Analysis of tricarboxylic acid-cycle metabolism of hepatoma cells by comparison of $^{14}$CO$_2$ ratios

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The CO$_2$-ratios method is applied to the analysis of abnormalities of TCA (tricarboxylic acid)-cycle metabolism in AS-30D rat ascites-hepatoma cells. This method utilizes steady-state $^{14}$CO$_2$-production rates from pairs of tracers of the same compound to evaluate TCA-cycle flux patterns. Equations are presented that quantitatively convert CO$_2$ ratios into estimates of probability of flux through TCA-cycle-related pathways. Results of this study indicated that the ratio of $^{14}$CO$_2$ produced from [1,4-$^{14}$C]succinate to $^{14}$CO$_2$ produced from [2,3-$^{14}$C]succinate was increased by the addition of glutamine (5 mM) to the medium. An increase in the succinate CO$_2$ ratio is quantitatively related to an increased flux of unlabelled carbon into the TCA-cycle-intermediate pools. Analysis of $^{14}$C distribution in $[^{14}$C]citrate derived from [2,3-$^{14}$C]succinate indicated that flux from the TCA cycle to the acetyl-CoA-derived carbons of citrate was insignificant. Thus the increased succinate CO$_2$ ratio observed in the presence of glutamine could only result from an increased flux of carbon into the TCA cycle from citrate to oxaloacetate. This result is consistent with increased flux of glutamine to α-oxoglutarate in the incubation medium containing exogenous glutamine. Comparison of the pyruvate CO$_2$ ratio, steady-state $^{14}$CO$_2$ production from [2-$^{14}$C]pyruvate versus [3-$^{14}$C]pyruvate, with the succinate $^{14}$CO$_2$ ratio detected flux of pyruvate to C$_4$ TCA-cycle intermediates in the medium containing glutamine. This result was consistent with the observation that $[^{14}$C]aspartate derived from [2-$^{14}$C]pyruvate was labelled in C-2 and C-3. $^{14}$C analysis also produced evidence for flux of TCA-cycle carbon to alanine. This study demonstrates that the CO$_2$-ratios method is applicable in the analysis of the metabolic properties of AS-30D cells. This methodology has verified that the atypical TCA-cycle metabolism previously described for AS-30D-cell mitochondria occurs in intact AS-30D rat hepatoma cells.

INTRODUCTION

The tricarboxylic acid (TCA) cycle is the hub for a large number of biosynthetic and catabolic pathways in the cell. For this reason the textbook description of the TCA cycle, where carbon enters as acetyl-CoA, is oxidized to generate NADH, and exits as CO$_2$, may not accurately describe this pathway in biosynthetically active cells. An extremely interesting variation of the TCA cycle, the ‘truncated TCA cycle’, has been proposed for tumour cells (Coleman & Lavietes, 1981). The key elements of this hypothesis are: (1) glutamine is a major energy source for tumours, and (2) TCA-cycle citrate is not completely oxidized to CO$_2$ but leaves the mitochondria to provide a source of acetyl units for synthesis of cholesterol and fatty acid. Flux through the α-oxoglutarate-to-oxaloacetate span of the cycle may be greater than flux from citrate to α-oxoglutarate (Fig. 1). This hypothesis is supported by data indicating that glutamine oxidation is a prominent feature of many tumour preparations, including Morris hepatoma in vivo (Sauer & Dauchy, 1978), isolated Ehrlich ascites cells (Lazo, 1981) and isolated tumour mitochondria (Matsuno et al., 1986; Abou-Khalil et al., 1983). Glutamine oxidation is correlated with mitochondrial glutaminase activity in Morris hepatomas (Kovacevic & Morris, 1972). However, the importance of glutamine has been disputed (Nakashima et al., 1984). Once glutamine is deaminated to glutamate, it enters the tumour TCA cycle largely via transaminase reactions (Moreadith & Lehninger, 1984). Flux of C$_4$ TCA-cycle intermediates to pyruvate, acetyl-CoA and citrate (path A in Fig. 1) is required for complete oxidation of glutamine carbon to CO$_2$. In view of this requirement, it is interesting that mitochondrial NAD(P)$^+$-dependent malic enzyme (EC 1.1.1.39) has been identified in tumour mitochondria (Hansford & Lehninger, 1973). Malic enzyme is progression linked in Morris hepatoma, and is proposed to

Abbreviation used: TCA cycle, tricarboxylic acid cycle.

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catalyse the flux of malate to pyruvate required for oxidation of glutamine carbon to CO₂ (Sauer et al., 1980). Thus both mitochondrial glutaminase and malic enzyme contribute to the atypical TCA-cycle metabolism of tumour mitochondria (reviewed by Fiskum, 1986).

The present study utilizes the 'CO₂ ratios' methodology to investigate these phenomena in AS-30D rat ascites-hepatoma cells. A modified version (Kelleher, 1985; Kelleher & Bryan, 1985) of this classical method (Weinman et al., 1957) of steady-state carbon-tracer analysis is used. To avoid substrate depletion over a 2 h incubation period, media were selected containing concentrations of ketone bodies and glutamine substantially greater than those found in vivo. Thus, although the CO₂-ratios method allows investigators to utilize optimally information available from measurement of CO₂ production, the requirement for metabolic and isotopic steady state imposes some restrictions on the choice of media.

METHODS

Theoretical considerations

In metabolic and isotopic steady state the rate of CO₂ production from labelled compounds oxidized in the TCA cycle provides quantitative information on the pattern of TCA-cycle oxidation. The CO₂-ratios method utilizes CO₂-production data from pairs of tracers of the same compound (Weinman et al., 1957; Kelleher, 1985; Kelleher & Bryan, 1985). Pairs of tracers that generate either [1,4-14C]oxaloacetate or [2,3-14C]-oxaloacetate on the first circuit through the TCA cycle are especially useful. The original description of this method utilized [1-14C]acetate and [2-14C]acetate (Weinman et al., 1957). In the present study, [1,4-14C]succinate and [2,3-14C]succinate are used as tracers. The succinate CO₂ ratio is defined as:

\[
\frac{\text{rate of } 14\text{CO}_2 \text{ production from } [1,4-14\text{C}]\text{succinate}}{\text{rate of } 14\text{CO}_2 \text{ production from } [2,3-14\text{C}]\text{succinate}}
\]

Under steady-state conditions, linear rates of CO₂ production result. In general, CO₂ production is greater from [1,4-14C]succinate than from [2,3-14C]succinate, because [2,3-14C]succinate must traverse more TCA-cycle carbon pools to reach CO₂ and, at each pool, there is some probability that the labelled carbon will efflux from the cycle to form products other than CO₂ (Fig. 2). Because the system is in steady state, efflux from the TCA cycle is equalled by influx of carbon into the TCA-cycle intermediate pools. The greater the steady-state flux of carbon into the TCA-cycle intermediate pools, the larger the value of the succinate CO₂ ratio. Flux of glutamine to TCA-cycle α-oxoglutarate is an example of this type of carbon entry.

The succinate CO₂ ratio can be used to calculate the probability that TCA-cycle carbon will remain in the cycle for a complete turn. This probability refers to the fate of carbons that are not destined to become CO₂ in the next circuit of the cycle. For example, in the absence of efflux to products other than CO₂, 14C contained in C-2 and C-3 of succinate is destined to appear equally in each of the four carbons of succinate in the next circuit of the cycle (Fig. 2). However, as carbon moves around the cycle, there is opportunity for efflux from the TCA cycle to related metabolites such as aspartate and alanine. The probability that carbon will remain in the cycle has been designated (A + T) (Kelleher, 1985), because it is the sum of the probability of flux by two pathways. Pathway T represents that traditional TCA-cycle flux from citrate to succinate, oxaloacetate and citrate. Pathway A represents flux from citrate to succinate, pyruvate, acetyl-CoA and citrate. These paths are diagrammed in Fig. 1. Eqn. (2) below is used to calculate the probability (A + T):

\[
A + T = \frac{2}{1 + \text{succinate CO}_2 \text{ ratio}}
\]

To summarize these relationships, the greater the flux of glutamine carbon into the TCA cycle accompanied by equal efflux to products other than CO₂, the larger the succinate CO₂ ratio, and the lower the probability that carbon will remain in the cycle.

The studies described here utilize pyruvate tracers in addition to succinate tracers. The pyruvate CO₂ ratio is defined as:

\[
\frac{14\text{CO}_2 \text{ produced from } [2-14\text{C}]\text{pyruvate}}{14\text{CO}_2 \text{ produced from } [3-14\text{C}]\text{pyruvate}}
\]

Fig. 3. Flux of pyruvate into the TCA cycle via pyruvate dehydrogenase (PDH) and pyruvate carboxylation (PC) pathways

Via PDH pathway [2-14C]pyruvate (△) generates [1,4-14C]succinate, and [3-14C]pyruvate (■) generates [2,3-14C]succinate. In the PC pathway, tracer enters as C-2 and C-3 of oxaloacetate or malate, leading to pyruvate CO₂ ratio less than succinate CO₂ ratio (Kelleher & Bryan, 1985).
Pyruvate may enter the TCA cycle via either of two paths (Fig. 3). If all pyruvate entering the TCA cycle is converted into acetyl-CoA and then to citrate, the pyruvate dehydrogenase route, the pyruvate $\text{CO}_2$ ratio will equal the succinate $\text{CO}_2$ ratio, because via this path [2-$^{14}$C]pyruvate is a precursor of [1,4-$^{14}$C]succinate and [3-$^{14}$C]pyruvate is a precursor of [2,3-$^{14}$C]succinate. However, if pyruvate is carboxylated to form oxaloacetate or malate, the pyruvate carboxylation route, the pyruvate (Pyr.) $\text{CO}_2$ ratio will be lower than the succinate (Suc.) $\text{CO}_2$ ratio. An equation quantifying the fraction of pyruvate oxidized in the TCA cycle via the carboxylation path (PC') has been derived (Kelleher & Bryan, 1985):

$$\text{PC'} = \frac{2[\text{Suc.} \text{ CO}_2] - (\text{Pyr.} \text{ CO}_2)\text{ratio}}{1 + (\text{Pyr.} \text{ CO}_2)\text{ratio}}[2 - F][\text{Suc.} \text{ CO}_2] - 1$$

(4)

where $F$ is the fraction of oxaloacetate or malate derived from pyruvate that is converted into fumarate before forming citrate. A value of 0.8, estimated for $F$ in rat liver, has been used in these calculations [see Kelleher & Bryan (1985) for details]. It should be noted that flux of pyruvate to acetyl-CoA, citrate, and then to lipid via ATP citrate lyase, is not included in this modelling approach. Thus the fraction of pyruvate oxidized in the TCA cycle that enters via pyruvate dehydrogenase is correctly calculated as (1 – PC'); however, pyruvate flux to ketone bodies or lipid via pyruvate dehydrogenase is not included in this value.

**Cell incubation procedure**

AS-30D ascites-hepatoma cells (Smith et al., 1970) were maintained as described previously (Fiskum & Pease, 1986). Hepatoma cells were washed free of contaminating erythrocytes by multiple centrifugations at 4°C (180 g, 5 min each) in 10 mM-Hepes buffer (pH 7.4) containing 150 mM-NaCl and 5 mM-KCl. Freshly isolated cells were resuspended in Krebs–Henseleit bicarbonate buffer supplemented with metabolic substrates. The basic medium contained the following substrates: acetocetate (10 mM), β-hydroxybutyrate (10 mM), pyruvate (50 μM), succinate (20 μM) and acetate (50 μM). Two experimental media were used for cell incubations, the basic medium plus 5 mM-mannitol (control medium) and the basic medium plus 5 mM-glutamine (glutamine medium). All media were equilibrated with $\text{O}_2/\text{CO}_2$ (19:1) and adjusted to pH 7.2, 37°C. Cell suspension (3 ml) containing 0.3 g wet wt. of cells (approx. 4.2 × 10⁸ cells) were placed in 25 ml polycarbonate flasks filled with the above gas mixture and capped with a rubber stopper equipped with a suspended centre well (Kontes Glassware, Vineland, NJ, U.S.A.). Each flask contained one of the following $^{14}$C-labelled compounds: [1,4-$^{14}$C]succinate, [2,3-$^{14}$C]-succinate, [2-$^{14}$C]pyruvate or [3-$^{14}$C]pyruvate. The chemical composition of the medium was not altered by including the radiosotope. Cell incubations were carried out at 37°C in a Dubnoff shaking water bath (60 oscillations/min). After the appropriate incubation period, metabolism was terminated by injection of 0.3 ml of 20% (v/v) HClO₄ into the flask and 0.3 ml of 4 M-NaOH into the centre well. $^{14}$CO₂ collection was continued for 3 h. Flasks containing label but no cells were used as blanks. Radioactivity (c.p.m.) in $^{14}$CO₂ was converted into d.p.m. by using a channels-ratio quench correction.

Identical cell suspensions, without label, were used for $\text{O}_2$-consumption determinations with a Clark oxygen electrode. $\text{O}_2$ consumption was determined in both experimental media at the start of the incubation ($t = 0$) and at 30 min intervals for the subsequent 2 h.

**Metabolite determinations**

Radiolabelled metabolites derived from succinate and pyruvate tracers were isolated and identified by ion-exchange chromatography. Anion-exchange chromatography was performed essentially by the method of Bush et al. (1952). Neutralized cell extracts were applied to columns (1 cm × 4 cm) of Dowex 1X8-400 anion-exchange resin (formate form). Elution was carried out with a linear gradient of 0–2.0 M-formic acid, total volume 400 ml; 3 ml fractions were collected. Compounds were identified by comparison with the elution profile of standards and by enzymic conversion into specific products (Bergmeyer, 1974). Citrate was enzymically degraded to acetate and malate by the method of Kent (1972). Malate and acetate derived from [1-$^{14}$C]-citrate were separated by anion-exchange chromatography and counted for radioactivity. $^{14}$C-labelled amino acids were isolated from cell extracts exposed to [2-$^{14}$C]pyruvate and [3-$^{14}$C]pyruvate by using an AG50W cation-exchange resin. Aspartate in this mixture was converted into malate with glutamate–oxaloacetate transaminase (EC 2.6.1.1) and malate dehydrogenase (EC 1.1.1.37). [14C]Malate produced by this procedure was separated from the remaining amino acids by a second AG50W column. Malate was enzymically degraded to acetate and $\text{CO}_2$ (Kent et al., 1977). This procedure provided an estimate of the $^{14}$C in aspartate C-1+C-4 (recovered as $\text{CO}_2$) and in C-2+C-3 (recovered as acetate). For both citrate and aspartate degradation, standards were used to estimate the fraction of recovery at each step.

**RESULTS**

Initial studies with AS-30D cells selected incubation conditions yielding a linear rate of $^{14}$CO₂ production from both succinate and pyruvate tracers. The profile of $^{14}$CO₂ production from pyruvate tracers (Fig. 4) displays three interesting features of the time course of pyruvate oxidation by AS-30D cells. First, all conditions are characterized by a lag phase before linear $^{14}$CO₂ production is achieved. The lag phase corresponds to the time required for the system to reach metabolic and isotopic steady state. Second, for each medium, the slope of the tracer profile for [2-$^{14}$C]pyruvate is greater than the slope for [3-$^{14}$C]pyruvate, indicating that the pyruvate $\text{CO}_2$ ratios are greater than 1.0. Third, addition of glutamine to the incubation medium decreased $^{14}$CO₂ production from tracer pyruvate. A similar pattern was observed with succinate tracers (results not shown), except that linear $^{14}$CO₂ production did not occur until after 30 min of incubation. For this reason, subsequent analysis comparing $^{14}$CO₂ production from succinate and pyruvate utilized incubations of at least 30 min.

The effect of 5.0 mM-glutamine on the production of $^{14}$CO₂ by succinate and pyruvate tracers is shown in Table 1. Decreased $^{14}$CO₂ production in the glutamine medium was not the result of a decreased oxidative metabolism in the presence of glutamine. $\text{O}_2$ consumption, measured at 30 min intervals over the 2 h
incubation, was enhanced by approx. 30% in the presence of glutamine. For example, the 60 min O₂ consumption was 2.72 ± 0.15 ng-atoms of O/min per mg wet wt. for the glutamine medium, compared with 2.06 ± 0.22 for control medium (n = 6).

Steady-state ratios of ¹⁴C⁰₂ production from succinate and pyruvate tracers were compared by using a one-way analysis of variance (Table 2). In the presence of glutamine, the succinate CO₂ ratio was approximately twice the pyruvate ¹⁴C⁰₂ ratios. The lower value for the pyruvate ¹⁴C⁰₂ ratio reflects pyruvate carboxylation, as indicated by eqn. (4). The pyruvate-carboxylation fraction calculated for the glutamine medium at 60 min incubation was 0.39. This calculation utilized eqn. (4), with a value of 0.8 for F, and indicates that 39% of pyruvate entering the TCA cycle travels by the pyruvate carboxylation route. The calculation further indicates that the remaining 61% of pyruvate oxidized in the TCA cycle is incorporated into citrate via the pyruvate dehydrogenase route. Since there was no significant difference in the succinate and pyruvate CO₂ ratios in control medium, eqn. (4) was not applied to these data. Further evidence for pyruvate carboxylation in the glutamine medium was provided by an analysis of the ¹⁴C distribution pattern in aspartate derived from [2-¹⁴C]pyruvate (Table 3). Labelling of C-2 and C-3 of aspartate from this tracer indicates carboxylation of pyruvate.

The probability of carbon completing a circuit of the TCA cycle was estimated by applying eqn. (2) to the data in Table 2. The results (Table 4) indicate that addition of glutamine to the medium decreased the value of (A + T). In addition, the value of A + T was less than 1.0 in control media indicating flux of carbon into TCA-cycle pools in the absence of glutamine. The relative flux of tracer via paths A and T was assessed by degradation of [¹⁴C]citrate formed from [2,3-¹⁴C]succinate (Table 4). Less than 3% of the label in citrate was recovered in the acetate moiety in either the presence or the absence of glutamine, indicating that flux through the path

\[ \text{succinate} \rightarrow \text{pyruvate} \rightarrow \text{acetyl-CoA} \rightarrow \text{citrate} \]

is substantially less than flux through the path

\[ \text{succinate} \rightarrow \text{oxaloacetate} \rightarrow \text{citrate}. \]

In addition to ¹⁴C⁰₂, other radiolabelled metabolites were detected. After 1 h of incubation, approx. 10% of either [2-¹⁴C]pyruvate or [3-¹⁴C]pyruvate was detected in the HClO₄-precipitable chloroform-soluble lipid fraction. No radioactivity was detected in this fraction when the tracer was either [1,4-¹⁴C]succinate or [2,3-¹⁴C]succinate. Anion-exchange chromatography of the HClO₄-soluble fraction revealed that malate, fumarate, citrate, alanine and aspartate were the major labelled metabolites.
Table 3. Distribution of $^{14}$C in aspartate carbon

<table>
<thead>
<tr>
<th>Tracer</th>
<th>Radioactivity in C$^-2$ + C$^-3$ (%)</th>
<th>Radioactivity in C$^-1$ + C$^-4$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2-14C]Pyruvate</td>
<td>43 ± 2.0</td>
<td>57 ± 3.0</td>
</tr>
<tr>
<td>[3-14C]Pyruvate</td>
<td>76 ± 0.6</td>
<td>24 ± 0.5</td>
</tr>
</tbody>
</table>

Table 4. Probabilities of TCA-cycle flux via paths A and T

<table>
<thead>
<tr>
<th>Medium</th>
<th>Time (min)</th>
<th>Control</th>
<th>Glutamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>A + T</td>
<td>60</td>
<td>0.68</td>
<td>0.42</td>
</tr>
<tr>
<td>A + T</td>
<td>120</td>
<td>0.74</td>
<td>0.41</td>
</tr>
<tr>
<td>A/T</td>
<td>60</td>
<td>≤ 0.02</td>
<td>≤ 0.03</td>
</tr>
</tbody>
</table>

generated in incubation with [14C]succinate tracers (Fig. 5). Alanine, lactate and aspartate were the major labelled products detected from incubation with [14C]pyruvate tracers (results not shown).

DISCUSSION

The results of this study indicate that the CO$_2$-ratios method is applicable in metabolic investigations of tumour-cell energy metabolism. Conditions of linear 14CO$_2$ production were obtained from pairs of tracers required for this analysis. Although 20 µM-succinate was metabolized less rapidly than 50 µM-pyruvate, sufficient 14CO$_2$ for the CO$_2$-ratios method was produced from succinate tracers in 60 min of incubation (Table 1). The lower rate of succinate oxidation is likely to be due to the low permeability of most mammalian cells to this dicarboxylic acid.

The succinate CO$_2$ ratio increased in the presence of glutamine (Table 2), indicating that the probability of TCA-cycle carbon remaining in the cycle (A + T, eqn. 2) is decreased. Flux of glutamine to α-oxoglutarate will generally produce a decrease in this probability, owing to the requirement that this flux is matched by equal efflux from the cycle in metabolic steady state. This statement is true unless carbon leaving the TCA cycle is 'recaptured' as acetyl-CoA (path A, Figure 1). In the present study, the value of A was less than 3% of T, and was not affected by addition of glutamine to the medium (Table 4). Thus the observed decline in the value of A + T was due to a decrease in the value of T. These results indicate that addition of 5 mM-glutamine increased flux of carbon into the span of the TCA cycle between citrate and oxaloacetate.

CO$_2$ ratios alone are not adequate to demonstrate glutamine oxidation under physiological conditions. Exchange of glutamine or glutamate carbon with TCA-cycle carbon without net oxidation of glutamine could provide the observed change in CO$_2$ ratios. In the present study, the observation that aspartate and alanine derived from [2,3-14C]succinate are detected on the anion-exchange column (Fig. 5) indicates that fluxes to these amino acids, rather than glutamate or glutamine exchange, are major carbon exit paths from the TCA cycle. In addition, CO$_2$-ratio data do not rule out the possibility that addition of 5 mM-glutamine to the medium increases the flux of a compound other than glutamine into the citrate-to-oxaloacetate span of the TCA cycle. This possibility was considered unlikely, because the incubation medium contained only one metabolite capable of entering this span of the cycle, succinate, and succinate oxidation was not stimulated by glutamine (Table 1). Thus the CO$_2$-ratio data obtained in this study support the hypothesis that AS-30D cells possess a capacity for conversion of extracellular glutamine into TCA-cycle intermediates that is quantitatively significant relative to TCA-cycle flux. The oxidation pattern of [14C]succinate by AS-30D cells in the present study is similar to that observed for isolated intestinal jejunal epithelial cells, another preparation reported to use glutamine as a source of energy (Mallet et al., 1986). These workers found that the succinate CO$_2$ ratio increased from 4.5 in media without added glutamine to 14 in the presence of 18 mM-glutamine. As with AS-30D cells, the value of A was far lower than T, indicating little flux of TCA-cycle carbon to the acetyl-CoA-derived carbons of citrate. The
similarity in the effect of glutamine on these cells is interesting, since it has been shown that small-intestinal epithelium is one of a class of rapidly renewing non-neoplastic cells that share with rapidly growing neoplastic cells two enzyme activities, mitochondrial glutaminase and NAD(P)⁺-dependent malic enzyme (Nagel et al., 1980).

Complete oxidation of glutamine requires flux of C₄ TCA-cycle intermediates to acetyl-CoA and the acetyl-CoA-derived carbons of citrate. Malic enzyme has been proposed as the catalyst for flux from malate to pyruvate in this pathway (Sauer et al., 1980). In the present study evidence was obtained for partial oxidation of glutamine via this pathway. Since [¹⁴C]alanine was formed from [¹⁴C]sucinate (Fig. 5), flux of TCA-cycle carbon to pyruvate does occur. However, complete oxidation of glutamine was not observed, since isotopic analysis of ¹⁴C distribution in citrate derived from [2,3-¹⁴C]sucinate (Table 4) indicated that the A/T ratio is very low and not stimulated by addition of glutamine. If TCA-cycle carbon was the only source of acetyl-CoA for citrate synthesis, an A/T value of 1.0 would be expected (Kelleher, 1985). Therefore these data support the hypothesis that the major source of carbon for acetyl-CoA used in citrate synthesis was not derived from the TCA cycle and that glutamine carbon was not oxidized completely to CO₂. These conclusions apply only to AS-30D cells under the incubation conditions of this study. The stoichiometry of glutamine flux into the TCA cycle may explain the incomplete oxidation of glutamine in this preparation. In AS-30D mitochondria glutamine is deaminated to form glutamate, but glutamate is converted into α-oxoglutarate almost entirely by transamination (Moreadith & Lehninger, 1984). Thus, for each glutamine molecule entering the TCA cycle α-oxoglutarate, an amino acid, either aspartate or alanine, is formed, leaving no surplus of TCA-cycle carbon for acetyl-CoA formation. Furthermore, both incubation media contain β-hydroxybutyrate and acetoacetate at 10 mM. Acetoacetate is readily oxidized by AS-30D-cell mitochondria (G. Fiskum, unpublished work). The availability of acetyl-CoA from acetoacetate may inhibit pyruvate dehydrogenase, diverting pyruvate derived from TCA-cycle intermediates to alanine.

The results of this study suggest that both pyruvate carboxylation and conversion of TCA-cycle intermediates into pyruvate occurred in the glutamine medium used with this AS-30D-cell preparation. Evidence for pyruvate carboxylation includes the CO₂-ratio data (Table 2) and ¹⁴C distribution in aspartate derived from pyruvate tracers (Table 3). Flux of TCA-cycle carbon to pyruvate is indicated by the conversion of [¹⁴C]sucinate into [¹⁴C]-alanine (Fig. 5). Flux of malate to pyruvate has been previously demonstrated in AS-30D-cell mitochondria (Moreadith & Lehninger, 1984). Although pyruvate carboxylation has not been previously demonstrated in this cell line, Parlo & Coleman (1984) hypothesized that pyruvate carboxylation via malic enzyme occurs in 3924A-Morris-hepatoma mitochondria in the presence of 5.0 mM-pyruvate. These findings suggest that the direction of flux between pyruvate and C₄ TCA-cycle intermediates may depend on the medium used. Although methods used in the present study can detect pyruvate carboxylation, a more sophisticated analysis is required to determine the direction of net carbon flux in intact cells.

The second element of the truncated TCA-cycle hypothesis, conversion of citrate-derived acetyl units into cholesterol, was not directly tested in the present study. However, the data presented in Table 1 indicated a substantial conversion of [¹⁴C]pyruvate into CO₂. For example, approx. 42% of the total [²-¹⁴C]pyruvate tracer was converted into CO₂ at 60 min in the medium without glutamine. If [²-¹⁴C]pyruvate is oxidized primarily to acetyl-CoA and enters the TCA cycle as [¹⁴C]-citrate (Fig. 1), at least half of the citrate must be metabolized to α-oxoglutarate rather than to lipid, to produce the amount of CO₂ observed. Additional support for the flux of citrate to α-oxoglutarate is the finding that C-1 and C-4 of aspartate were labelled when the tracer was either [²-¹⁴C]pyruvate or [³-¹⁴C]pyruvate (Table 3). Flux from citrate to α-oxoglutarate is required to label these carbons, regardless of the entry route to the TCA cycle (pyruvate carboxylation or pyruvate dehydrogenase; Fig. 3). These observations suggest that the TCA cycle is not completely truncated for the AS-30D cell preparation used here. Comparing this finding with the extensive flux of citrate-derived acetyl units to cholesterol reported for Morris-hepatoma 3924A slices metabolizing 10 mM-pyruvate (Parlo & Coleman, 1986) suggests that the extent to which tumour metabolism is altered may vary with the preparation.

In summary, the CO₂-ratios method is applicable in the analysis of tumour-cell TCA-cycle metabolism. Four major findings resulted from this investigation of AS-30D cell metabolism. (1) Addition of 5 mM-glutamine to isolated cells increases flux of carbon into the TCA cycle between citrate and oxaloacetate. (2) Flux of C₄ TCA-cycle intermediates to the acetyl-CoA derived portion of citrate is low. (3) Flux between pyruvate and C₄ TCA-cycle intermediates occurs in both directions. (4) Synthesis of aspartate and alanine from TCA-cycle carbon, previously described only for isolated AS-30D-cell mitochondria, occurs in intact AS-30D cells.

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