of the cytosolic Ca^{2+} mobilization induced by extracellular ATP in AS-30D hepatoma cells using Indo-1 Proc West Pharmacol Soc 1991, 34 399
36. Fleschner CR, Martin PA, Vorbeck ML, Darnold JR, Long JW Ca^{2+} release from energetically coupled tumor mitochondria Biochem Biophys Res Commun 1983, 115 430.
37. Evtodienko YV, Teplova VV, Duszynski J, Bogucka K, Wojtczak L The role of cytoplasmatic $[Ca^{2+}]$ in glucose-induced inhibition of respiration and oxidative phosphorylation in Ehrlich ascites tumour cells: a novel mechanism of the Crabtree effect Cell *Calcium 1994, 15.439*

; Moreno-Sánchez R, Torres-Márquez ME, López-Gómez FJ. La determinación de la concentración de calcio libre en células y mitocondrias con Indicadores Fluorescentes. Ciencia 1993, 44:383.

). Bogucka K, Teplova VV, Wojczaka L, Evtodienko YV. Inhibition by Ca^{2+} of the hydrolysis and the synthesis of ATP in Ehrlich ascites tumour mitochondria: relation to the Crabtree effect Biochim Biophys Acta 1995; 1228.261

0. Moreno-Sánchez R. Inhibition of oxidative phosphorylation by Ca^{2+}-induced diminution of the Adenine nucleotide translocator. Biochim Biophys Acta 1983, 724:278.

.1. Tuena de Gómez-Puyou M, Gavilanes M, Gómez-Puyou A, Ernster L. Control of activity states of heart mitochondrial ATPase Role of the *proton-motive force and* Ca^{2+}. *Biochim* Biophys Acta 1980; 592:396.

42. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and *nutrient supply, and metabolic microenvironment of human* tumors: A review Cancer Res 1989; 49.6449.

43 Sutherland RM Cell and environment interactions in tumor *microregions: the multicell spheroid model Science 1988,* 240.177.

44. Gorlach A, Acker H. pO_2 and pH-gradients in multicellular spheroids and their relationship to cellular metabolism and radiation sensitivity of malignant human tumor cells Biochim Biophys Acta 1994; 1227:105.

45. Hockel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res 1996;* 56:4509.

46 Nishikawa M, Sato EF, Utsumi K, Inove M. Oxygen-dependent regulation of energy metabolism in ascites tumor cells by nitric oxide. Cancer Res 1996; 56:4535

47. Wanders RA, Groen AK, Van Roermund CWT, Tager J Factors determining the relative contribution of the adenine-nucleotide translocator and the ADP-regenerating system to the control of oxidative phosphorylation in isolated rat liver mitochondria. Eur J Biochem 1984; 142 417

48. López-Gómez FJ, Torres-Márquez ME, Moreno-Sánchez R Control of oxidative phosphorylation in AS-30D hepatoma mitochondria Int J Biochem 1993; 25:373.

49. Lau BWC, Chan SHP. Efflux of adenine nucleotides in mitochondria from rat tumor cells of varying growth rates Cancer Res 1984; 44:4458.

50 *Segel IH. Enzyme Kinetics New York. John Wiley and Sons;* 1975. p 3.

51. Gellerich FN, Kunz WS, Bohnensack R. Estimation of flux *control-coefficients from inhibitor titrations by non-linear re*gression. FEBS Lett 1990; 274.167

52. Brand MD, Murphy MP Control of electron flux through the respiratory chain in mitochondria and cells Biol Rev 1987; 62:141.

53. White MT and Nandi S Biochemical studies on mitochondria isolated from normal and neoplastic tissue of the mouse mammary gland J Natl Cancer Inst 1976, 56.65

Una conclusión importante que emerge de la revisión anterior son las modificaciones en el metabolismo intermediario que incluye cambios en la actividad de ciertas vías catabólicas, todas ellas relacionadas con el suministro de ATP. Sin embargo, el ATP no es el único metabolito esencial en la proliferación tumoral: obviamente se requieren otros intermediarios (sustratos y/o señales) involucrados en la progresión del ciclo celular. A continuación se mencionan cuales son los intermediarios esenciales y su relación con las etapas de proliferación tumoral.

1.2 Cambios metabólicos durante la proliferación tumoral.

Tanto la glucólisis como la glutaminólisis son esenciales para los procesos proliferativos [2,3]. Al parecer, la presencia de ambas vías en los tumores de rápido crecimiento es una estrategia metabólica para sobrevivir y crecer en condiciones limitantes de oxígeno, nutrientes o de pobre vascularización [3,4]. Esta habilidad no es necesaria en las células proliferantes no-tumorigénicas, debido a que crecen en tejidos organizados y ricamente vascularizados [3,5].

La glucólisis, desde hace varias décadas, ha sido considerada la vía a partir de la cual la mayor parte de las células tumorales obtienen el ATP celular. Sin embargo, se ha documentado ampliamente que precisamente las líneas tumorales que muestran defectos en su capacidad oxidativa son menos tumorigénicas que las que mantienen una fosforilación oxidativa activa. Lo anterior sugiere una estrecha relación entre la fosforilación oxidativa y el grado de malignidad tumoral [6,7]. En el caso de la vía glutaminolítica, su principal función es la obtención de energía y la producción de glutamato, citrato y aspartato [8]. El citrato es necesario para la formación de acetil CoA, de ácidos grasos e isoprenoides; el aspartato es útil para la síntesis de pirimidinas. Existen dos características que permiten una glutaminólisis activa en tumores: (A) la alta disponibilidad de glutamina en los tejidos y tumores sólidos (cerca de 2 mM) probablemente debida a una activa degradación proteica durante el proceso post-inflamatorio; y (B) ser una vía activa que permite a las células tumorales crecer y proliferar en condiciones limitantes de glucosa sólo si el oxígeno está

disponible [4]. Unido a lo anterior, existen numerosos reportes que proponen que la glutaminólisis y la fosforilación oxidativa son requeridas para el desarrollo del fenotipo tumoral [4,6].

A pesar de que las enzimas glucolíticas se sobreexpresan manteniendo exacerbada a la vía durante la duplicación celular, la relación producción de lactato/ consumo de glucosa frecuentemente es menor a 2, lo que indica que una fracción de la glucosa es utilizada para fines sintéticos produciendo precursores (ribosa-5-fosfato) para la síntesis de ácidos nucleicos [4,6] y en algunos casos, para fines oxidativos [9]. Cuando las células tumorales se siembran en condiciones limitantes de glucosa, la energía es abastecida por la degradación aeróbica de piruvato o por la conversión de glutamina a lactato [10]. La adición de glucosa promueve un incremento en los niveles de fosfometabolitos y de algunos de sus derivados sintéticos. Se ha propuesto que estos compuestos actúan como señales reguladoras de la proliferación tumoral, ya sea induciendo la progresión de la fase G_0 a la fase G_1, y de ésta a la fase S del ciclo celular o bien actuando como represores o inhibidores de ciertas enzimas replicativas. A continuación se describen los principales aminoácidos y fosfometabolitos involucrados en los procesos replicativos de las células tumorales de rápido crecimiento [4,11-13].

Intermediarios esenciales para la proliferación tumoral

A) AMINOÁCIDOS

1) ***Aspartato***. *Uno de los metabolitos que se generan en mayor proporción a través de la vía glutaminolítica durante la proliferación tumoral es el aspartato;* sin embargo, una fracción de glutamina puede también convertirse en lactato y citrato [2]. Durante la duplicación celular, la concentración intracelular de aspartato disminuye drásticamente, sugiriendo que el catabolismo de aspartato hacia pirimidinas y purinas es mayor que su biosíntesis a partir de oxaloacetato. Esta reducción citosólica de aspartato tiene una ventaja metabólica: disminuye la actividad de la lanzadera aspartato/malato, y como consecuencia, la relación NADH/NAD^+ del citosol aumenta, la cual es una de las señales que inducen la progresión de la fase G_0 a la fase G_1 del ciclo celular [11]

2) *Serina.* La serina se forma a partir de 3-fosfoglicerato (un intermediario de la glucólisis). Su catabolismo provee de (a) glicina utilizada en la biosíntesis de purinas y pirimidinas; y (b) de grupos metilos para la formación de adenosil homocisteína y metionina, el primero requerido para la proliferación celular [12]. De hecho, se ha propuesto que la inhibición de la enzima que transfiere los grupos metilos al tetrahidrofolato para formar serina a partir de glicina (serina hidroximetiltransferasa), con análogos de serina (hidroxilaminas), detiene la proliferación celular en fase G1 [13]. La serina también es precursora de algunos lípidos como etanolamina, colina, y esfingomielina [14]. Durante la proliferación celular, la glucólisis ajusta la producción de serina, dependiendo de la demanda requerida por la célula durante el ciclo celular.

(B) FOSFOMETABOLITOS

1) *Fosforibosa pirofosfato (PRPP).* Este metabolito se sintetiza a partir de ribosa 5-fosfato en una reacción dependiente de ATP, catalizada por la P-ribosa-PP sintetasa. El nivel intracelular de PRPP en las células tumorales frecuentemente es mayor (1-3 mM)que el reportado en células no tumorigénicas (0.04-0.5 mM) [1,14] debido a que es un precursor directo de bases púricas y pirimídicas. Dependiendo de la disponibilidad de carbohidratos, la ribosa 5-fosfato puede sintetizarse de dos maneras: (1) en condiciones limitantes de glucosa, toda la ribosa 5-fosfato se forma oxidativamente a través de la vía de las pentosas a partir de glucosa 6-fosfato [15]. En cambio, cuando existe un exceso de carbohidratos, la ribosa 5-fosfato se obtiene preferencialmente de la ruta no-oxidativa, es decir, a partir de fructosa 6-fosfato + gliceraldehído 3-P, por reacciones de transcetolación y transaldolación. La P-ribosa-PP no sólo es precursora de la síntesis de novo de purinas y pirimidinas; también es precursora de nucleótidos de purina y pirimidina De hecho, existe evidencia de que el incremento en P-ribosa-PP así como purinas, pirimidinas y la suma de NADH + NAD inducen una disminución en el flujo de 3-fosfoglicerato a piruvato, lo que se interpreta como una estrategia para mantener siempre elevados los precursores de ácidos nucleicos [16]

2) ***Fructosa-1,6-bifosfato.*** Aunque no es un precursor directo para vías sintéticas, se ha descrito que a concentraciones milimolares estimula la síntesis de proteína a través de la activación de la vía de las pentosas, e inhibe la fosforilación de la piruvato cinasa tumoral manteniéndola en estado activo [17,18]. Durante la proliferación tumoral la concentración de fructosa-1,6-bifosfato se incrementa de 30-70 μM a 2.8 mM (18) en comparación con el estado quiecente. A estas concentraciones, la glucosa-6-fosfato deshidrogenasa se inhibe competitivamente lo cual acelera el flujo glucolítico. Se ha sugerido que el aumento en fructosa-1,6-bifosfato es una señal para que la célula progrese en el ciclo celular de la fase G_0, G_1 a la fase S [1].

3) ***AMP.*** Este metabolito, derivado del metabolismo de PRPP y de la activación de ácidos grasos, tiene un efecto inhibitorio sobre la PRPP sintetasa, lo que provoca una disminución en los niveles de NAD, NADH y en la síntesis de DNA [4, 19]. En células tumorales humanas glicolíticas (MCF-7), a diferencia de los tejidos diferenciados no tumorigénicos y de los tumores oxidativos (MDA-MB-453), la adición de AMP (3 mM) inhibe la glucólisis. Al parecer el efecto inhibitorio se debe a que MCF-7 carece de la glicerol-3 fosfato deshidrogenasa y por tanto la eficiencia de la lanzadera de glicerol-3fosfato disminuye. Al carecer de este sistema de transferencia, la masa de NADH generada por la gliceraldehído-3 fosfato deshidrogenasa se drena hacia la producción de lactato. En presencia de AMP, la relación NADH/NAD^+ disminuye 85% (de $63x10^{-4}$ a $9x10^{-4}$) por lo que la actividad de la lactato deshidrogenasa se atenúa. Por tanto, no hay suficiente NADH en el citosol y la gliceraldehído-3 fosfato deshidrogena se inhibe [3,4].

4) ***NADH Y NADPH.*** Recientemente se ha encontrado que un incremento en los niveles de equivalentes reductores promueve la proliferación tumoral [4, 20]. Lo anterior puede explicarse porque el NADPH se requiere para la síntesis de lipídos, colesterol e isoprenoides [21]. Se ha determinado que el AMP es un potente inhibidor de la P-ribosa-PP sintetasa (y por ende los niveles de equivalentes reductores disminuyen), por lo que en su presencia varias vías que utilizan como sustrato los equivalentes reductores (síntesis de colesterol y

triglicéridos) disminuyen su actividad. Además de los equivalentes reductores, los niveles de otros compuestos como el CTP y la UDP-N-acetilglucosamina disminuyen en presencia de AMP, abatiendo la proliferación celular [20,21]. Mazurek también ha propuesto que los equivalentes reductores y algunos fosfometabolitos pueden activar proteinas cinasas o inhibir proteínas fosfatasas e interferir en la cascada de cinasas que regula la proliferación celular [4].

1.3 Planteamiento del problema

En la mayor parte de las líneas tumorales de rápido crecimiento estudiadas (Ehrlich, Ehrlich Lettré, hepatoma de Novikoff, carcinoma de Walker-256) se ha propuesto que siendo la glucólisis una vía activa (varios órdenes de magnitud mayor a la reportada en hepatocitos), ésta es capaz de suministrar el ATP requerido para sus funciones celulares [1,2,4].

En algunas líneas tumorales esta aseveración es correcta ya que presentan un metabolismo oxidativo deficiente. Sin embargo, no existe evidencia concluyente de que todos los tumores dependan exclusivamente de la glucólisis. En el caso de las células del hepatoma AS-30D, la línea tumoral de estudio, dos características bioquímicas la pueden ubicar como un tumor con mayor dependencia del metabolismo oxidativo que del glucolítico: la elevada oxidación de piruvato [22,23] y la existencia de un ciclo de Krebs completo y funcional capaz de suministrar el NADH necesario para la síntesis de ATP [23]. Si esta hipótesis es correcta y las células tumorales de AS-30D tienen mayor dependencia a la FO, la modulación de esta vía (ya sea manipulando la fuente de carbono externa y/o utilizando inhibidores o fármacos selectivos) promoverá una disminución en su duplicación celular.

La glucosa a concentraciones fisiológicas inhibe parcialmente la respiración celular (efecto Crabtree) de diferentes líneas tumorales. En AS-30D no se ha explorado si la glucosa también modula la capacidad oxidativa de la célula. Además no existe una explicación bioquímica satisfactoria para tal efecto.

CAPITULO 2

OBJETIVO E HIPOTESIS DEL PROYECTO

OBJETIVOS

Objetivo General

Determinar la dependencia de la proliferación celular del aporte de ATP que proviene de la fosforilación oxidativa, en la célula tumoral de rápido crecimiento AS-30D.

Objetivos Particulares

1) ***Dilucidar el metabolismo energético de las células tumorales AS-30D en suspensión, para determinar la contribución parcial de cada vía (fosforilación oxidativa, glucólisis) al aporte total de ATP.***

a) Determinar la concentración de metabolitos glucolíticos y oxidativos en el líquido de ascitis (fracción libre de células).

b) Determinar el consumo endógeno y exógeno de los sustratos glucolíticos (glucosa) y oxidativos (glutamina y glutamato).

c) Estimar las velocidades celulares de síntesis de ATP provenientes tanto de glucólisis como de fosforilación oxidativa.

d) Determinar la distribución de control que ejercen las enzimas de la vía que aporta la mayor cantidad de ATP.

e) Identificar los mecanismos involucrados en la inhibición de la fosforilación oxidativa por adición de glucosa exógena (efecto Crabtree).

2) *Establecer la interrelación del metabolismo energético con la proliferación celular en cultivos primarios.*

a) Determinar la densidad y la velocidad de duplicación celular de AS-30D en función de la actividad de FO

b) Determinar los cambios en los flujos glucolíticos y oxidativos.

c) Determinar la variación en la concentración intracelular de glucosa, glucógeno, glutamato, glutamina, ATP.

HIPOTESIS DEL PROYECTO

En la línea tumoral AS-30D, la eficiente oxidación de piruvato mitocondrial [22], el alto suministro de equivalentes reductores a partir del ciclo de Krebs [23] y la degradación activa de sustratos mitocondriales como glutamina [24] y acetoacetato [25], son evidencias que sugieren fuertemente que dependen metabolismo mitocondrial, por tanto se predice que el abatimiento de la fosforilación oxidativa puede disminuir su duplicación celular.

CAPITULO 3

RESULTADOS

3.1 SUBSTRATE OXIDATION AND ATP SUPPLY IN AS-30D HEPATOMA CELLS*

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Oxidación de sustratos y suministro de ATP en las células de hepatoma AS-30D.

En este manuscrito demostramos que al igual que en otras líneas tumorales de rápido crecimiento, las células AS-30D son altamente glicolíticas aún en presencia de concentraciones saturantes de oxígeno. Sin embargo, otras vías involucradas en la degradación de sustratos que alimentan la FO se encuentran activas como son (1) el catabolismo de cuerpos cetónicos (acetoacetato y β–hidroxibutirato), (2) la oxidación de glutamato, (3) y la fosforilación oxidativa. De hecho, esta vía es la que aporta el 99% del ATP celular requerido por la célula para mantener sus funciones celulares. Otra evidencia que indica que en AS-30D prevalece un metabolismo mitocondrial más que glucolítico es que en el líquido de ascítis (fracción líquida del cual se nutre la célula) se registra una alta concentración de glutamina y oxígeno mientras que la glucosa se mantiene en baja concentración. En paralelo determinamos, utilizando la teoría moderna de control metabólico [27,28], que la FO se encuentra regulada en mayor proporción por la NADH-oxidoreductasa (sitio I de la cadena respiratoria) y por el bloque de enzimas consumidoras de ATP. Una observación importante que surgió en este trabajo fue que la adición de glucosa externa inhibió parcialmente la respiración celular de AS-30D, sensible a oligomicina, lo cual demuestra la incidencia de efecto Crabtree en tumores dependientes del metabolismo oxidativo. Esta observación indicó que la glucosa podría ser un freno fisiológico en los tumores dependientes del metabolismo mitocondrial

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Substrate Oxidation and ATP Supply in AS-30D Hepatoma Cells

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The oxidation of several metabolites in AS-30D tumor cells was determined. Glucose and glycogen consumption and lactic acid production showed high rates, indicating a high glycolytic activity. The utilization of ketone bodies, oxidation of endogenous glutamate, and oxidative phosphorylation were also very active: tumor cells showed a high respiration rate (100 ng atoms oxygen (min × 10^7 cells)$^{-1}$), which was 90% oligomycin-sensitive. AS-30D tumor cells underwent significant intracellular volume changes, which preserved high concentrations of several metabolites. A high O_2 concentration, but a low glucose concentration were found in the cell-free ascites liquid. Glutamine was the oxidizable substrate found at the highest concentration in the ascites liquid. We estimated that cellular ATP was mainly provided by oxidative phosphorylation. These data indicated that AS-30D hepatoma cells had a predominantly oxidative and not a glycolytic type of metabolism. The NADH-ubiquinol oxido reductase and the enzyme block for ATP utilization were the sites that exerted most of the control of oxidative phosphorylation (flux control coefficient = 0.3–0.42).

Key Words: **glycolysis; oxidative phosphorylation; AS-30D cells; metabolism; control analysis.**

The metabolism of fast-growth and largely dedifferentiated tumoral cells, such as Ehrlich, Ehrlich Lettré, and Walker-256 carcinosarcoma, differs markedly from that of their tissue of origin (1–3) The fast oxidation of glucose, even under aerobic conditions, and the active production of lactic acid are some of the main characteristics of these cells. It has been proposed that in fast-growth tumor cells there is a correlation between the degree of differentiation and the growth rate, with the rate of glucose catabolism (1) However, glucose consumption is not the only catabolic pathway with a high rate in fast-growth tumor cells (1, 4–11). For example, some amino acids such as glutamine, in Ehrlich and Ehrlich Letreé cells (5, 6), and ketone bodies, in AS-30D cells, also appear to be rapidly oxidized (8, 9). In addition, fatty acids and cholesterol synthesis, in Morris 3924A and AS-30D cells (8, 12), are anabolic active pathways in fast-growth tumor cells

In AS-30D hepatoma cells, a fast-growth ascites tumor cell line, only few oxidizable substrates such as ketone bodies (8, 9) and cholesterol (8, 12) have been quantified, and in fact, the consumption of glutamine and glutamate (mitochondrial substrates) has not been documented as yet. The rate of pyruvate oxidation by the pyruvate dehydrogenase complex is faster in AS-30D (11) than in Ehrlich and Ehrlich Lettré cells (5). Ehrlich and Ehrlich Lettré cells have an incomplete Krebs cycle, resulting in a low oxidation of mitochondrial substrates (13) In contrast, the Krebs cycle in AS-30D cells is complete and functional (11), providing sufficient reducing equivalents [>5% mitochondrial NADH in the steady-state (14)] to drive ATP synthesis. However, the concentrations of oxidizable substrates have not been determined in the cell-free ascites liquid This seems relevant since there is no evident reason to assume that the metabolite concentrations in the ascites liquid are similar to those found in the blood of tumor-bearing animals.

The tumor cell ATP levels, which do not change during the resting phase, diminish during cellular duplication (15) It has been proposed that in spite of maintaining an accelerated glycolysis, the ATP required for supporting the most important cellular re-

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actions in fast-growth tumor cells, i.e., protein synthesis and active ion transport (Na^+/K^+, Ca^{2+}) in tumor cells, proceeds from oxidative phosphorylation, with glycolysis playing only a minor role (15, 16). Hexokinase is located close to the mitochondrial external membrane in AS-30D and other tumor cell types, and is functionally associated to the ATP/ADP carrier, which suggests that the ATP used for the first glycolytic reaction derives from oxidative phosphorylation (17).

Specific blockade of oxidative phosphorylation by lipophylic cationic drugs such as rhodamines 123 (18, 19), 6G (20), and MKT-077 (21) brings about a significant diminution of cell growth in several tumoral lines. Simultaneous inhibition of glycolysis and oxidative phosphorylation induces a more drastic reduction in tumor cell growth and size (18–20).

Therefore, it would appear that oxidative phosphorylation is the principal source of ATP for cell growth in a variety of tumoral lines. In order to identify the type of metabolism (oxidative or glycolytic) prevalent in fast-growth AS-30D cells, we determined the substrates that are preferentially consumed as well as the intracellular and ascites liquid concentrations. Thereafter, we estimated the contribution of glycolysis and oxidative phosphorylation to the cell supply of ATP, and the degree of control exerted by various steps of oxidative phosphorylation.

MATERIALS AND METHODS

Preparation of tumor cells The AS-30D ascites tumor was propagated in female Wistar rats (200–250 g body weight) by intraperitoneal transplantation (22). Tumor cells were obtained by peritoneal puncture 5–7 days after inoculation. The cells were washed four times by dilution and centrifugation for 2 min at 3500*g* at 4°C in a modified Krebs–Ringer medium that contained the following (in mM): 125 NaCl, 5 KCl, 25 Hepes, 1 $MgCl_2$, 1 KH_2PO_4, 1.4 $CaCl_2$, pH 7.4. A final washing was carried out with the same medium, supplemented with 2% (w/v) albumin, which removed remaining erythrocytes and dead cells (22). The viability of AS-30D cells, estimated by 0.5% trypan blue exclusion, was 98–99%, with fewer than 0.1% erythrocytes.

Cellular respiration The rate of oxygen consumption in cell suspensions incubated in the modified Krebs–Ringer medium described above was measured polarographically by means of a Clark-type oxygen electrode at 37°C. The oxygen solubility at 2240 m altitude and 37°C was determined to be 330 ng atoms oxygen/ml (190 μM O_2).

Determination of metabolites. Glucose, pyruvate, lactate, glutamate, glutamine, β-hydroxybutyrate, acetoacetate, and ATP were determined in aliquots of the neutralized perchloric acid cell extract by standard enzymatic methods (23).

Determination of O_2 concentration in ascites liquid This was done as described elsewhere (24). Aliquots of ascites liquid were taken from the intraperitoneal fluid using an insulin syringe previously filled with 100 μl of 30% (v/v) perchloric acid and transferred to the oxymeter chamber, which contained medium with a low concentration of dissolved O_2, adjusted, by titration, with sodium dithionite.

Determination of intracellular volume An aliquot of cells (50–150 × 10^6 cells) was incubated in 0.5 ml of modified Krebs–Ringer medium containing ^3H_2O (sp act 13,300 cpm/ml) at 37°C. Aliquots of the same cell preparation were incubated in parallel for 45 s, and then 0.3 mg/ml of [^{3}H]inulin (sp act 660–720 cpm/μg) was added and further incubated for 15 s. Thereafter, the cell suspensions were carefully layered into microcentrifuge tubes containing, from bottom to top, 30% (v/v) perchloric acid (0.3 ml), 1-bromododecane (0.3 ml) (25), and fresh Krebs–Ringer medium (0.3 ml). The radioactivity of the top and bottom layers was measured and the intracellular volume was estimated following the formulations described by Rottenberg (26).

Determination of the flux control coefficient Cell respiration (1 × 10^7 cells/ml) was inhibited by either rotenone (1.5–500 pmol/10^7 cells), antimycin A (1–100 pmol/10^7 cells), myxothiazol (1.5–250 pmol/10^7 cells), KCN (0.2–20 mM), sodium azide (1–60 mM), oligomycin (5–500 pmol/10^7 cells), venturicidin (10–700 pmol/10^7 cells), carboxyatractyloside (0.6–2 nmol/10^7 cells), or 3-hydroxy-cyanocinnamate (0–500 μM). The estimation of the flux control coefficients (Co) was made by using the equation for noncompetitive tightly bound inhibitors proposed by Gellerich *et al.* (27),

$$J = \left[\frac{n(J_o - J_i)^2 \times E^n}{C_o \times J_o \times E_o^n + [(n - C_o) \times J_o - n \times J_i] \times E^n}\right] + J_i$$

$$\times E^2 + (K_d + I - E_o) \times E - K_{di} \times E_o = 0,$$

where J_o and J_i are the fluxes in the non-inhibited ($E = E_o$) and completely inhibited system ($E = 0$), K_d is the dissociation constant of the enzyme–inhibitor complex, and n is an empiric component that establishes the relation between substrate concentration and the catalyzed reaction by enzyme E (when $n = 1$, the reaction is a linear function of the substrate concentration).

RESULTS

Oxidation of Endogenous Substrates

AS-30D hepatoma cells incubated at 37°C oxidized endogenous glucose, glutamate, acetoacetate, β-hydroxybutyrate, and glycogen at significant rates (Table I). Under these conditions, the rate of lactate formation (Table II) matched the rates of glucose plus glycogen consumption: lactate formation was 0.48 nmol (min × 10^7 cells)$^{-1}$ in the first 25 min of incubation versus 0.06 nmol (min × 10^7 cells)$^{-1}$ of glucose consumption plus 0.28 nmol (min × 10^7 cells)$^{-1}$ in glucose residues units of glycogen disappearance, which should produce a maximum of 0.68 nmol lactate (min × 10^7 cells)$^{-1}$.

Estimation of the intracellular volume yielded the following values: 2.28 ± 0.39 μl internal water/10^7 cells (mean ± standard deviation, $n = 4$) for cells kept on ice (t_0) and 1.41 ± 0.38 μl/10^7 cells ($n = 5$; t_{25}) and 1.43 ± 0.28 μl/10^7 cells ($n = 4$; t_{40}) for cells incubated at 37°C, in the absence of external oxidizable substrates, during 25 and 40 min, respectively. The significant diminution in intracellular volume, after 25 ($P < 0.01$) and 40 min of incubation ($P < 0.025$), was very likely due to the loss of oxidizable substrates, which resulted in a net diminution in the intracellular concentration of glucose, acetoacetate, glutamate, and glycogen (Table I). In contrast, the concentrations of β-hy-

TABLE I

Substrates Oxidized by AS-30D Hepatoma Cells and their Concentrations in the Cell-Free Ascites Liquid

Metabolites	nmol/10^7 cells			Cell-free ascites liquid (μM)
	Basal (t_0)	t_{25}	t_{40}	
Glucose	3.2 ± 1.2 [1.4 ± 0.5 mM] (7)	1.6 ± 0.6* [1.1 ± 0.42 mM] (6)	1.1 ± 0.6** [0.76 ± 0.4 mM] (6)	26 ± 4 (7)
Acetoacetate	0.21 ± 0.07 [92 ± 30 μM] (5)	0.09 ± 0.04*** [63 ± 28 μM] (4)	0.03 ± 0.01** [20 ± 7 μM]** (4)	900 ± 600 (4)
β-Hydroxybutyrate	0.3 ± 0.09 [131 ± 40 μM] (5)	0.2 ± 0.03 [142 ± 21 μM] (4)	0.15 ± 0.06*** [104 ± 41 μM] (5)	40 ± 10 (3)
Glutamate	24 ± 3 [10.5 ± 1.3 mM] (4)	17 ± 4† [12 ± 2.8 mM] (4)	11.5 ± 3** [8 ± 2.1 mM] (4)	150 ± 95 (4)
	μg/10^7 cells			
Glycogen	2.3 ± 0.7 (3)	1.3 ± 0.4 (3)	0.7 ± 0.3*** (3)	

Note. Tumor cells (3×10^6 cells/ml) were incubated in a plastic flask in 1 ml of modified Krebs–Ringer medium under orbital shaking (100 rpm/mn) at 37°C. At the indicated incubation times, the reaction was stopped by adding 3% (v/v) cold perchloric acid. The content of metabolites was determined in the neutralized extracts as described under Materials and Methods. The freshly extracted ascites liquid was centrifuged at 510*g* for 5 mn at 4°C and the supernatant was immediately mixed with 3% perchloric acid, the neutralized ascites liquid extract was used for determination of metabolites. The values represent mean ± SD with the number of cell preparations assayed in parentheses. The data shown in square brackets represent the estimation of the metabolite concentrations using the values of the intracellular water volumes. After 40 mn of incubation, the viability diminished only 6%, from 98 ± 0.01% (t_0) to 92 ± 0.03% (t_{40}) ($P < 0.005$). Student t test for nonpaired samples.

* $P < 0.01$ versus t_0.
** $P < 0.005$ versus t_0.
*** $P < 0.025$ versus t_0.
† $P < 0.05$ versus t_0.

TABLE II

Substrates Not Oxidized by AS-30D Hepatoma Cells and their Concentrations in the Cell-Free Ascites Liquid

Metabolites	nmol/10^7 cells			Cell-free ascites liquid (mM)
	Basal (t_0)	t_{25}	t_{40}	
Lactate	13 ± 2 [5.7 ± 0.9 mM] (5)	25 ± 10* [17.7 ± 7 mM]** (5)	30 ± 9** [20 ± 6 mM]** (5)	3.3 ± 0.3 (3)
Glutamine	0.37 ± 0.1 [162 ± 43 μM] (5)	0.32 ± 0.1 [227 ± 71 μM] (5)	0.24 ± 0.17 [170 ± 30 μM] (5)	4 ± 1.3 (4)
Pyruvate	0.2 ± 0.06 [87 ± 26 μM] (4)	0.2 ± 0.05 [142 ± 35 μM] (5)	0.2 ± 0.1 [138 ± 70 μM] (5)	0.16 ± 0.04 (3)
ATP	1.9 ± 0.4 [0.83 ± 0.2 mM] (4)	2.1 ± 0.5 [1.5 ± 0.3 mM]* (4)	2.1 ± 0.2 [1.45 ± 0.13 mM]** (3)	3.5 μM (1)
$[O_2]$				50.2 ± 6 μM (4)

Note. See note to Table I for experimental details.
* $P < 0.05$ versus t_0.
** $P < 0.005$ versus t_0.

TABLE III

Cytosolic and Mitochondrial NADH/NAD$^+$ Ratios in AS-30D Cells Incubated in the Absence of Oxidizable Substrates

NADH/NAD$^+$	Basal (t_0)	t_{25}	t_{40}
Cytosolic	0.0071 ± 0.003 (4)	0.014 ± 0.002* (4)	0.016 ± 0.009 (4)
Mitochondrial	0.071 ± 0.06 (4)	0.108 ± 0.036 (4)	0.245 ± 0.2 (4)

Note. The values represent the mean (±SD) obtained with four different preparations. The cytosolic NADH/NAD$^+$ ratio was calculated from the lactate dehydrogenase reaction, where $K_{eq} = 1.11 \times 10^{-4}$, and the mitochondrial NADH/NAD$^+$ ratio from the D-β-hydroxybutyrate dehydrogenase reaction, where $K_{eq} = 0.049$ (31)

* $P < 0.01$ as compared with control (t_0).

droxybutyrate (Table I) and glutamine remained constant (Table II), despite the net loss of these metabolites through oxidation. The result with glutamine was somewhat surprising since it has been suggested that some fast-growth tumor cells are predominantly glutaminolytic (3, 7). During the incubation period examined, the total content and intracellular concentration of pyruvate did not change and the ATP concentration increased (Table II).

The concentrations of acetoacetate, glutamate, lactate, and pyruvate in the cell-free ascites liquid (Tables I and II) were in agreement with reported values for rat blood (6, 28, 29). However, the concentrations of glucose and β-hydroxybutyrate were lower, and the concentration of glutamine in ascites liquid was higher than those values reported for arterial rat blood (6, 28, 29). To our knowledge, there are no available data on the concentrations of metabolites in the cell-free ascites liquid which can be used for comparative purposes. The concentration of O_2 dissolved in the ascites liquid was relatively high, with values comparable to those found in rat arterial blood (30).

Cytosolic and Mitochondrial NADH/NAD$^+$ Ratios in AS-30D Hepatoma Cells

Without added substrates, the NADH/NAD$^+$ ratios increased during incubation at 37°C (Table III). It is possible that this was due to an increase in the mitochondrial and cytosolic dehydrogenase activities. Values of the NADH/NAD$^+$ ratios of a similar magnitude have been found in hepatocytes (31) and other tumor cell lines (32).

Oxidation of Exogenous Substrates

In the presence of added glucose, the intracellular steady-state concentration of glucose diminished more than in its absence ($P < 0.005$), in 40 min of incubation at 37°C (Table IV) A similar diminution in intracellular glucose was observed in the presence of external glutamine or glutamate. It should be noted that the

TABLE IV

Oxidation of Exogenous Metabolites in AS-30D Hepatoma Cells

Metabolites	t_{40} without substrate added	Exogenous substrates		
		+5 mM Glucose	+4 mM Glutamine	+10 mM Glutamate
Glucose	1.12 ± 0.1 [800 ± 70 μM] (4)	1.3 ± 0.5 [530 ± 210 μM] (4)	1.05 ± 0.03 [630 ± 10 μM]* (4)	1.15 ± 0.02 [660 ± 10 μM]* (5)
Glutamine	0.29 ± 0.01 [210 ± 7 μM] (4)	0.56 ± 0.03** [240 ± 10 μM]* (4)	0.37 ± 0.09 [220 ± 50 μM] (4)	0.32 ± 0.03 [180 ± 10 μM]* (4)
Glutamate	13.3 ± 2 [9.5 ± 1 mM] (7)	13.2 ± 4 [5.6 ± 2 mM]* (7)	12.6 ± 4.5 [7.6 ± 3 mM] (7)	12.9 ± 8 [7.45 ± 5 mM] (7)
Glycogen	0.79 ± 0.3 (6)	1.22 ± 0.01* (6)	0.78 ± 0.2 (6)	0.84 ± 0.3 (6)

Note The values are in nmol/10^7 cells, except for glycogen, which is in μg/10^7 cells. AS-30D cells were incubated for 40 min in 1 ml of modified Krebs–Ringer medium with the indicated substrate concentration under orbital shaking (100 rpm/min) at 37°C in a plastic container The intracellular water volumes were 2.35 ± 0.3 μl (4), 1.66 ± 0.16 μl (4) and 1.73 ± 0.2 μl (5) for cells incubated for 40 min with either 5 mM glucose, 4 mM glutamine, or 10 mM glutamate, respectively The values represent the mean ± SD. *n* is shown in parentheses The data shown inside brackets represent the estimation of the metabolite concentrations using the above described intracellular water volumes

* $P < 0.005$ versus no added substrate

** $P < 0.05$ versus no added substrate

TABLE V

Contribution of Glycolysis and Oxidative Phosphorylation to Cellular ATP Production

	Incubation condition	Without substrate	+5 mM Glucose	+4 mM Glutamine	+10 mM Glutamate
Rate of external substrate consumption (nmol/min/10^7 cells)	A		0.85 ± 0.06 (4)	0.58 ± 0.16 (5)	0.75 ± 0.3 (5)
	B		1.1 (2)	0.55 (2)	0.46 (2)
Rate of lactate production (nmol/min/10^7 cells)	A, B	0.46 ± 0.07 (4)	1.35 ± 0.28* (7)	0.47 ± 0.02 (7)	0.51 ± 0.01 (7)
% ATP produced	A, B	0.19–0.21	1.8–2	0.14–0.21	0.15–0.24
Rate of oligomycin-sensitive respiration (ng atoms oxygen/min/10^7 cells)	A	85 (2)	26 (2)*	86 (2)	82.5 (2)
	B	95 ± 7 (5)	28.7 ± 6* (3)	134 ± 12* (5)	133 ± 19* (5)
% ATP produced	A, B	99.8	98	99.8	99.8

Note (A) The rate of consumption of external substrates was determined in cell suspensions (3×10^6 cells) incubated at 37°C for 40 min under gentle stirring with the indicated concentrations of oxidizable substrates. The reaction was stopped by layering the cell suspension into microcentrifuge tubes which contained, from bottom to top, 30% perchloric acid (0.3 ml), 0.3 ml 1-bromododecane, and 0.3 ml modified Krebs–Ringer medium. The tubes were centrifuged at 16,000*g* for 4 min. The top layer was used for determination of external substrates. (B) To reduce the contribution of endogenous substrates to the rate of O_2 uptake, the cells were also incubated for 60 min at 37°C, in the absence of added substrates. Then, the cells were further incubated in the presence of the indicated substrate concentrations for 10–15 min at 37°C and the rates of O_2 uptake and substrate consumption measured as described in A. The rate of ATP produced by glycolysis and oxidative phosphorylation was determined as described by Nakashima *et al.* (48). ATP production by glycolysis was calculated assuming that 1 mol of ATP is generated per mol of lactate formed. The rate of lactate formation was essentially the same under experimental condition A or B. ATP production from oxidative phosphorylation was calculated from the rate of oxygen consumption by AS-30D cells, which was corrected by subtracting the oligomycin-resistant respiration; the fraction of oligomycin-resistant respiration was constant (12 ± 6%) under the different experimental conditions. A P/O ratio of 2.5 was used; this value is in the range of the measured value in purified tumor mitochondria (48).

* $P < 0.005$ versus without substrate.

intracellular glucose content became stable at similar levels under the conditions detailed in Table IV, but the intracellular water volume was much higher in the presence of external glucose than in its absence, i.e., the intracellular volume remained rather constant for 40 min incubation in the presence of external glucose, whereas it decreased in its absence. A larger intracellular volume was also attained by the addition of glutamate or glutamine (see legend to Table IV for values). The intracellular concentrations of glutamine were not modified by addition of external substrates (Table IV). Regardless of the significant elevation in the internal glutamine level in the presence of external glucose, the larger intracellular volume resulted in identical intracellular concentration of glutamine. Similarly to internal glucose, the intracellular concentrations of glutamate were also lowered by addition of external substrates. This observation suggests that external substrates increased the rates of glucose and glutamate consumption to a greater extent than the rates of glucose and glutamate uptake. As expected, the glycogen content was higher in the presence of external glucose, after 40 min incubation. Thus, the analysis of both metabolite content and intracellular volumes provides a more accurate description of the variations in the steady-state metabolite concentrations brought about by changes in metabolic fluxes in tumor cells. This has not been systematically determined by other groups.

The rate of glycolysis was significantly stimulated by the addition of external glucose ($P < 0.005$), but it was not affected by glutamine or glutamate (Table V). The enhanced glycolytic rate correlated well with the rate of external glucose consumption: a lactate production/glucose consumption ratio of 1.59 was obtained. In contrast, the rate of oxidative phosphorylation was markedly suppressed (68%) by external glucose. However, analysis of the total production of cell ATP revealed that even in the presence of external glucose, oxidative phosphorylation was the predominant pathway for ATP supply, while the contribution of glycolysis was negligible (Table V). Although the glycolytic ATP may be underestimated, because some of the consumed glucose (+ glycogen) generated pyruvate instead of lactate, the contribution of glycolysis to ATP supply would still be small (2.5%), even if glycolytic ATP were directly estimated from total glucose (+ glycogen) consumption. Oxidative phosphorylation stimulated by external glutamine (from 95 to 134 ng atoms oxygen (min × 10^7 cells)$^{-1}$) was inhibited by glucose only 31% ($n = 2$), in contrast to the 64% inhibition without added glutamine. These observations clearly

indicate that in AS-30D cells an oxidative type of metabolism predominates.

Control of Oxidative Phosphorylation

The analysis of metabolic control establishes that the variation in the activity or effective concentration of an enzyme [E] can change the metabolic flux [J], [$dJ/J = dE/E$] (33). The *flux control coefficient* [C_E^J] is a quantitative variable that defines the degree of control that a given enzyme in the pathway exerts on the flux (33):

$$C_E^J = (E_0/J_0)(dJ/dE).$$

The flux control analysis of oxidative phosphorylation, in liver and other non-tumoral mitochondria, has shown that the control of this pathway is distributed among several steps (34). The ATP/ADP translocator, the cytochrome oxidase, the dicarboxylate carrier, the ATP synthase, and the bc_1 complex are the main control steps and their control coefficients are in the range of 0.2–0.5 (34). The control analysis has also been applied to AS-30D isolated mitochondria (22) López-Gómez *et al* (22) found that the main points of control in these mitochondria incubated at 30°C were the ATP synthase ($C_E^J = 0.3$) and the ATP/ADP translocator ($C_E^J = 0.6$), when using saturating concentrations of either glutamate-malate, pyruvate–malate, or succinate–rotenone. Therefore, it was judged relevant to determine the flux control exerted by the enzymes involved in the pathway in *in situ* mitochondria, i e , in intact cells that oxidize physiological concentrations of substrates

Control distribution of oxidative phosphorylation was initially analyzed in AS-30D hepatoma cells incubated at 37°C, in the absence of added substrates The rate of respiration was 80–100 ng atoms oxygen (min $\times$ 10^7 cells)$^{-1}$. These respiratory rates were similar to those reported for perfused liver (35)· 2.25 nmol O_2/mg wet weight in liver versus 3 nmol O_2/mg wet weight in AS-30D cells, assuming that 1×10^7 AS-30D cells have a wet weight of 17.5 ± 6 mg ($n = 4$). This indicates that AS-30D tumor cells consume oxygen at high rates.

The flux control coefficients were determined by the inhibition titration method, using specific mitochondrial inhibitors (36) and measuring the resulting respiratory rates after appropriate incubation times (Fig 1) Oligomycin was the slowest inhibitor; for this reason, this inhibitor was allowed to equilibrate for several minutes (2–4 min at 37°C), until a new, inhibited steady state was reached.

It should be noted that, even at saturating inhibitor concentrations, there was a residual respiration (10–20%), that indicated the existence of O_2 uptake reactions not linked to oxidative phosphorylation The percentage of inhibitor-resistant respiration found in the presence of several respiratory chain inhibitors was similar to that found with ATP synthase inhibitors Hence, it was evident that 80–90% of cellular respiration in AS-30D (Table VI) was mitochondrial and used for ATP synthesis. The lesser inhibition of cell respiration induced by carboxyatractyloside and 3-hydroxycyanocinnamate was very likely due to a limited permeability of these hydrophilic inhibitors.

In contrast to other cell types, site I of the respiratory chain exerted the main control of oxidative phosphorylation, while the translocator of ATP/ADP showed a C_E^J value lower than 0.1 Estimation of C_E^J values for sites II and III and ATP synthase with two different specific inhibitors showed similar results (Table VI), indicating that the estimation of C_E^J was independent of the type of inhibitor used. The enzyme contents estimated for site III, ATP synthase, and ATP/ADP translocator were similar to those reported for isolated AS-30D and brain mitochondria (22, 37), while the values of E_e for sites I and II were lower. The values of the dissociation constant (K_d) found for in situ AS-30D mitochondria were very similar to those reported in other types of isolated mitochondria (37) These observations indicated that the enzyme–inhibitor interaction was not altered by the presence of the additional permeability barriers found in the cell The sum of C_E^J values for the evaluated steps was lower than 1.0 ($\Sigma C_E^J = 0.43$), indicating that significant control flux is exerted at other sites

With added glutamine (4 mM) or glutamate (10 mM), the C_E^J of site I did not change (the $C_{site\ I}$ value was 0.33 ($n = 2$) or 0.30 ($n = 2$) for glutamine or glutamate, respectively); however, in the presence of 5 mM glucose, the $C_{site\ I}$ increased to 0.47 ± 0.1 ($n = 5$; $P < 0.025$), suggesting an inhibition of site I activity, induced by external glucose catabolism.

To determine whether the ATP utilization represents a block of enzymes that exerts significant control on oxidative phosphorylation, the elasticity-based control analysis was applied to estimate its flux control coefficient. This approach consists of the experimental determination of the elasticity coefficients of the two segments of the pathway that produce and consume ATP (33, 38) By applying the theorems of control analysis, the flux control coefficients of the two enzyme blocks are calculated The elasticity coefficients of the ATP producers and ATP consumers were attained from titrations of the steady-state rate of oligomycin-sensitive cell respiration, and of the ATP levels, with streptomycin (0.1–0.6 mg/10^7 cells) or oligomycin (50–200 pmol/10^7 cells) The values of the elasticity coefficients were −0 24 ± 0 05 (3) and 0.58 ± 0 25 (5) (mean ± SE), whereas the flux control coefficients were 0.7 ± 0 1 (3) and 0 3 ± 0 1 (3) for producers and consumers, respectively

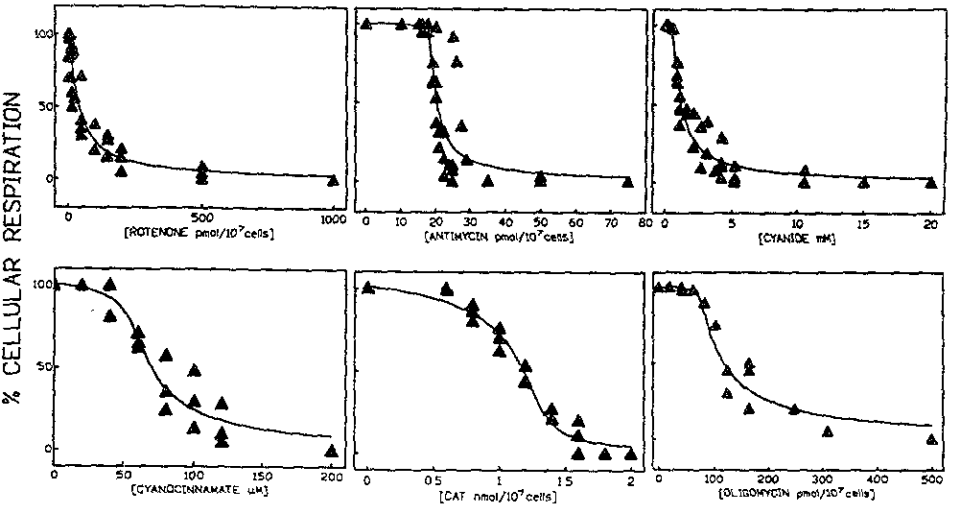

FIG. 1. Inhibition of respiration in AS-30D cells. Cells (1×10^7 cells/ml) were incubated, without substrate added, at 37°C in 1.9 ml of air-saturated Krebs–Ringer medium. The indicated inhibitor concentrations were added and the new steady-state rate of respiration was measured. The rate of respiration was normalized by subtracting the rate of respiration resistant to each inhibitor, to calculate the flux control coefficient by a non-linear regression analysis (37), according to the formulations proposed by Gellerich *et al.* (27). The solid curves represent the best fitting lines, derived from the non-linear regression analysis of the whole set of titrations made with each inhibitor for three to four independent preparations. The absolute rate of respiration in the absence of inhibitors was 92 ± 15 ng atoms oxygen (min × 10^7 cell)$^{-1}$ (n = 24). To achieve a significant inhibition of cell respiration with carboxyatractyloside (CAT) or α-cyano-4-hydroxycinnamate, 3×10^8 cells/ml were incubated with the indicated concentrations of these inhibitors for 1 h under orbital shaking (100 rpm) at 30°C. Then, an aliquot was transferred to the oxymeter chamber to measure the rate of respiration at 37°C. This procedure did not alter cell viability. The rate of respiration measured at 37°C, in cells incubated in the absence of inhibitors for 1 h at 30°C, was 87 ± 7.5 ng atoms oxygen (min × 10^7 cells)$^{-1}$ (n = 6).

DISCUSSION

Intermediary Metabolism in AS-30D Tumor Cells

The high rate of glycolysis of AS-30D (fast oxidation of glucose and accumulation of lactic acid) is in agreement with data reported in other tumoral cell lines, i.e., Morris and Novikoff hepatomas (1), Ehrlich and Ehrlich Letreé (2), and Walker carcinosarcoma (39). In addition to glucose, other metabolites were oxidized by AS-30D tumoral cells (Table I), indicating active ketone bodies and glutamate consumption and glycogenolysis.

Despite the diminution in the content of metabolites through oxidation, in cells incubated in the absence of external oxidizable substrates, the intracellular concentrations of glucose, glutamate, glutamine, pyruvate, and ATP were maintained at high values by adjustments in the intracellular water volume. This mechanism of homeostasis probably preserves the ability of these tumor cells to duplicate rapidly, when exposed to less stringent conditions. Modulation of the intracellular volume as a mechanism of control of proliferation has been proposed for neuroblastoma cells (40).

In contrast to hepatocytes, AS-30D hepatoma cells consumed ketone bodies. The liver cannot oxidize ketone bodies due to low levels of succinyl CoA–acetoacetyl CoA transferase (8). The utilization of acetoacetate in AS-30D cells was documented by Briscoe *et al.* (8) and our values agreed with their data, but β-hydroxybutyrate consumption found in AS-30D was not observed in other tumoral lines such as Walker carcinosarcoma (39) or Morris hepatoma (41). β-Hydroxybutyrate and acetoacetate consumption in AS-30D cells may be associated to the fast generation of acetyl-CoA required for lipogenesis and cholesterol synthesis (10, 12).

AS-30D tumor cells also consumed mitochondrial substrates such as glutamate, which could supply the carbon skeletons that feed the Krebs cycle as a compensatory

TABLE VI

Flux Control Coefficients of Several Steps of Oxidative Phosphorylation in AS-30D Cells

Complex	C_E^i	K_i pmol/10^7 cells	E_o pmol/10^7 cells	% Resistant respiration	Inhibitor
Site I (NADH dehydrogenase)	0.30 ± 0.1 (4)	9 ± 1 (4)	43 ± 5 (4)	13 ± 4 (4)	Rotenone
Site II (b-c$_1$ cytochrome complex)	0.023 ± 0.008 (4)	0.007 ± 0.004 (4)	21 ± 3 (4)	16 ± 4 (3)	Antimycin
	0.01 ± 0.007 (3)	0.08 ± 0.05 (3)	65 ± 0.05 (3)	9 ± 2 (3)	Myxothiazol
Site III (cytochrome c oxidase)	0.008 ± 0.004 (3)	0.047 ± 0.01 mM (3)	0.7 ± 0.1 mM (3)	18 ± 6 (3)	Azide
	0.04 ± 0.01 (4)	0.025 ± 0.018 mM (4)	0.6 ± 0.05 mM (4)	8 ± 3 (4)	Cyanide
ATP synthase	0.02 ± 0.006 (3)	0.3 ± 0.1 (3)	76 ± 11 (3)	13 ± 8 (3)	Oligomycin
	0.023 ± 0.01 (3)	0.9 ± 0.04 (3)	115 ± 26 (3)	20 ± 9 (3)	Venturicidine
ATP/ADP Translocase	0.088 ± 0.01 (3)	3.7 ± 0.1 (3)	980 ± 130 (3)	41 ± 13 (5)	Carboxyatractyloside
Pyruvate carrier	0.026 ± 0.01 (3)	0.41 ± 0.05 μM (3)	61 ± 17 μM (3)	55 ± 13 (13)	3-Hydroxycinnamate

Note. The values represent mean ± SD. n is shown in parentheses.

mechanism for a low pyruvate oxidation (11); this is probably related to the high transaminase and low glutamate dehydrogenase activities in tumors (42).

In the absence or in the presence of exogenous substrates, glycogen was another metabolite rapidly oxidized by AS-30D cells (Tables I, IV). This can be attributed to a high glycogenolytic activity, although the phosphorylase a activity has not yet been determined in AS-30D cells. The active glycogen consumption in AS-30D cells indicates the presence of a constant supply of glucose for glycolysis and pentose phosphate pathway.

It is remarkable that the glucose concentration found in AS-30D hepatoma ascites liquid (26 μM) was much lower than the value measured in blood from rats with developed hepatoma (4 ± 0.25 mM, n = 3), which suggests the following possibilities: (a) the presence of an extracellular glucose oxidase that transforms glucose to gluconate, consequently lowering glucose in the ascites liquid; (b) an intimate relationship between blood capillaries and tumor cells, which would not allow the equilibration of glucose with the ascites liquid, or (c) a faster transport of glucose from ascites liquid to the cells than from blood capillaries to ascites liquid.

The first possibility may be discarded since intrinsic or added glucose degradation was not observed in the cell-free ascites liquid (data not shown). The second and third alternatives have not been tested in AS-30D. However, Bading *et al.* (43) reported that cell glucose uptake, through a Na^+-dependent glucose transporter, is higher in tumor cells than in normal tissues.

The oxygen concentration in the ascites liquid was high (50 μM) (Table II), implying that the respiratory chain was not limited by O_2 concentration, since the apparent K_m of cytochrome c oxidase for O_2 is 1–2 μM, in normal mitochondria and cells (44), and ascites tumor cells (45). Hence, AS-30D cells may drive oxidative phosphorylation and other oxygen-dependent metabolic processes at high rates. Moreover, oxygen concentration was very similar to that of hepatic artery blood (30). Apparently, there might be a diminished Pasteur effect in AS-30D cells, because an active glycolysis was found even under a saturating oxygen concentration. However, there is no report in which the Pasteur effect has been directly assessed in fast-growth tumor cells.

The increase in the cytosolic and mitochondrial NADH/NAD^+ ratio induced by incubating at 37°C, without added substrates, suggested activation of both cytosolic α-glycerophosphate and glyceraldehyde 3-phosphate dehydrogenases, the cytosolic and mitochondrial $NADP^+$-dependent malic enzymes, and the Krebs cycle dehydrogenases.

The evaluation of the pyridine nucleotide redox state in AS-30D tumor cells has not been previously reported. The cytosolic and mitochondrial NADH/NAD^+ ratios were slightly higher in AS-30D cells than in hepatocytes (31) and breast cancer cells (32). It has been proposed that the elevated cytosolic NADH/NAD^+ ratio in tumor cells is the signal that triggers the progression from G_0 to G_1 in the cellular cycle (46). Other authors have proposed (47) that an increase in the NADH pool in hepatocytes, due to either hormonal stimulation or alterations in the oxidizable substrates, could promote the duplication process.

Cell ATP Supply

With 5 mM glucose, 1 × 10^7 cells/ml consumed 33 nmol glucose/ml in 40 min, during this time, lactate

formation was 43 nmol/10^7 cells. Thus, 21.5 nmol/10^7 cells of external glucose were transformed into lactate in 40 min. The remaining of external glucose (11.5 nmol/10^7 cells) could have fed oxidative phosphorylation and pentose phosphate pathway. With 4 mM glutamine or 10 mM glutamate, 1×10^7 cells/ml consumed 23.6 nmol glutamine/ml or 30.3 nmol glutamate/ml, respectively, in 40 min. The production of lactate was not increased by external glutamine or glutamate, suggesting that these mitochondrial substrates could be preferentially used for oxidative phosphorylation.

Analysis of the rates of glycolysis and oligomycin-sensitive respiration indicated that oxidative phosphorylation was the prevalent pathway in the cell supply of ATP under several near-physiological incubation conditions. In consequence, ATP utilization also depended on the rate of ATP supplied by oxidative phosphorylation. For instance, ATP utilization was 335.5 nmol/min/10^7 cells ($134 \times 2.5 + 0.47 \times 1$ in Table V) with glutamine, whereas glucose disminished ATP utilization down to 73 nmol/min/10^7 cells ($287 \times 2.5 + 1.35 \times 1$), because of its inhibitory effect on oxidative phosphorylation. Nakashima *et al.* (48) also estimated that AS-30D cells produced ATP almost entirely through oxidative phosphorylation (95%) in the absence of added substrate. However, they also reported that the participation of oxidative phosphorylation diminished drastically in the presence of glycolytic or mitochondrial substrates (48), for instance, in the presence of 6 mM glucose, the contribution of oxidative phosphorylation declined to 67%, although it also diminished to 85% of control in the presence of 0.75 mM glutamine.

These authors (48) proposed that the diminution in the proportion of ATP supplied by oxidative phosphorylation was due to a high glycolytic activity in the presence of added glucose: 25 nmol lactate/min/mg protein. This is in contrast with the values of glycolysis found in the present study (1.35 ± 0.28 nmol/min/10^7 cells or 1 nmol/min/mg protein, because 1×10^7 cells correspond to 1.34 ± 0.3 mg protein, $n = 3$, Table V) under similar, but not identical, conditions. In addition, the respiratory activity reported by Nakashima *et al.* (48) was three to four times lower than the value reported in the present study. Nakashima *et al.* experiments were done at 30°C using a medium that contained 6.2 mM KCl, 154 mM NaCl, and 11 mM sodium phosphate, pH 7.4, but which lacked Ca^{2+} and Mg^{2+}.

The measurement of the respiratory activity in AS-30D cells incubated in the Nakashima *et al.* medium yielded lower values (26.4 and 38.8 ng atoms oxygen/min/mg protein at 30 and 37°C, respectively) than those we have found in the modified Krebs–Ringer medium (36.3 and 51.7 ng atoms oxygen/min/mg protein at 30 and 37°C, respectively, see Table V). The production of lactate attained at 37°C in the Nakashima *et al.* medium was 1.1 nmol (min × 10^7 cells)$^{-1}$ or 0.82 nmol (min × mg protein)$^{-1}$. Apparently, the values we obtained for glycolysis and respiration at 37°C could be considered more physiological since the medium we used resembles more closely the composition of the extracellular milieu. It is worth noting that the viability of the cells in the aforementioned medium was lower (87%) than in the modified Krebs–Ringer medium (98%) and that erythrocyte contamination was higher too (1.5% vs 0.1%, respectively).

Control of Oxidative Phosphorylation

Site I of the respiratory chain was a major controlling step of oxidative phosphorylation (Table V), probably because of the lower enzyme content in reference to that of normal hepatocytes. Likewise, site I exerted a negligible control of flux in AS-30D isolated mitochondria incubated at 30°C (22). The difference found between the C_E^J values in cells and mitochondria is not surprising, since the C_E^J values depend on the experimental conditions, i.e., the extracellular and extramitochondrial concentrations of substrates and the NADH availability. Moreover, López-Gómez *et al.* (22) assumed that rotenone was an irreversible inhibitor instead of a noncompetitive tightly bound inhibitor. Analysis of titration curves of ADP-stimulated respiration with rotenone, in isolated AS-30D mitochondria incubated with 10 mM 2-oxoglutarate at 37°C, using the Gellerich *et al.* (27) equation for tightly bound inhibitors, showed C_E^J values for site I of 0.12 ± 0.06 ($n = 3$).

The elasticity-based control analysis gave a flux control coefficient for the ATP producers of 0.70, whereas the sum of coefficients from the inhibitor titration resulted in a value of 0.43 (cf. Table VI). Therefore, the difference between these two values (i.e., 0.27) indicates the control exerted by the nonevaluated steps in the ATP-producing segment: the passive H^+ permeability of the mitochondrial inner membrane, the Krebs cycle enzymes, and the P, and substrate carriers.

The finding that site I of the respiratory chain exerts significant control of oxidative phosphorylation, particularly in the presence of glucose, suggests that this enzyme may be a potential therapeutic target for development of antitumorigenic drugs. Along this line, it was recently described that relatively low doses of rotenone promoted a strong growth inhibition and apoptosis in HL-60 leukemia cells (49).

In conclusion, AS-30D tumor cells are able to maintain a very active glycolysis and oxidative phosphorylation. However, (i) the high concentrations of O_2 and glutamine and the low concentration of glucose determined in the cell-free ascites liquid; (ii) the high oligomycin-sensitive respiratory rates, even in the presence of saturating concentrations of glucose; and (iii) the estimated ATP provided by oxidative phosphorylation indicate a predominantly oxidative type of metabolism.

in these cells. In consequence, cellular processes with high energy demand such as growth, protein and nucleic acid biosynthesis, and formation and maintenance of ion gradients across the plasma membrane should be extremely sensitive to oxidative phosphorylation inhibitors in AS-30D hepatoma cells.

ACKNOWLEDGMENTS

This work was partially supported by Grant 25274-M from CONACyT, Mexico, and Grant 201373 from PAEP, UNAM. The authors thank Drs. A. Gómez-Puyou and G. Mac Carthy for their critical reading of the manuscript.

REFERENCES

1. Pedersen, P. L. (1978) *Prog. Exp. Tumor Res.* **22,** 190–274.
2. Eigenbrodt, E., Fister, P., and Reinacher, M. (1985) *in* New Perspectives on Carbohydrate Metabolism in Tumor Cells (Beitner, R., Ed.), Vol. 2, pp. 141–179, CRC Press, Boca Raton, FL.
3. Baggetto, L. G. (1992) *Biochimie* **74,** 959–974.
4. Pedersen, P. L. (1997) *J. Bioenerg. Biomembr.* **29,** 301–302.
5. Lazo, P. A. (1981) *Eur. J. Biochem.* **117,** 19–25.
6. Sauer, L. A., and Dauchy, R. T. (1983) *Cancer Res.* **43,** 3497–3503.
7. Medina, M. A., Quesada, A. R., and Núñez de Castro, I. J. (1991) *Bioenerg. Biomembr.* **23,** 689–697.
8. Briscoe, D. A., Fiskum, G., Holleran, A. L., and Kelleher, J. K. (1994) *Mol. Cell. Biochem.* **136,** 131–137.
9. Ohe, K., Morris, H. P., and Weinhouse, S. (1967) *Cancer Res.* **27,** 1360–1371.
10. Baggetto, L. G., and Testa-Parussini, R. (1990) *Arch. Biochem. Biophys.* **283,** 241–248.
11. Dietzen, D. J., and Davis, E. J. (1993) *Arch. Biochem. Biophys.* **305,** 91–102.
12. Dietzen, D. J., and Davis, E. J. (1994) *Arch. Biochem. Biophys.* **309,** 341–347.
13. Parlo, R. A., and Coleman, P. S. (1986) *Biochem. Biophys. Acta* **886,** 169–176.
14. Moreno-Sánchez, R., Hogue, B. A., and Hansford, R. (1990) *Biochem. J.* **268,** 421–428.
15. Schmidt, H., Siems, W., Muller, M., Dumdey, R., and Rapoport, S. M. (1991) *Exp. Cell Res.* **194,** 122–127.
16. Muller, M., Siems, W., Buttgereit, F., Dumdey, R., and Rapoport, S. M. (1986) *Eur. J. Biochem.* **161,** 701–705.
17. Bustamante, E., and Pedersen, P. L. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3735–3739.
18. Lampidis, T. J., Bernal, S. D., Summerhayes, I. C., and Chen, L. B. (1983) *Cancer Res.* **43,** 716–720.
19. Jaroszewski, J. W., Kaplan, O., and Cohen, J. S. (1990) *Cancer Res.* **50,** 6936–6943.
20. Fearon, K. C. H., Plumb, J. A., Burns, H. J. G., and Calman, K. C. (1987) *Cancer Res.* **47,** 3684–3687.
21. Koya, K., Li, Y., Wang, H., Ukai, T., Tatsuta, N., Shishido, T., and Chen, L. B. (1996) *Cancer Res.* **56,** 538–543.
22. López-Gómez, F. J., Torres-Márquez, M. E., and Moreno-Sánchez, R. (1993) *Int. J. Biochem.* **25,** 373–377.
23. Bergmeyer, H. U. (1983) in Methods of Enzymatic Analysis (Bergmeyer, H. U., Ed.), Vols. 3–9, Weinheim, Verlag Chemie.
24. Vanderkooi, J. M., Erecinska, M., and Silver, I. A. (1991) *Am. J. Physiol.* **260,** C1131–C1150.
25. Altshuld, R. A., Jung, D. W., Phillips, R. M., Narayan, P., Whitaker, T. E., Hensley, J., Hohl, C. M., and Brierly, G. P. (1994) *Am. J. Physiol.* **266,** H1103–H1111.
26. Rottenberg, H. (1979) *Methods Enzymol.* **55,** 547–569.
27. Gellerich, F. N., Kunz, W. S., and Bohnensack, R. E. (1990) *FEBS Lett.* **274,** 167–170.
28. Owen, O. E., Felig, P., Morgan, A. P., Wahren, J., and Cahill, G. F. (1969) *J. Clin. Invest.* **48,** 574–583.
29. Salway, J. G. (1969) *Clin. Chim. Acta* **25,** 109–116.
30. Jungermann, K., and Katz, N. (1982) *Hepatology* **2,** 385–395.
31. Arnze, I. J., and Hanson, R. W. (1980) *in* Principles of Metabolic Control in Mammalian Systems (Herman, R. H., Cohn, R. M., and McNamara, P. D., Eds.), pp. 495–534, Plenum, New York.
32. Mazurek, S., Michael, A., and Eigenbrodt, E. (1997) *J. Biol. Chem.* **272,** 4941–4952.
33. Kacser, H., and Burns, J. (1973) *Symp. Soc. Exp. Biol.* **27,** 65–104.
34. Moreno-Sánchez, R., and Torres-Márquez, M. E. (1991) *Int. J. Biochem.* **23,** 1163–1174.
35. Kashiwagura, T., Deutsch, C. J., Taylor, J., Erecinska, M., and Wilson, D. F. (1984) *J. Biol. Chem.* **239,** 237–243.
36. Tager, J. M., Groen, A. K., Wanders, R. J., Duszynski, J., Westerhoff, H. V., and Vervoorn, R. C. (1983) *FEBS Lett.* **151,** 1–9.
37. García, C., Pardo, J. P., and Moreno-Sánchez, R. (1996) *Biochem. Arch.* **12,** 157–176.
38. Moreno-Sánchez, R., Bravo, C., and Westerhoff, V. (1999) *Eur. J. Biochem.* **264,** 427–433.
39. Argilés, J. M., and López-Soriano, F. J. (1991) *J. Biol. Chem.* **266,** 2978–2982.
40. Rouzaire-Dubois, B., and Dubois, J. M. (1998) *J. Physiol.* **510,** 93–102.
41. Fenselcau, A., Wallis, K., and Morris, H. P. (1975) *Cancer Res.* **35,** 2315–2320.
42. Moreadith, R. W., and Lehninger, A. L. (1984) *J. Biol. Chem.* **259,** 6215–6221.
43. Bading, R. J., Mircheff, A. K., and Kan-Mitchell, J. (1996) *Life Sci.* **58,** 1445–1452.
44. Brand, M. D., and Murphy, M. P. (1987) *Biol. Rev. Camb. Philos. Soc.* **62,** 141–193.
45. Ferreira, J. (1992) *Eur. J. Biochem.* **119,** 857–866.
46. Schwartz, J. P., Passonneau, J. V., Johnson, G. S., and Pastan, J. (1979) *J. Biol. Chem.* **249,** 4138–4143.
47. Koretsky, A. P., and Balaban, R. S. (1987) *Biochim. Biophys. Acta* **893,** 398–408.
48. Nakashima, R. A., Paggi, M. G., and Pedersen, P. L. (1984) *Cancer Res.* **44,** 5702–5706.
49. Matsunaga, T., Kudo, J., Takahashi, K., Dohmen, K., Hayashida, K., Okamura, S., Ishibashi, H., and Niho, Y. (1996) *Leuk. Lymph.* **20,** 487–494.

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From: Joe.McCord@UCHSC.edu Save Address Block Sender
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Subject: RE: ABB manuscript
Date: Fri, 27 Aug 1999 16:12:12 -0600

Dear Dr. Rodriguez-Enriquez:

The comments of the reviewer should have been mailed to you on July 27. Perhaps you have not yet received this mail, if you were responding only to my e-mail message. Here are the reviewer's comments:

Comments:

This paper describes a thorough analysis of energy metabolism in AS-30D hepatoma cells under different substrate conditions. Strengths of the paper include the use of a variety of substrate conditions, the analysis of metabolite concentrations corrected for cellular volume changes, estimation of the ATP supplied by glycolysis and oxidative phosphorylation, and an estimate of the control distribution for oxidative phosphorylation by inhibitor titrations. The authors make the point that a knowledge of the control of energy metabolism of fast-growing tumor cells may help devise more targeted drug treatment protocols. Be this as it may, this type of analysis has gone out of fashion several years ago and it is rare and refreshing nowadays to come across a paper that goes into so much detail to analyze the metabolic performance of a specific cell type.

Despite this generally favorable assessment, there are a few issues that raise some concerns, which the authors need to address.

1. There is a question of how comparable the conditions are under which O2 consumption rates and metabolite levels were measured from which metabolic activities were calculated in Table V. From the description in the legend to Table V, it appears that substrate consumption was measured over 40 minutes without preincubation, but that O2 uptake was determined over 10-15 minutes after a 60 minute preincubation to deplete endogenous substrates. How linear are metabolic activities under these conditions? The rates of glucose, utilization and the NADH/NAD+ ratios appeared to be changing under the
incubation conditions used in Table I, but it is not clear how much of this would also apply to the conditions with exogenous substrates used.

This is a matter of concern particularly with regard to the estimates of ATP production. As the calculations come out now, it would appear that glucose suppresses not only the rate of respiration, but also markedly decreases the total ATP utilization of the cells, from an estimated 238 nmol/min/10^7 cells with endogenous substrate (95 x 2.5 + 0.42 x 1) to 73 nmol/min in the presence of glucose (29 x 2.5 + 1.35 x 1), whereas glutamine and glutamate increase the apparent rate of ATP utilization by about 30%. This effect of glucose is unlikely to be correct Only about 2/3 of glucose utilization is

only a fraction of the remainder is oxidized through the Krebs cycle. If a substantial proportion of glucose is used for synthetic purposes as suggested by the authors, the rate of ATP utilization would be expected to go up, not down. Alternatively, it may suggest that there is some discrepancy in the rates that are being compared here. These considerations may also be important to explain the apparent discrepancies with the findings of Nakashima et al discussed on p. 16.

2. The estimates of control coefficients by inhibitor titrations place an unexpected emphasis on site I as a dominant site of control. However, the fact that considerably less than 50% of control can be accounted for raises the question where the major portion of flux control is located in these cells. The authors suggest (p. 12) proton permeability, Krebs cycle activity and metabolite transporters, but these suggestions are not obviously compatible with the data. One issue raised by the data is the high residual respiration obtained with CAT titrations, which may affect the estimates of ANT control contributions. Did the authors try to correct their estimates for the apparently incomplete ANT inhibition? Another, possibly more likely site of the major part of flux control might be the ATP utilization by the cell. If this were correct the mitochondria in the intact cells may be operating away from State 3 conditions. The authors might consider some form of top-down control analysis, to assess to what extent the major control of respiration is located at the downstream rather than upstream sites. Alternatively, the effect of uncouplers or activation of energy demanding reactions may provide some insight.

Minor Comments

1. There are some defects of presentation in tables and figure, e.g, concentration units are omitted for glutamate in columns 3 and 4 in Table IV; or numbers are provided with excessive precision, e.g., O2 uptake in the presence of glucose in Table V; cyanide is misspelled in Fig. 1.

2. The paper needs some editorial and minor grammatical corrections. Several suggestions are noted in the margins of the manuscript, which is sent by regular mail for that reason. The authors insist on referring to Rinser-Krebs buffer, presumably of the some composition of what is usually referred to as Krebs-Ringer buffer (i.e., Ringer's solution as modified by Krebs). Other suggestions for minor corrections are suggested in the manuscript.

I am sorry for the delay, and for the fact that you have comments from only one reviewer. However, if you can accommodate these remarks into your manuscript, it will become acceptable.

Sincerely,

Joe M. McCord

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Dear Dr McCord.

Thank you for your E-mail of August 27, 1999 concerning the manuscript (No Ref ABB 99-0266-Mc 026) entitled "Substrate oxidation and ATP supply in AS-30D hepatoma cells". We were informed that the paper may become acceptable, if we could accomodate the referee's remarks into the manuscript We have tried to comply with these observations as follows.

1 We agree with the referee that the consumption used for the determination of the rates of respiration and substrate consumption in Table V were somewhat different In the present version show new data in Table V, obtained under the same experimental conditions, however, not modify the original conclusion drawn from the Table: oxidative phosphorylation is the major provider of cellular ATP in this tumor line Hence, the legend to Table V was slightly changed

The referee points out correctly that exogenous glucose decreases both oligomycin-sensitive respiration and cellular ATP utilization, but she (he) concludes that this glucose effect is unlike to be correct We argue, on the contrary, that this is precisely the expected effect induced y glucose in this tumor cell line. Since inhibition of oxidative phosphorylation by glucose (Crabtree effect) brings about a diminution in ATP utilization, the glucose should affect cell processes, such as growth, that strongly depend on ATP supply In experiments with AS-30D cell cultures, growth is about 50% lower with glucose that with glutamine as carbon source Because these data on cell cultures will be part of another work, we decided not to include this information in the present manuscript A paragraph discussing this referee's observation was included on p 16, lines 3-7 Our suggestion that a fraction of glucose could be used for triglyceride synthesis was erased as requested by the referee

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2. Estimation of flux control coefficients from the inhibitor titrations was actually made from curves from which the inhibitor resistant activity was substracted This was stated on lines 4-5 of the figure legend.

We have estimated the flux control coefficient of the ATP utilization segment, following the referee´s suggestion. Accordingly, to describe these results, paragraphs were added on p. 12 (3^{th}) and 18 (1^{st}) and the text was slightly modified in the Abstract (p.2, lines 12-13) and on p. 12, lines 3-4. We would like to thank the referee for this particular observation. We think that the paper has been strengthened by the incorporation of this new information.

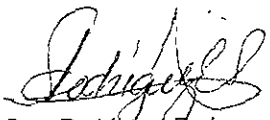
Minor Comments

1. The defects of presentation in tables and figure have been corrected.
2. We have tried to amend the grammatical errors throughout the text.

We agree to refer to the buffer used as "Krebs-Ringer".

Except for the changes specified in this letter, the rest of the manuscript has not been altered. We hope that in its present form, the manuscript will be found acceptable for publication in *Archives of Biochemistry and Biophysics*

Sincerely yours,



Sara Rodríguez-Enríquez

3.2 MULTISITE CONTROL OF THE CRABTREE EFFECT IN ASCITES HEPATOMA CELLS*

*Este artículo se encuentra en prensa en la revista *European Journal of Biochemistry*

Control multisitio del efecto Crabtree en células de hepatoma AS-30D.

El efecto Crabtree o inhibición de la fosforilación oxidativa por glucosa o fructosa exógenas es un fenómeno frecuentemente encontrado en los tumores glucolíticos. Sin embargo, a pesar de que ha sido estudiado en varias líneas tumorales no existe explicación satisfactoria acerca de cual es la naturaleza del factor o factores responsables. En AS-30D, la glucosa o fructosa promovieron una inhibición parcial de la respiración celular acoplada a la síntesis de ATP (respiración resistente a oligomicina) que osciló alrededor del 50%, la cual fue restaurada por la adición de un desacoplante clásico. Esta observación indica que el sistema fosforilante es el que se encuentra afectado. La adición de glucosa o fructosa provocó una disminución en la concentración intracelular de fosfato (40%), y de ATP (53%) así como un cambio en el pH citosólico de 7.2 a 6.8. Determinamos que en esta condición, parte del fosfato fue incorporado a glucosa 6-fosfato, fructosa 6-fosfato y fructosa 1,6-bifosfato debido a que su contenido intracelular aumentó considerablemente (15,13 y 50 veces, respectivamente. A diferencia de otras líneas tumorales de rápido crecimiento como Ehrlich, la concentración intracelular de calcio no cambió en presencia de glucosa o fructosa, sugiriendo que en AS-30D la variación en este factor no está relacionado con el efecto Crabtree. Para dilucidar si la disminución en el fosfato y pH citosólicos eran los únicos factores que promovían un abatimiento de la FO, utilizamos mitocondrias aisladas de AS-30D las cuales fueron expuestas a un medio con baja concentración de fosfato (0.6 mM) y pH 6.8. Solo en la condición en la que se disminuyó simultáneamente el fosfato y el pH, la fosforilación oxidativa disminuyó 40% su actividad. Lo anterior puede explicarse ya que el pH y el fosfato son potentes reguladores de varias enzimas de la vía. Los resultados anteriores sugieren que el efecto Crabtree es el resultado de cambios metabólicos a diferentes niveles, que dan como consecuencia una inhibición parcial de la respiración utilizada para la síntesis de ATP.

Eur J Biochem 268, 1–9 (2001) © FEBS 2001

Multisite control of the Crabtree effect in ascites hepatoma cells

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AS-30D hepatoma cells, a highly oxidative and fast-growing tumor line, showed glucose-induced and fructose-induced inhibition of oxidative phosphorylation (the Crabtree effect) of 54% and 34%, respectively. To advance the understanding of the underlying mechanism of this process, the effect of 5 mM glucose or 10 mM fructose on the intracellular concentration of several metabolites was determined. The addition of glucose or fructose lowered intracellular P_i (40%), and ATP (53%) concentrations, and decreased cytosolic pH (from 7.2 to 6.8). Glucose and fructose increased the content of AMP (30%), glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate (15, 13 and 50 times, respectively). The cytosolic concentrations of Ca^{2+} and Mg^{2+} were not modified. The addition of galactose or glycerol did not modify the concentrations of the metabolites. Mitochondria isolated from AS-30D cells, incubated in media with low P_i (0.6 mM) at pH 6.8, exhibited a 40% inhibition of oxidative phosphorylation. The data suggest that the Crabtree effect is the result of several small metabolic changes promoted by addition of exogenous glucose or fructose.

Keywords: Crabtree effect; fast-growth tumor cells; multisite control, oxidative phosphorylation.

Fast-growing tumor cells are characterized by a high glycolytic activity, even in the presence of saturating oxygen. In these cells it is also known that cellular oxidative phosphorylation is strongly inhibited by exogenous glucose [1]. A diminished or absent Pasteur effect would explain the former phenomenon. However, for the glucose-induced inhibition of oxidative phosphorylation, known as the Crabtree effect [2], there is no satisfactory explanation. The Crabtree effect is not restricted to malignant cells as it is also observed in normal proliferative cells [3]. In fact, in several nontumoral tissues (pig platelets, hamster embryos, proliferating thymocytes, retina, intestinal mucosa) [4–7] and in bacteria and yeast [8,9], it has been reported that oxidative phosphorylation is inhibited by exogenous hexoses.

Eigenbrodt *et al.* proposed that this process could be a selective advantage for tumor cells, as the consequence of a glucose-dependent accumulation of essential metabolic intermediates, such as serine, phosphoribosyl-pyrophosphate, fructose 1,6-bisphosphate, and glycerol 3-phosphate, which can trigger the mitogenic events [10]. Several mechanisms have been suggested to explain the Crabtree effect in tumor cells: (a) a glucose-induced increase in cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) would inhibit oxidative phosphorylation through the increased association of the inhibitory subunit to the ATP synthase [11–13]; (b) competition between oxidative phosphorylation and glycolysis for ADP and P_i [14]; (c) a decrease in the cytosolic pH, as a consequence of lactic acid formation, that diminishes the activity of oxidative enzymes [15]; and (d) damage of the mitochondrial membranes by free radicals, produced as a consequence of glucose catabolism [16].

In this work, we have evaluated some of these hypotheses, using the oxidative ascites tumor AS-30D cell line as the experimental model. We show that several factors are involved in the onset of the Crabtree effect.

MATERIALS AND METHODS

Cell preparation

AS-30D ascites tumor cells were propagated by inoculation of 2×10^8 cells into the peritoneal cavity of female Wistar rats of 200–250 g weight. The cells were harvested, and washed as described previously [17]. They were stored in ice at a density of $2–3 \times 10^8$ cells mL^{-1} until use.

Isolation of mitochondria

AS-30D mitochondria were isolated by the digitonin permeabilization procedure described by Moreadith and Fiskum [18], with some minor modifications. The final concentration of digitonin (Sigma Chemicals) used for plasma membrane solubilization was 40 ± 10 μg mg cellular protein^{-1}. The mitochondrial pellet was incubated with 0.5% (w/v) fatty acid free-albumin and 1 mM ADP for 10–15 min at 4 °C before the final centrifugation.

Determination of metabolites

Cell suspensions were incubated in Krebs–Ringer medium under gentle stirring (100 r.p.m.) at 37 °C, in plastic centrifuge tubes. After 1 min, 5 mM glucose, 10 mM fructose, 10 mM galactose or 10 mM glycerol was added

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Abbreviations: BCECF, 2′,7′-bis(carboxyethyl) 5(6) carboxyfluorescein; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; $[Ca^{2+}]_c$, cytosolic free Ca^{2+}; $[Ca^{2+}]_m$, mitochondrial matrix free Ca^{2+}; $[Mg^{2+}]_c$, cytosolic free Mg^{2+}; $[Mg^{2+}]_m$, mitochondrial matrix free Mg^{2+}; PFK-1, phosphofructokinase-1.

(Received 25 January 2001, accepted 6 March 2001)

liquots were withdrawn 5 min later and quenched with e-cold $HClO_4$ (3%, v/v, final concentration) For the etermination of intracellular P_i, aliquots of the cell spension were centrifuged through a 1-bromododecane ayer into 3% (v/v) $HClO_4$ solution at 16 000 *g* for 4 min he $HClO_4$ extracts were neutralized with 10 M KOH/0.1 M ris and the precipitated $KClO_4$ was removed by centriugation. The neutralized samples were used for determination of ATP, ADP, AMP, P_i, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, fructose 1,6-isphosphate and lactate by standard enzymatic assays [19,20]

Determination of O_2 consumption

The rate of respiration of intact cells ($1–2 \times 10^7$ cells mL^{-1}) was determined with a Clark-type oxygen electrode at 37 °C, in 1.9 mL of air-saturated Krebs–Ringer medium Respiration of isolated mitochondria (1–3 mg protein mL^{-1}) was determined at 30 °C, in 1.9 mL of a medium containing 120 mM KCl, 20 mM Mops, 1 mM EGTA (KME medium), plus 1 mM $MgCl_2$, 0.05% (w/v) albumin, at different pH values (7.2 or 6.8), and phosphate concentrations (1.5 or 0.6 mM).

Determination of $[Mg^{2+}]_c$, $[Ca^{2+}]_c$ and cytosolic pH

AS-30D cells were loaded for 20 min at 30 °C with either 3 μM Indo-1/AM, 3 μM Mag-Indo-1/AM or 5 μM 2',7'-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) by incubating 1×10^8 cells mL^{-1} in 3 mL of Krebs–Ringer medium, which also contained 1.3 mM EGTA and 2% albumin, pH 7.4 Dye-loaded cells were washed once by centrifugation, resuspended in Krebs–Ringer medium with 1 mM EGTA and kept on ice until use. This loading protocol did not affect cell viability Aliquots of Indo-1, Mag-Indo-1 or BCECF-loaded cells were added to 2.5 mL Krebs–Ringer medium to give a density of 2.5×10^6 cells mL^{-1} The basal fluorescence was recorded in a dual-wavelength spectrofluorometer equipped for continuous gassing with 100% O_2, and magnetic stirring at 37 °C After 1–2 min, 5 mM glucose or another hexose was added To determine Ca^{2+} in Indo-1-loaded cells [21], the excitation wavelength was 350 nm and emission was simultaneously recorded at 395 and 485 nm To determine Mg^{2+} in Mag-Indo-1-loaded cells [22], the excitation wavelength was 336 nm and emission was recorded at 400 and 500 nm To determine pH in BCECF-loaded cells [23], the excitation wavelengths were 450 and 500 nm, fluorescence was measured at 550 nm

$[Ca^{2+}]_c$ and cytosolic free $[Mg^{2+}]_c$ were determined from the fluorescence ratio signal (R, 350/395 nm or 336/400 nm, respectively, of Indo-1- and Mag-Indo-1-loaded cells) Maximum (R_{max}) and minimum (R_{min}) signals were obtained at the end of each experiment For both cations, R_{min} was generated by addition of 160 pmol digitonin 10^7 cells^{-1} EGTA/Tris (2 mM) or EDTA/Tris (2 mM), pH 8.0 was included, to chelate all Ca^{2+} or Mg^{2+} ions present in the incubation medium, Triton X-100 (0.02%, v/v) was added to ensure complete ion equilibration across the cellular membranes R_{max} was obtained after addition of $CaCl_2$ (10 mM) or $MgCl_2$ (30 mM) Calculation of $[Ca^{2+}]_c$ and $[Mg^{2+}]_c$ was made using the following equations [21,22]:

$$[Ca^{2+}]_c = \frac{K_{d_{Ca^{2+}}} R - R_{min}}{R_{max} - R} \cdot \frac{S_f}{S_b} \quad (1)$$

$$[Mg^{2+}]_c = \frac{K_{d_{Mg^{2+}}} R - R_{min}}{R_{max} - R} \cdot \frac{S_f}{S_b} \quad (2)$$

where $K_{d_{Ca^{2+}}}$ and $K_{d_{Mg^{2+}}}$ are the dissociation constants for the cation–dye complex For the former case, the K_d used was 179 nM at pH 7.2 and 37 °C [24]. The K_d for the Mg–dye complex determined experimentally at 37 °C was 2.7 ± 0.4 mM ($n = 3$) S_f and S_b are the dye fluorescence intensities at 485 nm (or 500 nm) in the absence or with an excess of Ca^{2+} (or Mg^{2+}), respectively.

A plot of pH values (measured with a pH glass electrode) versus the fluorescence ratio signal was used to calibrate the BCECF fluorescence signal The fluorescence was measured in BCECF-loaded cells (2.5×10^6 cells mL^{-1}) incubated in Krebs–Ringer medium, at the desired pH, and treated with 10 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), 160 pmol digitonin 10^7 cells^{-1} and 0.02% (v/v) Triton X-100 to equilibrate all ion gradients The plot yielded a straight line between pH 6.8 and 8.0, in agreement with a previous report [25]

Determination of free Ca^{2+} and Mg^{2+} in the mitochondrial matrix

Hepatoma AS-30D mitochondria were loaded with Fluo-3 or Mag-Fura-2 by incubating 40 mg of mitochondrial protein with either 5 μM Fluo-3/AM or Mag-Fura-2/AM at 25 °C for 20 min The loading medium (2 mL) contained 250 mM sucrose, 10 mM Hepes, 1 mM EGTA (SHE medium), 1 mM $MgCl_2$, 1 mM ADP, 0.2% fatty acid-free bovine serum albumin, pH 7.4 Mitochondria, washed once by dilution and centrifugation in ice-cold SHE medium, were kept on ice until use The respiratory control values of dye-loaded mitochondria were slightly lower than those of nonloaded mitochondria, using 0.5 mM pyruvate (+0.3 mM malate) as substrate

Free Mg^{2+} in the mitochondrial matrix ($[Mg^{2+}]_m$) was determined as previously described [25] To determine free Ca^{2+} in the mitochondrial matrix ($[Ca^{2+}]_m$), Fluo-3-loaded mitochondria (0.5 mg protein mL^{-1}) were incubated at 30 °C, in KME medium plus 10 mM succinate, and 5 μM rotenone, at different pH (7.2 or 6.8), and phosphate concentrations (1.5 or 0.6 mM). To avoid interference by matrix NADPH fluorescence, pyruvate was not used as an oxidizable substrate for determinations of $[Ca^{2+}]_m$ The excitation wavelength for Fluo-3-loaded mitochondria was 506 nm and emission was measured at 526 nm F_{max} and F_{min} were obtained at the end of each experiment F_{min} was generated by addition of 800 pmol A23187 mg protein^{-1} and sufficient EGTA/Tris, pH 8.0, to chelate all the Ca^{2+} present in the incubation medium, 0.01% (v/v) Triton X-100 was added to ensure complete Ca^{2+} equilibration across the membrane F_{max} was obtained after further addition of 10 mM $CaCl_2$ Calculation of $[Ca^{2+}]_m$ was made using a K_d of 516 nM at pH 7.0 and 22 °C for the Ca^{2+}–dye complex [24]

Q4 ✓ Q5 ✓ Q6 ✓ Q7 ✓ Q8 OK Q9 OK

Maximum (F_{max}) and minimum (F_{min}) fluorescence

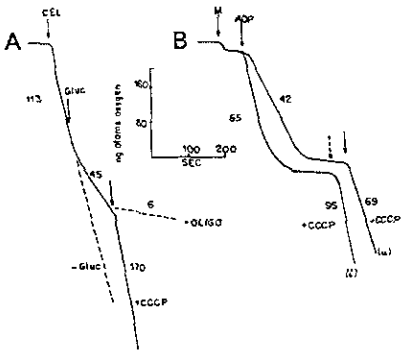

Fig 1 Inhibition of oligomycin-sensitive respiration by glucose. (A) AS-30D cells (1.7×10^7 cells mL^{-1}) were incubated at 37 °C, in air-saturated Krebs–Ringer medium. Once a steady-state rate of *endogenous respiration was established, 5 mM glucose* was added and the new steady-state respiration rate was measured. Other additions were oligomycin (1 nmol 10^7 cells^{-1}) or CCCP (10 μM). (B) AS-30D mitochondria (2.5 mg protein mL^{-1}) were incubated at 30 °C in air-saturated KME medium with 0.5 mM pyruvate plus 0.3 mM malate as substrates, and 1.5 mM P_i, pH 7.2 (trace 1) or 0.6 mM P_i, pH 6.8 (trace 2). State 3 respiration was started by addition of 500 nmol ADP. CCCP (3 μM) was added at the end of each experiment. The numbers on the traces indicate the rates of respiration in ng atoms oxygen (min × 10^7 cells or mg protein)$^{-1}$. CEL, xxxxx, glucose, M, xxxxx. CEL, cells ; M, mitochondria.

RESULTS

Oxygen uptake in AS-30D cells in presence of exogenous glucose and other carbohydrates

The rate of respiration of freshly isolated AS-30D cells was 90–95% oligomycin-sensitive, thus, most of the cellular O_2 uptake was associated with mitochondrial ATP synthesis. The addition of 5 mM glucose (Fig. 1A) or 10 mM fructose (*Table 1*) *induced a significant inhibition of cellular*

Table 1. *Effect of* D-glucose and other carbohydrates on the oligomycin-sensitive respiratory rate in AS-30D ascites tumor cells. The rate of respiration was normalized by subtracting the rate of respiration resistant to oligomycin (0.5–1 nmol 10^7 cells^{-1}). The values represent mean ± SD with the number of different preparations assayed between parentheses. AO atoms oxygen

Condition	Respiration rate (ng AO min^{-1} 10^7 cells^{-1})	% Inhibition
No added substrate	104 ± 15 (19)	
Glucose 5 mM	47.5 ± 10 (5) *	54
Fructose 10 mM	69 ± 12 (5) *	34
Galactose 10 mM	90 ± 26 (5)	13
Glycerol 10 mM	95.5 ± 16 (7)	8

* $P < 0.005$ versus no added substrate.

Table 2 $[Ca^{2+}]_c$ and $[Mg^{2+}]_c$ in AS-30D cells. Indo 1-(fCa^{2+}) or Mag-Indo 1-(Mg^{2+}) loaded cells (1×10^7 mL^{-1}) were incubated in 2 mL Ringer Krebs medium at 37 °C. The fluorescence ratio signal was measured and calibrated as described in Materials and methods. The data shown represent the mean ± SD with the number of different preparations assayed between parentheses

Condition	$[Ca^{2+}]_c$ (nM)	$[Mg^{2+}]_c$ (mM)
No added substrate	82.5 ± 8 (5)	0.9 ± 0.2 (4)
Glucose 5 mM	94 ± 7 (5)	1.2 ± 0.4 (4)

respiration. The hexose-induced inhibition of oxidative phosphorylation reached a steady state after 1.5–3 min, which was stable for at least 15 min. The reversal of the glucose-induced respiratory inhibition by the H^+ ionophore CCCP (Fig. 1A), which stimulates the activity of the respiratory chain, suggested a specific effect on the phosphorylating system, *i.e. the ATP synthase, the ATP/ADP* exchanger, and/or the P_i carrier. Addition of galactose or glycerol did not promote inhibition of cellular respiration (data not shown), suggesting a low activity of the enzymes involved in the metabolism of these compounds.

In turn, the rate of lactate formation was 77 ± 16 nmol min^{-1}·g wet weight^{-1} or 1 ± 0.3 nmol min^{-1} mg protein^{-1} in cells incubated at 37 °C with *5 mM glucose, whereas the activity of the phospho*-fructokinase-1 (PFK-1) was 180 ± 16 nmol fructose 1,6-bisphosphate min^{-1} mg protein^{-1} ($n = 7$). These rates of glycolysis and PFK-1 activity are within the range of values reported for AS-30D [26,27] and other tumor cells [14].

Effect of exogenous glucose on $[Ca^{2+}]_c$ and $[Mg^{2+}]_c$

It has been reported that changes in $[Ca^{2+}]_c$, promoted by added glucose, induce a strong diminution of oxidative phosphorylation in Ehrlich and Ehrlich Letre cells [11–13]. *However, we observed that in AS-30D cells, the* addition of exogenous glucose did not modify the intracellular concentration of Ca^{2+}, which remained at a constant level for at least 30 min (Table 2). The $[Mg^{2+}]_c$ was also not modified by glucose. These results indicate that these cations are not involved in the mechanism of the Crabtree effect, as has been suggested for Ehrlich and Ehrlich Lettré cells [11–13].

Changes in the content of adenine nucleotides, P_i and phosphorylation potential

The ATP content diminished significantly after the addition of glucose, this diminution was still observed after an incubation of 15 min. The ADP content remained constant, but the AMP content increased slightly after 5 min from the addition of glucose or fructose (Table 3). After 15 min of incubation with glucose, a significant decrease in the ADP content (0.4 ± 0.2, $n = 8$) was observed. The intracellular P_i *concentration remained constant after 15 min* of incubation in the absence of added substrates (data not shown). P_i concentration diminished more than 50% in the presence of glucose after 5 min of incubation (Table 3), but it slowly returned to 85% of control values after 15 min. In consequence, the addition of glucose or fructose induced

Table 3. Effect of D-glucose and other carbohydrates on adenine nucleotides and P_i contents. AS-30D cells (1×10^8 cells mL^{-1}) were incubated for 5 min as described in Materials and Methods. The indicated substrates were added to initiate the reaction. The values represents the mean ± SD, with the number of different preparations assayed between parentheses. The data shown inside square brackets represent the estimation of the metabolite concentration using the intracellular water volume of 2.3 μL 10^7 cells^{-1} obtained in presence of glucose [17].

Condition	[Adenine nucleotide] (nmol 10^7 cells^{-1}) ATP	ADP	AMP	Total	[ATP/ADP] (nmol 10^7 cells^{-1})	P_i
No added substrate	1.7 ± 0.2 (9)	1.4 ± 0.3 (7)	1.3 ± 0.1(6)	4.4 ± 0.6	1.21	3.8 ± 0.6 (10) [1.5 mM]
Glucose 5 mM	0.9 ± 0.1[a] (7)	1.0 ± 0.4 (7)	1.7 ± 0.1[a] (6)	3.6 ± 0.6	0.9	1.5 ± 0.3[a] (8) [0.6 mM][a]
Fructose 10 mM	0.9 ± 0.2[a] (6)	0.9 ± 0.4 (6)	1.9 ± 0.4 (5)	3.7 ± 1	1.0	1.4 ± 0.2[a] (8)
Galactose 10 mM	1.9 ± 0.2 (6)	1.4 ± 0.1 (5)	1.5 ± 0.2 (6)	4.8 ± 0.2	1.35	3.2 ± 0.3 (4)
Glycerol 10 mM	1.8 ± 0.1 (6)	1.5 ± 0.2 (9)	1.3 ± 0.1 (5)	4.6 ± 0.1	1.2	2.8 ± 0.2[b] (4)

[a] $P < 0.005$ versus no added substrate. [b] $P < 0.01$ versus no added substrate.

Table 4. Content of monophosphate and bisphosphate hexoses in AS-30D ascites tumor cells. The values represents the mean ± SD, with the number of different preparations assayed between parentheses.

Condition	Content (nmol 10^7 cells^{-1}) Glucose 6-phosphate	Fructose 6-phosphate	Fructose 1,6 bisphosphate	Glucose 1-phosphate (pmol 10^7 cells^{-1})
No added substrate	0.04 ± 0.01 (6)	0.03 ± 0.02 (6)	0.3 ± 0.05 (3)	8 ± 1 (4)
Glucose 5 mM	0.5 ± 0.2[a] (7)	0.4 ± 0.2[a] (8)	15 ± 1.6[a] (4)	7 ± 1 (4)
Fructose 10 mM	0.7 ± 0.3[a] (8)	0.4 ± 0.1[a] (8)	13 ± 3[a] (4)	9 ± 1 (4)
Galactose 10 mM	0.04 ± 0.009 (4)	0.02 ± 0.01 (5)	0.9 ± 0.4 (4)	8 ± 2 (4)
Glycerol 10 mM	0.5 ± 0.2 (5)	0.03 ± 0.02 (9)	0.5 ± 0.1 (4)	7 ± 3 (4)

[a] $P < 0.005$ versus no substrate added.

a diminution in the ATP/ADP ratio and an increase in the phosphorylation potential (ATP [ADP × P_i]$^{-1}$) from 0.32 to 0.6–0.7 (Table 3). The content of adenine nucleotides was unchanged by addition of galactose or glycerol, however, the P_i content was slightly diminished by glycerol.

Effect of glucose and fructose on levels of monophosphate and bisphosphate hexoses

The addition of glucose or fructose led to an increase in the cytosolic levels of glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate, and no change in glucose 1-phosphate content (Table 4). This indicates that a significant fraction of P_i was used in the synthesis of the monophosphate and bisphosphate hexoses, albeit P_i incorporation into other phosphorylated compounds, such as fructose 2,6-bisphosphate or phosphoribosyl-pyrophosphate, could also have taken place. The increase in the concentrations of glucose 6-phosphate, fructose 6-phosphate and fructose 1,6-bisphosphate induced by glucose reached a steady state, that is, no change was observed between 5 min (Table 4) and 15 min of incubation (data not shown). Indeed, glucose addition also increases the level of these metabolites in Sarcoma 180 and other tumor cell lines [10,14].

Table 5. Cytosolic pH in AS-30D hepatoma cells in presence of glucose and other carbohydrates. The values represent the mean ± SD, with the number of different preparations assayed between parentheses.

Condition	Cytosolic pH (5-min point)
No added substrate	7.2 ± 0.05 (7)
Glucose 5 mM	6.8 ± 0.2[a] (7)
Fructose 10 mM	7.01 ± 0.14 (7)
Galactose 10 mM	7.1 ± 0.1 (4)
Glycerol 10 mM	7.0 ± 0.2 (6)

[a] $P < 0.005$ versus no substrate added.

Changes in cytosolic pH in the presence of glucose and other carbohydrates

During glucose catabolism, cytosolic pH decreases as a consequence of a high lactate and H^+ production [15]. In the absence of added substrate, cytosolic pH was 7.2 ± 0.05 ($n = 7$) and remained constant for 5–10 min or longer (Table 5). The addition of glucose or fructose induced an immediate decrease of 0.4 in the cytosolic pH. This was slowly restored to the cytosolic pH of the control after approximately 15 min (data not shown).

Effect of the diminution of P_i concentration and pH on oxidative phosphorylation of isolated AS-30D mitochondria

In a simulation of the factors involved in the glucose-induced inhibition of oxidative phosphorylation, isolated

Table 6. Effect of lowering the extramitochondrial P_i and pH on the rate of ATP synthesis in isolated AS-30D mitochondria. Tumor mitochondria (1 mg mL^{-1}) were isolated and incubated as described in Materials and methods with 0.5 mM pyruvate, 0.3 mM malate, and the indicated P_i concentrations and pH values, at 30 °C. The rate of state four respiration was measured before the addition of ADP. After 1–2 min, state three respiration was started by addition of 500 nmol ADP. The values represent mean ± SEM of the four different preparations assayed. The theoretical ATP synthesis values were calculated from state three × ADP/O. AO, atoms oxygen.

Condition	ng AO min^{-1} mg $protein^{-1}$		ADP/O	ATP synthesis	% Inhibition
	State four	State three			
P_i 1.5 mM + pH 7.2	17 ± 3	68 ± 4	2.4 ± 0.2	163 ± 7.5	–
P_i 0.6 mM + pH 7.2	20 ± 3	47 ± 4 *	2.2 ± 0.2	106 ± 6 *	30–35
P_i 1.5 mM + pH 6.8	17 ± 1.5	57 ± 5	2.4 ± 0.06	137 ± 5	15
P_i 0.6 mM + pH 6.8	17 ± 2	41 ± 3 *	2.4 ± 0.3	99 ± 5 *	40

* $P < 0.05$ versus condition (1).

mitochondria from AS-30D cells were incubated at pH values and P_i concentrations similar to those of intact cells (Tables 3 and 5). At pH 7.2, the steady-state rate of state three respiration, with 0.5 mM pyruvate (+ malate) and 0.6 mM P_i, was 30–35% lower than that obtained with 1.5 mM P_i (Table 6). When AS-30D mitochondria were exposed to 1.5 mM P_i at pH 6.8, there was a small decrease in state three respiration (10–15%). The simultaneous diminution in pH and P_i concentration provoked an additive diminution of state three respiration (and ATP synthesis) of 40% ($P < 0.05$, Fig. 1B). The constant ADP/O ratio (2.2–2.4) and state four (basal) respiration, together with the full respiratory activation by CCCP, indicated that oxidative phosphorylation was inhibited, but not uncoupled, by the conditions described above.

The same diminution in state three respiration was obtained with 1 mM glutamine (+ malate) or 0.5 mM α-ketoglutarate (+ malate) as substrates (data not shown), indicating that independent of the oxidizable substrate, the decrease in extramitochondrial P_i concentration and pH inhibits oxidative phosphorylation. Lowering the external ADP concentration from 1 to 0.2 mM, which was the estimated cytosolic concentration of ADP in cells incubated with glucose for 15 min, did not affect the rate of state three respiration. This indicated that such low ADP concentrations are saturating for oxidative phosphorylation. In these experiments low concentrations of malate (0.3 mM) were used in order to reduce the contribution of the mitochondrial $NADP^+$-malic enzyme. A similar decrease of state three respiration by lowering P_i and pH was achieved at 37 °C. However, at this temperature, AS-30D mitochondria showed unstable respiratory rates, low ADP/O ratios, and high rates of state four respiration (data not shown).

In an attempt to fully reconstitute the 54% inhibition of oxidative phosphorylation induced by glucose in intact cells, several compounds were added to isolated mitochondria. However, 1 mM glucose 6-phosphate, 1 mM fructose 6-phosphate, 1 mM fructose 1,6-bisphosphate, 1 mM phosphoenolpyruvate, or 5 mM acetoin did not affect oxidative phosphorylation. $[Ca^{2+}]_m$ and $[Mg^{2+}]_m$ were not modified by variations in the P_i or pH, suggesting that the concentrations of these cations were constant, and that they were not involved in the diminution of oxidative phosphorylation by exogenous glucose or fructose.

DISCUSSION

The rate of glycolysis in AS-30D cells is about 50 times higher than that found in normal hepatocytes [28], and similar to that of other tumor cells [14,30]. Interestingly, the tumor cell lines with high glycolytic rates also exhibit the Crabtree effect [2–4,10]. Moreover, thymocytes in the resting state do not have the Crabtree effect, whereas thymocytes in the proliferative state show a significantly higher rate of glycolysis and a strong glucose-induced inhibition of cellular respiration [5].

The inhibition of the oligomycin-sensitive respiration by added glucose or fructose in AS-30D tumor cells was in the same range (40–60%) of the values reported for Ehrlich Lettré [10], and other nontumorigenic cells, such as cultured hamster eight-cell embryos [4], proliferating thymocytes [5], neural tissue of rat embryos [16], renal proximal tubule cells [29], and different strains of *Escherichia coli* [8]. It has been suggested that the Crabtree effect is apparent only in tumors with comparable glycolytic and respiratory capacities for ATP synthesis (Sarcoma 180, Ehrlich or hyperdiploid Ehrlich Lettré) [14,30]. A decreased mitochondrial function in tumor cells may be due to a deficiency of their respiratory chain or oxidative phosphorylation enzymes [1,31]. However, the present study shows that, in the ascites tumor cell line AS-30D, in which oxidative phosphorylation is the main source of ATP supply [17], this effect is also observed. Similarly, some human tumor cell lines such as KB3-1 cervical carcinoma, FEMX melanoma, and A2780 ovarian carcinoma also show an oxidative type of metabolism and fast growth rates [32].

In contrast to Sarcoma 180 [14], in AS-30D cells (this work), and Ehrlich ascites cells [33], oxidative phosphorylation was also inhibited by fructose. Very probably, the fructose concentration (4 mM) that was added to Sarcoma 180 [14] was low; indeed, in our experiments 4 mM fructose did not affect cellular respiration (data not shown). This may imply that tumor cells have low activities of fructokinase-1 and fructose 1-phosphate aldolase, and a hexokinase with low affinity for fructose. In fact, depending on their differentiation state, some types of Morris hepatomas show isoenzyme alterations of some of their glycolytic enzymes. For example, glucokinase and aldolase type B are replaced by hexokinase and aldolase type A, respectively, and it has been documented that this emerging

hexokinase has a lower affinity for fructose than for glucose [33,34]

In agreement with data reported for Sarcoma 180 [14], other carbohydrates such as galactose and glycerol did not affect oxidative phosphorylation in AS-30D cells. Moreover, it has been described that the activity of glycerol 1-kinase is negligible in some tumor lines [35]. However, the activity of this enzyme has not yet been evaluated in AS-30D cells

In Ehrlich ascites cells [11,12], it has been observed that $[Ca^{2+}]_c$ levels increase from a basal level of 170 nM to 300 nM in response to added glucose The authors proposed that mitochondrial ATP synthesis and hydrolysis and the activity of the ATP/ADP carrier were inhibited due to the Ca^{2+}-induced association of the inhibitory subunit to the ATP synthase and to the formation of Ca^{2+}–ATP or Ca^{2+}–ADP complexes, respectively. However, the magnitude of similar $[Ca^{2+}]_c$ fluctuations induced by glucose is commonly found in normal and tumor cells [36,37] Furthermore, the activities of the Ca^{2+}-sensitive mitochondrial dehydrogenases were stimulated by the same range of Ca^{2+} concentrations in isolated AS-30D mitochondria [38]

AS-30D cells express the multidrug resistance phenotype through the production of P-glycoprotein 170, an ATP-driven plasma membrane pump that can extrude a large variety of lipophilic compounds [39] It is conceivable that the fluorescent dyes used in this study may be expelled by P-glycoprotein 170 and, in consequence, interfere with the measurements of Ca^{2+}, Mg^{2+}, and pH However, the amount of dye intracellularly trapped after 5 or 40 min incubation at 37 °C was essentially identical (not shown), indicating that at least during the course of a typical experiment, extrusion of dyes was negligible

It should be noted that the ATP/ADP ratio and the phosphorylation potential represents the metabolic link between glycolysis and oxidative phosphorylation Therefore, the changes observed in these parameters induced by glucose suggest that these factors may be related to the Crabtree effect. Although the glucose effect on the cell content of adenine nucleotides depends on the cell line used [13,40], in AS-30D cells the presence of glucose promoted a diminution in the ATP content and the ATP/ADP ratio This was presumably due to the activation of a fraction of hexokinase, which is bound to the external mitochondrial membrane [27]. In turn, the diminution in the ATP/ADP ratio, together with a high concentration of fructose 1,6-bisphosphate should bring about PFK-1 activation, furthermore, fructose 1,6-bisphosphate prevents the inhibition of this enzyme by ATP, and also activates pyruvate kinase These changes should lead to a very active glycolysis [10] Although PFK-1 may be inhibited by acidic pH in some solid tumors [41], PFK-1 from other tumor cells is less sensitive to pH [42] This suggests that the low pH could affect enzymes involved in oxidative phosphorylation more drastically than those in glycolysis

The events involved in the Crabtree effect may be triggered by a glucose-induced activation of hexokinase PFK-1 and pyruvate kinase should be subsequently activated by the elevation in fructose 1,6-bisphosphate, followed by stimulation of the glycolytic flux, which leads to a diminution in the P_i content and accumulation of monophosphate and bisphosphate hexoses It is noted that the increase in phosphorylated hexoses induced by the addition of glucose was not matched by the decrease in the contents of ATP and P_i The source of phosphate for the over-production of hexose phosphate is unknown; incubation of AS-30D cells with high external concentrations of P_i (20 mM) did not prevent the drop in the contents of intracellular ATP and P_i (data not shown) A similar overproduction of phosphorylated hexoses was reported for some yeast mutants when they were exposed to high glucose [43], apparently, the supply of P_i for the hyper-accumulation of phosphorylated hexoses came from the polyphosphate pool [44]

The lowering in the P_i concentration and the more acidic cytosolic pH induced by glucose may affect oxidative phosphorylation at several levels. These changes in P_i and pH could inhibit enzymes that use P_i as a cofactor or substrate, such as α-ketoglutarate dehydrogenase, ATP synthase and P_i-dependent glutaminase [25,45], or that are highly pH-dependent such as cytochrome bc_1 [46,47].

In summary, the results of this study suggest that there is more than a single factor that responds to the addition of glucose or fructose in AS-30D cells. Thus, it would seem that the Crabtree effect is the result of several small changes induced by glucose or fructose that affect several of the enzymes related to oxidative phosphorylation.

One question that arises from the present results is whether the Crabtree effect has any physiological relevance for tumor cells Glycolysis is a metabolic pathway with turbo design [48], in which ATP is initially expended to obtain, from the downstream reactions, a profit This surplus of ATP, in turn, drives further the oxidation of glucose In such metabolic design, the existence of the starting enzyme with insensitivity to product inhibition (hexokinase is not inhibited by glucose 6-phosphate or fructose 6-phosphate) brings about the possibility of ATP and P_i depletion when the cells are exposed to high concentrations of glucose Under such conditions, cells may accumulate inviable amounts of phosphorylated hexoses [48,49] In AS-30D cells [27] and also in other tumor cells [50], hexokinase exhibits lower sensitivity to product inhibition In yeast mutants, this is accompanied by the generation of higher steady levels of glucose 6-phosphate, fructose 6-phosphate, and fructose 1,6-bisphosphate when the cells are exposed to high glucose [43,48,49,51,52,] (see also Table 4) However, tumor cells exposed to high glucose do not undergo ATP or P_i depletion and they reach stable steady-state rates of glycolysis

How do tumor cells deal with the danger of the turbo design of glycolysis when hexokinase is weakly modulated by its product? The answer to this question seems to be the inhibition of oxidative phosphorylation by extracellular glucose Thus, the availability of ATP to hexokinase, the only mechanism of regulation left in tumor cells for hexokinase, is limited by the Crabtree effect Similarly, the failure of *Saccharomyces cerevisiae* mutant cells to grow on glucose or fructose was relieved by inhibition of oxidative phosphorylation with antimycin [53] Another complementary way to deal with the glycolysis turbo design is to use preferentially nonsaturating concentrations of glucose, as observed for ascites AS-30D tumor cells [17] Therefore, a better understanding of the biochemical mechanism of the Crabtree effect in tumor cells may open new ways of approaching the diminution of their accelerated rate of duplication

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REFERENCES

1 Pedersen, P.L. (1978) Tumor mitochondria and the bioenergetics of cancer cells *Prog Exp Tumor Res* 22, 190–274
2 Ibsen, H K (1961) *The Crabtree effect a review Cancer Res.* 21, 829–841
3 Wang, T., Marquat, C & Feker, J. (1976) *Aerobic glycolysis* during lymphocyte proliferation *Nature* 261, 702–705.
4 Seshagiri, P.B. & Bavister, B.D. (1991) Glucose and phosphate inhibit respiration and oxidative phosphorylation in cultured hamster eight-cell embryos evidence for the 'crabtree effect'. *Mol Report Dev.* 30, 105–111.
5 Guppy, M., Greiner, E & Brand, K. (1993) The role of the Crabtree effect and an endogenous fuel in the energy metabolism of resting and proliferating thymocytes *Eur J Biochem* 212, *95–99*
6. Nishimura, J. & Minakami, S (1975) Effect of hydrogen ion *concentration on energy metabolism in pig platelets Biochim. Biophys Acta* 396, 1–10
7 Leese, H J & Bronk, J R (1975) Lactate *formation by rat small* intestine *in vitro Biochim Biophys. Acta* 404, 40–48
8 Mustea, I. & Muresian, T (1967) Crabtree effect in some bacterial culture *Cancer* 20, 1499–1501
9 Postma, E., Verduyn, C., Scheffers, W A & Van Dijken, J.P (1989) Enzymic analysis of the Crabtree effect in glucose-limited chemostat cultures of *Saccharomyces cerevisiae. Appl Environ. Microbiol* 55 468–477.
10 Eigenbrodt, E., Fister, P & Reinacher, M. (1985) New perspectives on carbohydrate metabolism in tumor cells In *Regulation of Carbohydrate Metabolism* (Pestner, R., ed.), Vol. 2, pp 141–179. CRC Press, Boca Raton, FL, *USA*
11 Teplova VV, Bogucka, K., Czyz, A., Evtodienko, YV, Duszynski J & Wojtczak, L (1993) *Effect of glucose and* deoxyglucose on cytoplasmic $[Ca^{2+}]$ in Ehrlich ascites tumor cells *Biochem Biophys Res. Commun.* 196, 1148–1154
12 Wojtczak, L (1996) The Crabtree effect a new look at the old problem *Acta Biochim Pol.* 43, 361–368
13 Wojtczak, L., Teplova, VV, Bogucka, K., Czyz, A., Makowska, A., Wieckowski, M.R., Duszynski, J. & Evtodienko, YV (1999) Effect of glucose and deoxyglucose on the redistribution of calcium in Ehrlich ascites tumour and Zajdela hepatoma cells and its consequence for mitochondrial energetics *Eur J Biochem* 263, 495–501
14 Sussman, I., Erecińska, M & Wilson, D F (1980) Regulation of cellular energy metabolism. the Crabtree effect *Biochim Biophys Acta* 591, 209–223
15. Heinz, A Sachs, G & Schafer, J.A (1981) Evidence for activation of an active electrogenic proton pump in Ehrlich ascites tumor cells during glycolysis *J Membrane Biol* 61, 143–153
16. Yang X., Borg, L A H & Eriksson U J (1997) Altered metabolism and superoxide generation in neural tissue of rat embryos exposed to high glucose *Am. J Physiol* 272, E173–E180
17 Rodríguez-Enríquez, S., Torres-Márquez, M E & Moreno Sanchez, R (2000) Substrate oxidation and ATP supply in *AS 30D* hepatoma cells *Arch Biochem Biophys* 375, 21–30
18 Meredith, R & Fiskum, G (1984) Isolation of mitochondria from *ascites tumor cells permeabilized with digitonin Anal Biochem* 137, 360–367
19 Bergmeyer, H U & Bernt, E (1974) In *Methods of Enzymatic Analysis* (Bergmeyer H U ed.) Academic Press, Orlando, FL, USA
20 Bergmeyer, H U (1983) In *Methods of Enzymatic Analysis* (Bergmeyer, H U., ed.), Weinheim Verlag Chemie, Germany
21 Grynkiewicz, G., Poem, TJ & Tsien, R Y (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol. Chem.* 260, 3440–3450
22 London, R E (1991) Methods for measurement of intracellular magnesium *NMR* and fluorescence *Annu Rev. Physiol* 53, 241–258
23. *Boyarsky, G., Hanssen, C & Clyne, L (1996) Inadequacy of high* K^+/nigericin for calibrating BCECF I Estimating steady-state intracellular pH *Am. J Physiol* 271, C1131–C1145
24. Lattanzio, F.A. (1990) The effect of pH and temperature on fluorescent calcium indicators as determine with Chelex-100 and EDTA buffer systems *Biochem Biophys Res Commun.* 171, 102–108.
25 Rodríguez-Zavala, J S & Moreno-Sánchez, R (1998) Modulation of oxidative phosphorylation by Mg^{2+} in rat heart mitochondria. *J. Biol. Chem.* 273, 7850–7855
26. Hollerand, A L., Fiskum, G & Kelleher, J K. (1997) Quantitative analysis of acetoacetate in AS-30D hepatoma cells with ^{13}C and ^{14}C isotopic techniques. *Am. J. Physiol* 272, E945–E951
27. *Nakashima, R A., Paggi, M G., Scott, L J & Pedersen, P L (1988)* Purification and characterization of a bindable form of mitochondrial bound hexokinase from the highly glycolytic AS-30D rat hepatoma cell line *Cancer Res* 15, 913–919.
28 Xu, Z X., Meléthil, S., Winberg, L. & Badr, M (1990) Mechanism of aluminium-induced inhibition of hepatic glycolysis inactivation of phosphofructokinase *J Pharmacol Exp Ther* 254, 301–305
29 Brazy, P & Chobanian, M C (1996) Interaction between D-glucose and phosphate in renal proximal tubule cells *Kidney Int Suppl* 57, S30–S34
30 Sauer, L A (1977) On the mechanism of the Crabtree effect in mouse ascites tumor cells *J Cell Physiol* 93, 313–316
31. Baggetto, L G (1992) Deviant energetic metabolism of glycolytic cancer cells *Biochimie* 74, 959–974
32 Jaroszewski, J W., Kaplan, O & Cohen, J S (1990) Action of *gossypol and rhodamine 123 on wild type multidrug-resistant* MCF-7 human breast cancer cells ^{31}P nuclear magnetic resonance and toxicity studies *Cancer Res* 50, 6936–6943
33 Kun, E., Talalay P & Williams-Ashman, G (1959) Studies on the Ehrlich ascites tumor The enzymic and metabolic activities of the ascitic cells and the ascitic plasma. *Cancer Res* 11, 855–862
34 Weinhouse, S. (1983) Isoenzyme alterations, gene regulation and the neoplastic transformation *Adv Enzyme Regul* 21, 369–383.
35 Harding J W., Pyeritz, E A., Morris, H P & White, H B (1975) Proportional activities of glycerol kinase and glycerol 3-phosphate dehydrogenase in rat hepatomas *Biochem J.* 148 545–550
36 Berling, J R & Konishi, M (1983) Ca^{2+} transient in cardiac myocytes measured with high and low affinity Ca^{2+} indicators. *Biophys J 65*, 1632–1647
37. Zhu, Z., Tepel, M., Spieker, C & Zidek, W. (1995) Effect of extracellular Mg^{2+} concentration on agonist-induced cytosolic free Ca^{2+} transient *Biochim Biophys Acta* 1265, 89–92.
38 Murphy, A N., Kelleher, J K & Fiskum, G (1988) Calcium sensitive isocitrate and 2-oxoglutarate dehydrogenase activities in rat liver and AS-30D hepatoma mitochondria *Biochem Biophys Res Commun* 157 1218–1225
39 Baggetto, L G (1997) Biochemical, genetic and metabolic adaptations of tumor cells that express the typical multidrug-resistance phenotype Reversion of new therapies *J Bioenerg Biomembr* 29, 401–413
40. Hess, B & Chance B (1961) Metabolic control mechanisms VI *Chemical events after glucose addition to ascites tumor cells J Biol Chem* 236, 239–246
41 Meldolesi, M F., Macchia, V & Lacetti, P (1976) *Differences in* phosphofructokinase regulation in normal and tumor rat thyroid cells *J Biol Chem* 251, 6244–6251

42 Sumi, T & Ui, M (1972) Purification and some properties of phosphofructokinase from Ehrlich ascites tumor cells *Biochim Biophys Acta* 268, 354–363
43 Ernandes, J R, De Meirsman, C, Rolland, F, Winderickx, J, De Winde, J, Lopes Brandao, R & Thevelein, J M (1998) During the initiation of fermentation overexpression of hexokinase PII in yeast transiently causes a similar deregulation of glycolysis as deletion of Tps1 *Yeast* 14, 255–269.
44 Van Aelst, L, Hohmann, S, Zimmermann, F K, Jans, A W H & Thevelein, J M (1991) A yeast homologue of the bovine lens fibre MIP gene family complements the growth defect of a *Saccharomyces cerevisiae* mutant in fermentable sugars but not its defect in glucose-induced RAS-mediated cAMP signalling *EMBO J* 10, 2095–2104
45 Medina, M A, Sánchez-Jiménez, F, Márquez, J, Quesada, A R & Núñez de Castro, I (1992) Relevance of glutamine metabolism to tumor cell growth *Mol. Cell Biochem.* 113, 1–45
46 Brandt, U & Okun, J G (1997) Role of deprotonation events in ubihydroquinone cytochrome *c* oxidorreductase from bovine heart and yeast mitochondria *Biochemistry* 36, 11234–11240
47 Kashiwagura, T, Deutsch, C J, Taylor, J., Ereciñska, M & Wilson, D F (1984) Dependence of gluconeogenesis, urea synthesis, and energy metabolism of hepatocytes on intracellular pH *J Biol Chem.* 259, 237–243.
48 Teusink, B, Walsh, M C, Van Dam, K & Westerhoff, H V (1998) The danger of metabolic pathways with turbo design *Trends Biochem Sci.* 23, 162–169
49 Bakker, B M, Mensonides, F I C, Teusink, B, Van Hoek, P, Michels, P A M & Westerhoff, H V (2000) Compartmentation protects trypanosomes from the dangerous design of glycolysis *Proc Natl Acad Sci USA* 97, 2087–2092
50 Bustamante, E & Pedersen, P L (1977) High aerobic glycolysis of rat hepatoma cells in culture: role of mitochondrial hexokinase *Proc Natl Acad Sci USA* 74, 3735–3739
51. Teusink, B, Mensonides, F I C, Bakker B M, Walsh, M C, Snoep, J L, Van Dam, K & Westerhoff, H (1998) Regulation through moiety conservation: the role of production sensitivity in glycolysis of yeast and trypanosomes In *Biothermokinetics in the Post Genomic Era* (Larsson, C Påhlman, I -L & Gustafsson, L, eds), pp 240–245 Chalmers Reproservice Goteborg, Fiskebackskil, Sweden
52 Hohmann, S, Neves, M J, De Koning, W, Alijo, R, Ramos, J & Thevelein, J M (1993) The growth and signalling defects of the *ggs1* (*fdp1*/*byp1*) deletion mutant on glucose are suppressed by a deletion of the gene encoding hexokinase PII *Curr Genet* 23, 281–289
53. Neves, M J, Hohmann, S, Bell, W, Dumortier, F, Luyten, K, Ramos, J, Cobbaert, P, De Koning, W, Kaneva, Z & Thevelein, J M. (1995) Control of glucose influx into glycolysis and pleiotropic effects studied in different isogenic sets of *Saccharomyces cerevisiae* mutants in trehalose biosynthesis *Curr Genet* 27, 110–122

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22 February 2001

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Reference no.: 01-0129
Title: Multisite control of the Crabtree effect in ascites hepatoma cells
Authors: Sara Rodríguez-Enríquez, Oscar Juárez, José S Rodríguez-Zavala, Rafael Moreno-Sánchez
Editor: Girard

Dear Dr Rodríguez-Enríquez

Thank you for submitting your paper to the European Journal of Biochemistry. I am pleased to inform you that your manuscript has been accepted for publication

Please send a new disk, or the text of the manuscript as an attachment to email, as soon as possible The disk that you sent with your manuscript was infected with a virus and we are unable to use it. In addition, if possible, please send an electronic copy of figure 1 in TIFF or EPS format.

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Kind regards
Vanessa Wilkinson

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Assistant Editorial Manager
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The authors have studied the Crabtree effect in AS-30D hepatoma cells where they state that oxidative phosphorylation is the main source of ATP supply; they show that this effect is present in these cells and they correlate it with an intracellular decrease of Pi, ATP, and pH, an intracellular increase of G6P, F6P, F1,6BP, and a partial inhibition of the mitochondrial oxidative phosphorylation (40%), in an effort to propose an explanation to this apparent controversial effect in these cells.

AS-30D cells derive from a cell line described in 1970 as a highly glycolytic cancer cell line. Several authors invariably described important abnormalities at the level of their energetic and central metabolism. The present study brings some more details to further characterize this particular cell line. However, even though the discussion section is rather well documented, in the absence of a certain number of necessary controls, this study sounds rather poor and with little interest in the field of cancer research except for researchers who work more particularly with AS-30D cells. Moreover, a number of caveats need to be addressed.

GENERAL COMMENTS.

1- Because of its "old age", AS-30D cells may have derived from their original form in many points. More particularly, it would be necessary to evaluate the actual glycolytic level of these fast growth cells. In the same manner, what is the activity of phosphofructokinase and of other key enzymes of glycolysis in these cells?

2- The features the authors describe and especially the tentative explanation of the Crabtree effect may only be relevant to AS-30D cells. How would these features be placed in a scale of cancer cells spanning a normal rate to a high rate glycolysis (*i.e.* the Morris hepatoma cell lines) as compared to normal hepatocytes?

3- AS-30D cells derive from hepatocytes, which naturally produce the P-Glycoprotein (Pgp170), an ATP-driven membrane pump known to extrude a large variety of lipophilic compounds (and to be therefore the main cause of the multidrug resistance (MDR) phenotype of cancers). This cell line has recently been described to express the MDR phenotype through the production of Pgp170. The authors sit of most of their results on the use of lipophilic dyes such as BCEFCF, Indo-1, Mag-Indo-1, Fluo-3, Mag-Fura-2, which are substrates of the Pgp170 and susceptible to be incompletely loaded into their respective targets and create bias in the measurement. It would be of a prime necessity to address this question.

4- Explain ALL abbreviations (*i.e.* $[Mg^{2+}]m$, page 3, line 16, and following)

SPECIFIC COMMENTS.

1- Oxygen uptake of intact cells is presented in figure 1. It would be interesting to appose a curve of O_2 uptake of mitochondria isolated from AS-30D cells, and incubated in the presence of substrates such as malate+pyruvate and succinate.

2- Figure 1 does not clearly show that oligomycin induces an 85-90% inhibition, as stated in page 6, first line of the Results section.

3- Legend of table 1, line 2: correct "*substracting*"

4- Page 7, line4 and following: the correct orthography is "Ehrlich Lettré".

5- Provide a legend for table 2

6- Page 7, line 18 and Table 3 I am not able to figure out how the authors calculated the phosphorylation potential. Usually, this potential represents the energetic state of a cell and is defined as the ratio $\frac{[ATP]}{[ADP]\bullet[Pi]}$. According to that formula I found values ranging from 0 33 to 0 42 with a slight increase from 0 33 to 0.36-0.39 in the presence of glucose or fructose, and not from 0.35 to 0 6-0.7 as mentioned by the authors Under the authors' conditions, the phosphorylation potential

increases even more when cells are incubated with compounds such as galactose and glycerol. How can they explain such a behavior?

The expected value under normal conditions for a normal cell reaches 0.85. In every case studied by the authors, the phosphorylation potential is far below 0.85 meaning that the ATP-generating sequences are highly accelerated, probably as a response to adenylate-depending regulatory enzymes. This is obviously another reason for which it is necessary to evaluate the kinetic constants and the metabolic state of key enzymes, the regulation of which depends on the concentration of ATP, ADP, and AMP.

7- Provide dimensions for the values in the column entitled "Σ adenosine nucleotides".

8- As mentioned above, a curve representing the respiration of AS-30D mitochondria would illustrate the results presented in page 8, line 11, and visually show their coupling state.

Report 2

Report on manuscript No. 00-1136 by Rodríguez-Enríquez et al. entitled "Multisite control of the Crabtree effect ..."

Despite seven decades that passed since the inhibitory effect of glucose on the respiration of malignant tissues was first observed, the mechanism of the Crabtree effect is not fully understood. The paper by Rodriguez-Enriquez et al. is a further attempt to elucidate the problem. In brief, the positive findings of these authors are: (1) decrease of cytosolic pH, (2) decrease of intracellular content of ATP and ADP and of the total pool of adenine nucleotides, (3) decrease of cytosolic inorganic phosphate concentration, and (4) increase of hexose phosphates in AS-30D hepatoma cells supplemented with glucose or fructose. Events (1) and (3) have been found to decrease coupled respiration of isolated mitochondria from the cell line under study, whereas events (2) and (4) were without effect.

Although these results provide some new information, in my opinion they do not allow for far-reaching speculations that are confined in the second part of the Discussion.

I suggest to supplement the experimental part with the following information:

1. Is the inhibition of cell respiration by glucose reversed by uncouplers?
2. Is the inhibition of mitochondrial respiration by low pH and low [P_i] abolished by uncouplers as well?
3. The authors say (p. 6) that "the hexose-induced inhibition of oxidative phosphorylation reached a steady-state after approximately 5 min". This is not depicted by the corresponding inhibition of the rate of respiration (Fig. 1) which is practically instantaneous. I propose to show a real experimental trace instead of an 'idealized' one.
4. Table 1. Inhibition by 10 mM fructose is not 50%.

In contrast to Wojtczak et al. (Refs. 11 and 12), the authors of the present paper observed no change of cytosolic calcium concentration after addition of glucose or fructose to the medium. This may be due to a difference in the cell line used. However, Wojtczak et al. have recently found an increase of $[Ca^{2+}]_c$ in Zajdela hepatoma as well [Eur. J. Biochem. 263, 495-501 (1999)]. By the way, the inhibition of coupled respiration by increased $[Ca^{2+}]_c$ and $[Ca^{2+}]_m$ is interpreted by these authors as being the result of an increased association of the natural protein inhibitor with the F_oF_1 complex and not as complexing by Ca^{2+} of ADP and ATP, as stated in the present manuscript (pp. 3 and 10).

Similarly, the finding of Cittadini et al. (Ref. 13) on Mg^{2+} efflux is misinterpreted (pp. 3 and 10) in the present manuscript. The Italian authors observed potentiation by glucose of Mg^{2+} efflux only in respiration-inhibited cells. In fully active cells, glucose had a slight inhibitory effect, if any.

The authors of the present manuscript should also note that glucose may have different effects on intracellular ATP and ADP, depending on the cell line. For example, Hess & Chance [J.B.C. 236, 239-246 (1961)] found a short, transient, change of ADP and ATP concentrations and Wojtczak et al. (1999, reference cited above) observed no change at all after supplementation with glucose of Ehrlich ascites tumour cell.

Full name of CCCP (p. 2) is incomplete.

12 January 2001

Dr. Richard Perham
Chairman of the Editorial Board
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Dear Dr. Perham:

Thank you for your letter of November 14, 2000 concerning our manuscript (No. 00-1136) entitled "Multisite control of the Crabtree effect in ascites hepatoma cells". We were informed that a new version of the manuscript, which takes into account the objections raised by the referees, would be considered for evaluation. We have amended the manuscript following the referees' observations. The changes made are described below.

Referee No. 1

The reviewer made the general comment that *"in the absence of a certain* number of necessary controls" our study was of "little interest in the field of cancer research except for researchers who work with AS-30D cells". We think, on the contrary, that the present work may be of great interest in the cancer research field since AS-30D cells represent a model of tumor cells with an oxidative type of metabolism, a high rate of growth, and with expression of the P-glycoprotein, as the same referee pointed out. Moreover, there is a number of human tumor lines that show similar characteristics to AS-30D. We have emphasized this matter on p. 2, line 1; p. 3, 1st paragraph; p. 9, 3th and 4th paragraph; p.10, 4th paragraph. We hope that *the referee agrees with our point of view on this matter.*

General comments

1. Data on the rates of glycolysis and PFK-1 activity are now included on p. 7, 1st paragraph.
2. We are thankful to the referee for calling our attention to the correlation between the rate of glycolysis and the presence of the Crabtree effect. This is now stated on p. 9, 3th paragraph.

3. We have addressed the question of the potential effect of the P-glycoprotein on the measurements of Ca^{2+}, Mg^{2+}, and pH using fluorescent dyes on p. 10, 4th paragraph.

   It should be noted, however, that most of our results are rather based on the determination of the rates of O_2 uptake (Fig. 1, Table 1) and the concentration of several relevant metabolites (Tables 3 and 4).
4. All abbreviations have been fully described.

Specific comments

1,2, and 8.- A new figure 1 is now shown with measurements in cells and mitochondria, as suggested by the referee.

3.- OK

4.- OK

5.- OK

6.- The referee found lower values for the phosphorylation potential because he (she) used, in the calculations, the **AMP** instead the **ADP** content shown in Table 3.

7.- OK

Referee No. 2

1, 2.- Figure 1 now shows the CCCP effect on cellular and mitochondrial respiration. Accordingly, the text on p. 6, lines 3-6 from bottom, and p. 8, lines 7-8 from bottom has been modified to incorporate these new experiments.

3.- Figure 1A shows a 'real' *experimental trace with cells.*

4.- The degree of inhibition by fructose has been corrected in Table 1.

The paper by Wojtczak et al (1999) has also been cited on p. 3 and 7. The interpretation by these authors that the inhibitor protein association to the ATPase is increased by Ca^{2+} has been included on p. 3, and p.10.

The comments based on the paper by Cittadini et al (1994) regarding Mg^{2+} efflux have been deleted from the present version.

A comment on the different effect of glucose on the ATP and ADP contents was added on p. 11, 1st paragraph.

Except for the changes specified in this letter, the rest of the manuscript has not been altered. We hope that in its present form the manuscript may be found acceptable for publication in the European Journal of Biochemistry.

Yours sincerely,

Sara Rodríguez-Enríquez

3.3 CONTROL OF THE RATE OF CELLULAR DUPLICATION BY MODULATION OF OXIDATIVE PHOSPHORYLATION IN OXIDATIVE TUMOR CELL LINES *

*Este artículo se encuentra en preparación y se pretende enviarlo a *Cancer Research* o *Cancer Letters*. Se incluirán resultados con líneas tumorales humanas.

Disminución de la duplicación tumoral en tumores oxidativos por inhibición de la fosforilación oxidativa.

Los estudios anteriores indicaron que el metabolismo mitocondrial es el que prevalece en el hepatoma AS-30D. Por tanto, una estrategia para disminuir la proliferación tumoral podría ser la supresión del metabolismo oxidativo ya sea manipulando la fuente de carbono externa o bien utilizando inhibidores selectivos o farmacos cuyo blanco es el metabolismo mitocondrial. Para demostrar la hipótesis utilizamos como herramienta los cultivos primarios de AS-30

AS-30D mantiene un crecimiento diferencial asociado a la fuente de carbono externa. El crecimiento óptimo se alcanzó en presencia de sustratos glucolíticos (glucosa 25 mM) y mitocondriales (glutamina 4 mM). Sin embargo, el destino de ambos sustratos fue diferente: la glutamina se utilizó para proveer el ATP requerido para los procesos dependientes de ATP durante la proliferación tumoral mientras que la glucosa fue necesaria para proveer otros metabolitos esenciales en la proliferación (NADH, NAD, PRPP). En presencia de sólo glutamina o glutamato, las células alcanzaron una densidad menor (20%), aunque la concentración de ATP fue el doble que en la condición glucosa+glutamina, lo cual indica que el sustrato limitante podría ser otros metabolitos utilizados para la progresión del ciclo celular (PRPP, NADH, bases púricas y pirimídicas). Con sólo glucosa las células fueron incapaces de proliferar por la baja disponibilidad de ATP registrada en esta condición.

En presencia de inhibidores de la fosforilación oxidativa (oligomicina, rodamina 123, 6G, clofazimina), la proliferación tumoral cesó drásticamente mientras que en presencia de fármacos que afectan sólo al metabolismo glucolítico (gosipol, iodoacetato), la proliferación disminuyó ligeramente. Lo anterior indica la alta dependencia de este tipo tumoral al ATP mitocondrial. De los fármacos ensayados proponemos que los compuestos de la familia de las rodaminas son los más potentes para ser utilizados en tumores oxidativos debido a su alto índice de toxicidad a concentraciones menores.

DISMINUCION DE LA DUPLICACIÓN TUMORAL EN TUMORES OXIDATIVOS POR INHIBICION DE LA FOSFORILACION OXIDATIVA.

INTRODUCCION

Las células tumorales de rápido crecimiento (CTRC) desarrolladas experimentalmente en roedores (Ehrlich, Ehrlich-Lettré, carcinoma de Walker-256) y las líneas tumorales humanas como HeLa, SiHa o Hep-2, son metabólicamente diferentes al compararlas con su tejido de origen [1-3]. Entre sus características bioquímicas más relevantes se encuentran: (1) la elevada actividad glicolítica en presencia de concentraciones saturantes de oxígeno (efecto Pasteur disminuído) [4]; (2) la deficiencia en el metabolismo oxidativo (baja oxidación de piruvato [5] y la presencia de un ciclo de Krebs incompleto, incapaz de suministrar suficientes equivalentes reductores para impulsar la síntesis de ATP [6]); y (3) la inhibición parcial de la fosforilación oxidativa (FO) por glucosa y otras hexosas exógenas (efecto Crabtree) [7]. Por tanto, se propuso que el ATP utilizado para la biosíntesis de proteínas, ácidos nucleicos, activación de ATPasas (ATPasa de Na^+/K^+) y fenómenos de transporte (Ca^{2+}) provenía exclusivamente del metabolismo glicolítico [8-11]. Además, en la mayor parte de los tumores sólidos de bajo crecimiento la glucólisis es la única vía productora de energía, ya que la FO se encuentra limitada por la baja disponibilidad de oxígeno en el microambiente del tumor [12].

Nosotros hemos demostrado que el hepatoma de rápido crecimiento AS-30D, además de mantener una glucólisis muy elevada, mantiene otras vías catabólicas muy activas como la glucogenólisis, la degradación de sustratos mitocondriales como glutamato y cuerpos cetónicos (β-hidroxibutirato y acetoacetato) y la fosforilación oxidativa [13].

La alta velocidad respiratoria registrada en AS-30D (100 ngAO/min/10^7cels), es 90-95% sensible a oligomicina (un inhibidor específico de la ATP sintetasa), lo que indica que la mayor parte del consumo de oxígeno celular está asociado a la síntesis de ATP. En líneas tumorales humanas (cáncer de colon HT29, carcinoma de pulmón de Lewis [14,15]), y en la cadena hiperdiploide Ehrlich Letreé [16] también se han registrado actividades respiratorias elevadas; sin embargo, no se reporta en ninguna de ellas la sensibilidad a oligomicina o a otros inhibidores mitocondriales. Un consumo de oxígeno resistente a

oligomicina, pero sensible a inhibidores respiratorios como rotenona, antimicina o cianuro, sería indicativo de desacoplamiento de la fosforilación oxidativa.

Durante el estado proliferativo de las células de Ehrlich, Ehrlich-Lettré y de algunos carcinomas (Walker-256 y MCF-7) existe una activación tanto de la glicólisis como de la FO, lo cual sugiere que durante esta fase ambas vías son importantes para el desarrollo del tumor [17-20]. En consecuencia, una estrategia bioquímica para disminuir la proliferación tumoral ha sido la manipulación simultánea de las dos vías productoras de ATP [19-21]. Por ejemplo, en el carcinoma de Walker-256 se utilizó 3-mercaptopicolinato (inhibidor de la fosfoenolpiruvato carboxicinasa) para bloquear la gluconeogénesis en el hospedero; de esta manera se disminuyó la concentración de glucosa circulante y en consecuencia la glucólisis tumoral; para bloquear específicamente a la FO, se utilizaron colorantes catiónicos como la rodamina 6G, que inhibe la síntesis de ATP mediante el colapso del gradiente electroquímico de H^+. Cuando se inhibió simultáneamente a la FO y a la glucólisis, el crecimiento celular se redujo en un 50% [19]. El tratamiento por separado con rodamina 6G o 3-mercaptopicolinato no alteró la velocidad de crecimiento, indicando que cualquiera de las dos vías puede sostener la proliferación celular.

Otro compuesto que se ha utilizado para inhibir la glucólisis en la línea MCF-7 (carcinoma de pecho humano) es el gosipol, un aldehído bisnaftaleno polifenólico, que inhibe específicamente a las enzimas citosólicas dependientes de NAD^+ como la gliceraldehído 3-fosfato deshidrogenasa [20, 20a]. La inhibición simultánea de la glucólisis (por gosipol) y de la FO (por rodamina 123), disminuyó la proliferación celular en un 60% [20]. También se han realizado estudios *in vivo* e *in vitro* con agentes antilepróticos como la clofazimina. Este compuesto provoca una reducción del 40% en el tamaño de la línea tumoral WIL, un carcinosarcoma bronquial humano resistente a la quimioterapia, debido a su acción como desacoplante [21]. En células y mitocondrias aisladas de AS-30D se han empleado algunos antiinflamatorios no esteroideos como el nimesulide, el diclofenac, el meloxicam y la nabumetona, para colapsar el gradiente de H^+ y por tanto la síntesis de ATP [22] Lo anterior sugiere que el uso de estos fármacos en cultivos de células tumorales, podría disminuir selectivamente el aporte de ATP mitocondrial.

El uso de colorantes catiónicos (familia de las rodaminas) o de fármacos como los antiinflamatorios no esteroideos, puede resultar ventajoso con respecto a los inhibidores

selectivos mitocondriales como oligomicina y rotenona, debido a que se ha reportado que a las concentraciones utilizadas de algunos de ellos (rodamina 6G y rodamina 123) solo afectan al tumor sin perturbar el metabolismo del hospedero [19,20]. Lo anterior se puede explicar porque la membrana tanto plasmática como mitocondrial de las células tumorales mantiene un potencial transmembranal más negativo que el de las células no-tumorigénicas, lo que favorece el acarreo y retención de estas moléculas catiónicas [20,23, 24].

Los compuestos anteriormente descritos no son los únicos que se han encontrado como inhibidores de la glucólisis y la fosforilación oxidativa en células tumorales. Al parecer, la ceramida [25] (un segundo mensajero que modula al factor alfa de necrosis tumoral, y a ciertos reguladores del crecimiento celular y la diferenciación) inhibe al sitio III de la cadena respiratoria en mitocondrias tumorales. Otros fármacos como las N-tiadiazolil anilinas, citotoxinas mitocondriales, son capaces de inhibir el crecimiento tumoral en concentraciones micromolares, inhibiendo la síntesis de ATP y actuando como desacoplantes clásicos [26]; ó como la suramina que presenta actividad antitumoral por su efecto desacoplante mitocondrial [27]. Otros compuestos de la familia de la norcantarina son bloqueadores del transporte de glucosa y por lo tanto inhibidores de la glucólisis [28].

En la línea ascítica AS-30D [3,13], la FO es la vía que suministra el ATP durante la fase proliferativa pero no la glucólisis. Por tanto es de suponerse que los procesos celulares con mayor gasto de energía, como la biosíntesis de proteínas y de ácidos nucléicos, el mantenimiento de gradientes iónicos (ATPasa de Na^+/K^+, y de Ca^{2+}) y la duplicación celular, dependen de la velocidad a la cual se sintetiza el ATP mitocondrial.

El desarrollo de cultivos primarios de células tumorales AS-30D es fundamental para manipular la velocidad de duplicación celular, utilizando dos estrategias: a) el control externo de la fuente de carbono y la disponibilidad de factores esenciales para la célula y b) el uso de inhibidores selectivos.

El análisis del metabolismo energético en AS-30D, una línea tumoral desarrollada en roedores, provee información acerca de los cambios metabólicos que pudieran llevarse a cabo en otros tumores oxidativos, particularmente en los humanos. Sin embargo, es más conveniente que este análisis se extienda en líneas tumorales humanas de rápido crecimiento: HeLa, SiHa (ambos carcinomas de cérvix) y Hep-2 (un tumor de laringe), en las cuales el análisis metabólico y la manipulación energética no se han descrito.

MATERIAL Y METODOS

Modelos de estudio. El modelo utilizado para este estudio es la línea tumoral ascítica AS-30D. Los cultivos primarios de AS-30D se realizaron a partir de una alicuota de hepatoma, el cual se propaga intraperitonealmente en ratas Wistar hembras de 250-300 g; el tiempo de desarrollo del tumor es de 5-7 días [29]. El hepatoma se siembra en tubos de centrifuga cónicos con medio Dulbecco-MEM modificado sin sustratos (GIBCO), suplementado con 10% (v/v) de suero bovino fetal , estreptomicina/penicilina (10,000 U/ml) y fungizona (anfotericina B) para evitar el desarrollo de pirógenos. Todo el procedimiento se realiza en una campana de flujo laminar. Las células se incuban a 37°C, en una atmósfera de 5 % CO_2 y 95% O_2 con agitación constante (20 oscilaciones/minuto) para permitir el intercambio de gases. Las células se cosecharon durante la fase estacionaria de crecimiento.

Determinación de la proliferación celular y del índice de toxicidad (IC_{50}). Las células AS-30D ($1x10^6$cels/ml) se expusieron (i) a diferentes sustratos oxidables glucolíticos y mitocondriales: glucosa 25 mM, glutamato 5 mM, glutamina 4 mM o la combinación de glucosa + glutamina ([Gluc+Gln]; (ii) a bloqueadores metabólicos oxidativos como rotenona (5 μM) y oligomicina (5 μM) o glucolíticos como iodoacetato (100 μM) y iii) diferentes drogas como rodamina 123 (10 μM), rodamina 6G (10 μM), clofamizimina (100 μM); nimesulide (0.4 μM), gosipol (100 μM); se ha documentado que algunos de estos fármacos, a las concentraciones utilizadas, pueden disminuir al tumor sin afectar la integridad de otros tipo celulares no-tumorigénicos [19, 20].

En cada condición se determinó a) densidad celular con un hematocitómetro y b) viabilidad utilizando el método de exclusión del azul tripano.

Para la determinación del IC_{50} (concentración del inhibidor o fármaco al cual se obtiene el 50% de muerte celular), las células AS-30D fueron expuestas a concentraciones variables de cada droga En todos los casos, el índice de toxicidad se determinó a las 48 hrs de cultivo, tiempo al cual se alcanza la fase estacionaria de crecimiento.

Determinación de los flujos oxidativo y glucolítico y contenido de ATP celular. Las células tumorales se cosecharon en fase estacionaria, y se lavaron con medio Krebs-Ringer frío (NaCl 125 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.4 mM, H_2PO_4 1 mM, HEPES 25 mM, pH 7.4). Para determinar el flujo oxidativo, se determinó el consumo de oxígeno sensible a oligomicina con un electrodo tipo Clark. La velocidad de glucólisis se determinó por análisis enzimático, determinando la velocidad de aparición de piruvato y lactato [30]. El ATP celular se determinó enzimáticamente utilizando el sistema acoplado del par hexocinasa/glucosa –6fosfato deshidrogenasa [31].

oxidativa por el sustrato mitocondrial. Con glucosa, el contenido de ATP disminuyó un 63% *vs* Gln + Gluc, indicando una baja producción de ATP por la glucólisis.

Proliferación celular, fosforilación oxidativa y glucólisis en cultivo primario de AS-30D.

Las células cultivadas con Gln + Gluc se expusieron a diferentes inhibidores respiratorios (oligomicina o rotenona) ó inhibidores glucolíticos (iodoacetato) y a drogas que sólo afectan la fosforilación oxidativa ó la glucólisis tumoral (Tabla 2). El crecimiento celular, la actividad de fosforilación oxidativa y glucólisis fueron drásticamente abatidos en presencia de oligomicina (Tabla 1), rotenona, rodamina 123, rodamina 6G, clofazimina, baicaleína; registrándose muerte celular a partir de las 22 hrs; sin embargo, en presencia de iodoacetato o gosipol, el crecimiento tumoral solo disminuyó un 30% manteniendo alta viabilidad (Tabla 2). La fosforilación oxidativa de las células expuestas a iodoacetato (AIA) no se modificó, mientras que la glucólisis disminuyó un 25%. En cambio, el gosipol abatió drásticamente la glucólisis y no causó una disminución abrupta en la densidad celular. Lo anterior sugiere que la glucólisis no sostiene la duplicación celular en AS-30D. El nimesulide disminuyó ligeramente la proliferación celular manteniendo la viabiliad alta, indicando que este compuesto es ineficaz para abatir la FO, a pesar de que se ha demostrado que es un potente desacoplante mitocondrial [22].

En la Tabla 3 se indican los índices de toxicidad de diferentes drogas oxidativas y glucolíticas en AS-30D. Los compuestos de la familia de las rodaminas (123, 6G), fueron los que exhibieron mayor toxicidad en comparación con las demás drogas analizadas. Los valores de IC_{50} calculados con rodamina 123 se encuentran entre los reportados para otras líneas tumorales humanas oxidativas como la línea A 2780 [33].

DISCUSION

Cambios metabólicos inducidos por la exposición a diferentes fuentes de carbono.

Las células AS-30D mantienen un crecimiento diferencial asociado al metabolismo de uno u otro sustrato, y solo crecen óptimamente en un medio enriquecido con Gln+Gluc en comparación con cualquiera de los sustratos solos. En este caso, la oxidación de glutamina provee (a) de un alto suministro de ATP (Tabla 1) y (b) de sustratos para la biosíntesis de purinas y pirimidinas, así como una fuente de nitrógeno constante utilizada para la biosíntesis de ácidos nucleicos. La oxidación de glucosa no es utilizada en forma de piruvato o poder reductor por la mitocondria, debido a que en presencia de solo Gluc, la actividad respiratoria asociada a la síntesis de ATP es nula (Tabla 1). Al parecer, el catabolismo de glucosa está destinado al abastecimiento de metabolitos intermediarios de la biosíntesis de ácidos nucleicos, como puede ser el fosforibosil pirofosfato (PRPP), bases puricas y pirimídicas, así como equivalentes reductores.

En presencia de un sustrato oxidativo como la glutamina, la fosforilación oxidativa se incrementa un 50% en comparación con la condición Gln+Glc, lo cual indica una activación de los procesos oxidativos, y una mayor disponibilidad de ATP. Sin embargo, bajo estas condiciones no se alcanza el crecimiento óptimo encontrado en presencia de ambos sustratos (hubo una disminución del 20% en el crecimiento). Lo anterior se puede deber a que en ausencia de glucosa exógena, la concentración endógena de PRPP y de NAD(P)H citosólico (probablemente derivado del glucógeno endógeno) no son suficientes para alcanzar un crecimiento óptimo. La actividad respiratoria registrada con Gln + Gluc es menor que con solo Gln debido al efecto Crabtree (inhibición parcial de la fosforilación oxidativa por glucosa) que presenta AS-30D [32].

Con Gluc como única fuente de carbono las células no proliferan pero se mantienen viables; su fosforilación oxidativa se encuentra suprimida y el contenido de ATP es insuficiente para impulsar el crecimiento y la biosíntesis de metabolitos de carácter proliferativo (Fig. 1 , Tabla 1). Es posible que un cultivo sin glutamina limite a la célula de una fuente de nitrógeno para la biosíntesis nucleica, sin embargo, la adición de oligomicina a células cultivadas con los dos sustratos (Gln+Glc) disminuye drásticamente el

crecimiento tumoral (Tabla 1), sugiriendo que no es la limitación de nitrógeno el factor que aminora el crecimiento de las células con Gluc, sino la disponibilidad de ATP mitocondrial.

Debe notarse que las células en cultivos se cosecharon durante la fase estacionaria, es decir cuando las velocidades de duplicación y muerte celular son iguales. Para establecer una correlación más rigurosa entre la proliferación celular y la FO será conveniente extender los estudios de este trabajo a células cosechadas en la fase logarítmica (28 hrs de cultivo).

Inhibición del crecimiento tumoral por la supresión del metabolismo oxidativo.

Los resultados anteriores indican que las células AS-30D son esencialmente oxidativas y que una estrategia para disminuir la proliferación de este tipo tumoral es el uso de compuestos que afecten el metabolismo oxidativo.

La adición de inhibidores clásicos de la fosforilación oxidativa, como oligomicina y rotenona, abatió drásticamente la proliferación tumoral. Aunque cualquiera de los dos disminuyó la proliferación en su totalidad, la adición de rotenona indujo la mortalidad de todas las células después de 48 hrs (dato no mostrado) Este comportamiento no se observó con oligomicina, ya que su adición al cultivo celular en las primeras horas sólo disminuyó un 50% la viabilidad. Nosotros hemos reportado en el ascitis de AS-30D que una de las enzimas que ejercen mayor control sobre el flujo de fosforilación oxidativa es el complejo I mitocondrial; por tanto, la inhibición de esta enzima en los cultivos de AS-30D con rotenona ocasiona que la vía aminore drásticamente su actividad. Este resultado apoya la propuesta de que la supresión del metabolismo oxidativo puede ser una estrategia experimental para la disminución del crecimiento tumoral. De hecho, la adición de iodoacetato (un inhibidor glucolítico) no perturba grandemente el crecimiento tumoral, ni la FO (el crecimiento disminuye 30% y la viabilidad se mantiene alta) (Tabla 2), lo que sugiere que el metabolismo glucolítico no es esencial para que la célula continúe proliferando, mientras que el metabolismo oxidativo permanezca activo. Se ha reportado que la concentración que hemos ensayado de iodoacetato (100 μM) es suficiente para inhibir la glucólisis, sin embargo, podría estar inhibiendo inespecíficamente otras vías.

Los inhibidores específicos mitocondriales y glucolíticos que hemos empleado en este estudio afectan también a cualquier célula no-tumorigénica. Por lo anterior ensayamos compuestos o fármacos que se ha reportado que afectan sólo al tumor sin perturbar al metabolismo del hospedero [20,23]. Nuevamente, la adición de compuestos como rodamina 123, rodamina 6G, clofazimina (todos ellos desacoplantes de la fosforilación oxidativa) abatieron drásticamente el crecimiento tumoral. En algunos reportes se ha determinado que a las concentraciones ensayadas, ninguno de estos fármacos afectan otras vías metabólicas en células tumorales [19-21]. En el caso del gosipol, que inhibe la glucólisis actuando como un inhibidor específico de las deshidrogenasas celulares, no hubo una disminución tan drástica del crecimiento tumoral, aunque la glucólisis fue severamente disminuída.

Nosotros hemos reportado que dos de los sustratos que se encuentran en mayor concentración en el líquido de ascitis (fracción líquida que nutre fisiológicamente a las células tumorales en el abdomen de la rata) son la glutamina (4 mM) y el oxígeno (50 μM), mientras que la glucosa se encuentra a bajas concentraciones (26 μM). Lo anterior sugiere que el mejor modelo de comparación entre las células asciticas y los cultivos primarios es la condición con Gln, donde el metabolismo oxidativo prevalece. Por tanto, los resultados obtenidos en cultivos primarios pueden ser extrapolados a las células asciticas que se desarrollan intraperitonealmente en el roedor.

De los fármacos ensayados, los compuestos de la familia de las rodaminas son los más potentes y los que exhibieron mayor toxicidad a bajas dosis. Las IC_{50} calculadas en presencia de estos fármacos se encuentran en los intervalos reportados para otras líneas humanas tumorales, al parecer de carácter oxidativo. Estas concentraciones de droga no afectan algunos tejidos normales proliferativos (líneas mioepiteliales de pecho) y no-proliferativas (líneas renales), por lo que pueden ser considerados como agentes anticancerígenos para disminuir el crecimiento de tumores oxidativos [29]. En estudios *in vivo* utilizando ratas portadoras con carcinoma de Walker-256, la rodamina 123 a bajas dosis (0.8mg/kg peso) no afecta al hospedero. Sin embargo, para reducir considerablemente el tamaño del tumor se administró simultáneamente un inhibidor de la glucólisis tumoral, debido a que este tumor, particularmente, depende de ambas fuentes de energía [19]. En roedores que porten tumores oxidativos, la estrategia consistirá en administrar la rodamina a dichas dosis para garantizar la muerte tumoral sin poner en riesgo al hospedero.

La conclusión que emerge del presente estudio es que las células tumorales AS-30D en **cultivo primario** son esencialmente oxidativas: a) tienen un mejor crecimiento en presencia de sustratos mitocondriales que glucolíticos, b) la fosforilación oxdativa se mantiene con alta actividad, tal como hemos documentado en el ascitis de AS-30D [13], y c) la adición de fármacos o inhibidores del metabolismo oxidativo abaten el crecimiento tumoral.

BIBLIOGRAFIA

1. Pedersen PL. (1978). Prog. Exp. Tumor Res. 22:190-274.
2. Eigenbrodt E, Fister P, Reinacher M. In Regulation of Carbohydrate Metabolism. Pteiner R. Boca Raton Fl. CRC Press (1984). 6(4) 141-179.
3. Rodríguez-Enríquez S, y Moreno-Sánchez R. (1998). Arch.Med.Res. 29,1-12.
4. Dills WL .(1993) Parasitol. 107:S177-S186.
5. Baggeto, LG. (1992). Biochimie 74: 959-974.
6. Parlo RA y Coleman PS. (186). Biochem. Biophys. Acta 886:169-176.
7. Ibsen KH. Cancer Res. (1961) 21:829-841
8. Hume AD, Radik JL, Ferber E, y Weidemann MJ. (1978). Biochem.J. 174:703-710.
9. Pedersen,P. (1978) Prog. Exp. Tumor Res. 22, 190-274.
10. Lazo, PA . (1981) Eur. J. Biochem. 117:19-25.
11. Nakashima RA, Paggi MG y Pedersen PL (1984) Cancer Res. 44:5702-5706.
12. Görlach A, y Acker H. (1994). Biochem. Biophys. Acta 1227: 105-110.
13. Rodríguez-Enríquez S, Torres-Márquez,ME y Moreno-Sánchez R. (2000). Arch. Biochem. Biophys. 375:21-30.
14. Kelly DA y Storey KB. (1988). Am. J. Physiol. 255: R774-779.
15. Holleran AL, Fiskum G, y Kelleher JK. (1997) Am J.Physiol. 272:E945-E951.
16. Gauthier T, Denix-Pouxviel C, y Murat JC. (1990) Int. J. Biochem. 22:411-417.
17. Schmidt H, Siems W, Muller M, Dumdey R, y Rapoport S. (1991). Exp. Cell Res. 194:122-127.
18. Muller M, Siems W, Buttgereit F, Dumdey R y Rapoport S (1986). Eur. J. Biochem. 161: 701-706
19. Fearon KCH, Plumb JA, Burns HJG, y Calman KC. (1987). Cancer Res. 47: 3684-3687
20. Jaroszewski JW, Kaplan O, y Cohen JS. (1990). Cancer Res. 50, 6936-6943.

20a. Lambeir AM, Kuntz DA, Vellieux FM, Michels PA, y Opperdoes FR. (1991). Eur. J Biochem. 198, 429-435

21. Sri-Pathmanathan RM, Plumb JA, y Fearon KCH (1994). Int.J. Cancer 56, 900-905.
22. Moreno-Sánchez R, Bravo C, Vásquez C, Ayala G, Silveira L, y Martínez-Lavín M (1999).Biochem Pharmacol 57, 743-752.

23. Lampidis TJ, Bernal SD, Summerhayes IC, y Chen L B. (1983). Cancer Res. 43:716-720.

24. Chen,L.B. (1988). Mitochondrial membrane potential in living cells. Annu. Rev. Cell. Biol. 4:155-181.

25. Gudz TI,Tserng KY, y Hoppel CL. (1997). J. Biol. Chem. 272:24154-24160.

26. Hori H, Noguchi N, Yokoyama H, Ise H, Jin CZ, Kasai S, Goto T, y Taira Z. (1996). Bioorg. Med. Chem. 4:247-253.

27. Rago RP, Brazy PC, y Wilding G. (1982). Cancer Res. 52:6953-6960.

28. Mack P, Ha XF, y Cheng LY. (1996). HPB Surg. 10:65-68.

29. López-Gómez FJ, Tórres-Márquez ME, y Moreno-Sánchez R. (1993). Int. J. Biochem. 25:373-377.

30. Bergmeyer HU (1983). In Methods of Enzymatic Analysis (Bergmeyer, H.U., Ed.), vols 3-9, Weeinheim, Verlag Chemie.

31. Bergmeyer HU (1983). In Methods of Enzymatic Analysis (Bergmeyer, H.U., Ed.), vol 4, Weeinheim, Verlag Chemie.

32 Rodríguez-Enríquez S, Juárez O, Rodríguez-Zavala JS,y Moreno-Sánchez R. *Eur. J Biochem.* 268:1-9.

33. Jaroszewski JW, Kaplan O, y Cohen JS. (1990). Cancer Res. 50:6939-6943.

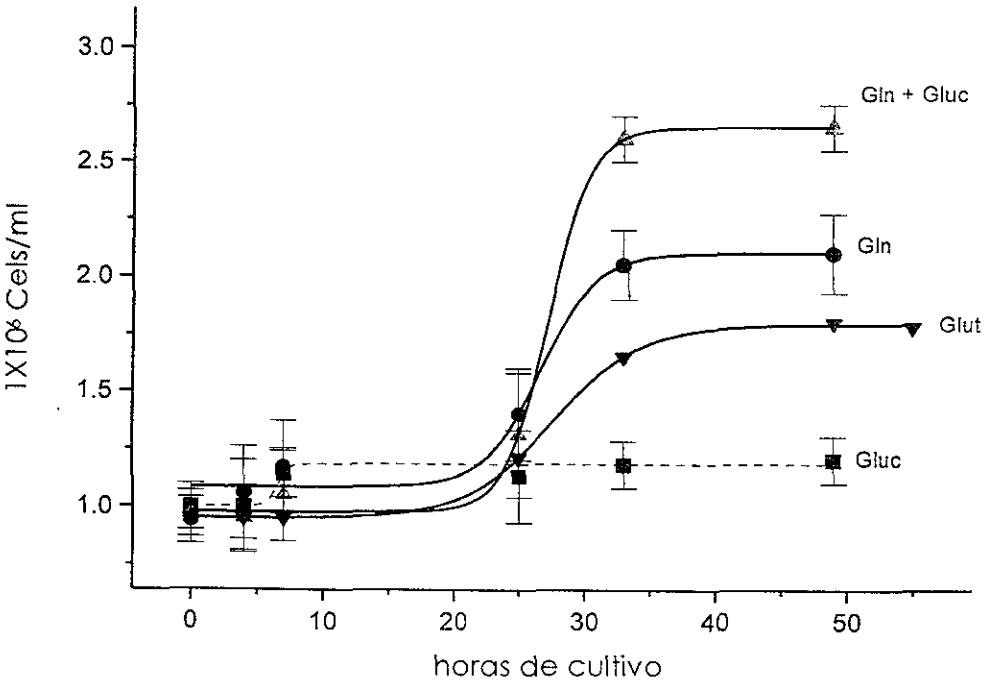

Figura 1. Crecimiento de las células AS-30D cultivadas en presencia de diferentes fuentes de carbono externas. Las células tumorales (1 x10⁶ cels/ml) se sembraron en el medio Dulbecco-MEM enriquecido con glucosa 25 mM + glutamina 5 mM (Gluc + Gln); glutamina 4 mM (Gln), glutamato 5 mM (Glut) o glucosa 25 mM (Gluc). Cada valor representa el promedio de 3 experimentos diferentes ± D E. La viabilidad en cada punto experimental fue de 98 ± 1%, a excepción de la condición Gluc que fue de 83 ± 3%.

Tabla 1. Cambios en la densidad celular, fosforilación oxidativa y contenido de ATP en cultivos primarios de AS-30D en presencia de diferentes fuentes de carbono externas.

Condición	Densidad celular (x 10^6cels/ml)	Respiración celular (ngAO/min/10^7cels)		ATP (nmol/10^7cels)
		A	B	
Gln+Gluc	2.65 ± 0.2 (6)	91 ± 5 (4)	12 ± 0.6 (4)	1.2 ± 0.8 (4)
Gln+Glc+oligo	0.9± 0.1* (4)	ND	ND	ND
Gln	2.1± 0.2* (3)	138 ±20* (4)	21 ± 3 (4)	2.8 ± 1* (4)
Glc	1.2 ± 0.1* (3)	0	21 ± 7* (4)	0.45 ± 0.1* (3)

Las células se cosecharon a las 48 hrs (fase estacionaria) y se lavaron con medio Krebs-Ringer frío. A) respiración sensible a oligomicina. B) insensible a oligomicina.

Abreviaciones: 5 mM glutamina + 25 mM glucosa (Gln+Gluc); glutamina 5 mM (Gln); glucosa 25 mM (Gluc); oligo, 10 µM oligomicina. La viabilidad de las células cultivadas con oligomicina fue del 8%. La respiración resistente a oligomicina fue menor al 15 % en todos los casos, excepto con glucosa donde no hubo sensibilidad al inhibidor. Las determinaciones se realizaron en ausencia de sustrato oxidable. Los valores representan la media ± D.E. El número de experimentos se presenta entre paréntesis. *$p<0.05$ vs. condición Gln+Gluc. ND. No determinados.

Tabla 2. Cambios en la densidad celular, fosforilación oxidativa y glucólisis en AS-30D inducidos por diferentes fármacos del metabolismo energético.

		drogas					
	Gln+Gluc	AIA	gosipol	rodamina 123	rodamina 6G	nimesulide	clofazimina
Densidad celular (x10⁶cels/ml)	2.65 ± 0.2 (6)	1.82 ± 0.1* (3)	1.9 ± 0.5* (3)	0.8 ± 0.1* (3)	0.5 ± 0.1* (3)	1.6 (2)	1.1 (2)
% viabilidad	97.5 ± 2	86 ± 4	93 ± 8	2	0.5	100	30
Fosforilación oxidativa (ngAo/min/ 10⁷cels)	91 ± 5 (4)	101 ±22 (3)	70 (2)	0 (3)	0 (1)	ND	ND
Glucólisis (nmol lactato/ min/10⁷cels)	1.6 ± 0 4 (3)	1 2 ±0.5 (3)	0.16 (2)	0.1 (1)	0 (1)	ND	ND

Las células tumorales (1x10⁶cels/ml) se cultivaron en presencia de Gln + Gluc + las diferentes drogas. La densidad celular fue determinada a las 48 hrs de iniciado el cultivo celular (fase estacionaria). Inhibidores oxidativos: rodamina 123 10 µM, rodamina 6G 10 µM, clofazimina 10 µM, nimesulide 0.4 µM (81 nmol/10⁷). Inhibidores *glucolíticos: gosipol* 100 µM, AIA 100 µM Los valores representan la media ± D.E. El número de experimentos se muestra entre paréntesis

*p<0 05 vs condición Gln + Gluc

Tabla 3. Indice de toxicidad [IC_{50}] para algunos fármacos que afectan el metabolismo energético de AS-30D.

Fármacos	IC_{50} (μM)
Gosipol	198 ± 60 (3)
AIA	98.7 ± 21(3)
Rodamina 123	2 ± 1 (3)
Rodamina 6G	0.9 ± 0.7 (3)
Nimesulide (nmol/10^7cels)	78.5 ± 25 (3)
Clofazimina	10 (1)

Las células AS-30D se cosecharon en fase estacionaria (48 hr). El número de experimentos se muestra entre paréntesis.

CAPITULO 4

DISCUSION GENERAL

Esta tesis enfatiza la importancia de la FO en el suministro de ATP en la línea tumoral ascítica de rápido crecimiento AS-30D. Las células tumorales de rápido crecimiento se han considerado esencialmente glucolíticas debido a fallas en sus sistemas oxidativos:

i) *contener un número reducido de mitocondrias [1],*

ii) la baja actividad en algunos complejos mitocondriales [1,2],

iii) *la existencia de un ciclo tricarboxílico irregular [28].*

Sin embargo a excepción del primer punto, que no se ha evaluado en esta línea, AS-30D presenta un ciclo de Krebs activo y completo [23], y al parecer sus complejos respiratorios son funcionales lo que dan como resultado una elevada tasa respiratoria, tan alta como la reportada en hígado perfundido (2.25 *vs* 3 nmol O2/mg peso seco, respectivamente) [29] y exclusivamente utilizada para la síntesis de ATP (Capítulo 3.1). Por otro lado, se ha descrito en mitocondrias de hepatoma de Zadjela un aumento en la expresión de genes (de 4-6 veces) que codifican para enzimas de la FO como son la translocasa de adenín nucleótidos, las subunidades COX_I y COX_{II} de la citocromo oxidasa y la subunidad II de la NAD oxido-reductasa [30,31]. Sin embargo no se reportan las velocidades de respiración y de síntesis de ATP de este tipo tumoral, por lo que no es posible correlacionar el aumento en los transcritos de estos complejos con la actividad de fosforilación oxidativa.

La actividad glucolítica en AS-30D es muy alta como sucede en numerosas líneas tumorales (ascitis de Ehrlich [2], carcinoma de Lewis [32]); sin embargo a diferencia de las anteriores, en AS-30D la fosforilación oxidativa se encuentra activa y suministra prácticamente todo el ATP celular. De hecho, la degradación activa de glutamato y de cuerpos cetónicos (acetoacetato y β-hidroxibutirato), así como la alta concentración de oxigeno y glutamina, y la baja concentración de glucosa en el liquido de ascitis de AS-30D respaldan fuertemente esta

aseveración (Tablas I y II del Capítulo 3.1). Lo anterior sugiere que es el metabolismo oxidativo el que sustenta el desarrollo del tumor.

En este contexto fue de nuestro interés dilucidar cuales son las enzimas "controladoras" del flujo de fosforilación oxidativa en este hepatoma. A la fecha no se había reportado en ningún tipo tumoral cuál es o son la(s) enzima(s) clave(s) en las vías energéticas tumorales. Lo anterior toma relevancia ya que conociendo las enzimas regulatorias se pueden considerar como sitios de acción terapéutica para el desarrollo de fármacos anticancerígenos. Usando la ecuación de Gellerich *et al.* [27] determinamos que el sitio I de la cadena respiratoria (NADH ubiquinona oxido-reductasa) y el bloque de enzimas consumidoras de ATP son los sitios que ejercen más control (~30%) en el flujo oxidativo de AS-30D. Debido a que la síntesis de proteínas, la activación de ATPasas (Na^+/K^+), la síntesis de ácidos nucleicos y el transporte de Ca^{2+} son altamente demandantes de energía [33], es probable que estos procesos formen parte del bloque de enzimas consumidoras de ATP. La rotenona (inhibidor del sitio I) a concentraciones bajas (0.1-0.5 μM) disminuye el crecimiento tumoral de algunas líneas de osteosarcoma humano promoviendo apoptosis [34]. Estos estudios apoyan la idea de que el sitio I es un punto de regulación dentro de la vía, tal como lo demostramos en la línea AS-30D. Por tanto, es lógico pensar que los procesos celulares con alto gasto energético, como la duplicación tumoral, la síntesis de proteínas y ácidos nucléicos, y el mantenimiento de gradientes iónicos a través de la membrana plasmática y mitocondrial, sean sensibles a inhibidores o fármacos de la fosforilación oxidativa, primordialmente inhibidores del sitio I.

Una observación interesante en el estudio del metabolismo intermediario de AS-30D en ascitis se describe en el Capítulo 3.2: la inhibición parcial de la fosforilación oxidativa por glucosa o fructosa a concentraciones fisiológicas (efecto Crabtree). Los modelos tumorales utilizados para estudiar el efecto Crabtree son básicamente glucolíticos. Por tanto, dilucidar cuál o cuáles son los intermediarios (productos de la catálisis de la glucosa) que promueven la inhibición parcial en la respiración celular y la síntesis de ATP es simplemente una caracterización mecanística del efecto; *i e*, siendo la fosforilación oxidativa una

vía poco eficiente en los tumores glicolíticos no tendría relevancia alguna de que ésta operara o no en presencia de glucosa. Sin embargo, en tumores oxidativos este tipo de estudios no sólo son mecanísticos, sino que pueden tener relevancia clínica; es decir, la adición de glucosa puede ser utilizada para disminuir la duplicación tumoral en líneas oxidativas experimentales (AS-30D) o humanas (carcinoma cervical KB3-1, melanoma FEMX y carcinoma ovárico A 2780). Esta manipulación nutricional para reducir el crecimiento tumoral sólo se ha demostrado en tumores glucolíticos, pero no en oxidativos [35,36]. Por ejemplo, en modelos *in vivo* (ratas portadoras de carcinoma de Walker-256) se demostró que la actividad glucolítica se reduce drásticamente si las ratas portadoras del tumor se someten a una dieta deficiente en carbohidratos [38]; sin embargo, la consecuencia es dañina para el hospedero puesto que puede desarrollar hipoglicemia [35]. En modelos *in vitro* tales como cultivos tumorales, la glucólisis puede ser abatida con 2-desoxiglucosa un análogo no metabolizable de la glucosa [36].

Desde el punto mecanístico, diferimos de la idea de que el efecto Crabtree se deba a un solo factor, como se ha reportado para otras líneas tumorales [37-39]. Nuestros resultados sugieren un control multisitio donde diferentes factores a niveles diferentes en conjunto abaten la fosforilación oxidativa. Un punto de discusión en este estudio es si el efecto Crabtree tiene relevancia fisiológica en las células tumorales (ver sección de discusión del capítulo 3.2). Es probable que fisiológicamente el efecto Crabtree no se manifieste en AS-30D debido a la baja concentración de glucosa en el líquido ascítico, aunque intracelularmente la concentración de glucosa sea 2 mM (Tabla I, capítulo 3.1).

Los capítulos 3.1 y 3.2 de esta tesis arrojaron luz sobre la importancia del metabolismo oxidativo y el fuerte control que el catabolismo de la glucosa ejerce sobre éste. Sin embargo, para demostrar concluyentemente la hipótesis fue necesario recurrir a los cultivos primarios de AS-30D. Con este modelo demostramos que las células AS-30D son incapaces de crecer en presencia de glucosa como única fuente de carbono exógena. Esta observación concuerda con la aseveración de Masurek [3] de que no existe una relación estricta entre la velocidad glucolítica y la proliferación celular como se ha descrito para los

tumores dependientes del metabolismo glicolítico [2,3]. En éstos, la adición de hexosas promueve una acumulación de fosfometabolitos como glucosa 6-P, la cual es canalizada hacia la vía de las pentosas para generar precursores de ácidos nucleicos, nucleótidos y equivalentes reductores. En ausencia o presencia de concentraciones muy bajas de glucosa (0.1 mM), el ATP se produce por la oxidación de piruvato o por la conversión de glutamina a lactato [2, 40]. En el caso de AS-30D, aunque la glucosa promueve acumulación de fosfometabolitos para los precursores de ácidos nucléicos, el factor limitante podría ser la disponibilidad de ATP (Tabla 1, capítulo 3.3). Con glutamina la célula tiene mayor ventaja para proliferar: la oxidación de glutamina provee de ATP, sustrato para la síntesis de purinas y pirimidinas, y equivalentes reductores citosólicos.

Desde hace varias décadas se ha intentado utilizar el metabolismo energético como un sitio para la terapia antineoplástica y aunque es concebible pensar que ciertos tumores dependen de ambas vías energéticas, en la mayor parte de las líneas tumorales el metabolismo glucolítico prevalece sobre el oxidativo. En este contexto es sorprendente encontrar una serie de estudios donde los tumores glucolíticos son sensibles a compuestos que afectan sólo al metabolismo oxidativo. Por ejemplo en algunas líneas glucolíticas experimentales (carcinoma de Walker 256 [41]) y humanas (carcinoma de páncreas CRL1420, carcinoma de pecho MCF-7, tumor de cérvix CRL1550 [42,43]), el empleo de compuestos de la familia de las rodaminas (rodamina 123, 6G, MKT-077) abaten drásticamente el crecimiento tumoral. A bajas concentraciones estos cationes lipofílicos se concentran en las mitocondrias tumorales debido a su alto potencial transmembranal disipando el gradiente electroquímico de protones utilizado para la síntesis de ATP [44]. Por otro lado, la adición de dosis no tóxicas de oligomicina (< 1 nM) a tumores glucolíticos ha revelado que las células se detienen en la fase G_1 del ciclo celular. En estas células se registra una abrupta disminución en el contenido de ATP (20-30%), lo cual indica que el ATP mitocondrial es necesario para la progresión de la fase G_1 a la fase S del ciclo celular [45]. Estas dos observaciones son similares en cuanto a su efecto: alteran el funcionamiento mitocondrial.

Aunque los estudios que hemos realizado se han desarrollado en una línea

experimental derivada de hígado de rata, este modelo puede ser útil en el esclarecimiento de los procesos bioquímicos asociados a la pérdida de control del crecimiento celular en los tumores humanos oxidativos, debido que metabólicamente no son diferentes. Sin embargo, un punto a consideración es la utilización de ciertos fármacos donde la sensibilidad de un tipo tumoral podría cambiar con respecto a otro.

Desde el punto de vista experimental, cualquier tipo de estudio bioquímico requiere de una cantidad considerable de material biológico. El uso de roedores para desarrollar líneas experimentales tumorales (AS-30D) proporciona la gran cantidad del material biológico requerido. Lo anterior se visualiza como una ventaja técnica que no es proporcionada por la utilización de cultivos celulares, donde la disponibilidad de material es limitada.

CAPITULO 5

CONCLUSIONES

1. La línea tumoral AS-30D mantiene una actividad glucolítica elevada, sin embargo otras vías catabólicas activas son; i) la degradación de glucógeno, la cual mantiene un suministro constante de glucosa; ii) la degradación de metabolitos mitocondriales: glutamato, cuerpos cetónicos (acetoacetato y β-hidroxibutirato) y iii) la fosforilación oxidativa.

2. La fosforilación oxidativa de AS-30D provee el 99% del ATP celular requerido para realizar funciones de alta demanda energética.

3. Las altas concentraciones de glutamina (4 mM), glutamato (10 mM), cuerpos cetónicos, y oxígeno (50 μM), y la baja concentración de glucosa (26 μM) en el líquido de ascitis indican que esta línea tumoral se nutre de metabolitos mitocondriales y no de metabolitos glucolíticos.

4. El sitio I de la cadena respiratoria y el bloque de enzimas consumidoras de ATP (como son la duplicación celular, la síntesis de proteínas y de ácidos nucleicos) son las enzimas que controlan el flujo de la fosforilación oxidativa. Ambos sitios ejercen un 30% de control cada uno, por lo que el 40% restante se encuentra distribuido entre los complejos restantes de la cadena respiratoria, la ATP sintetasa, los acarreadores de adenín nucleótidos y de piruvato, el acarreador de fosfato, las deshidrogenasas mitocondriales y la difusión pasiva de los protones. A este respecto se propone al sitio I como un sitio de sensibilidad terapéutica.

5. La glucosa y la fructosa exógenas promovieron un abatimiento en la actividad de FO (54 y 34%, respectivamente). Esta inhibición parcial fue revertida por la adición de desacoplantes, lo que indica que el sistema fosforilante (ATP sintetasa, acarreador ATP/ADP, acarreador de fosfato) es el sitio del efecto Crabtree.

6. Los factores responsables del efecto Crabtree en la línea AS-30D son la disminución en el pH citosólico y la disminución en la concentración intracelular de fosfato. En mitocondrias aisladas de AS-30D, sólo la disminución simultánea en ambos factores (fosfato de 1.5 a 0.6 mM y pH de 7.2 a 6.8) promovió un abatimiento significativo en la fosforilación oxidativa, lo que indica que el efecto Crabtree es multifactorial.

7. La duplicación celular de AS-30D en cultivos primarios exhibió mayor dependencia energética a intermediarios oxidativos (glutamina) que a glucolíticos (glucosa). En presencia de ambos sustratos el crecimiento es óptimo ya que el destino de cada sustrato es diferente: la glucosa es utilizada para un fin sintético mientras que la glutamina es utilizada para un fin energético.

8. La duplicación de AS-30D fue drásticamente abolida por inhibidores o fármacos que disminuyen la actividad de la fosforilación oxidativa, mientras que los inhibidores glucolíticos no tuvieron un efecto significativo.

9. De los fármacos oxidativos evaluados, los compuestos de la familia de las rodaminas (123 y 6G) fueron los que presentaron valores de IC_{50} menores, en el intervalo de 1-2 µM, lo que indica que este tipo tumoral es altamente sensible a estos compuestos.

CAPITULO 6

PERSPECTIVAS

A pesar de que hemos dilucidado parte del metabolismo intermediario de un modelo tumoral oxidativo como AS-30D, aún falta por explorar otros fenómenos involucrados en el desarrollo de su metabolismo atípico.

Concluímos puntualmente que la NADH oxido-reductasa es una de las enzimas regulatorias de la fosforilación oxidativa. La causa aparente es la baja cantidad de enzima cuantificada en las células aisladas, lo que implica que es un sitio de control. Sin embargo, no se han realizado experimentos donde se haya aislado y caracterizado cinética y molecularmente a este complejo respiratorio en células de AS-30D, el cual promete ser un sitio blanco para agentes antitumorales. De hecho, el complejo I se encuentra afectado en varias enfermedades de origen mitocondrial no sería sorprendente que en el desarrollo del cáncer también lo esté [32]. No se han explorado aún los cambios a nivel molecular de la enzima, desde la posible supresión en la expresión de estos genes hasta la activación de los procesos de degradación enzimática. Tampoco se han realizado estudios cinéticos en esta enzima, por lo que se desconoce si presenta mayor afinidad por NADH que la enzima de tejidos no tumorigénicos, de ser así, las células tumorales serían más sensibles a agentes que alteren el potencial redox mitocondrial.

Por otro lado hemos avanzado en el estudio del metabolismo energético en cultivos primarios de AS-30D un tumor experimental, y aunque es concebible pensar que metabólicamente pueden ser similares, sería interesante ampliar estos estudios utilizando diferentes líneas de tumores, los tumores oxidativos serán más susceptibles a fármacos cuyo blanco es la mitocondria. Es importante entonces caracterizar energéticamente a las líneas humanas seleccionadas provenientes de tejidos diferentes. De hecho, a pesar de que se ha descrito que algunos tumores humanos (HeLa y Hep-2) son glucolíticos, no existe evidencia sobre la dependencia de los tumores humanos al metabolismo oxidativo. Görlach y Acker,

utilizando dos tumores de colon humano en multiesferoides, uno oxidativo y el otro glucolítico, encontraron que el tumor glucolítico es menos sensible a la radiación y al empleo de ciertos fármacos anticancerígenas, *i.e.* el metabolismo glucolítico (bajo pH e hipoxia) se asocia con la resistencia tumoral [46]. Estos autores sugirieron una estrategia bioquímica para cambiar el metabolismo de los tumores glucolíticos (más resistentes) a oxidativos (menos resistentes), la cual consistió en el empleo de oxamato, un análogo estructural del piruvato que dependiendo de la concentración puede inhibir competitivamente a la lactato deshidrogenasa o al transportador de piruvato [46]. Lo anterior puede ser empleado en otros modelos tumorales para cambiar su metabolismo energético.

De hecho, es posible pensar que en tumores glicolíticos, la manipulación metabólica puede realizarse de otras maneras: i) el uso de cualquier inhibidor del catabolismo de la glucosa (a concentraciones que no afecten el metabolismo oxidativo) podrá estimular el flujo de la fosforilación oxidativa para compensar la pérdida energética que proviene de la vía citosólica; o bien ii) el empleo de sustratos esencialmente mitocondriales como glutamina ó glutamato, forzará la activación del metabolismo oxidativo. De esta manera, también se puede hacer evidente el cambio en la dependencia de un metabolismo a otro.

Una conclusión importante en nuestro estudio fue que AS-30D mantiene un crecimiento casi óptimo en presencia de metabolitos mitocondriales como glutamina (Figura 1, capítulo 3.3). Sin embargo, AS-30D en suspensión oxida preferencialmente otros metabolitos mitocondriales que la glutamina como los cuerpos cetónicos (acetoacetato y β-hidroxibutirato). Briscoe *et al.* [25] han reportado que la actividad de la acetoacetil CoA transferasa de AS-30D, enzima involucrada en la formación de acetoacetil CoA a partir de acetoacetato más succinil CoA, es 40 veces más alta que la de hígado. Esto concuerda con nuestros resultados de que la degradación de acetoacetato es activa en este tipo tumoral; con respecto al β-hidroxibutirato los mismos autores argumentan que su oxidación es menor que el acetoacetato, pero no cuantifican la actividad de la β-hidroxibutirato deshidrogenasa. En AS-30D observamos una degradación significativa de β-hidroxibutirato, lo que significa que la enzima es más activa que

la reportada en algunos tipos de hepatoma [47,48]. Con este antecedente se pretende realizar cultivos primarios de AS-30D en presencia de estos metabolitos para dilucidar la participación de la cetólisis en el suministro de intermediarios oxidativos como acetil CoA para la síntesis de ATP.

Por otro lado, una gran mayoría de tumores humanos malignos (como el carcinoma de pulmón [WIL]) son resistentes al tratamiento con quimioterápia y radiación. Debido a lo anterior, el desarrollo de alternativas que permitan combatir el crecimiento y propagación del tumor toma relevancia. El enfoque metabólico que se maneja en esta tesis es una de las alternativas fuertes para combatir este tipo de tumores, donde los tratamientos convencionales no son efectivos. Aunado a lo anterior, el estudio de la bioenergética del tumor es fundamental para proponer la estrategia a seguir: el tratamiento dependerá de la vía energética que prevalezca en el tumor.

Aunque se ha estudiado ampliamente el metabolismo tumoral, no existe literatura (más que la presentada en esta tesis) acerca del grado de control que ejercen las enzimas sobre un flujo importante en el suministro de ATP en tumores oxidativos como AS-30D. Exponemos, con el análisis de las elasticidades, que las vías consumidoras de ATP también ejercen un control evidente. Sin embargo, este análisis puede ser útil para determinar la sensibilidad de estas vías (síntesis de proteínas, ácidos nucléicos) a otros intermediarios de la proliferación celular. Para lo anterior se pueden utilizar células cultivadas en presencia de diferentes sustratos, y a concentraciones variables de cada uno de ellos. Por ejemplo, las células cultivadas con glutamina + glucosa exhibirán una mayor producción de NAD(P)H que aquellas cultivadas con sólo uno de los dos sustratos. Estos cambios en la concentración del intermediario se reflejará en una respuesta diferente en el proceso duplicativo.

Otro punto interesante que emerge de este estudio es la alta sensibilidad de AS-30D a los diferentes fármacos oxidativos Sin embargo, el análisis debe extenderse al estudio del efecto de estos mismos compuestos, a las mismas

concentraciones que afectan al tumor, pero sobre líneas celulares normales proliferativas y no-proliferativas. Esta comparación podría arrojar luz en la consideración de estos compuestos como posibles "estrategias clínicas" en un tipo específico de tumor.

CAPITULO 7

BIBLIOGRAFIA

1. Pedersen,PL. (1978). Tumor mitochondria and the bioenergetics of cancer cells. Prog. Exp. Tumor Res. 22:190-274.
2. Eigenbrodt, E., Fister, P., Reinacher, M. (1985) *in* New Perspectives on Carbohydrate Metabolism in Tumor Cells. (Reitner, R. Ed.), Vol 2, pp 141-179, *CRC Press, Boca Raton, FL.*
3. Mazurek, S., Boschek, C.B., Eigenbrodt, E. (1997). The role of phosphometabolites in cell proliferation energy metabolism, and tumor therapy. J. Bioenerg. Biomembr. 29:315-330.
4. Mazurek, S., Michel, A., Eigenbrodt, E. (1997). Effect of extracellular AMP on cell proliferation and metabolism of breast cancer cell lines with high and low glycolytic rates. J. Biol. Chem. 272:4941-4952.
5. Kallinowski, F., Schlenger, KH., Runkel,S., Kloes,M. Stohrer,M., Okunieff,P., Vaupel, P. (1989). Blood flow, metabolism, cellular miroenvironment, and growth rate of human tumor xenografts. Cancer Res. 49:3759-3764.
6. Soderberg, K., Nissien, E., Bakay, B., Scheffler, I.E. (1980). The energy charge in wild-type and respiration-deficient Chinese hamster cell mutants. J. Cell. Physiol. 103:169-172.
7. Lanks, K.W., Li, P.W. (1988). End products of glucose and glutamine metabolism by cultured cell lines. J. Cell. Physiol. 135:151-155.
8. Board, M., Humm, S., Newsholme, E.A. (1990). Maximum activities of key enzymes of glycolysis, glutaminolysis, pentose phosphate pathway and tricarboxylic acid cycle in normal, neoplastic and suppressed cells. Biochem. J. 265:503-509.
9. Rodríguez-Enríquez, S., Torres-Márquez, M.E., Moreno-Sánchez, R. (2000) Substrate oxidation and ATP supply in AS-30D hepatoma cells. Arch. Biochem. Biophys. 375:21-30.
10. Moreadith, R.W, Lehninger, A L (1984). The pathways of glutamate and glutamine oxidation by tumor cell mitochondria. Role of mitochondrial NAD(P)+-dependent malic enzyme. Biol. Chem 259.6215-6221

11. Schwartz, J.P., Passonneau, J.V., Johnson, G.S., Pastan, I. (1974). The effect of growth conditions o NAD^+ and NADH concentrations and the NAD/NADH ratio in normal and transformed fibroblasts. J. Biol.Chem., 249:4138-4143.

12. Pazmiño, N., Doherty, D.G., Reagan, J.D. (1973). Inhibition of DNA and RNA synthesis, but minimally of protein synthesis, in human leukemia cells by a serine antimetabolites. J. Natl. Cancer Inst., 51, 761-765.

13. Girgis, S., Suh, J.R., Jolivet, J., Stover, P.J. (1997). 5-Formyltetrahydrofolate regulates homocysteine remethylation in human neuroblastoma. J. Biol. Chem., 272:4729-4734.

14. Warden, C.H., Friedkin, M., Geiger, P.J. (1980). Acid-soluble and derivatives of phospholipids increase after stimulation of quiescent Swiss 3T3 mouse fibroblast with serum. Biochem. Biophys. Res. Commun. 94:690-696.

15. Reitzer, L.J., Wice, B.M., Kennell, D. (1980). The pentoses cycle. Control and esential function in HeLa cells . J. Biol. Chem. 255:5616-5626.

16. Eigenbrodt, E., Glossman, H. (1980). Glycolysis –one of the keys to cancer. Trends Pharmacol. Sci. 1:240-250.

17. Ravid, K., Diamant, P., Aji-Dor, Y. Glucose-dependent stimulation of protein synthesis in cultured heart muscle cells. (1980). FEBS Lett. 119.20-24.

18. Lenz, J.R., Chaterjee, G.E., Maroney, A., Baglioni, C. (1978). Phosphorylated sugars stimulate protein synthesis and Met-tRNA binding activity in extracts of mammalian cells. Biochemistry 17:80-85.

19. Hugo, F., Mazurek, S., Zander, U., Eigenbrodt (1992). In vitro effect of extracellular AMP on MCF-7 breast cancer cells: inhibition of glycolysis and cell proliferation. J. Cell Physiol. 153:539-549.

20. Wice, B.M., Trugnan, G., Pinto, M., Rousset, M., Chevalier, G., Dussaulx, E., Lacroix, B., Zweibaum, A. (1985). The intracellular accumulation of UDP-N-acetylhexosamines is concomitant with the inability of human colon cancer cells to differentiate. J. Biol. Chem. 260.139-146.

21. Schwartz, A.G., Lewbart, M.L., Pashko, L.L. (1988). Novel dehydroepiandrosterone analogues with enhanced biological activity and reduced side effects in mice and rats. Cancer Res. 48:4817-4822.

22 López-Gómez, F.J., Torres-Márquez, M.E , Moreno-Sánchez, R. (1993). Control of oxidative phosphorylation in AS-30D hepatoma mitochondria. Int. J. Biochem 25 373-377

23. Dietzen, D.J., Davis, E.J. (1993). Oxidation of pyruvate, malate, citrate, and cytosolic reducing equivalents by AS-30D hepatoma mitochondria. Arch. Biochem. Biophys. 305:91-102.

24. Nakashima, R.A., Paggi, M.G., Pedersen, P.L. (1984). Contributions of glycolysis and oxidative phosphorylation to adenosine 5'-triphosphate production in AS-30D hepatoma cells. Cancer Res. 44:5702-5706.

25. Briscoe, D.A., Fiskum, G., Holleran, A.L., Kelleher, J.K. (1994). Acetoacetate metabolism in AS-30D hepatoma cells. Mol. Cell. Biochem. 136:131-137.

26. Tager, J.M., Wanders, R.J., Groen, A.K., Kunz, M., Bohsensack, R., Küster, U., Letko, G., Böhne, G., Duszynsky, J., Wotczak, L. (1983). Control of mitochondrial respiration. FEBS Lett. 151:1-9.

27. Gellerich, F.N., Kunz, W.S., Bohsensack, R.E. (1990). Stimation of flux control-coefficients from inhibitior titrations by non-linear regression. FEBS Lett. 264:167-170.

28. Parlo, R.A., Coleman, P.S. (1986). Continuos pyruvate carbon flux to newly synthesized cholesterol and the suppressed evolution of pyruvate-generated CO_2 in tumor: further evidence for a persistent truncated Krebs cycle in hepatomas. Biochem. Biophys. Acta 886:169-176.

29. Kashiwagura, T., Deutsch, C.J., Taylor, J., Erecinska, M., Wilson, D.F. (1984). Dependence of gluconeogenesis, urea synthesis, and energy metabolism of hepatocytes on intracellular pH. J. Biol. Chem. 239:237-243.

30. Luciakova, K., Kuzela, S. (1992). Increased steady-state levels of several mitochondrial and nuclear gene transcripts in rat hepatoma with a low content of mitochondria. Eur. J. Biochem. 205:1187-1193.

31. Capuano, F., Guerrieri, F., Papa, S. (1997). Oxidative phosphorylation enzymes in normal and neoplastic cell growth. J. Bioenerg. Biomembr. 29: 379-384.

32. Miralpeix, M., Azcon-Bieto, R., Argilés J.M. (1990). The impairment of respiration by glycolysis in the Lewis lung carcinoma Cancer Lett. 50:173-178.

33. Schmidt, H., Siems, W., Buttgereit, F., Dumdey, R, Rapoport, S. (1991). ATP-producing and consuming processes of Ehrlich mouse ascites tumor cells in proliferating and resting phases Exp Cell Res. 194:122-127

34. Barrientos, A., Moraes, C. (1999). Titrating the effects of mitochondrial complex I. Impairment in the cell physiology. J. Biol. Chem. 274:16188-16197.

35. Dills, W.L., and Block, E.F.J. (1992). Differential effects of hexose analogs on the rat Walker tumor *in vivo* and *in vitro*. Biochem. Arch. 8:221-225.

36. Chen, S.C.H., Tsai, S., Neishem, M.C. (1980). Response of rats fed diets low in glucose and glucose precursors to low levels of starch and chemically modified starch. J. Nutrition. 110:1023-1031.

37. Sussman, I., Erecinska, M., Wilson, D.F. (1980). Regulation of cellular energy metabolism: The Crabtree effect. Biochem. Biophys. Acta. 591:209-223.

38. Gabai, V.L. (1992). Glucose decreases respiratory control ratio in EL-4 tumor cells. FEBS. 313:126-128.

39. Evtodienko, Y.V., Teplova, V.V., Duszynski, J., Bogucka, K., Wojtczak, L. (1994). The role of cytoplasmic [Ca^{2+}] in glucose-induced inhibition of respiration and oxidative phosphorylation in Ehrlich ascites tumour cells: a novel mechanism of the Crabtree effect. Cell Calcium. 15:439-446.

40. Pouysségur, J., Franchi, A., Salomon, J.C., Silvestre, P. (1980). Isolation of a Chinese hamster fibroblast mutant defective in hexose transport and aerobic glycolysis: its use to dissect the malignant phenotype. Proc. Natl. Acad. Sci. USA 77:2698-2701.

41. Fearon, K., Plumb, J.A., Burns, H., Calman, K. (1987). Reduction of the growth rate of the Walker 256 tumor in rats by rhodamine 6G together with hypoglycemia. Cancer Res. 47:3684-3687.

42. Koya, K., Li, Y., Wang, H., Ukai, T., Tatsuta, N., Kawakami, M., Shishido, T., Chen, L.B. (1996). MKT-077, a novel rhodacyanine dye in clinical trials, exhibits anticarcinoma activity in preclinical studies based on selective mitochondrial accumulation. Cancer Res. 56:538-543.

43. Summerhayes, I., Lampidis, T., Bernal, S., Nadakavukaren, J., Nadakavukaren, K., Shepherd, E., Chen, L.B. (1982). Unusual retention of rhodamine 123 by mitochondria in muscle and carcinoma cells. Proc. Natl. Acad. Sci. USA 79:5292-5296.

44. Chen,L.B. (1988). Mitochondrial membrane potential in living cells. Annu. Rev. Cell. Biol 4:155-181

45. Sweet, S., Singh, G. (1995). Accumulation of human promyelocytic leukemic (HL-60) cells at two energetic cell cycle checkpoints. Cancer Res. 55:5164-5267

46. Görlach, A., Acker, H. (1994). pO_2 and pH-gradients in multicellular spheroids and their relationship to cellular metabolism and radiation sensitivity fo malignant human tumor cells. Biochim. Biophys. Acta 1227:105-110.

47. Nakashima, R.A., Paggi, M.G., Scott, L.J., Pedersen, P.L. (1988). Purification and characterization of a bindable form of mitochondrial bound hexokinase from the highly glycolytic AS-30D rat hepatoma cell line. Cancer Res. 48:913-919.

48. Ohe, K., Morris, H.P., Weinhouse, S. (1967). D-β Hydroxybutirate dehydrogenase activity in liver and tumors. Cancer Res. 27:1360-1371.

49. Zhang, W.W., Churchill, S., Lindahl, R., Churchill, P. (1989). Regulation of D-β Hydroxybutirate dehydrogenase in rat hepatoma cell lines. Cancer Res. 49:2433-2437.

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Oxidative phosphorylation supported by an alternative respiratory pathway in mitochondria from *Euglena*

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Abstract

The effect of antimycin, myxothiazol, 2-heptyl-4-hydroxyquinoline-*N*-oxide, stigmatellin and cyanide on respiration, ATP synthesis, cytochrome *c* reductase, and membrane potential in mitochondria isolated from dark-grown *Euglena* cells was determined. With L-lactate as substrate, ATP synthesis was partially inhibited by antimycin, but the other four inhibitors completely abolished the process. Cyanide also inhibited the antimycin-resistant ATP synthesis. Membrane potential was collapsed (< 60 mV) by cyanide and stigmatellin. However, in the presence of antimycin, a H^+ gradient (> 60 mV) that sufficed to drive ATP synthesis remained. Cytochrome *c* reductase, with L-lactate as donor, was diminished by antimycin and myxothiazol. Cytochrome bc_1 complex activity was fully inhibited by antimycin, but it was resistant to myxothiazol. Stigmatellin inhibited both L-lactate-dependent cytochrome *c* reductase and cytochrome bc_1 complex activities. Respiration was partially inhibited by the five inhibitors. The cyanide-resistant respiration was strongly inhibited by diphenylamine, *n*-propyl-gallate, salicylhydroxamic acid and disulfiram. Based on these results, a model of the respiratory chain of *Euglena* mitochondria is proposed, in which a quinol-cytochrome *c* oxidoreductase resistant to antimycin, and a quinol oxidase resistant to antimycin and cyanide are included.

Keywords: ATP synthesis; Antimycin; Cyanide-resistant respiration; *Euglena*

1. Introduction

Mitochondria isolated from dark-grown *Euglena gracilis* have respiratory components that are resistant to antimycin and cyanide [1–3]. The cyanide-resistant respiratory pathway is inhibited by diphenylamine (DPA) [2,4], preferentially oxidizes L-lactate [4,14], and builds up a small, uncoupler-sensitive membrane potential [4,5], that supports the energy-dependent uptake of Ca^{2+} [6]. This pathway is partially inhibited by salicylhydroxamic acid (SHAM) [4,7], a potent inhibitor of alternative respiratory pathways in plant mitochondria [8]. Cell growth in the presence of antimycin [1,2,9], cyanide [10] or ethanol [10] as carbon source, induces an increase in the content of a *b*-type cytochrome, that reacts with carbon monoxide. This last observation has been interpreted in terms of an adaptable enhancement of an alternative oxidase, which is resistant to the stress conditions of the culture [1,2,10]. However,

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Re: JBB 1032

Dear Dr. Moreno-Sanchez:

Your manuscript entitled "Metabolic Changes Induced by Cold Stress in Rat Liver Mitochondria" has been reviewed by an expert in your area of research. I am pleased to say that the reviewer only found minor criticisms of your paper. I am sending you his review and asking you to take care of these minor points. You can then send two copies of the corrected pages back to me at which time the manuscript will be considered officially accepted.

Sincerely yours,

Peter L. Pedersen

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INVOLVEMENT OF FREE FATTY ACIDS AND UNCOUPLING PROTEIN 2 IN THE METABOLIC CHANGES INDUCED BY COLD STRESS IN LIVER MITOCHONDRIA

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Key Words. Uncoupling protein 2, decoupling, free fatty acids, ATP synthesis, respiration.

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PROBLEMA BIOQUÍMICO

TEMA: Control Metabólico

Las vías metabólicas pueden simplificarse para su análisis de control, en bloques de enzimas que trabajan en un estado estacionario, donde E_1 y E_2, respectivamente, representan al conjunto de enzimas que producen y consumen a un intermediario. Por ejemplo, la regulación de la concentración de ATP es función de la actividad de las vías productoras de ATP (glicólisis, fosforilación oxidativa) y también de las vías consumidoras de ATP (síntesis de proteínas, ácidos nucleicos, lípidos, polisacáridos, etc).

$$SH_2 \xrightarrow{E_1} ATP \xrightarrow{E_2} \text{Trabajo Celular} \begin{cases} \text{Procesos anabólicos} \\ \text{Fenómenos de Transporte} \end{cases}$$

Para determinar cuál de los bloques de enzimas, el sistema consumidor o el productor de ATP, ejerce un mayor control sobre el flujo de la vía, se puede utilizar el análisis de control de las elasticidades. Esta aproximación consiste en determinar los coeficientes de elasticidad [e] de los 2 segmentos de la vía que producen y consumen ATP. Una manera sencilla de determinar experimentalmente los e consiste en modificar el flujo de la vía que cataliza E_1 y E_2, y medir el intermediario (ATP):

En células tumorales de AS-30D, el flujo de E_1 y E_2 (medido como respiración celular) fue titulado con inhibidores de los dos segmentos de la vía: oligomicina (100-500 pmol/10^7cels) ó estreptomicina (0.1-0.5 mg/10^7cels) respectivamente. En cada condición experimental se cuantificó la cantidad de ATP.

Condición	Respiración celular ngAO/min/10^7cels		ATP nmol/10^7cels	
		*%		*%
Control	60	100	1.88	100
Oligomicina				
100	54.6	91	1.43	76.0
200	50.7	84.5	1.29	68.5
300	49.4	82.3	1.12	59.5
400	47.6	79.4	1.01	53.7
500	47.0	78.3	1.00	53.1
Estreptomicina				
0.1	58.8	98	2.20	113
0.2	57.6	96	2.25	120
0.3	55.8	93	2.44	130
0.5	54.6	91	2.63	140

*% con respecto al control

Determinar los coeficientes de elasticidad y los coeficientes de control de flujo de cada bloque de enzimas.

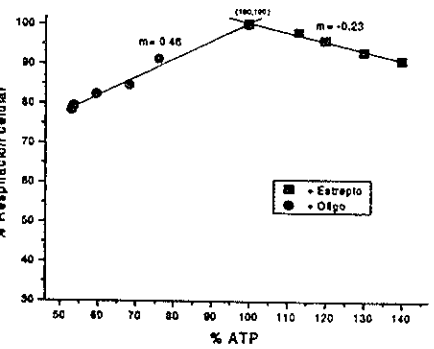
PROBLEMA BIOQUÍMICO

RESPUESTA:

Los coeficientes de elasticidad (grado de respuesta) de los 2 bloques de enzimas al variar la concentración de ATP se calculan a partir de la siguiente ecuación:

$$\varepsilon = \frac{Mo}{Vo}\left[\frac{\delta V}{\delta M}\right]_{ss}$$

Donde *Mo* es la concentración inicial del metabolito y *Vo* es la velocidad inicial; *Mo* y *Vo* están normalizados al 100% y tienen el mismo valor, por lo que el cociente Mo/Vo es igual a 1. $(\delta V/\delta M)_{ss}$ es la variación infinitesimal de la actividad enzimática cuando se varía la concentración del intermediario *M* y se calcula experimentalmente de la pendiente de la gráfica del flujo (% respiración celular) *vs* el intermediario (% ATP), tal como se muestra en la siguiente figura:



Como se observa, se obtienen 2 rectas desde el punto de origen (100,100). Este punto se obtuvo de los valores de respiración y concentración de ATP en estado estacionario, en ausencia de inhibidor. De este gráfico se pueden obtener los coeficientes de elasticidad de los 2 bloques de enzimas participantes en el sistema:

Al disminuir el flujo de E_2 con estreptomicina, se puede determinar el grado de respuesta (coeficiente de elasticidad) del bloque E_1 hacia el intermediario que comparte con E_2. El coeficiente de elasticidad para E_1 se calcula de la pendiente de la recta obtenida al titular el flujo de E_2, con signo negativo porque denota que es producto de E_1= -0.23. De la misma forma, el coeficiente de elasticidad del bloque E_2 se obtiene de la pendiente de la recta al titular el flujo con oligomicina (inhibidor del bloque E_1). En este caso, el valor del coeficiente de elasticidad es positivo porque denota que es sustrato de E_2 (0.46).

Los coeficientes de control de flujo (Co) pueden cuantificarse utilizando el teorema de la conectividad [ecuación (1)], que establece un formalismo entre ε y Co; y la propiedad de la sumatoria [ecuación (2)] que establece que la suma de los coeficientes de control de flujo de las enzimas que integran la vía metabólica es igual a la unidad (100%).

$$C_{E1}^{J}\varepsilon_{ATP}^{E1} = - C_{E2}^{J}\varepsilon_{ATP}^{E2} \quad \text{.....(1)}$$
$$C_{E1}^{J} + C_{E2}^{J} = 1 \quad \text{..... (2)}$$

De tal manera que se tiene un sistema de ecuaciones con 2 incógnitas.

Sustituyendo los valores de los coeficientes de elasticidad de cada segmento:

$$C_{E1}^{J}(-0.23) = - C_{E2}^{J}(0.46) \quad \text{.....(1)}$$

Sustituyendo en términos de una sola variable (ecuación 2):

$$C_{E1}^{J}(0.23) = 0.46\,[1 - C_{E1}^{J}] = 0.46 - 0.46\,C_{E1}^{J}$$

$$0.23\,C_{E1}^{J} + 0.46\,C_{E1}^{J} = 0.46$$

$$C_{E1}^{J} = \frac{0.46}{0.69}$$

$$C_{E1}^{J} = 0.66$$

Sustituyendo en la ecuación (2):

$$C_{E2}^{J} = 0.34$$

El coeficiente de elasticidad de E_1 es menor que el obtenido para E_2 (-0.23 *vs* 0.46, respectivamen-

te). Lo anterior significa que E_1 tiene una menor respuesta (es menos sensible) a las variaciones en la [ATP] que E_2; es decir, la actividad de E_1 se encuentra más cerca de la saturación por ATP, en este particular estado estacionario. Por lo tanto, la actividad de E_1 varía poco al modificar la [ATP]; es decir, se trata de un bloque de enzimas que controla notoriamente el flujo (su coeficiente de control es alto). En cambio, E_2 puede responder más rápidamente a las variaciones de ATP (ε más alto), compensando más fácilmente cualquier cambio en la [ATP] con cambios en su velocidad. Como consecuencia, la actividad de E_2 tiene un efecto menor en el flujo; es decir; su coeficiente de control es pequeño (0.34).

Entonces, E_1 es el bloque de enzimas más limitante en células tumorales AS-30D en estado proliferativo, porque presenta un Co más alto (0.66), controlando un 66% del flujo total de la vía; mientras que E_2 controla el 34% restante.

Bibliografía

1. Westerhoff HV, Groen AK, and Wanders RJA (1984). Biosci. Rep. *4*: 1-22.
2. Fell D (1992). Biochem. J. *286*: 313-330.
3. Rodríguez-Enríquez S., Torres-Márquez ME, and Moreno-Sánchez R. (2000). Arch. Biochem. Biophys. *375*: 21-30.

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