ABSTRACT

These experiments were performed to determine the patterns of substrate utilization for energy production in tumors in vivo. A method is described for the growth of "tissue isolated" rat tumors. Implants of transplantable tumors are attached to a surgically prepared vascular pedicle composed of the superficial epigastric artery and vein. The vascular pedicle and tumor implant are enclosed in a paraffilm envelope and placed in the inguinal fossa, and the skin incision is closed. Subsequent tumor growth is contained within the envelope, and vascularization of the tumor is entirely via the vascular pedicle. Tumors (1 to 6 g, wet weight) are harvested within 1 week to 2 months, depending on the growth rate. Blood samples may be collected by catheter from the tumor vein (superficial epigastric and femoral) and the carotid artery, or the tumor and pedicle may be removed and perfused. The overall success rate of the method, that is, tumor implants brought to successful blood sampling, is about 50%.

Arteriovenous differences for whole-blood amino acids, glucose, and lactic acid were measured in vivo in Morris hepatomas 5123C, 7777, and 7288CTCF and Walker carcinosarcoma 256 grown by this method. Most whole-blood amino acids were utilized with mean utilization rates of 5 nmol/min/g tumor, wet weight, or less. Glutamine, the most abundant whole-blood amino acid, was also the most extensively utilized (mean utilization rates were 4 to 10 nmol/min/g). Ammonia was released into the venous blood by all tumors. The glucose utilization rate was directly proportional to the rate of glucose supply. Lactic acid was either produced or utilized, depending on the arterial blood lactic acid concentration. Lactate production occurred at arterial blood lactate concentrations less than 2 to 3 mM, and utilization occurred at higher concentrations. The results show that new information on tumor-host organ interrelationships and on the energy metabolism of tumors in vivo can be obtained using these techniques.

INTRODUCTION

In a previous publication (31), we suggested that glutamine and ketone bodies are important respiratory fuels in undifferentiated tumors. Energy production resulting from the oxidation of these substrates is envisaged as occurring simultaneously and in addition to that derived from aerobic glycolysis. While this proposal is supported by the results of experiments performed in vitro (4, 6, 17, 18, 21, 22, 24, 29), there is, to our knowledge, no information available on the use of these substrates for energy production by tumors in vivo. Demonstration of glutamine and ketone bodies utilization by tumors in vivo would be important support for this concept. In this report, we describe the results of the first series of experiments designed to determine the respiratory fuels used by rat tumors in vivo. A new method for growing transplantable tumors in male and female rats which restricts the tumor blood supply and drainage to a single artery and vein is described. The technique adopts some of the features of the pioneering method developed by Guillino and Grantham (10). We have used the tumor preparations to measure arteriovenous differences for whole-blood amino acids, ammonia, glucose, and lactic acid in vivo. Ketone body and fatty acid utilization will be described in a subsequent paper.

MATERIALS AND METHODS

Animals, Tumors, and Reagents. Adult male and female Buffalo rats were either purchased from Microbiological Associates, Walkersville, Md., or were obtained from a colony established here. The rats were fed a standard laboratory chow (Charles River Rat, Mouse, Hamster Formula; Agway, Inc., Syracuse, N. Y.) and water ad libitum and were subjected to alternate 12-hr periods of dark and light. Morris hepatomas (5123C, 7777, and 7288CTCF) were originally supplied by Dr. Harold P. Morris, Department of Biochemistry, Howard University Cancer Center, Washington, D. C., and have been carried in this laboratory for 35 months. Walker carcinosarcoma 256 was obtained from E. G. and G. Mason Research Institute, Worcester, Mass., and was maintained in Sprague-Dawley rats purchased from Harlan Sprague-Dawley, Madison, Wis. Animals bearing the fast-growing tumors (hepatomas 7777 and 7288CTCF, and Walker carcinosarcoma 256) had a depressed food intake relative to ad libitum-fed non-tumor-bearing rats; therefore, pair-fed non-tumor-bearing rats were used for determination of control arterial whole-blood amino acid, glucose, and lactate levels. The daily ration of the pair-fed non-tumor-bearing animals was the same as that amount of food consumed by the tumor-bearing animals on the preceding day.

Enzymes, buffers, nucleotides, and other chemicals used in the enzymatic assays were purchased from Sigma Chemical Co. Glutaminase (Pseudomonas spp.) was obtained from Dr. Lawrence Pinkus, Nassau County Medical Center, East Meadow, N. Y. Buffers used in the amino acid analyses were purchased from Dionex Corporation, Sunnyvale, Calif. Ninhydrin, hydridantin, lithium acetate, dimethyl sulfoxide, and the amino acid standard mixture were purchased from Pierce Chemical Co., Rockford, Ill.

Animal Preparation, Tumor Implantation, Growth, and Sample Collection. A 150- to 200-g rat was anesthetized with Nembutal (2.5 mg/100 g body weight) i.p., and the fur was shaved from the inguinal region (right or left) and lower abdomen. A 2-cm incision was made in the inguinal crease, and the femoral vessels were exposed by blunt dissection. All subsequent steps were performed with an operating microscope. Use of this instrument facilitates the dissection and aids meticulous andatraumatic surgical technique. The femoral vein and its major branches were exposed and cleaned of adipose and connective tissue (Chart 1A). The femoral vein was ligated just distal to the origin of the superficial epigastric vein (Chart 1B), and the great
saphenous vein (which usually arises from the femoral vein distal to the origin of the superficial epigastric vein but may arise near the origin of the superficial epigastric vein) was also ligated, if necessary. The femoral artery was not ligated. Two deep venous branches (the muscular branch and the superficial circumflex iliac) which drain the medial surface of the thigh muscles and enter the femoral vein proximal to the first major bifurcation. Both artery and vein were cleared of adipose and connective tissue to the surface of the thigh muscles and enter the femoral vein proximal to the first major bifurcation. Both artery and vein were cleared of adipose and connective tissue to the surface of the thigh muscles and enter the femoral vein proximal to the first major bifurcation. Both artery and vein were cleared of adipose and connective tissue to the surface of the thigh muscles and enter the femoral vein proximal to the first major bifurcation. Both artery and vein were cleared of adipose and connective tissue to the surface of the thigh muscles and enter the femoral vein proximal to the first major bifurcation. Both artery and vein were cleared of adipose and connective tissue to the surface of the thigh muscles and enter the femoral vein proximal to the first major bifurcation. Both artery and vein were cleared of adipose and connective tissue to the surface of the thigh muscles and enter the femoral vein proximal to the first major bifurcation.
RESULTS AND DISCUSSION

Amino Acids and Ammonia. Whole-blood amino acid levels in pair-fed control and tumor-bearing Buffalo rats are listed in Table 1. Tumor growth decreased the food intake of the tumor-bearing rats relative to ad libitum-fed non-tumor-bearing animals, and consequently, pair-fed control animals were partially fasted. In this setting, tumor growth had no effect on the concentrations in host blood of the amino acids examined. These results contrast with those of Moyer and Pitot (23) who found several amino acids, especially glutamine, threonine, alanine, and lysine, to be decreased in plasma of Morris hepatoma-bearing rats. The control non-tumor-bearing rats in their study did not appear to have been pair fed. This difference in experimental design may explain the discrepancies observed.

On the other hand, the differences between plasma and whole-blood levels could result from specific effects of the Morris hepatomas studied. Also, the relative sizes of the plasma and erythrocyte pools in tumor-bearing rats are unknown. Shifts in compartmentation of specific amino acids between plasma and erythrocytes due to tumor growth could explain why we were unable to demonstrate in whole blood the plasma changes found by Moyer and Pitot (23).

Tumor utilization and production rates for whole-blood amino acids and ammonia in vivo are shown in Chart 2. Utilization of blood amino acids predominated. Glutamine, the most abundant amino acid in the whole blood of tumor-bearing rats (Table 1), was also the most extensively consumed, especially in the fast-growing tumors. As indicated by the bars, the faster-growing tumors showed the largest range in utilization rates. Glutamine utilization rates ranged from ~0.6 to 35 nmol/min/g tumor for hepatoma 7288CTCF and from 0 to 29.7 nmol/min/g tumor for Walker carcinosarcoma 256. Two tumors in the hepatoma 5123C series and one in the hepatoma 7777 group showed small glutamine production rates. The mean glutamine utilization rate was the slowest in hepatoma 5123C, the slowest-growing tumor, and was exceeded by the rate of alanine consumption. Glutamine utilization appeared to be dependent on the rate of glutamine supply to the tumor (Chart 3). About 75% of the tumors sampled showed utilization and supply rates below 16 and 50 nmol/min/g tumor, respectively, and the distribution was somewhat skewed toward the lower supply and utilization rates. Data points for glutamine utilization and supply rates collected from tumors in fasted rats were grouped about a nearly identical linear regression line.

Table 1. Amino acid levels in arterial whole blood of normal and tumor-bearing Buffalo rats

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Normal&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hepatoma 5123C bearing&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hepatoma 7288CTCF bearing&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>35 ± 2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>31 ± 7&lt;sup&gt;e&lt;/sup&gt;</td>
<td>40 ± 6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Threonine</td>
<td>142 ± 17</td>
<td>135 ± 12</td>
<td>171 ± 11</td>
</tr>
<tr>
<td>Serine</td>
<td>289 ± 30</td>
<td>219 ± 9</td>
<td>242 ± 14</td>
</tr>
<tr>
<td>Asparagine</td>
<td>40 ± 9</td>
<td>29&lt;sup&gt;d&lt;/sup&gt;</td>
<td>23&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>233 ± 26</td>
<td>250 ± 25</td>
<td>254 ± 28</td>
</tr>
<tr>
<td>Glutamine</td>
<td>580 ± 31</td>
<td>719 ± 58</td>
<td>646 ± 41</td>
</tr>
<tr>
<td>Glycine</td>
<td>331 ± 43</td>
<td>356 ± 24</td>
<td>311 ± 24</td>
</tr>
<tr>
<td>Alanine</td>
<td>519 ± 24</td>
<td>486 ± 50</td>
<td>334 ± 34</td>
</tr>
<tr>
<td>Citruline</td>
<td>31 ± 8</td>
<td>50&lt;sup&gt;e&lt;/sup&gt;</td>
<td>50&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>63 ± 7</td>
<td>72 ± 5</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>Leucine</td>
<td>112 ± 10</td>
<td>133 ± 11</td>
<td>104 ± 8</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>40 ± 7</td>
<td>70 ± 7</td>
<td>56 ± 4</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>43 ± 9</td>
<td>77 ± 7</td>
<td>64 ± 7</td>
</tr>
<tr>
<td>Lysine</td>
<td>394 ± 20</td>
<td>354 ± 41</td>
<td>348 ± 14</td>
</tr>
<tr>
<td>Ammonia</td>
<td>218 ± 34</td>
<td>243 ± 90&lt;sup&gt;f&lt;/sup&gt;</td>
<td>177 ± 24&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Pair-fed animals (n = 6).
<sup>b</sup> n = 8.
<sup>c</sup> n = 10.
<sup>d</sup> Mean ± S.E. for n determinations.
<sup>e</sup> n = 6.
<sup>f</sup> n = 2.

<sup>3</sup> The abbreviation used is: TCA, trichloroacetic acid.

<sup>4</sup> L. A. Sauer and R. T. Dauchy, unpublished data.
Aspartic and glutamic acids, major products of glutamine utilization in Morris hepatomas and ascites tumor cells in vitro (17, 18), were removed from the arterial blood in vivo. The rate of aspartate utilization was very low, which suggested that the decreased rate of aspartate uptake (relative to liver) observed in Morris hepatomas in vivo and in vitro by Koch et al. (15, 16) was occurring here. On the other hand, aspartate utilization was low in all 3 Morris hepatomas, and in the experiments of Koch and Lea (16), hepatoma 5123C had shown a rate of aspartate uptake similar to that of host liver and much faster than the uptake into hepatomas 7288CTCF and 7777. We have no explanation for this discrepancy. It seems likely that adequate amounts of aspartate are formed intracellularly from the glutamine utilized by hepatomas 7288CTCF and 7777. Aspartate (and glutamate) generated intracellularly during glutamine catabolism could explain why the intracellular pools of these amino acids are maintained at near normal levels (23), relative to host liver, in the face of a diminished rate of glutamate and aspartate uptake from the blood (15). In contrast to the hepatomas, Walker carcinosarcoma 256 removed glutamic acid from the arterial blood and released aspartic acid. Utilization of the other amino acids was about 5 nmol/min/g tumor or less. The extent to which anabolism and catabolism contributed to these utilization rates has not yet been determined. Hepatoma 5123C is known to synthesize and secrete albumin (28), and hepatoma 7777 is known to contain the albumin mRNA (33).

Several of the fast-growing tumors released amino acids to the venous blood (Chart 2). Glycine, alanine, and aspartic acid were released by 3, 3, and 5, respectively, of the 5 Walker 256 carcinosarcomas examined. Three of the Walker 256 tumors also released threonine. Glycine was produced by 3 of the 4 hepatomas 7777 and by 4 of the 10 hepatomas 7288CTCF. In contrast, alanine, glycine, aspartic acid, and threonine were utilized by all 7 of the slower-growing hepatomas 5123C. Since these 4 amino acids are potential carbon sources for host liver gluconeogenesis, their apparently preferential release from some of the fast-growing tumors is of special interest. Assuming that the 4 amino acids released are derived from the catabolism of other amino acids, the function of their formation and release by fast-growing tumors might be to increase net glucose production during the cachexia caused by these tumors.

Ammonia was released into the venous blood by all 4 tumor types, indicating that some of the utilized amino acids were deaminated. As judged by the prominent glutamine utilization rates, it seems likely that a portion of the ammonia released was derived from glutamine deamidation. Several of the Morris hepatomas contain carbamyl phosphate synthetase I (19) and can synthesize urea (20), although often at diminished activities relative to liver, and the ammonia production rates measured across the hepatomas may be the differences between production and urea synthesis. Because individual tumors of the same strain show different metabolic responses to apparently identical in vivo conditions, a detailed analysis of nitrogen and carbon balance across a single tumor will be needed to resolve these questions.

The results shown in Chart 2 provide the first direct quantitative evidence in support of glutamine (and other amino acid) utilization by tumors in vivo. Results of previous investigations were indirect and were based on plasma glutamine (and other amino acid) concentration differences between tumor-bearing...
and normal humans (30), animals (23), and human limbs (26). Direct evidence for amino acid uptake by tumors in vivo was obtained from incorporation of injected radioactive amino acids (15, 25, 27). However, the tumor blood flow rate and the specific activity of the circulating amino acid were not known, and utilization (or production) rates could not be calculated. With the use of the tumor preparation described here, it will be possible to obtain direct information on substrate utilization and production in vivo and to determine the metabolic pathways used in the utilization or production.

Glucose and Lactic Acid. The mean arterial glucose concentration in pair-fed control Buffalo rats was 6.6 ± 0.2 mm (n = 6; range, 6.0 to 7.4 mm). In arterial blood of tumor-bearing animals, the mean value was 7.4 ± 0.4 mm (n = 26; range, 4.4 to 10.8 mm). With a single exception, glucose was utilized by all of the tumors examined. Mean glucose utilization rates and the ranges for each tumor group were: for hepatomas 5123C (n = 7), 116 ± 32 nmol/min/g tumor, wet weight, range, −5 to 234; for hepatomas 7777 (n = 4), 151 ± 57 nmol/min/g, range, 46 to 306; for hepatomas 7288CTCF (n = 10), 177 ± 40 nmol/min/g, range, 34 to 409; and for Walker carcinosarcoma 256 (n = 5), 228 ± 71 nmol/min/g, range, 78 to 460. One tumor in the hepatoma 5123C group released 5 nmol glucose per min per g tumor during the period of arterial and venous blood sampling. As shown in Chart 4, the tumor glucose utilization rates were directly proportional to the rate of glucose supply to the tumor. Depending on the glucose supply rate, a tumor may utilize from 25 to 35% of the glucose supplied in vivo. An essentially identical relationship was described by Guillon et al. (12) for in vivo glucose utilization and supply in rat tumors, and the data shown in Chart 4 confirm their finding.

A plot of glucose utilization against glutamine utilization suggested that the rates at which these tumors removed glucose and glutamine from the arterial blood were related (Chart 5). About 70% of the tumors were clustered at glucose and glutamine utilization rates under 150 and 15 nmol/min/g, respectively. The remainder were spread over higher values, and a regression line fit to the data by the least-squares method had a positive slope and indicated that glutamine utilization rates could average 5 to 10% of the glucose utilization rate. This was an unexpected finding, because experiments performed in vitro have shown an inverse relationship between glutamine and glucose oxidation in tumors (17, 18) and utilization and oxidation in fast-growing normal cells (37). An inverse relationship between glucose and glutamine utilization might also be expected in vivo if the substrates were used solely for energy production. The results reported here, however, counter that idea and demonstrate that tumors in vivo have a large capacity to utilize glucose, glutamine, and lactate (see below) and that the rate of utilization depends on the supply rate only. Davis and Busch (3) described a stimulation by glucose of amino acid uptake in Walker carcinosarcoma 256 slices in vitro, but the stimulation may have been dependent on an increased energy production due to glycolysis.

The apparent relationships between glutamine supply and utilization rates (Chart 3), between glucose supply and utilization rates (Chart 4), and between the rates of glutamine and glucose utilization were defined by linear regression analysis. Although the correlation coefficient values suggest that the relationships are significant, only about 25% of the data points in Charts 3 and 5 were distributed at the higher supply and utilization rates (see Ref. 5, pp. 56–61, for caveats for interpreting scattered data by linear regression). The supply and utilization rates were calculated from the results of one and 2 analytical determinations, respectively, and from measurements of tumor wet weight and rate of blood flow. The error in the supply and utilization rates is unknown, since we were unable to estimate the error in the tumor blood flow measurement. Tumor blood flow was underestimated to the extent that blood passed around the venous catheter. On the other hand, the blood flow rates were quite uniform, and we have no reason to
At this point, it seems reasonable to consider that the positive correlations shown in Charts 3 to 5 are real. Clearly, other interpretations are possible, and more data need to be collected before we can be sure that the relationships are different from those shown in the charts.

Lactic acid was produced by 22 tumors, utilized by 10 tumors, and neither produced nor utilized by one tumor (Chart 6). The cross-over point between lactate production and utilization was about 2 to 3 mM arterial lactic acid; below this value, production occurred, and above this value, utilization occurred. Glucose utilization ranged from 19 to 460 nmol/min/g [215 ± 41 (S.E.)] among the lactate-utilizing tumors and from —5 to 409 nmol/min/g [134 ± 23 (S.E.)] among the lactate-producing tumors. Lactate production or utilization depended entirely on the arterial lactate concentration and not on either the arterial glucose concentration or the glucose utilization rate. Spencer and Lehninger (32) have shown that mouse ascites tumor cells will transport lactate at a high rate in either direction, and the rates were salurable and had an apparent $K_m$ of 4.7 mM. The results shown in Chart 6 indicate that similar processes occur in tumors in vivo. The metabolic fate of the lactic acid removed from the arterial blood by the tumors is not known but is of great interest, since these tumors were utilizing glucose at the same time and presumably were generating both NADH and pyruvate intracellularly. No associated alanine (or other amino acid) release was detected in the tumors that were utilizing arterial lactate. More detailed experiments will be required to determine the chemical fate of the lactic acid. Small intestinal mucosa, another tissue capable of high rates of aerobic lactic acid production, also utilized lactate when the arterial lactate concentration was greater than 1.2 to 1.6 mM (36). Lactate and glucose were utilized simultaneously, and the utilized lactate was used for other amino acids.

It seems likely that studies of tumor metabolism in vivo will permit us to answer several specific questions of host-tumor interactions. If, as we have suggested (31), tumors develop a common type of energy metabolism during progression, the "biochemical strategy" (34) of the tumor cell may be for integration with the energy supplies (and needs) of the host. Two aspects of this initial research support this idea. (a) Tumors show remarkable ability to utilize whatever substrate is in most abundant supply. Glucose, glutamine, and lactate utilizations were directly correlated with either supply or the arterial blood concentration. (b) There is a striking similarity between the pattern of substrate utilization in tumor and intestinal mucosa. This finding implies that a form of energy metabolism qualitatively comparable to the gut develops in undifferentiated tumors. Gut metabolism has been studied in vivo in greater detail than has tumor metabolism, and significant differences may yet be found, but at this stage more similarities than differences are evident. In fasted animals, ketone bodies were removed from the arterial blood by these tumors, presumably to be activated (6) and oxidized to CO$_2$ in the mitochondria. Utilization of glutamine, ketone bodies, and lactate as well as oxidation of glucose via aerobic glycolysis suggests that the undifferentiated tumor fits into the energy metabolism of the host in the same manner as does the gut. Utilization of fatty acids by tumors in vivo has yet to be measured but is of interest because they are little utilized as respiratory fuels in intestinal mucosa (36). Bloch-Frankenthal et al. (1) and Fields et al. (7) have shown that fatty acid oxidation is decreased in undifferentiated Morris hepatomas in vitro.

ACKNOWLEDGMENTS

Thanks are due Dr. Theodore Peters, Jr., for help with the amino acid analyses and Dr. Walter O. Nagel for many helpful discussions and for help with the figures. Dr. Lawrence Pinkus, Nassau Medical Center, kindly supplied the glutaminase used in the glutamine assays.

Note Added in Proof

After this report was in proof, we became aware of the Brief Communication by F. H. Grantham, D. M. Hill and P. M. Gullino entitled, "Primary Mammary Tumors Connected to the Host by a Single Artery and Vein." (J. Natl. Cancer Inst., 50: 1381–1383, 1973). Their procedure, which also used the superficial epigastric artery and vein, has similarities to that described in this paper.

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Moyer, G. H. in rat liver in 2653, 1974.


Fig. 1. A vascular pedicle composed of the superficial epigastric artery and vein dissected free from surrounding connective and adipose tissue and ligated as described in "Materials and Methods." The pedicle (about 15 mm long) is prepared for attachment of the tumor implant.

Fig. 2. Vascular pedicle with attached 3-mm cube of tumor enclosed in paraffin envelope and ready for closing of the envelope.

Fig. 3. Hepatoma 5123C tumor (about 2.5 g, wet weight) growing on a superficial epigastric vascular pedicle. The pedicle stump is visible above the paper underlay.

Fig. 4. Photomicrograph of a cross-section through a hepatoma 7288CTCF (3.1 g, wet weight; outside dimensions, 26 x 22 x 14 mm). Small focal necrotic areas are visible in the upper central and right areas. x 2.6.

Fig. 5. Photomicrograph of a cross-section through a hepatoma 5123C (4.8 g, wet weight; outside dimensions, 25 x 18 x 18 mm). Small focal necrotic areas are visible on the cut surface in the lower central and right areas. x 3.
Amino Acid, Glucose, and Lactic Acid Utilization in Vivo by Rat Tumors

Leonard A. Sauer, J. Webster Stayman III and Robert T. Dauchy


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