Energy Substrate Utilization in Freshly Isolated Morris Hepatoma 7777 Cells

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ABSTRACT

Freshly isolated Morris Hepatoma 7777 cells were prepared for a study of the utilization of palmitate and \( \beta \)-hydroxybutyrate as metabolic fuels compared to other major energy substrates (glucose and glutamine). These cells were capable of oxidizing both \( [U-14C] \)palmitate and \( \beta \)-\( \beta-3-14C \)hydroxybutyrate although the rates accounted for only 10 ± 3 (SD) and 9 ± 4% of total oxygen consumed, respectively; \( n = 4 \). Incorporation of \( [U-14C] \)glutamine and \( [U-14C] \)glucose carbon into \( 14\text{CO}_2 \) made up for 38 ± 13 and 9 ± 2% of oxygen consumed by these cells, respectively. The conversion of glucose carbon into lactate was estimated to supply 26 ± 6% of ATP. Thus, glutamine oxidation and lactate formation from glucose were the major contributors to estimated ATP needs. Conversion of these substrates into lipids was also studied and compared with oxidized products. Incorporation of glucose, glutamine, and \( \beta \)-hydroxybutyrate into non-saponifiable lipids and fatty acids was only 6.0 ± 2.9, 0.8 ± 0.2 and 17.7 ± 6.65% (\( n = 3 \)) of their respective rates of \( \text{CO}_2 \) formation. This suggests that in freshly isolated Morris Hepatoma 7777 cells, citrate export from the mitochondria for cholesterogenesis and lipogenesis is a minor fate of substrate carbon entering the mitochondria for oxidation.

INTRODUCTION

It is widely held that many tumors rely mainly on glycolysis to meet their energy requirements. This theory, first proposed over 50 years ago by Otto Warburg, was based on the observation that several tumors formed lactate from glucose at substantial rates even in the presence of oxygen (1). Evidence for parallel increases in activities of key glycolytic enzymes and degree of differentiation in neoplasms (2-4) tended to support this notion. Yet, data which compare the relative importance of various energy substrates available to tumor cells are lacking.

One particular controversy is over the ability of poorly differentiated hepatomas to oxidize fatty acids and ketones for energy. Fields et al. (5) observed no detectable oxidation of palmitate or acetate in Morris Hepatoma 7777 homogenates, which tended to confirm the earlier report of Bloch-Frankenthal et al. (6) indicating decreased ability of hepatomas with higher growth rates to oxidize fatty acids. The rate of uptake of fatty acids by a poorly differentiated hepatoma was also found to be lower than that of host liver (7). However, these same investigators observed 40% of radioactivity in tumors from i.v. administered palmate in the aqueous fraction, which suggested that oxidation may represent an important fate of fatty acids taken up by this hepatoma. The inability of such neoplasms to utilize ketone bodies seemed questionable after Sauer and Dauchy (8) reported \( \text{in vivo} \) uptake of ketone bodies by numerous neoplasms, including poorly differentiated hepatomas, at rates which depended on their arterial concentration. Other investigators had also earlier observed an increase in activity of the first enzyme required for the utilization of acetocetate (succinyl-CoA:acetocetyl-CoA transferase) in hepatoma mitochondria as growth rate increased (9). One goal of the work reported here was to determine whether a poorly differentiated rat hepatoma (Morris Hepatoma 7777) was capable of oxidizing fatty acids and ketone bodies. Second, their relative contribution to meeting the energy requirements of this tumor was sought.

Another important fate for energy substrates in rapidly growing hepatomas is the synthesis of cholesterol. Cholesterogenesis in hepatomas has been demonstrated to be high and unregulated compared to that in liver (10-12). A persistent truncated Krebs cycle in hepatomas which results in increased mitochondrial efflux of citrate and its subsequent incorporation into cholesterol has recently been hypothesized (13). In fact, greater conversion of radiolabeled pyruvate carbon into sterols than into \( \text{CO}_2 \) was observed in slices of a poorly differentiated hepatoma. Thus, a third goal of this research was to determine whether a greater proportion of carbon atoms from major energy substrates were directed toward cholesterogenesis than to \( \text{CO}_2 \) formation.

Freshly isolated cells may better characterize tumors \( \text{in vivo} \) than tissue slices, subcellular organelles, or cells maintained in culture. Although a few previous investigators have utilized freshly isolated hepatoma cells (14, 15), viability based on trypan blue exclusion was either not reported or low. Documentation of metabolic viability by other indicators is therefore needed. To our knowledge, a generally accepted procedure to isolate fresh hepatoma cells with demonstrated metabolic viability has not been reported. A procedure was developed in our laboratory to address this problem, as well as the questions of energy substrate utilization posed earlier.

MATERIALS AND METHODS

Cell Isolation. MH 7777 cells originally obtained from the laboratory of H. C. Pit, University of Wisconsin, were maintained in our laboratory by serial s.c. transplantation of approximately 2 × 10⁶ cells into hind limbs of male Buffalo rats (150-200 g). Hepatoma cells were isolated 3 to 4 weeks after transplantation from tumors measuring approximately 1 to 15 cm³.

Initially, we attempted to use a method previously reported for isolation of hepatoma cells for subsequent cell culture (16). Cell yield and viability were improved by use of a two-step incubation procedure described for the dissociation of rat mammary adenocarcinoma cells (17). We found that a good yield of viable hepatoma cells could be obtained with similar collagenase concentrations but included Mg²⁺ and excluded hyaluronidase from the incubation medium and shortened incubation times. The final collagenase concentration was approximately 10% of that used to isolate hepatoma cells by Barra et al. (14). Increasing the collagenase concentration approximately 7-fold or excluding Mg²⁺ from the incubation medium did not increase cell yield or viability. Consequently, the following procedure was used to isolate...
with 2% perchloric acid, dissolved in 1.5 ml 0.3 M NaOH, and added were 3 ml. Reactions were stopped by the addition of 1 ml 10% perchloric acid. The acid-insoluble material was washed three times in a new flask containing 15 ml fresh Hanks’ balanced salt solution with collagenase, gassed, and incubated at 37°C with shaking for an additional 45 min. The filtrate from the first incubation which contained a considerable quantity of RBC was discarded. The suspension from the second incubation was filtered through 80-μm nylon mesh with gentle stirring using a rubber policeman. Cells were centrifuged for 3 min at 50 X g and washed three times with cold EMEM containing 25 mM HEPES and 1% BSA. Cells were counted in a hemocytometer and viability was determined by trypan blue exclusion. Mean cell yield using this procedure was 4 ± 1 (SD) × 10^7 cells/g viable tissue. Ninety-four % of cells ± 2 (n = 19) excluded trypan blue.

**Metabolic Viability Studies.** Approximately 2 × 10^8 cells in 25-ml stopped flasks were preincubated in EMEM containing 25 mM HEPES and 1% BSA, pH 7.4, for 15 min in a shaking water bath (37°C) after gassing with 95% O_2-5% CO_2. Leucine incorporation into protein was then estimated at 30, 60, and 90 min in triplicate flasks following the addition of 0.5 μCi [1-14C]leucine (final specific activity, 0.5 mCi/μmol), a known inhibitor of protein synthesis, was added to parallel flasks during some experiments. Uridine incorporation into RNA was estimated at 20, 40, and 60 min in triplicate flasks after the addition of 0.5 mCi [3H]uridine (specific activity, 26 Ci/μmol). Final incubation volumes were 3 ml. Reactions were stopped by the addition of 1 ml 10% perchloric acid. The acid-insoluble material was washed three times with 2% perchloric acid, dissolved in 1.5 ml 0.3 M NaOH, and added to aqueous scintillation fluid (Aquasol; Amersham, Arlington Heights, IL). Parallel flasks in which reactions were stopped at zero time served as blanks. Radioactivity was counted in a Packard liquid scintillation spectrometer.

ATP and ADP were quantitated in duplicate cell suspensions after isolation and following 30, 60, and 90 min of incubation as described above. Cells were transferred rapidly to test tubes, frozen in liquid nitrogen, and stored at −70°C. Adenine nucleotides were extracted by vortexing ice cold samples in 0.6 M perchloric acid. After centrifugation (30,000 × g, 10 min) supernatants were brought to pH 6 by the addition of 0.12 volume of 1.5 M KH_2PO_4, pH 6.0, and approximately 0.2 volume of 4 N KOH. Samples were centrifuged to remove potassium perchlorate salts and filtered. Analyses of ADP and ATP concentrations were done by anion exchange high performance liquid chromatography using a modification of the procedure described by DeBoer et al. (18). Nucleotides were sequentially eluted from a Whatman SAX strong anion exchange column in a Z module (Waters Associates, Milford, MA) using a rising concentration of ammonium phosphate buffer (initial concentration, 0.05 mM increased to 0.7 mM between 22 and 23 min), pH 4.4, and quantitated spectrophotometrically at 256 nm with simultaneous comparison at 250 and 280 nm using external ADP and ATP standards. Cell adenine nucleotide levels were compared with those in rapidly frozen tissue. Samples of viable tumor tissue from separate animals were rapidly frozen in liquid nitrogen and stored at −70°C. Frozen samples were homogenized in 0.6 M perchloric acid and further processed as described above for cell extracts.

Cells from each experiment were washed 4 times in Hank’s BSS, pH 7.4, for subsequent protein analysis. Cell and tissue protein concentrations were determined by the Hartree (19) modification of the Lowry method using BSA as a standard.

**Oxygen consumption.** Cells were measured utilizing a Gilford model KM (Gilford, Middleton, WI) oxygraph with a Clark oxygen electrode. Since the relationship between oxygraph deflection and change in oxygen concentration is affected by the ionic strength of a solution it was necessary to determine the nature of this relationship in the media used. This was accomplished by the xanthine oxidase reaction.

Xanthine + 2O_2 → xanthine oxidase → uric acid + 2H_2O_2
to consume known quantities of oxygen by the addition of known quantities of xanthine. Xanthine at final concentrations between 14 and 44 μM was added to EMEM containing 25 mM HEPES and 1% BSA, heated to 37°C, and added to the oxygraph cell. The oxygraph deflection after addition of 0.2 μM xanthine oxidase was recorded. In this medium the mean ± SE deflection after full scale calibration with air-saturated water was 1.83 ± 0.16% per μmol O_2 (n = 6).

After a 15-min preincubation, oxygen consumption was measured in at least duplicate 1.5-ml aliquots of cells (2 × 10^8 cells/ml) at 0, 30, and 60 min of incubation as described earlier. Oxygen consumption was monitored for approximately 10 min and the linear portion of the oxygen consumption curve was utilized to calculate oxygen consumption rates.

**Substrate Incorporation into CO_2.** The production of CO_2 from glucose, glutamine, β-hydroxybutyrate, and palmitate was estimated using substrate concentrations which are similar to those in blood of tumor-bearing rats in the fasted state (8, 20, 21). Approximately 2 × 10^8 hepatoma cells were incubated with shaking at 37°C for 0, 30, and 60 min after a 5-min preincubation in 3 ml EMEM, pH 7.4, containing 25 mM HEPES and 1% fatty acid-free BSA which was modified to contain the following concentrations of substrates: 4.5 mM glucose, 0.7 mM glutamine, 2 mM β-hydroxybutyrate, and 0.6 mM palmitate complexed to essentially fatty acid-free BSA (molar ratio, 4:1). One substrate was radio labeled in each set of triplicate flasks. Final medium specific activities (μCi/nmol) were approximately 35, 210, 85, and 280 for glucose, glutamine, β-hydroxybutyrate, and palmitate, respectively. Reaction flasks were fitted with rubber stoppers from which center wells containing 0.2 ml Hyamine hydroxide were suspended. Reactions were stopped by the addition of 1 ml 20% perchloric acid. Flasks were incubated for an additional 60 min to trap CO_2. Center wells containing Hyamine hydroxide were transferred to scintillation vials to which liquid scintillation fluid was added, and radioactivity was counted as described previously. Oxygen consumption of cells in this medium was determined at 0 and 60 min after a 5-min preincubation as described earlier.

The incorporation of glucose carbon into lactate and palmitate carbon into acid-soluble products was determined. Perchloric acid supernatants from cells incubated with [U-14C]glucose or [U-14C]palmitate were neutralized with 4 N KOH and centrifuged to remove potassium perchlorate salts. Radioactivity in an aliquot of supernatant...
from cells incubated with \([U^{-14}C]\) palmitate was counted. Aliquots of supernatant from cells incubated with \([U^{-14}C]\) glucose were applied to 0.5-cm columns containing 1 ml Dowex 1-X8-400 (Cl form) resin to isolate lactate using a modification of the procedure described by Longmore and Mourning (22). Glucose was eluted with 6 ml of distilled water in 2-mi fractions. All of the glucose was present in the first two fractions. Lactate was then eluted with 6 ml 0.1 N HCl in 3 fractions. This elute was added to liquid scintillation fluid and radioactivity was counted.

Substrate Incorporation into Lipids. In a series of separate experiments, glucose, glutamine, and \(\beta\)-hydroxybutyrate carbon incorporation into lipids and palmitate carbon incorporation into esterified lipids was compared with their incorporation into CO\(_2\). Incubations were performed in duplicate as described for substrate carbon incorporation into CO\(_2\). Final specific activities of approximately 100, 200, 260, and 390 \(\mu\)Ci/\(\mu\)mol for glucose, glutamine, \(\beta\)-hydroxybutyrate, and palmitate were used, respectively. After 0 and 60 min of incubation, 2 ml of the mixture for cells incubated with radioactive glucose, glutamine, or \(\beta\)-hydroxybutyrate were transferred to glass-stopped centrifuge tubes containing 7.5 ml of methanol:chloroform (2:1, \(v/v\)). Lipids were extracted according to a modification of the procedure of Bligh and Dyer (23) described by Kates (24). An aliquot of the final chloroform phase was washed 3 times with water, evaporated under \(N_2\) and saponified at 73°C in 5 ml 0.5 N NaOH in 50% methanol containing 7.5 ml of methanol:chloroform (2:1, \(v/v\)). Lipids were used, respectively. After 0 and 60 min of incubation, 2 ml of the mixture for cells incubated with \([U^{-14}C]\) glutamine in 3 experiments were pooled for saponification due to low radioactivity in this lipid fraction. Nonsaponifiable lipids, assumed to represent predominantly cholesterol, were extracted with 3 x 4 ml petroleum ether. The remaining lipids were acidified by the addition of 1 ml 6 N HCl and extracted with 3 x 4 ml petroleum ether to recover radioactivity in fatty acids. Radioactivity remaining in the aqueous phase was assumed to represent that incorporated into glycerol moieties of lipids. Petroleum ether extracts were dried and radioactivity in these extracts and in the aqueous phase was counted as described previously. Radioactivity in fractions from cells incubated for 0 min was subtracted from that observed in fractions from cells incubated for 60 min.

An aliquot (1 ml) of the incubation mixture from cells incubated with \([U^{-14}C]\) palmitate for 0 and 60 min was added to Dole’s extraction mixture (25) and lipids were extracted by the method of Rodbell (26). The hexane phase was washed 3 times with 5 ml 50% ethanol containing 5% 1 N KOH and dried, and radioactivity was counted as described earlier to quantify radioactivity in the esterified fatty acid fraction. Extraction of \([U^{-14}C]\) palmitate is complete using this method and no radioactivity from palmitate is present in the hexane phase after washing.

Molar incorporation of substrate carbon into products measured was determined using initial specific activities of substrates in the medium. Data were analyzed by analysis of variance. When significant \(F\) ratios were observed (\(P < 0.05\)), means for individual incubation times were compared using Tukey’s multiple comparison test (27).

Materials. Collagenase (grade IA), xanthine oxidase (grade II), cycloheximide, essentially fatty acid free bovine serum albumin (prepared from fraction V), and modified Eagle’s medium containing 25 mM HEPES were purchased from Sigma Chemical Co., St. Louis, MO. \([1^{-14}C]\) Leucine, \([5-3H]\) uridine, \([U^{-14}C]\) glucose, \([U^{-14}C]\) glutamine, and \((\alpha\rightarrow\beta)3\)-hydroxy\([3^{-14}C]\) butyrate (sodium salt) were obtained from Amersham, Arlington Heights, IL, and \([U^{-14}C]\) palmitate was from New England Nuclear, Boston, MA.

RESULTS

Amino acid and uridine incorporation into acid-insoluble material are commonly used indices to assess protein and RNA synthesis. Linear incorporation of radiolabeled leucine and uridine into acid-insoluble products was observed in isolated hepatoma cells (Fig. 2). The addition of cycloheximide limited leucine incorporation to 4 ± 1% of that observed after 90 min of incubation, confirming that the leucine label in acid-insoluble material was indicative of protein synthesis. Linear incorporation of radiolabeled leucine and uridine suggests that hepatoma cells isolated by this procedure retain metabolic viability at least over 90 min of incubation. The integrity of the cells is also supported by constant oxygen consumption rates, which at 0, 30, and 60 min after incubation were not significantly different (Fig. 3).

The hepatoma cells were found to retain adenine nucleotides following their isolation. ATP:ADP ratios in cells after isolation were significantly higher than in rapidly frozen hepatoma tissue and did not vary significantly in incubated cells (Table 1). Interestingly, ATP:ADP ratios in freshly isolated cells were actually higher than in rapidly frozen hepatoma tissue. ATP and ADP levels determined in hepatoma tissue in this study are comparable to those observed previously in poorly differentiated hepatomas (28). It is possible that ATP:ADP ratios declined in tissue samples during extraction and freezing, (accomplished within 1 min after disruption of the blood supply of the tumor). Several types of neoplastic cells have been shown to lose ATP more rapidly than normal cells when deprived of glucose (29). The isolation of cells in a balanced salt solution containing glucose may have increased ATP:ADP ratios. It is not clear whether ATP or ADP concentrations observed in these cells or tissue best represent \(in vivo\) concentrations.
Rates of labeled substrate incorporation into CO₂ and other products over 60 min of incubation are reported in Table 2. Rates of incorporation over 30 min of incubation were 5.8 ± 1.5, 26.4 ± 9.1, 9.8 ± 6.9, and 0.6 ± 0.6 nmol substrate/h/mg protein for glucose, glutamine, β-hydroxybutyrate, and palmitate into CO₂ and 487 ± 62 and 7.2 ± 2.2 nmol substrate/h/mg protein for glucose carbon incorporation into lactate and palmitate carbon incorporation into acid-soluble products, respectively. These rates were not significantly different from those observed over 60 min. Therefore, rates of labeled substrate incorporation into CO₂ and other products were approximately linear over 60 min. The incorporation of label from β-hydroxybutyrate into CO₂ and label from palmitate into CO₂ and acid-soluble products provides evidence that these cells are capable of oxidizing these substrates. The calculation of molar quantities of β-hydroxybutyrate incorporation into CO₂ was based on the assumption that the metabolic fate of C-3, the labeled carbon, was representative of the fate of all four carbon atoms in that molecule. However, oxidation of this molecule results in acetyl-CoA which is labeled at C-1. Since C-1 of acetate requires only two turns of the citric acid cycle to yield CO₂ versus three for C-2, the molar quantity of β-hydroxybutyrate incorporation into CO₂ (and lipids) may be overestimated.

Rates of oxygen consumption at 0 and 60 min were 598 ± 605 and 7.0 ± 3.3 nmol/h/mg protein for glucose, glutamine, β-hydroxybutyrate, and palmitate towards the energy requirement of the cell. Thus, if exogenous palmitate did not come into rapid equilibrium with endogenous fatty acids the contribution of palmitate to CO₂, respectively, and by 230 ± 11% of initial rates, indicating that the decline in oxygen uptake is approximately linear. Oxygen consumption attributable to net reactions (Table 3) measured was estimated by multiplying molar rates of substrate label incorporation into product by 6, 4.5, 4.5, and 23 for glucose, glutamine, β-hydroxybutyrate, and palmitate to CO₂, respectively. That attributable to palmitate incorporation into acid-soluble products (assumed to represent ketone bodies with equal distribution between acetoacetate and β-hydroxybutyrate) was estimated using a multiplication factor of 6.

Oxygen uptake which could be attributed to incorporation of substrate carbon into products measured (Table 3) was 65 ± 15% of average oxygen consumption. Since the media used contained other amino acids, their oxidation could have provided the remainder of the energy requirements of the cell. Utilization of endogenous substrate may have also explained our failure to account for all contributions to energy production. The assumption that exogenously supplied substrates came into rapid equilibrium with endogenous pools was made but may not be entirely valid. Since this hepatoma contains little glycogen (21) it is likely that lipids would account for most available endogenous fuel. Thus, if exogenous palmitate did not come into rapid equilibrium with endogenous fatty acids the contribution of palmitate towards the energy requirement of the cell may be underestimated. Finally, all products of oxidative catabolism were not measured and could account for some of the discrepancy between observed rates of oxygen consumption and the sum of oxygen uptake attributable to conversion of substrates to products measured. For example, partial catabolism of glutamine could result in the accumulation of radioactivity in lactate (30).

The total ATP production from respiration and conversion of glucose to lactate were calculated from rate of average oxygen consumption and radiolabeled glucose incorporation into lactate. This calculation was done assuming that during respiration, 5.4 ATP molecules were produced per molecule consumed (31) and that 2 ATP molecules are produced per glucose molecule converted to lactate. The contribution of net reactions measured to total ATP production (Table 3) was calculated by multiplying molar rates of substrate label incorporation into product by 38, 27, 21, and 129 for glucose, glutamine, β-hydroxybutyrate, and palmitate to CO₂, respectively, and by 2 and 30 for glucose to lactate and palmitate to acid-soluble products (making assumptions described in calculation of attributable oxygen consumption). The total ATP production was 3556 ± 947 nmol/h/mg protein with 77 ± 13% of the total estimated to be accounted for by net reactions measured (Table 3).
Incorporation of glucose, glutamine, and β-hydroxybutyrate into 14C-fatty acids and NSL was significantly less than incorporation into CO₂ (P < 0.001, 0.001, and 0.05, respectively) during 60-min incubations (Table 4). However, when incorporation of [U-14C]glucose into glycerol moieties was considered, no significant difference between glucose carbon incorporation into lipids and CO₂ was observed. There was no significant difference between incorporation of β-[3-14C]hydroxybutyrate carbon into total lipids and CO₂. β-Hydroxybutyrate carbon incorporation was significantly greater than glucose and glutamine carbon incorporation into NSL (Table 4). Furthermore, molar quantities incorporated into NSL and fatty acids expressed as a percentage of that incorporated into CO₂ is significantly greater for β-hydroxybutyrate than these other substrates.

As shown in Table 5, the incorporation of label from [U-14C]palmitate into esterified fatty acids was greater than into oxidized products (P < 0.001). This difference was approximately 5-fold.

DISCUSSION

Contrary to previous observations in MH 7777 homogenates (5), isolated MH 7777 cells were capable of oxidizing palmitate and β-hydroxybutyrate under the conditions studied. These rates of palmitate oxidation cannot be compared directly with those in hepatocytes since studies using the same incubation conditions have not been conducted. However, rates of palmitate oxidation to total oxidized products in this study were approximately 7 to 24% of rates observed by others in hepatocytes from fasted and fed rats, respectively, when palmitate was the sole exogenous carbon source (32). Capacity for ketone body utilization was suspected due to the previous demonstration of acetoacetate-CoA transferase in poorly differentiated rat hepatomas (9) and the observation of ketone body uptake by this and other poorly differentiated neoplasms (8).

Despite the ability of hepatoma cells to oxidize palmitate and β-hydroxybutyrate, their estimated contributions to total oxygen consumption were only 10 and 9%, respectively. It is also important to consider that concentrations of palmitate and β-hydroxybutyrate utilized in these experiments were in the high range of those encountered physiologically. Sauer and Dauchy (8, 20) have shown that the rates of uptake of ketone bodies, lactate, glucose, and glutamine by this and other neoplasms in vivo were directly proportional to rates of supply. Thus, oxidation of fatty acids and ketone bodies may be lower when blood concentrations are lower in vivo than estimated by this model.

These results confirm the importance of glutamine as a metabolic fuel previously observed in poorly differentiated hepatoma mitochondria (33). Complete glutamine oxidation was estimated to contribute similarly to total ATP production as oxidation products of glutamine metabolism may have led to an underestimate of the contribution of glutamine oxidation toward ATP production.

In vivo, where the oxygen supply to some tumor cells is more limiting, the conversion of glucose to lactate may provide a greater proportion of the energy needs of the cell. In mice with Ehrlich ascites carcinoma or in rats with ovarian ascites carcinoma i.p. introduction of oxygen decreases lactate production while stimulating protein synthesis (34). Thus, it appears that when oxygen availability is increased energy requirements increase and the proportion of energy provided by the conversion of glucose to lactate decreases. The effect of oxygen tension on energy substrate utilization in these isolated hepatoma cells requires investigation.

This pattern of energy metabolism characterized by high levels of glutamine utilization which often exceed the rate of complete glucose oxidation has also been observed in cultured human diploid fibroblasts (35), in Chinese hamster fibroblasts (36), in HeLa cells (34, 37, 38), and in perfused rat intestine (39). Intact Ehrlich ascites cells oxidize glutamine to CO₂ at higher rates than any other amino acid present (40). Furthermore, glutaminase activity has been found to be positively correlated with growth rate of rat hepatomas (41). Thus, it appears that this pattern of glutamine utilization for energy metabolism may be characteristic of rapidly growing cells.

It was expected that a large proportion of carbon from energy substrates which enter the citric acid cycle would be diverted into synthesis of non-esterifiable lipids (assumed to be predominantly cholesterol). This assumption was based on the previous observation of a greater proportion of pyruvate carbon incorporated into sterols than into CO₂ in slices of Morris Hepatoma 3924A (13) and the evidence for enhanced and unregulated cholesterol synthesis in numerous mouse (10, 11), rat, and human (12) hepatomas. Results from this study suggest that conversion of energy substrate carbon into cholesterol and fatty acids is a far less dominant fate in freshly isolated MH 7777 cells under the conditions studied. The lower incorporation of carbon from pyruvate-generating substrates (such as glucose) into sterols relative to CO₂ observed in this study may be due to metabolic differences between the Morris Hepatoma 3924A used by other investigators (13) and MH 7777 or the possibility that the isolation procedure utilized in this study selectively isolated particular MH 7777 cell types with characteristics which may not represent the total MH 7777 cell population.

### Table 4 Substrate label incorporation into lipids

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Non-esterifiable lipids</th>
<th>Fatty acids</th>
<th>Lipid glycerol</th>
<th>Total lipids</th>
<th>NSL + FAc</th>
<th>CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>[U-14C]Glucose</td>
<td>0.29 ± 0.22</td>
<td>0.39 ± 0.27</td>
<td>19.1 ± 7.4e</td>
<td>19.9 ± 7.7</td>
<td>6.0 ± 2.9</td>
<td>6.0 ± 2.9</td>
</tr>
<tr>
<td>[U-14C]Glutamine</td>
<td>0.24</td>
<td>0.30</td>
<td>0.19</td>
<td>0.74 ± 0.07</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>β-[3-14C]Hydroxybutyrate</td>
<td>0.88 ± 0.35</td>
<td>ND</td>
<td>2.72 ± 1.39</td>
<td>3.42 ± 1.46</td>
<td>17.7 ± 6.6e</td>
<td>17.7 ± 6.6e</td>
</tr>
</tbody>
</table>

* Data are expressed as mean ± SD; n = 3.
* NSL, incorporation into non-esterifiable lipids; FAc, incorporation into fatty acids.
* Significantly different than incorporation of other substrates (P < 0.05).
* Significantly different than glucose incorporation into non-esterifiable lipids or fatty acids, P < 0.001.
* ND, not detected.
Alternatively, isolated cells which are equally exposed to media components may behave differently than whole tumors or tissue slices. The use of a more physiological mixture of substrates in the current study than in the study of Morris Hepatoma 3924 slices (13), where pyruvate was the sole carbon source, may also account for differences in the relative flux of energy substrate carbon into sterols.

Of those studied, β-hydroxybutyrate appears to be the most important carbon source for synthesis of nonsaponifiable lipids. Although ketone bodies are not catabolized by normal liver, they have been identified as important precursors of brain (42) and lung lipids (43) in the developing rat. Data from this study also raise the possibility of cytosolic incorporation of ketone bodies into cholesterol. In most adult tissues it is generally accepted that the carbons for cholesterologenesis arise from mitochondrial conversion of ketone body carbon to citrate, subsequent citrate export from the mitochondria, and incorporation of acetyl-CoA generated from the ATP-citrate lyase reaction into sterol production. However, there is evidence for cytosolic pathways for the conversion of acetoacetate into cholesterol in developing rat brain (44) and of acetoacetate and β-hydroxybutyrate into cholesterol in rat neonatal liver (43). If only the mitochondrial pathways for conversion of β-hydroxybutyrate carbon into acetyl-CoA existed, one would expect that the ratio of carbon incorporation into CO₂ from substrates which form acetyl-CoA (glucose and ketone bodies) versus fatty acids and nonsaponifiable lipids would be equivalent (or slightly greater from glucose which is also oxidized to CO₂ in the hoxe monophosphate shunt pathway). However, in these hepatoma cells, the ratio of radiolabeled carbon recovered as cholesterol and fatty acids versus CO₂ was higher with β-hydroxybutyrate than glucose. The importance of ketone bodies as cholesterol precursors and the possible cytosolic involvement in these hepatoma cells deserve further study.

The dominant fate of palmitate in MH 7777 cells appears to be esterification as opposed to oxidation. This is contrary to the conclusions of Morton et al. (7) who observed 40% of the i.v. [¹⁴C]palmitate injection taken up by this tumor in the aqueous soluble fraction, 6 min after i.v. injection. These results also indicate that the cytosolic fatty acid-binding protein concentration may play less of a role in determining the metabolic fate of free fatty acids in these cells than in liver. In liver, low concentrations of this protein increased mitochondrial acyl-CoA synthetase and β-oxidation activities and reduced microsomal esterification (45). The concentration of the fatty acid-binding protein in this hepatoma was shown to be 20% of control liver (46), despite higher ratios of palmitate esterification to oxidation than previously found in hepatocytes isolated from fed rats (32, 47, 48).

The predominant route for incorporation of both glucose and β-hydroxybutyrate into lipids appears to be through their incorporation into glyceroide moieties. This is in contrast to normal liver where glucose carbon is recovered in greater quantities in fatty acids than lipid glyceride both in vitro (49) and in vivo (50). The relatively greater flux of glucose and β-hydroxybutyrate carbon into lipid glycerol and 5-fold higher rate of palmitate esterification over oxidation suggest that MH 7777 cells may rely less heavily on de novo fatty acid synthesis for the production of cellular lipids. However, this idea conflicts with the earlier observation of 5- to 10-fold higher activity of acetyl-CoA carboxylase activity observed in homogenates of this hepatoma relative to liver in the fasting state (51).

In summary, MH 7777 cells incubated under conditions which approximate those found in the fasted state in vivo derive most of their cellular energy requirements from the oxidation of glutamine and formation of lactate from glucose. Complete oxidation of glucose, palmitate, and β-hydroxybutyrate occurs but contributes less to the energy needs of the cell. The utilization of energy substrates for cholesterologenesis is less than their oxidation for ATP production. β-Hydroxybutyrate appears to contribute a larger proportion of carbon toward cholesterologenesis than other energy substrates studied. Hepatoma cells isolated by this procedure appear to maintain metabolic viability in incubations at least up to 90 min and should be useful in the future study of the biochemical characteristics of this tumor, particularly in studies which must consider the affect of the host metabolic milieu.

ACKNOWLEDGMENTS

We are grateful to Janet Arnold for excellent technical assistance and to Laurence DeBoer for analysis of adenine nucleotide content of hepatoma cells and tissue.

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