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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 span of 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

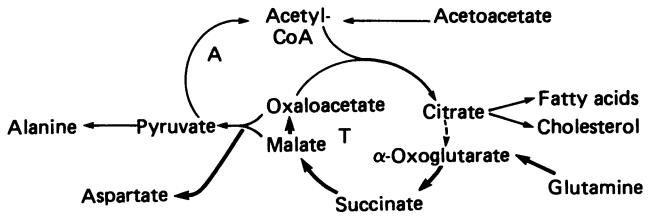

Fig. 1. TCA-cycle flux patterns hypothesized for tumour cells

Flux from citrate to α-oxoglutarate may be low, owing to cholesterol and fatty acid synthesis. Flux around the 'traditional' citrate-to-oxaloacetate-to-citrate portion of the cycle is designated 'T', and flux in the path citrate to oxaloacetate to acetyl-CoA to citrate is designated 'A'.

Abbreviation used: TCA cycle, tricarboxylic acid cycle.

catalyse the flux of malate to pyruvate required for oxidation of glutamine carbon to CO_2 (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 'CO_2 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 CO_2-ratios method allows investigators to utilize optimally information available from measurement of $^{14}CO_2$ production, the requirement for metabolic and isotopic steady state imposes some restrictions on the choice of media.

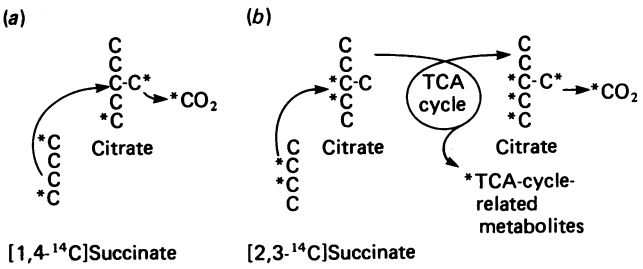

Fig. 2. Path for generation of $^{14}CO_2$ from (*a*) [1,4-^{14}C]succinate and (*b*) [2,3-^{14}C]succinate

Because [2,3-^{14}C]succinate carbon travels through more TCA-cycle carbon pools to reach CO_2, there is increased chance of exit to TCA-cycle-related compounds. Thus steady-state $^{14}CO_2$ production for [2,3-^{14}C]succinate is generally lower than for [1,4-^{14}C]succinate. Asterisks denote ^{14}C atoms.

METHODS

Theoretical considerations

In metabolic and isotopic steady state the rate of $^{14}CO_2$ production from labelled compounds oxidized in the TCA cycle provides quantitative information on the pattern of TCA-cycle oxidation. The CO_2-ratios method utilizes $^{14}CO_2$-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-^{14}C]oxaloacetate or [2,3-^{14}C]-oxaloacetate on the first circuit through the TCA cycle are especially useful. The original description of this method utilized [1-^{14}C]acetate and [2-^{14}C]acetate (Weinman *et al.*, 1957). In the present study, [1,4-^{14}C]succinate and [2,3-^{14}C]succinate are used as tracers. The succinate CO_2 ratio is defined as:

$$\frac{\text{rate of } ^{14}CO_2 \text{ production from [1,4-}^{14}\text{C]succinate}}{\text{rate of } ^{14}CO_2 \text{ production from [2,3-}^{14}\text{C]succinate}} \quad (1)$$

Under steady-state conditions, linear rates of $^{14}CO_2$ production result. In general, $^{14}CO_2$ production is greater from [1,4-^{14}C]succinate than from [2,3-^{14}C]succinate, because [2,3-^{14}C]succinate must traverse more TCA-cycle carbon pools to reach CO_2 and, at each pool, there is some probability that the labelled carbon will efflux from the cycle to form products other than CO_2 (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 CO_2 ratio. Flux of glutamine to TCA-cycle α-oxoglutarate is an example of this type of carbon entry.

The succinate CO_2 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 CO_2 in the next circuit of the cycle. For example, in the absence of efflux to products other than CO_2, ^{14}C 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):

$$A+T = \frac{2}{1+\text{succinate } CO_2 \text{ ratio}} \quad (2)$$

To summarize these relationships, the greater the flux of glutamine carbon into the TCA cycle accompanied by equal efflux to products other than CO_2, the larger the succinate CO_2 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 CO_2 ratio is defined as:

$$\frac{^{14}CO_2 \text{ produced from [2-}^{14}\text{C]pyruvate}}{^{14}CO_2 \text{ produced from [3-}^{14}\text{C]pyruvate}} \quad (3)$$

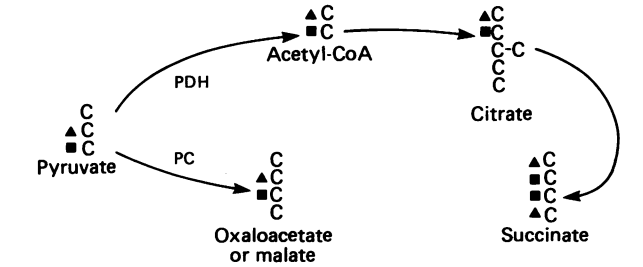

Fig. 3. Flux of pyruvate into the TCA cycle via pyruvate dehydrogenase (PDH) and pyruvate carboxylation (PC) pathways

Via PDH pathway [2-^{14}C]pyruvate (▲) generates [1,4-^{14}C]succinate, and [3-^{14}C]pyruvate (■) generates [2,3-^{14}C]succinate. In the PC pathway, tracer enters as C-2 and C-3 of oxaloacetate or malate, leading to pyruvate CO_2 ratio less than succinate CO_2 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 CO_2 ratio will equal the succinate 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.) CO_2 ratio will be lower than the succinate (Suc.) 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):

$$PC' = \frac{2[\text{Suc. } CO_2 \text{ ratio}) - (\text{Pyr. } CO_2 \text{ ratio})]}{[1 + (\text{Pyr. } CO_2 \text{ ratio})][2 - F][\text{Suc. } CO_2 \text{ ratio}) - 1]} \quad (4)$$

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: acetoacetate (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 O_2/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. 4.2×10^8 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 radioisotope. 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) $HClO_4$ into the flask and 0.3 ml of 4 M-NaOH into the centre well. $^{14}CO_2$ collection was continued for 3 h. Flasks containing label but no cells were used as blanks. Radioactivity (c.p.m.) in $^{14}CO_2$ was converted into d.p.m. by using a channels-ratio quench correction.

Identical cell suspensions, without label, were used for O_2-consumption determinations with a Clark oxygen electrode. 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 [^{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). [^{14}C]Malate produced by this procedure was separated from the remaining amino acids by a second AG50W column. Malate was enzymically degraded to acetate and CO_2 (Kent *et al.*, 1977). This procedure provided an estimate of the ^{14}C in aspartate C-1 + C-4 (recovered as 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 $^{14}CO_2$ production from both succinate and pyruvate tracers. The profile of $^{14}CO_2$ 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 $^{14}CO_2$ 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 CO_2 ratios are greater than 1.0. Third, addition of glutamine to the incubation medium decreased $^{14}CO_2$ production from tracer pyruvate. A similar pattern was observed with succinate traces (results not shown), except that linear $^{14}CO_2$ production did not occur until after 30 min of incubation. For this reason, subsequent analysis comparing $^{14}CO_2$ production from succinate and pyruvate utilized incubations of at least 30 min.

The effect of 5.0 mM-glutamine on the production of $^{14}CO_2$ by succinate and pyruvate tracers is shown in Table 1. Decreased $^{14}CO_2$ production in the glutamine medium was not the result of a decreased oxidative metabolism in the presence of glutamine. O_2 consumption, measured at 30 min intervals over the 2 h

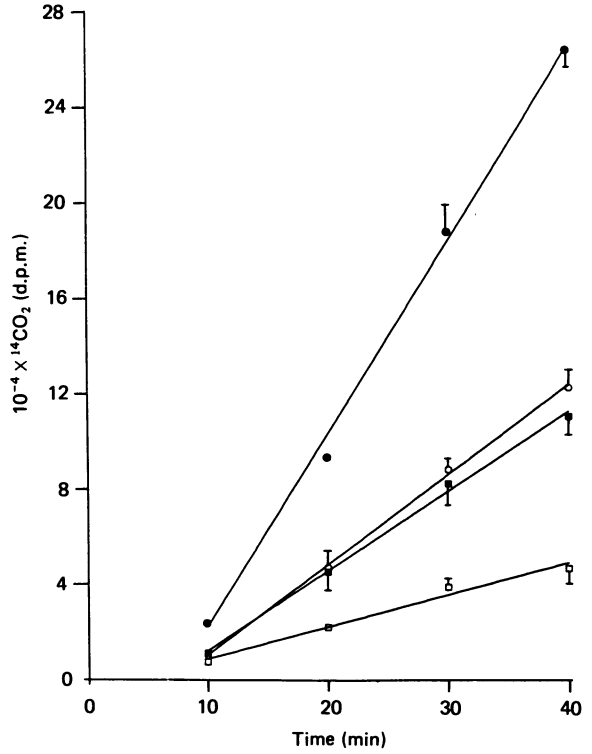

Fig. 4. Production of $^{14}CO_2$ from [^{14}C]pyruvate tracers by AS-30D cells

Mean values are plotted ($n = 3$): S.E.M. values are shown where larger than the symbol. Linear least-squares regression lines are drawn between points. Key: ●, [2-^{14}C]pyruvate, zero glutamine; ○, [3-^{14}C]pyruvate, zero glutamine; ■, [2-^{14}C]pyruvate, 5.0 mM-glutamine; □, [3-^{14}C]pyruvate, 5.0 mM-glutamine.

Table 1. $^{14}CO_2$ production by AS-30D hepatoma cells

Results are means ± S.E.M.; $*P < 0.05$ versus control medium ($n \geqslant 4$).

Tracer	Time (min)	$10^{-4} \times {}^{14}CO_2$ production (d.p.m.) Control medium	Glutamine medium
[1,4-^{14}C]Succinate	60	4.08 ± 0.38	3.48 ± 0.09*
[2,3-^{14}C]Succinate	60	1.94 ± 0.23	1.05 ± 0.06*
[2-^{14}C]Pyruvate	60	45.8 ± 2.6	29.0 ± 2.0*
[3-^{14}C]Pyruvate	60	33.7 ± 2.6	15.5 ± 1.3*
[2-^{14}C]Pyruvate	30	23.8 ± 2.9	11.7 ± 1.8*
[3-^{14}C]Pyruvate	30	12.2 ± 1.6	5.7 ± 0.83*

incubation, was enhanced by approx. 30% in the presence of glutamine. For example, the 60 min O_2 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 $^{14}CO_2$ 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 CO_2 ratio was approximately

Table 2. CO_2 ratios for AS-30D-cell experiments

CO_2 ratios were calculated individually for at least four experiments by using eqns. (1) and (3); results are means ± S.E.M. Means with the same letter are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$).

Medium	Tracer	Time (min)	$^{14}CO_2$ ratio	
Glutamine	Pyruvate	30	1.85 ± 0.13	B
Glutamine	Pyruvate	60	1.89 ± 0.14	B
Glutamine	Succinate	60	3.73 ± 0.17	A
Glutamine	Succinate	120	3.88 ± 0.51	A
Control	Pyruvate	30	1.79 ± 0.09	B
Control	Pyruvate	60	1.50 ± 0.05	B
Control	Succinate	60	1.95 ± 0.13	B
Control	Succinate	120	1.69 ± 0.13	B

twice the pyruvate $^{14}CO_2$ ratios. The lower value for the pyruvate $^{14}CO_2$ 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 via 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 CO_2 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 ^{14}C distribution pattern in aspartate derived from [2-^{14}C]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 [^{14}C]citrate formed from [2,3-^{14}C]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

succinate → pyruvate → acetyl-CoA → citrate

is substantially less than flux through the path

succinate → oxaloacetate → citrate.

In addition to $^{14}CO_2$, other radiolabelled metabolites were detected. After 1 h of incubation, approx. 10% of either [2-^{14}C]pyruvate or [3-^{14}C]pyruvate was detected in the $HClO_4$-precipitable chloroform-soluble lipid fraction. No radioactivity was detected in this fraction when the tracer was either [1,4-^{14}C]succinate or [2,3-^{14}C]succinate. Anion-exchange chromatography of the $HClO_4$-soluble fraction revealed that malate, fumarate, citrate, alanine and aspartate were the major labelled metabolites

Table 3. Distribution of ^{14}C in aspartate carbon

Cells were incubated in glutamine medium for 60 min. Aspartate was degraded as described in the Methods section. Results are means ± S.E.M. ($n = 6$).

Tracer	Radioactivity in C-2+C-3 (%)	Radioactivity in C-1+C-4 (%)
[2-^{14}C]Pyruvate	43±2.0	57±3.0
[3-^{14}C]Pyruvate	76±0.6	24±0.5

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

A+T values were calculated from means in Table 2 by using eqn. (2). A/T was calculated from citrate degradation as described in the Methods section ($n = 3$).

	Time (min)	Medium: Control	Medium: Glutamine
A+T	60	0.68	0.42
A+T	120	0.74	0.41
A/T	60	⩽0.02	⩽0.03

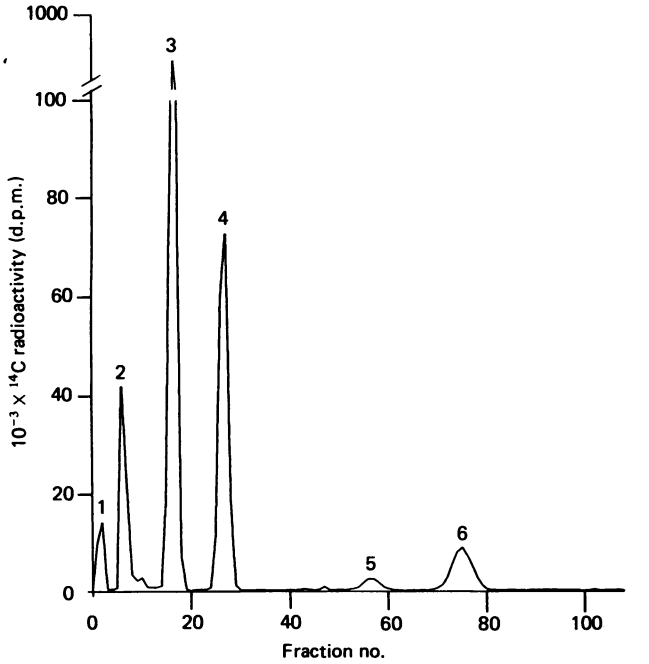

Fig. 5. ^{14}C profile from Dowex IX8-400 anion-exchange column of $HClO_4$-soluble radioactivity resulting from a 1 h incubation of AS-30D cells with [2,3-^{14}C]succinate in glutamine medium

The 'alanine' peak is approx. 50% alanine; all other peaks contain no detectable impurities. [^{14}C]Succinate accounts for 83% of $HClO_4$-soluble radioactivity. Peaks: 1, alanine; 2, aspartate; 3, succinate; 4, malate, 5, citrate; 6, fumarate.

generated in incubation with [^{14}C]succinate tracers (Fig. 5). Alanine, lactate and aspartate were the major labelled products detected from incubation with [^{14}C]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 $^{14}CO_2$ production were obtained from pairs of tracers required for this analysis. Although 20 μM-succinate was metabolized less rapidly than 50 μM-pyruvate, sufficient $^{14}CO_2$ 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-^{14}C]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 [^{14}C]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 C_4 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 [^{14}C]alanine was formed from [^{14}C]succinate (Fig. 5), flux of TCA-cycle carbon to pyruvate does occur. However, complete oxidation of glutamine was not observed, since isotopic analysis of ^{14}C distribution in citrate derived from [2,3-^{14}C]succinate (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 CO_2. 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 as 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 CO_2-ratio data (Table 2) and ^{14}C distribution in aspartate derived from pyruvate tracers (Table 3). Flux of TCA-cycle carbon to pyruvate is indicated by the conversion of [^{14}C]succinate into [^{14}C]-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 C_4 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 [^{14}C]pyruvate into $^{14}CO_2$. For example, approx. 42% of the total [2-^{14}C]pyruvate tracer was converted into $^{14}CO_2$ at 60 min in the medium without glutamine. If [2-^{14}C]pyruvate is oxidized primarily to acetyl-CoA and enters the TCA cycle as [^{14}C]-citrate (Fig. 1), at least half of the citrate must be metabolized to α-oxoglutarate rather than to lipid, to produce the amount of $^{14}CO_2$ 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-^{14}C]pyruvate or [3-^{14}C]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 CO_2-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 C_4 TCA-cycle intermediates to the acetyl-CoA derived portion of citrate is low. (3) Flux between pyruvate and C_4 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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