HUMAN TUMOR RESPIRATION[1]

Por

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Resumen

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71 tumores humanos malignos y 20 benignos fueron estudiados en relación con su capacidad de utilizar oxígeno con sustratos endógenos o con glucosa, glutamina o una mezcla de las dos como sustratos. Se encontró que los tumores consumen oxígeno en forma muy similar a la de los tejidos peritumorales que fueron ablados en la misma operación quirúrgica. No se encontró diferencia en el consumo de glucosa o de glutamina por los tumores, pero se constató que la glutamina disminuía la captación de glucosa, mientras que la glucosa aumentaba la captación de glutamina. Se demostró que la marca radioactiva de la glucosa podía aparecer en CO_2, constatando que no hay una ruptura completa entre glicólisis y ciclo de ácidos tricarboxílicos en tumores humanos. Se propone que el aumento en lactato típico de los tumores puede ser debido a inhibición de los transportadores de equivalentes reducidos del citoplasma a la mitocondria acompañado de un descontrol de la enzima fosfofructocinasa.

Palabras claves: Tumores humanos - respiración.

Abstract

71 malignant and 20 benign human tumors were tested as to their capability of oxygen utilization using endogenous substrates or either glucose or glutamine or a combination of these two energy substrates. It was found that tumor oxygen uptake did not differ significantly from that of peritumoral tissues excised in the same chirurgical procedure. No difference was found in glucose and glutamine uptake between tumoral and peritumoral tissues, but it was shown that glutamine decreases glucose uptake while glucose increases glutamine uptake in tumors. It was demonstrated that the radioactive label from glucose could be found in CO_2 showing that there is not a complete uncoupling of glycolysis and tricarboxylic acids cycle in human tumors. It is proposed that the typical increased lactate production in tumors might be due to inhibition of reduced equivalent shuttles between cytoplasm and mitochondria concomittant with a non-controlled phosphofructokinase.

Key words: Human tumor - respiration.

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Introduction

During close to two decades, **Warburg** (1930) carried out very careful experiments in order to establish the metabolic difference between normal and cancer cells. In the process he designed new methodology to measure oxygen uptake by manometric techniques and to determine the amount of lactate produced by tissues. Warburg mainly used animal tumors for his experiments, even though he reported a few with human tumors. His findings can be summarized saying that tumores differ from normal cells in that the former use significantly greater amounts of glucose and concommitantly produce larger amounts of lactate, i.e., tumors seem to be essentially glycolytic while normal cells are aerobic. This, of course, does not mean that tumors do not use oxygen, and in fact, Warburg did some experiments in which he showed tumor oxygen uptake. From his findings, **Warburg** (1956) advanced a theory according to which cancer was the result of a metabolic alteration in normal cells which somehow impaired their respiratory machinary. According to this theory, only cells which were able to switch from aerobic respiration to anaerobic glycolisis could survive and were released from normal growth control allowing them to proliferate uncontrolled. Even though this theory was soon proved to be invalid, the notion that tumors are essentially anaerobic has remained latent even to this day.

That tumor cells had the necessary enzymes for aerobic respiration was first shown by **Weinhouse** (1955). Several other workers confirmed these findings during the following years (**Wenner & Mackner**,1967; **Weber**, 1977). **Weber** (1971), working with a series of Morris hepatomas, was able to show that neither respiration nor glycolysis were altered in well differentiated slow-growth hepatomas. **Koukl** *et al.* (1977) showed that tumor mitochondria had bizarre forms and **Villalobos** and **Lehninger** (1979) showed that Ehrlich's ascites mitochondria had the same proton transport stoichiometry as heart and liver mitochondria.

The production of large amounts of lactic acid from glucose in the presence of oxygen, characteristic that has been called aerobic glycolysis, is not limited to cancer cells. In fact, some normal tissues, such as chicken and rat embryos, retina, brain cortex, renal medulla, bone marrow and placenta, may also exhibit this type of metabolic trait (**Pedersen**, 1978).

Aerobic glycolysis seems to be a metabollically wasteful process. A quick calculation shows that in the process of converting a mole of glucose aerobically to CO_2 and O_2, between 36 and 38 moles of ATP are formed. The difference, of course, depends on whether the glicerolphosphate or the malate shunts are used to reoxydize the NADH produced in glycolysis. Even if we take the lower ATP production and compare it with the two moles of ATP formed when a mole of glucose is converted to lactate, we find that the latter process is only 5.6% as efficient as respiration. For a tissue that is supposed to have high energy requirements, this seems to be a disadvantage *vis a vis* normal tissues.

This line of reasoning led some workers to propose that tumors must consume far greater amounts of glucose than normal tissues in order to meet their energy requirements, resulting in the starving of other tissues and contributing to cancer cachexia (**Gold**, 1974) . This is, of course, an attractive hypothesis. However, one can easily calculate that in order to meet basal metabolic needs a tumor tissue would have to consume almost 18 times as much glucose as a normal comparable tissue. This, however, has not found experimental confirmation (**Ahmed** *et al.*, 1993).

If the amount of glucose used by most tumors is not significantly different from that utilized by normal tissues and tumor cells have a normal and functional complement of respiratory enzymes, one must suppose that a) there must be some alteration in cytoplasmic enzymes or mitochondrial transporters that would somehow channel pyruvate to lactate at the expense of acetyl-CoA production and b) tumor cells must be able to use respiratory substrates such as fatty acids and aminoacids to meet their energy needs.

Several workers have addressed the issue of glycolytic enzymes in cancer cells. On the whole, there seem to be alterations in enzyme kinetics and make-up, most of which are somehow reminiscent of those found in early stages of development. **Newsholme** and his group (1990) studied maximal activities of key control enzymes in glycolysis, pentose-phosphate shunt and tricarboxylic acid cycle in normal and neoplastic cells. They showed that pyruvate kinase activity from the latter was far larger than that found in muscle. They also showed that neoplastic cells had a full complement of functional Krebs cycle enzymes. **Goldman** *et al.* (1964) studied the lactate dehydrogenase from a large amount of normal and neoplastic tissues. They found that the activity was greater in the tumor cells and that they all seemed to have M type subunits, which is consistent with the conversion of pyruvate to lactate. These, and other findings (**Rasschaert** and **Malaisse**, 1993; **Board** *et al.*, 1995) only show, how-

ever, that the enzymes concerned can handle an increased glycolytic flux, but do not explain what causes it.

It could be postulated that the increased lactate production could be due to an accumulation of pyruvate due to an impairment of either pyruvate dehydrogenase activity or of the mitochondrial membrane transport system for that ketoacid. **Lazo** and **Sols** (1980) showed that the pyruvate dehydrogenase complex in Ehrlich ascites tumor cells exhibited greatly reduced activity as compared to normal cells. He also showed (**Lazo**, 1981) that only 1.5% of the glucose taken up went to the tricarboxylic acid cycle and 2% to lipids. These findings, however, have not been substantiated for other tumors. For instance, **Portais** *et al.*, (1993), showed that 78% of the lactate and 57% of the acetyl CoA came from glucose in C6 glioma cells and there are reports (**Briscoe** *et al.*, 1994) that experimental hepatoma cells have both high glycolytic and lipid synthesis rates.

Newsholme (1990) also showed that glutaminase activity of tumor cells was just as large as that of glucokinase, suggesting that glutamine can be used quite well by tumor cells. In fact, several authors had shown that glutamine or glutamate could be used by tumor cells (**Kovacevic**, 1971; **Kovacevic & Morris**,1972; **Reitzer** *et al.*, 1979; **Lazo**, 1981; **Moreadith** and **Lehninger**, 1984) and **Souba** (1993) has suggested that tumors are actually "glutamine traps" which lead to depletion of the metabolite *in vivo* from other tissues. This is an interesting finding since it suggests that tumors can at the same time use glycolytic and respiratory substrates. The question raised by this realization is, of course, whether or not glycolysis and respiration are uncoupled or whether the tricarboxylic acid cycle is somehow truncated, and acetyl CoA from pyruvate cannot be oxidized in it. Evidence in this sense is not conclusive: **Reitzler** (1979) found that less than 5% of the glucose available to HeLa cells enters the tricarboxylic acid cycle, while 35% of the glutamine is converted to CO_2, 25% is incorporated into proteins and 13% go to lactate. On the other hand, **Dietzen** and **Davis** (1993) provided evidence that the tricarboxylic acid cycle is not truncated in hepatoma mitonchondria. **McKeehan** (1982) proposed that glutamine could be metabolyzed in a fashion similar to glucose in normal tissues: glutamine would be deaminated to glutamate which would be oxidized in turn to á-ketoglutarate. The keto acid would then continue through the cycle to malate which would go out of the mitochondria and be converted to pyruvate and lactate in cytoplasm. If this were to apply to tumors *in vivo*, it would be possible that circulating glutamine would contibute both to respiration and to the lactate production. **Moreadith** and **Lehninger** (1984), while finding that glutamine and glutamate were excellent respiratory substrates for Ehrlich ascites tumor cells, showed, however, that they did not contribute to lactate production and were rather converted to aspartate. **Ahmed** *et al.* (1993) suggest that the high rates of both glycolysis and glutaminolyisis in tumors, coupled with a depressed oxidative glucose-phosphate shunt are consistant with the hypothesis that neither glucose nor glutamine contribute greatly to energy metabolism but rather are precursors for nucleic acids and protein synthesis.

Most of the data available is derived from work with animal tumors or human tumoral cell-lines maintained *in vitro*. While it is legitimate to extrapolate such findings to human tissues, we thought that it was important to readdress the issue using human tumors. We first wanted to find out unequivocally whether or not human tumor cells were able to respire and whether their respiratory rate differed from that of normal tissues. In case that the respiratory rates were high enough, would the uptake of glucose, glutamine, or of a combination of these substrates account for the oxygen uptake? Finally, what is the extent of glucose and glutamine oxidation to CO_2? The experiments reported here were carried out in the biochemistry department of Universidad del Valle.

Materials and Methods

Tumors

71 malignant and 20 benign tumors were studied over this period, as follows: Carcinomas: mammary, 32; gastric 11; Thyroid papillary, 3; folicular, 3; Epidermoid, 3; Basocelular,2; meningioma,3; ovarian, 3 and one each from cervix, colon and vulva; one Edwin carcinoma and one adenofibroma. Sarcomas,3: fibrosarcoma, liposarcoma and osteosarcoma. The tumors were obtained from programmed surgical procedures at the Hospital Universitario del Valle. The excised tumoral tissue was divided in two portions at the operating room, one for Pathology and the other one for these studies. This portion was immediately placed in ice-cold Krebs Ringer Phosphate (KRP), pH 7.4. Some macroscopically healthy tissue surrounding the visible limits of the tumor was always excised, and this was used as control. The tumoral or "healthy" status of the tissues used was later confirmed by pathology. 41% of the tumors studied were mammary infiltrating ductal carcinomas, 15% were gastric carcinomas and the rest were mostly carcinomas from different tissues. Only 6% were sarcomas. No attempt

was made to study one single kind of tumoral tissue, because we wanted to have as large a representation of transformed tissues as possible.

Oxygen uptake

Oxygen uptake was measured manometrically in a Warburg apparatus (**Umbreit** *et al.*, 1957). All experiments were performed in triplicate, as long as there was enough tissue to place between 70 and a 100 mg in the flask. This was not always the case, particularly with peritumoral tissues which were normally a lot smaller than the tumors. This, of course, was to be expected, as the surgeon will excise as little normal tissue as possible, insuring the removal of all the malignant cells. All flasks contained 3 ml of KRP pH 7.4 containing enough substrate to give a final concentration of either D-glucose, 5 mM; L-glutamine, 0.58 mM or a combination of the two, depending on the experiment performed. When no substrate was used, the flask contained only KRP. The center well contained 40% KOH mostly absorved in a fluted filter paper, in order to catch any CO_2 produced. Approximately 100 mg of tissue was blotted dried, weighed and cut in 1 mm slices. The slices were placed in the Warburg flasks which were, then, connected tightly to the corresponding manometer and placed in the bath at 30° C. The two arms of the manometers were equilibrated, the valve connecting to the atmosphere shut and agitation at 80 r.p.m.was initiated. Readings were made at one hour intervals for 4 hours. After each reading, the valve connecting to the atmosphere was open and the manometer was allowed to equilibrate. In all cases there was a thermobarometer containing only KRP.

Substrate uptake

Substrate uptake was ascertained by determining the amount of substrate present in the incubation medium before and after four hours of incubation. Substrate uptake is not equivalent to substrate use, since some of it may be adsorved to the cell membranes and some may accumulate as such in the cell. However, it does reflect the amount left available in solution after incubation.

Glucose was measured by the glucose oxidase method, utilizing Merck´s commercial kit. The suggested procedure was modified in the following manner: 50 µl of the solution to be measured were added to 1 ml of the color reagent containing the enzymes, diluted 1:2 with bidistilled water. After mixing, it was incubated for 30 minutes at 37° C without shaking. Absorvancy at 510 nm was read in a spectrophotomer against a blanck prepared and incubated in exactly the same way, but with 50 µl bidistilled water instead of sample. Standard curves were prepared to ascertain that Beer-Lambert´s law held for the concentrations measured and the glucose concentration was read of the corresponding curve.

There is no good enzymatic method for the determination of glutamine. For this reason we finally had to develope a reliable coupled enzyme procedure based on the following reactions:

$$\text{L-glutamine} + H_2O \xrightarrow{\text{Glutaminase}} \text{L-glutamate} + NH^+_4$$

$$NH^+_4 + \alpha\text{-ketoglutarate} + \beta\text{-NADH} \xrightarrow{\text{Gldhase}} \text{L-glutamate} + \beta\text{-NAD} + H^+$$

$$\text{L-glutamine} + \alpha\text{-ketoglutarate} + \beta\text{-NADH} \longrightarrow 2\text{L-glutamate} + \beta\text{-NAD} + H^+$$

In this coupled enzyme reaction for each mole of glutamine converted to glutamate, a mole of NADH will be converted to NAD. The conversion of NADH to NAD can be followed spectrophotometrically at 340 nm. We found, however, that this was not a straight-forward procedure in which both enzymes could be present in the same reaction mixture, as it is usually the case with coupled enzyme systems, due to the fact that the pH and temperature optimum of the glutaminase and the glutaminate dehydrogenase are widely divergent. Therefore, we first incubated the assay mixture for one hour at 37° C with glutaminase after adjusting the pH to 4.0 with HCl. Next, the pH was raised to 8.0 with NaOH and both glutamate dehydrogenase, á-ketoglutarate and â-NADH were added and the resulting mixture was incubated at 25°C for 90 minutes. The procedure was as follows: In a final volume of 380 µl there were 100 µl of sample and the following substances to give final concentrations of 2 mM HCl; 0.22 M acetate buffer, pH 5.0, and 0.3 U glutaminase. The mixture was incubated in a spectrophotometer cell at 37° C for 1 hour. At that time, the reaction was stopped by addition of 20 µl concentrated NaOH. Next, the following reagents were added to obtain the final concentrations indicated: 0.1 M TRIS buffer, pH 8.0; 10 mM á-ketoglutarate and 0.24 mM â-NADH. The mixture was shaken and L-glutamate dehydrogenase was added to obtain a final concentration of 6 U/ml. Final reaction mixture was 1 ml. The reaction was followed by recording the absorvancy at 340 nm in a Gilford Response spectrophotometer at 25° C for 90 minutes. Standard curves were run at regular intervals. The procedure was found to be accurate for the small concentrations involved in the experiments.

CO_2 production

CO_2 production was measured by incubating the tissues with radioactive substrates as follows: 3.0 ml of KRP pH 7.4 containing either 5 mM U-C^{14} glucose or 0.58 mM U-C^{14} glutamine (2 x10^6 cpm) were added to 25 ml erlenmeyers that had been previously fitted with glass center wells. In the wells were placed smaller removable empty glass or hard plastic wells. 70-100 mg sliced tissue were then added to the reaction mixture, the flasks were tightly stoppered with vaccine rubber stoppers and incubated with shaking for 4 hours at 30° C in a Dubnoff shaking bath. At the end of the 4 hours, hyamine hydroxide was injected through the stopper into the removable center well and then the flasks were placed in an ice-cold bath overnight to insure that all CO_2 was taken up the the hyamine hydroxide. The center wells were then removed and placed in a scintillation vial containing 5 ml Bray's solution. The radioactivity was measured in a Beckman Scintillation Counter.

Results

In figure 1 we can observe the oxygen consumption of the tumors and of the corresponding peritumoral tissues studied in the absence of substrate or using glucose or glutamine as substrates after one hour of incubation. The most striking feature is the very great variation of values which, nevertheless, tend to group around certain values, with occasional greatly deviant figures. For this reason, our results will be presented as the median of the values found and their range. We believe that the great dispersion in O_2 uptake figures is due to several factors. There was a large variety in tumor types studied and in the degree of desdifferentiation. Most of the tumors studied were mammary

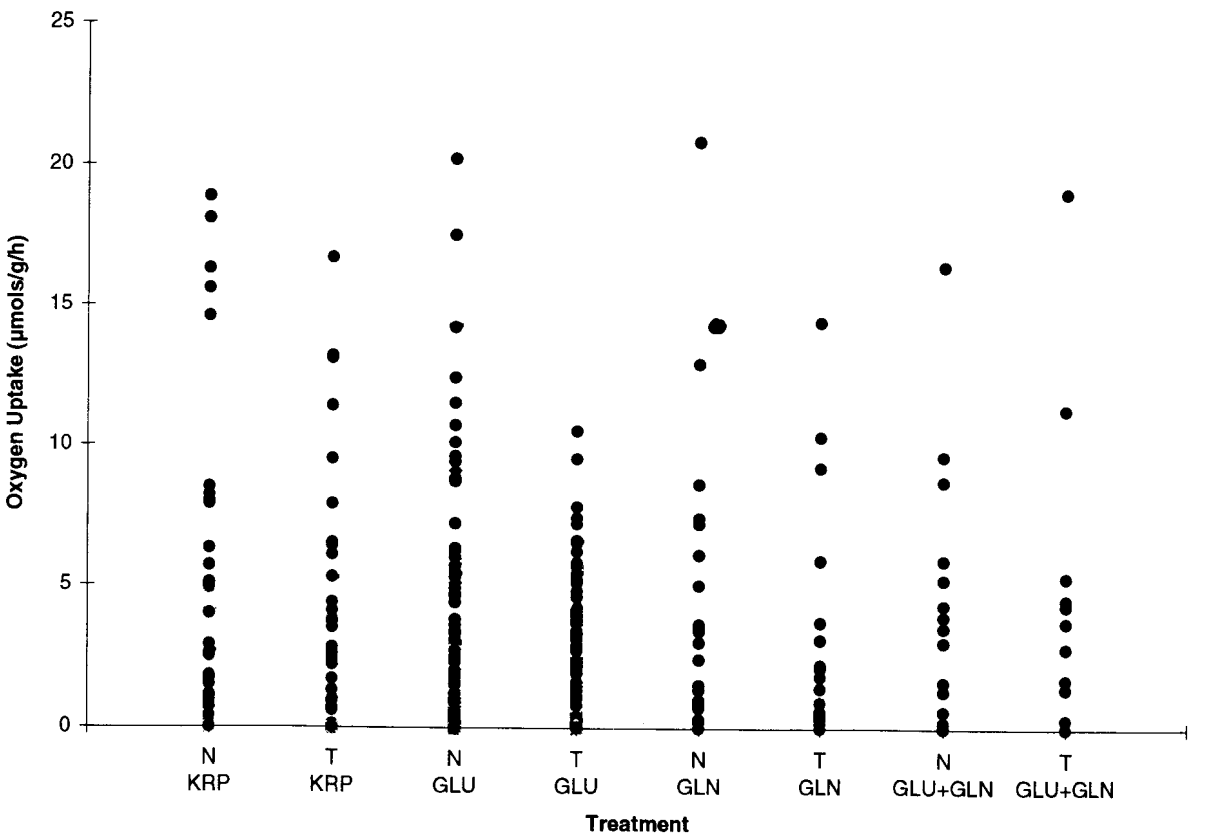

Figura 1. Oxygen consumption by different types of human tumors. Tumor slices were incubated in KRP buffer without any substrate or in buffer containing 5.0 mmoles glucose/ml or 0.58 mmoles glutamine/ml. Oxygen uptake was measured manometrically in a Warburg apparatus. (GLU = glucose; GLN=glutamine)

tumors in which there necessarilly it is found a mixture of normal and cancer cells. And in some of the solid tissues, there might be dead cells along with very active ones. Independent of this variation, which was to be expected, what one observes is that there is little if any differences in O_2 uptake between the peritumoral and tumoral tissues. The lack in statistically significant differences between normal and tumoral tissues was confirmed using the Mann-Whitney non parametric test which assumes that the events studied are independent. This test is generally used in cases where a great dispersal of values is observed.

In table I is shown the median of the O_2 uptake values found in all esperiments, and the range of the figures. As can be seen, there is no apparent significant difference in the medians. However, there is a tendency for the tumors to respire a little less than the peritumoral tissues. This tendency is more pronounced in the case when glutamine was used as substrate.

Table I. OXYGEN UPTAKE BY TUMORAL TISSUES

	Peritumoral	Tumoral	Peritumoral	Tumoral	Peritumoral	Tumoral
	KRP		GLUCOSE		GLUTAMINE	
	μmoles O_2/ g.h					
Median	3.5	3.6	3.0	2.8	3.4	2.1
Range	0.14 - 18.9	0.10 - 16.7	0.20 -20.2	0.03 - 10.5	0.20 - 20.8	0.16 - 18.1

In order to ascertain if the general tendency found with different types of tumors, both benign and malignant, held also for the two types, we grouped them as to malignancy or non-malignancy. In table II the medians and ranges for both types of tissues are shown. As can be seen, no significant differences are found. The only difference seems to be that the dispersion of values is less apparent in benign tumors.

Table II. O XYGEN UPTAKE BY MALIGNANT AND BENIGN TUMORS

	MALIGNANT				BENIGN			
	Peritumor	Tumoral	Peritumor	Tumoral	Peritumor	Tumoral	Peritumor	Tumoral
	KRP		GLUCOSE		KRP		GLUCOSE	
	μmoles O_2/ g.h							
Median	3.45	3.70	3.05	3.10	3.45	3.0	1.65	1.4
range	0 - 18.9	0.1- 16.7	0.2 -20.2	0.03 -10.5	0.4-15.6	0.7-6.4	0.5-7.2	0.3-5.2

We next wanted to ascertain whether or not one could find a different pattern of O_2 uptake for a single type of tumors. In order to do that, we grouped the 22 experiments in which only mammary tumors were used with both substrates. The results are shown in Table III. As can be seen, the tendency remains, and we found no statistically significant difference between the figures.

Table. III. OXYGEN UPTAKE BY MAMMARY CARCINOMA

	Peritumoral	Tumoral	Peritumoral	Tumoral	Peritumoral	Tumoral
	KRP		GLUCOSE		GLUTAMINE	
	μmoles O_2/ g.h					
Median (30)	2.75	3.7	2.4	2.75	1.65	3.4
Range	1.14 -18.1	0.10 - 13.2	0.20 - 11.5	0.11 - 10.5	0.30 - 20.8	0.16 - 18.1

We next wanted to know whether or not simultaneous incubation of glutamine and glucose resulted in a different pattern of oxygen uptake. As can be seen in Table IV, it would seem that tumors respire better with glutamine than with glucose alone. The dispersion of the values, however, does not permit to determine whether or not this tendency is statistically significant.

Table IV. OXYGEN UPTAKE IN THE PRESENCE OF BOTH GLUCOSE AND GLUTAMINE

	Peritumoral	Tumoral	Peritumoral	Tumoral	Peritumoral	Tumoral
	GLUCOSE		GLUTAMINE		GLUCOSE +GLUTAMINE	
	μmoles O_2/ g.h					
Median	2.4	2.75	1.65	3.4	3.7	4.0
Range	0.20 - 11.5	0.11 - 10.5	0.30 - 20.8	0.16 - 18.1	0.02 - 16.4	0.30 - 19.0

It was interesting to note that tumors respired seemingly well in the absence of exogenous substrate during the first hour. As can be seen in figure 2, however, this did not hold for longer incubations periods. In fact, both normal (not shown) and cancer tissues without substrate practically stopped respiring after the third hour, while those with substrate continued to respire, albeit not at the same rate. In fact, glucose maintained tumor respiration almost constant during the four hours. Glutamine did the same, but at a higher rate, while a mixture of glucose and glutamine resulted in maximal O_2 uptake.

The amount of substrate used was determined after four hours incubation. In table V we show the amount of glucose taken up by the tissues under these conditions.

Table V. GLUCOSE UPTAKE BY TUMORAL TISSUES

SUBSTRATE	GLUCOSE		GLUCOSE + GLUTAMINE	
	PERITUMORAL	TUMORAL	PERITUMORAL	TUMORAL
	μmoles/4 hrs/g tissue			
means (N)	10.21 (23)	6.17 (23)	7.24 (16)	4.66 (13)
S.D.	6.74	5.56	4.76	4.21

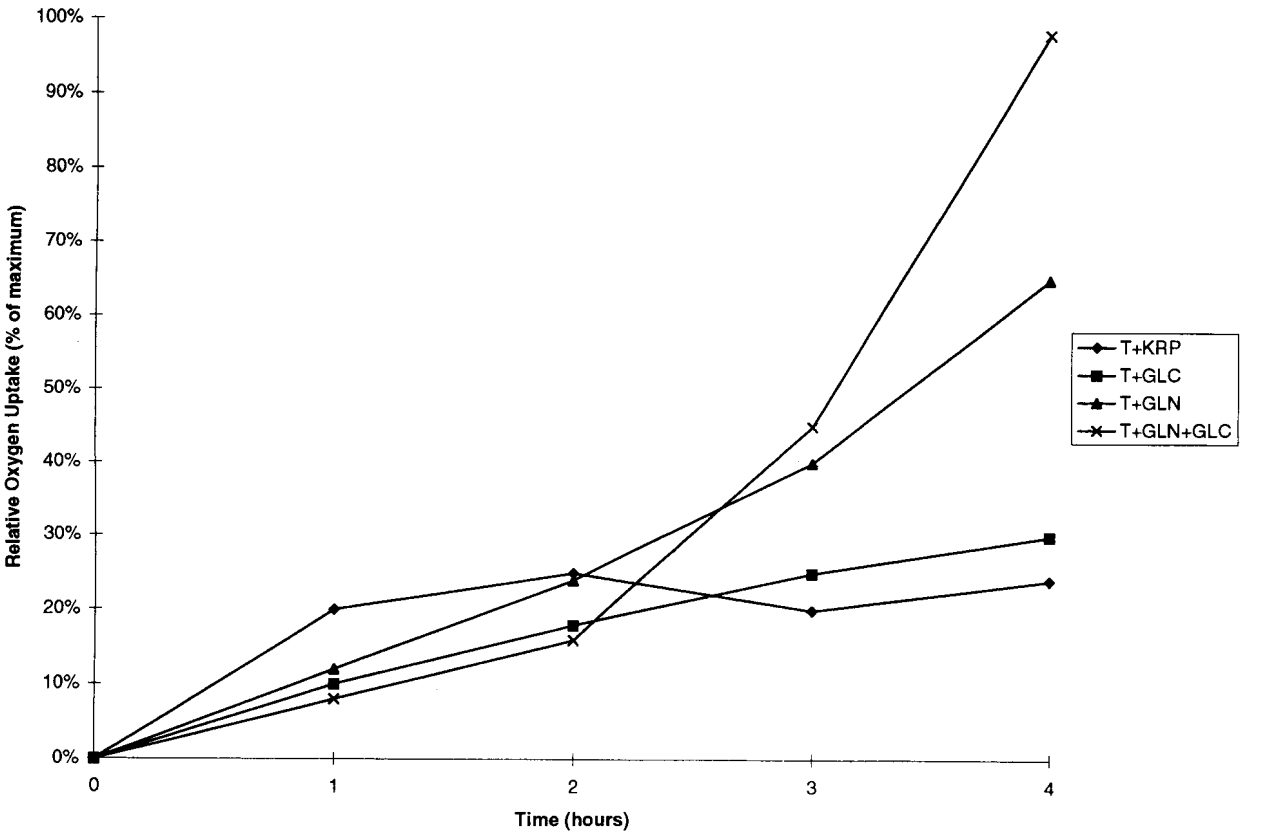

Figure 2. Tumoral tissues were incubated for four hours in KRP alone or with glucose, 5 mM, glutamine, 0.58 mM, or with a mixture of both. The oxygen uptake was measured each hour in a Warburg respirometer. Results are presented as percent of the maximal value obtained.

Even though the mean value of glucose uptake by the tumoral tissue when glucose is the only substrate seems to be less than that of the peritumoral one, the difference is not statistically significant The same is true when both glucose and glutamine are used by the tissues at the same time. However, in this case, the Student t test does show a significance at the 0.1 level.

In the same manner we determined the glutamine uptake by the tissues after four hours incubation. As can be seen in Table VI, tumoral tissues appeared to take up more glutamine than peritumoral tissues both in the presence and in the absence of glucose. This difference, however, is not statistically significant, and simply shows a trend.

Table VI. GLUTAMINE UPTAKE BY TUMORAL TISSUES

SUBSTRATE	GLUTAMINE		GLUTAMINE + GLUCOSE	
	PERITUMORAL	TUMORAL	PERITUMORAL	TUMORAL
	μmoles/4 hrs/g tissue			
means (N)	1.13 (11)	2.43 (11)	2.27 (10)	2.92(12)
S.D.	0.63	0.93	1.93	2.69

In order to find out whether or not tumors were able to convert glucose to CO_2 and in this way to ascertain if there was inhibition of pyruvate entrance into the Krebs cycle, we incubated several tumor tissues with U-C^{14}-glucose and collected the CO_2 produced. We also incubated them with U-C^{14}-glutamine to find out the proportion of the label appearing in CO_2 in relation to that produced by peritumoral tissues. In table VII we show a typical experiment using carcionoma from cervix. As expected, tumors took up more glucose than peritumoral tissues, even in the presence of non radioactive glutamine. However, it was surprising to find that tumors took up almost double the amount of glutamine than the peritumorals did. The most interesting thing that can be observed in the table is that tumors do produce CO_2 from glucose, and that even though the extent is less than the peritumoral tissue, it is a sizable proportion of the normal. In other experiments the pattern was reproduced, but the proportion of CO_2 produced by the tumor in relation to that produced by the peritumoral tissue varied greatly, even though it always was less. On the other hand, the proportion of glutamine that went to CO_2 in this experiment was similar for both peritumoral and tumoral tissues, just as it would be expected. It should be noted that glucose seemed to increase the CO_2 produced from glutamine, while this latter substrate seemed to reduce the amount of CO_2 produced from glucose.

Table VII. RADIOACTIVE SUBSTRATE UPTAKE AND CO_2 PRODUCTION BY CERVICAL CARCINOMA

	RADIOACTIVITY UPTAKE		$C^{14}O_2$ PRODUCED	
	PERITUMORAL	TUMORAL	PERITUMORAL	TUMORAL
SUBSTRATE	CPM		µmoles/g	
Glucose-U-C^{14}	49793	64188	16.2 ± 7.5	10.4 ± 2.2
Glutamine-U-C^{14}	57569	103722	1.6 ± 0.8	1.5 ± 0.3
Glucose-U-C^{14} + Glutamine	70107	107582	11.7 ± 6.3	8.2 ± 1.7
Glutamine-U-C^{14} + Glucose	54579	26257	1.5 ± 0.4	3.3 ± 1.0

Discussion

Ever since **Warburg** (1930) showed that tumors had a high rate of lactate production many workers have tried to find out whether or not they have the necessary metabolic respiratory machinery. **Weinhouse** (1955) was the first to show in a series of systematic experiments that the enzymes of the respiratory chain were present and active in tumor cells. Confirmation followed shortly thereafter (**Wenner & Mackner**,1967; **Weber**, 1977). However, some workers, based on **Koukl** *et al.* (1977) ultramicroscopic findings that tumor mitochondria had bizarre forms, proposed that due to morphological alterations the enzymes would not be active *in vivo*. **Villalobos** and **Lehninger** (1979) showed that this was not the case, since Ehrlich´s ascites mitochondria had the same proton transport stoichiometry as heart and liver mitochondria. Dietzen and **Davis** (1993) showed that mitochondria isolated from AS-30D hepatoma cells oxidized many NAD-linked respiratory substrates at rates 1.5 to 4 times faster than those from liver. These findings clearly demonstrate that there is nothing wrong with the respiratory machinery of tumors.

However, most experiments to date have been performed using animal tumors or human tumor cell lines. Due to the relative ease of obtaining animal desdifferentiated tissues and of maintaning cell lines *in vitro* as well as to the differences between tumor types, this approach is entirely appropriate and, in fact, preferable for certain purposes. Nevertheless, the question remains open as to whether or not human tumor tissues participate of the features found in animal ones or *in vitro* maintained cell lines, since the latter conceivebly could have acquired metabolic features not necessarily found in the original cells.

For this reason we decided to work with fresh human tumor tissues and first ascertain in them the rates of

respiration using either glucose or glutamine as substrates. Even though all tissues were obtained from programmed surgical procedures, it proved very difficult to obtain enough tumoral and apparently healthy peritumoral tissues for reasons that included from the tumors being so small that there was not enough tissue both for pathological examination and for our experiments to the placing of the tumor in formaldehyde immediately after excision by the surgeon helper, thus preventing further experimentation. However, we were able to collect 71 malignant and 20 benign tumors over a period of five years. Due to the differences reported in the literature about certain particular metabolic features between different animal tumors and cell lines, we thought that the one feature that seemed to cover all tumoral tissues was the fact that they exhibited aerobic glycolysis. Therefore, we felt that it would be important to ascertain the rate of oxygen uptake in the presence of glucose or glutamine regardless of the tumor type.

The next decission was a bit more difficult to take. For the past 30 years oxygen electrodes have been available to measure oxygen in solution. While highly appropriate for measuring O_2 uptake by mitochondria, we felt that it would be better to follow respiration for longer periods than those that could be possible using oxygen polarimetry. Therefore, we decided to use the Warburg manometric respirometer in order to measure O_2 uptake for hours. In some preliminary experiments we went up to 48 hours incubation, finding greatly increased O_2 consumption at about 16 hours, that, however, was finally shown to be due to bacterial contamination. For this reason, all experiments reported here were performed with sterilized materials and with solutions passed through micropore filters and were limited to 4 hour incubations since we could not find any contamination until this time. Even though we did not pursue the issue, one very strange feature was that peritumoral tissues were very little subject to bacterial contamination in exactly the same incubation conditions. This observation could mean that somehow tumors are more susceptible to bacterial infection.

Even though we measured O_2 uptake for four hours, we only present the results for the first hour. As can be seen in figure 2, O_2 uptake was maintained approximately constant only in tumors in the presence of glucose or glutamine. In all other cases O_2 uptake declined with time. We do not know whether this is due to some enzymic inactivation with time under our conditions. The interesting thing, however, is that glucose, glutamine or a combination of the two were able to increase or at least keep the rate of respiration nearly constant in the tumor, albeit at different rates. In this sense, the work of **Eskey** *et al.* (1993) may be relevant. In fact, these authors ascertained the relative contributions of glycolysis and respiration in tumor energy metabolism using *in vivo* P^{31} NMR spectroscopy. They found that the nucleotide triphosphate (NuTP)/P_i ratio is maintained in tumors in hypoxia, but it is not maintained when glucose is removed from the medium or is replaced by glutamine. Our findings, on the contrary, suggest that the increased oxidation could result in a higher NuTP/P_i, unless there was uncloupling of oxidative phosphorylation in tumors. This could be possible if tumor mitochondria had a lower Ca^{++} content than normal mitochondria, since **Gabai** (1993) has shown that endogenous and glutamine-supported respiration as well as succinate-supported respiration was Ca^{++} dependent. At any rate, we decided to report O_2 uptake for the first hour only in order to make all treatments more comparable.

As reported in Tables I, II and III, the most prominent feature found in tumors as relate to respiration is that there is no statistically significant differences in O_2 uptake between the peritumoral and the tumoral tissues, respiring with either endogenous or exogenous energy substrates. As could be expected, the O_2 uptake values varied a great deal from experiment to experiment and from tissue to tissue, but most values grouped around a median which was in the neighborhood of 2-3 μmoles O_2 per hour per gram of tissue, regardless of the substrate. We believe that this great disperssion in values is due to factors that could not be controlled, such as the type of tumor available, the degree of undifferentiation and the time between the surgical excission and the beginning of the experiment. These conditions were foreseen and accepted as inherent to the study of freshly obtained human tumors, since they reflect the biological reality. Besides, the study was designed precisely with the end of ascertaining if there was a general pattern of respiration in human tumors, and not in a particular one.

When all tumors, regarless as to whether they were malignant or benign, were grouped together, we found that the median O_2 uptake was 3.5 μmoles in peritumoral and 3.6 μmoles in tumoral tissues respiring with endogenous substrates. The range of uptake was similar in both tissues with values as low as 0.1 μmoles and as high as 19 μmoles for the first hour. This clearly shows that all human tumors studied are just as capable of oxygen utilization as presumibly normal ones. The median O_2 uptake of peritumoral tissues was a little lower with glucose (3.0 μmoles), but remained the same with

glutamine (3.4 μmoles) in relation to the values found with endogenous substrates. However, the median values for the tumoral tissues showed a tendency to lower values: 2.8 μmoles with glucose and 2.1 μmoles with glutamine. While the Mann-Whitney test shows that the values are not significantly different, the tendency seems interesting. However, when we separated the values obtained with malignant tumors from those found with leiomiomas, the tendency was not maintained. In this case, there were no differences between tumoral and peritumoral tissues in the absence or in the presence of glucose and the median O_2 uptake values remained around 3 μmoles, except in the case of the benign tumors with glucose in which both normal and tumoral tissues showed lower medians, which, nevertheless, were not statistically different.

When we considered only mammary tumors we found no differences from what was found with all tumors. We were a little surprised, however, to find the same dispersion in values, since we expected that with a single type of tumor the results would more homogeneous. Even though we do not find an explanation for the fact, it could be possible that the dispersion is due, at least in part, to the nature of the tumor, ductal infiltrating, and to differences in the degree of desdifferentiation.

We also wanted to know whether the tissues would change the rate of O_2 consumption if at the same time they had glucose and glutamine as substrates. We thought that if glycolysis and glutaminolysis were not independent, combination of the two substrates might somehow depress O_2 uptake. This was not the case. In fact, if anything, the combination of substrates resulted in a median O_2 uptake that was higher in the tumor, but, again, not significantly so. This is particularly striking when the results during 4 hours are observed. In fact, as pointed out above, a combination of glutamine and glucose maintained high rates of respiration during four hours incubation. Glutamine was able to maintain the rate to a lower extent, as was the case with glucose alone, but at much lower rates. This shows that some influence of one substrate over the other might actually exist in tumors. **Gonzalez-Mateos** *et al.* (1993), working with Ehrlich ascites tumor cells, showed that glutamine and asparagine decreased the glycolytic flux by about 80% and **Ahmed** *et al.* (1993) reported that glucose utilization was depressed by exogenous glutamine in human leukemic cell line HL60. These last authors showed, however, that glutamine utilization was enhanced by glucose. These findings could explain our results in the sense that even if glycolysis was depressed by glutamine, this would not be reflected in O_2 uptake while glucose might increase glutamine utilization over the long run maintaining high O_2 uptake dependent of the aminoacid.

This interpretation might also be substantiated by our own results. In fact, we found that glucose uptake by tumoral tissues was depressed by coincubation with glutamine. We also found some enhancement in glutamine utilization caused by glucose. While the values are consistent with the findings of **Ahmed** *et al.* (1993), they were not statistically different. It should be pointed out that the concentration of the substrates used in all cases was that usually found in blood, i.e., 5 mM for glucose and 0.58 mM for glutamine. In this sense, the tissues had almost ten times more glucose than glutamine available. This difference is reflected in the amount of substrates taken up reported here. The uptake of glucose and glutamine could be affected by the activity of enzymes that are important in the initial step of glycolysis and glutaminolysis. In this respect, Newsholme´s group (**Board** *et al*, 1995) has shown that glucokinase of several tumor lines seems to be the rate-limiting step in glycolysis and that its inhibition by mannoheptulose, a substance that can be found in avocados, dramatically decreases tumor growth in experimental animals. On the other hand, Rasschaert and **Malaisse** (1995) have found that hexokinase is increased in tumoral islet cells.

The issue of whether or not glucose can actually be oxidized in the tricarboxilic acid cycle can not be settled by CO_2 production alone, since CO_2 is given off in the operation of oxidative hexose-monophosphate shunt. Ahmed at al. (1993) showed that the activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase were lower in leukemic cell lines and that the oxidation of [1-^{14}C] glucose was significantly lower than that of [6-^{14}C] glucose. This observations imply that the activity of the oxidative branch of the hexose monophosphate shunt is depressed. This allows us to suggest that the $C^{14}O_2$ that we found upon incubation of U-C^{14}-glucose with tumoral tissues comes from oxidation of pyruvate derived from glucose. The same, of course, can not be said for the peritumoral tissue. In these conditions our results must be understood only in qualitative, rather than in quantitative terms, as far as glucose is concerned. The same does not apply to glutamine since all the CO_2 obtained after incubation of both peritumoral and tumoral tissues with the amino acid comes from respiration related oxidation. With this in mind, we find that some pyruvate derived from glucose was oxidized to at least acetyl CoA. This is in line with Portais et al. (1993) finding that up to 57% of the Acetyl CoA found in C6 glioma cells is derived from glucose. We do not know whether or not this acetyl CoA is further oxidized in the

tricarboxilic acid cycle or if it is used in lipogenesis. In this sense, **Briscoe** *et al.* (1994), while demonstrating a high rate of lipogenesis in AS-30D hepatoma cells, suggest that the substrate for lipid synthesis is acetoacetate rather than pyruvate derived acetyl CoA.

The experiments in which [U-^{14}C] glutamine was the substrate show no difference in CO_2 production between peritumoral and tumoral tissues. In this case, the glutamine label will appear only in CO_2 derived from the tricarboxylic acid cycle and there should be no difference in glutaminolysis in peritumoral and tumoral tissues, as found in this report. **Ahmed** *et al.* (1993) have observed that glutaminase activity was high in tumor cells, yet, the rate of uptake was low. On the other hand, **Souba** (1993) has compiled a lot of evidence that shows that many tumors are avid glutamine users and that *in vivo* they may act as a glutamine "trap" depleting the organism of this aminoacid. *In vivo* experiments reported by his group (**Chen** *et al.* 1993) showed that in an animal bearing a tumor, muscle glutamine was depleted because of an incresed release to the blood, despite the fact that its synthesis was greatly augmented while the fibrosarcoma took practically all of the aminoacid up from circulation. Glutamine depletion increased with tumor growth. Our own experiments in which there is no difference in CO_2 production are not in contradiction with Souba's data, since **Ahmed** *et al.* (1993) data suggest that neither glucose nor glutamine contribute much to energy metabolism and their role might be more directed to nuecleotide and protein synthesis in the tumor.

Our data also show that glutamine decreased the $C^{14}O_2$ recovered from incubation of tumors with [U-^{14}C] glucose, while glucose increased $C^{14}O_2$ derived from [U-^{14}C] glutamine. These results confirm the data reported on glutamine and glucose uptake, discussed above. **Ahmed** *et al.* (1993) suggest that the decreased glucose utilization in the presence of glutamine could be due to allosteric inhibition of phosphofructokinase. This is not likely since neither glutamine nor metabolites in its oxidative pathway affect phosphofructokinase directly. On the other hand, **Gonzalez-Mateos** *et al.* (1993) showed that the activity of the enzyme in the presence of effectors concentrations found in Ehrlich ascites tumor cells is sufficient to explain the low activity of the enzyme. The nucleotide effectors concentration would, of course, be due to glutamine oxidation.

Our experiments show that human tumors respire actively using endogenous substrates or glucose or glutamine. In this activity they do not differ from apparently normal tissues. They also show that glucose may be oxidized to CO_2, while a significant proportion is converted to lactate (results not shown). Our experiments do not provide any clues as to why there seems to be a separation between glycolysis and tricarboxylic acid cycle oxidation of pyruvate. In this respect it is interesting to note that there are reports that the malate shuttle of reduced equivalents seems to be inoperative in tumors *in vivo* (**Dietzen & Davis**, 1993) while the glycerol-phosphate shuttle might be inhibited (**Halder** *et al.*, 1993, **Rasschaert & Malisse**, 1995). The high lactate production of tumors may provide a comparative advantage *vis a vis* normal cells, since a decreased pH in the interstitial fluid might protect the cells against immune cells by altering the conformation of the recognition surface proteins. However, it is interesting to note that a low pH may also destroy the cancer cells, a feature that has found some therapeutic use (**Harguindey**, 1982). Regardless of its possible activity, there is a larger lactate production than in normal cells. Preliminary experiments from our laboratory have shown that this may be due to a non-controlled phosphofructokinase in some human tumors.

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