Journal of Cellular Biochemistry 68:213–225 (1998)

Oxidation of Glutamine in HeLa Cells: Role and Control of Truncated TCA Cycles in Tumour Mitochondria

Terrence J. Piva* and Edward McEvoy-Bowe

Department of Chemistry and Biochemistry, James Cook University, Townsville, Queensland, Australia

Abstract The oxidative metabolism of glutamine in HeLa cells was investigated using intact cells and isolated mitochondria. The concentrations of the cytoplasmic amino acids were found to be aspartate, 8.0 mM; glutamate, 22.2 mM; glutamine, 11.3 mM; glycine, 9.8 mM; taurine, 2.3 mM; and alanine, <1 mM. Incubation of the cells with [^{14}C]glutamine gave steady-state recoveries of ^{14}C-label (estimated as exogenous glutamine) in the glutamine, glutamate, and aspartate pools, of 103%, 80%, and 25%, respectively, indicating that glutamine synthetase activity was absent and that a significant proportion of glutamate oxidation proceeded through aspartate aminotransferase. No label was detected in the alanine pool, suggesting that alanine aminotransferase activity was low in these cells. The clearance rate of [^{14}C]glutamine through the cellular compartment was 65 nmol/min per mg protein. There was a 28 s delay after [^{14}C]glutamine was added to the cell before ^{14}C-label was incorporated into the cytoplasm, while the formation of glutamate commenced 10 s later.

Aspartate was the major metabolite formed when the mitochondria were incubated in a medium containing either glutamine, glutamate, or glutamate plus malate. The transaminase inhibitor AOA inhibited both aspartate efflux from the mitochondria and respiration. The addition of 2-oxoglutarate failed to relieve glutamate plus malate respiration, indicating that 2-oxoglutarate is part of a well-coupled truncated cycle, of which aspartate aminotransferase has been shown to be a component [Parlo and Coleman (1984): J Biol Chem 259:9997–10003]. This was confirmed by the observation that, although it inhibited respiration, AOA did not affect the efflux of citrate from the mitochondria. Thus citrate does not appear to be a cycle component and is directly transported to the medium. Therefore, it was concluded that the truncated TCA cycle in HeLa cells is the result of both a low rate of citrate synthesis and an active citrate transporter. DNP (10 μM) induced a state III-like respiration only in the presence of succinate, which supports the evidence that NAD-linked dehydrogenases were not coupled to respiration, and suggests that these mitochondria may have a defect in complex I of the electron transport chain. Arising from the present results with HeLa cells and results extant in the literature, it has been proposed that a major regulating mechanism for the flux of glutamate carbon in tumour cells is the competitive inhibition exerted by 2-oxoglutarate on aspartate and alanine aminotransferases. This has been discussed and applied to the data. J. Cell. Biochem. 68:213–225, 1998.

Key words: glutamine; glutamate; mitochondria; metabolism; HeLa cells

Glutamine has not only been shown to be a precursor for the synthesis of purines, pyrimidines, and proteins but also to function as a major substrate for the energy metabolism of rapidly growing tumour cells [Reitzer et al., 1979; Kovacevic and McGivan, 1983; Moreadith and Lehninger, 1984; Medina et al., 1992]. It has been proposed that the high rate of glutamine oxidation in tumour cells is related more to controlling the synthesis of biosynthetic precursors rather than that for energy metabolism [Newsholme et al., 1985; Newsholme and Board, 1991].

One of the major indicators of the transformation of control in neoplastic cells is an increase in the rate of glutamine consumption [Rivera et al., 1988]. This increase is probably due in part to the low level of glutamine synthetase coupled with a high level of glutaminase activity in tumour cells [Medina et al., 1992; Street et al.,

Abbreviations: BHM, bovine heart mitochondria; HCM, HeLa cell mitochondria; AOA, aminooxyacetate; BSA, bovine serum albumin; FBS, fetal bovine serum; PBS, phosphate-buffered saline; RCR, respiratory control ratio; DNP, 2,4-dinitrophenol; MIM, mitochondrial incubation medium; HPLC, high-performance liquid chromatography.

Dr. McEvoy's current address is 98 Wright St, Wulguru, Townsville, Queensland 4810, Australia (retired). E-mail: emcevoybowe@ultra.net.au

*Correspondence to: T.J. Piva, Cancer Unit, Queensland Institute of Medical Research, Post Office Royal Brisbane Hospital, Queensland 4029, Australia. E-mail: terryP@qimr.edu.au

Received 12 June 1997; Accepted 16 September 1997

1993]. The presence of glutaminase has been shown to be "progression-linked" in hepatomas [Knox et al., 1969]. Subsequent steps in the oxidation of glutamate vary with the type of tumour cell and the availability of exogenous metabolites [Reitzer et al., 1979; Moreadith and Lehninger, 1984; Matsuno, 1987; Medina et al., 1992; Piva and McEvoy-Bowe, 1992].

Reitzer et al. [1979] demonstrated that glutamine oxidation, rather than glycolysis, is the major source of energy in HeLa cells. These investigators were able to account for 75% of the total ^{14}C-label from cells exposed to [^{14}C]glutamine, the major product being CO_2 (35% label). Reitzer et al. did not investigate the fraction of label in glutamate and aspartate, which in other studies have been shown to account for a significant proportion of ^{14}C-label derived from [^{14}C]glutamine [Zielke et al., 1984; Lanks, 1987]. Cellular glutamate and aspartate levels are important in determining whether glutamate derived from glutamine is converted to 2-oxoglutarate by transamination or dehydrogenation. The importance of the relative levels of glutamate and aspartate in tumour cells is related to the proposal that tumour cells oxidise glutamate through a truncated tricarboxylic acid (TCA) cycle. This involves the insertion and replacement by aspartate aminotransferase of the span of reactions oxaloacetate to 2-oxoglutarate, thus displacing isocitrate synthesis and isocitrate dehydrogenase and the by-pass of glutamate dehydrogenase [Parlo and Coleman, 1984; Kovacevic et al., 1991]. Under these conditions, aspartate becomes a major product of glutamine (glutamate) oxidation. In a further development, it was shown that when certain tumour mitochondria are exposed to both glutamate and malate, there results a switch from aspartate to alanine efflux [Moreadith and Lehninger, 1984]. We propose that this arises from a modification of the truncated TCA cycle, in that alanine aminotransferase replaces aspartate aminotransferase as the truncating element and malic enzyme replaces malate dehydrogenase. This proposition is based on a study using AS-30D hepatoma cells, where [^{14}C]succinate was converted to [^{14}C]alanine [Kelleher et al., 1987].

In this study we examine the uptake and oxidative metabolism of glutamine in HeLa cells, including their mitochondria. This work may be considered an extension of an earlier study using intact HeLa cells [Reitzer et al., 1979]. The results obtained from this study and others were used to test the proposal that a major regulating mechanism of the metabolism of glutamate in tumour cells is the inhibitory effect of 2-oxoglutarate on aspartate and alanine aminotransferases.

MATERIALS AND METHODS

Materials

All chemicals and biochemicals were obtained from Sigma Chemicals (St. Louis, MO), except for the following: DMSO, lithium citrate tetrahydrate, lithium hydroxide monohydrate, hydrindantin, ninhydrin, bovine serum albumin (BSA), lactate dehydrogenase, and malate dehydrogenase were obtained from Merck (Melbourne, Australia); ADP and amino acids were obtained from Calbiochem-Behring (Sydney, Australia); citrate lyase, glutamate-oxaloacetate transaminase, NAD, and NADH from Boehringer Mannheim (Melbourne, Australia); fetal bovine serum (FBS), culture medium and culture vessels from Gibco (Grand Island, NY); L-[U-^{14}C]glutamine and liquid scintillant from Amersham Radiochemical Centre (Sydney, Australia); and Cytodex microcarrier beads from Pharmacia (Sydney, Australia).

[^{14}C]Glutamine Uptake

HeLa cells were grown in medium 199 containing 10% (v/v) FBS under sterile conditions. Cells grown in 250-ml culture flasks were used in radiolabel uptake studies. The day of the experiment, 20 ml fresh medium 199 containing 2 mM glutamine supplemented with 10% (v/v) FBS was added to each flask. After 2 h, this medium was removed, and the cells were exposed for specified times to 10 ml medium 199 containing glutamine (3 mM), and 5 µCi [^{14}C]glutamine. The experiment was terminated by removing the labelled medium and the cells washed thrice with ice-cold saline followed by three washes with ice-cold acetone. The acetone wash removed water from the cells and trapped the intracellular metabolites. Less than 5% of the labeled intracellular metabolites were lost during these washing steps (Data not shown). The cytoplasmic amino acid and protein fractions were extracted and isolated as previously described [McEvoy-Bowe, 1985]. ^{14}C-Label recovered in these fractions was measured using a liquid scintillation counter.

HeLa Cell Microcarrier Cultures

HeLa cells harvested by trypsin-EDTA treatment from 16–20 confluent 250-ml culture flasks were added to 6 g hydrated cytodex microcarrier beads suspended in 500 ml medium 199 (containing 10% (v/v) FBS and 20 mM Hepes) prior to being transferred to a 1.5-L microcarrier vessel (Techne, Cambridge, UK). Upon reaching confluency, the cells were harvested from the Cytodex beads as described [Piva and McEvoy-Bowe, 1992].

Isolation of Mitochondria

The harvested HeLa cells were washed with phosphate-buffered saline (PBS) and centrifuged (180*g* for 5 min) before being suspended in H-medium (210 mM mannitol, 70 mM sucrose, 0.5% (w/v) BSA and 5 mM K-Hepes at pH 7.2) containing 1 mM EGTA and the mitochondria isolated using the method of Moreadith and Fiskum [1984], except that the digitonin treatment step was omitted. The HeLa cell mitochondria (HCM) were finally resuspended at 1–2 mg mitochondrial protein/ml H-medium and stored on ice prior to use.

Hearts from cattle over 3 years of age were obtained from Swift Meatworks (Townsville, Australia), and the mitochondria were isolated using the method of Smith [1967]. The bovine heart mitochondria (BHM) were finally resuspended at 4 mg protein/ml in sucrose solution (250 mM sucrose, 10 mM Tris, and 0.2 mM EDTA at pH 7.8) prior to use.

Mitochondrial Morphology

Isolated HCM examined under transmission electron microscopy (TEM) appeared predominantly rounded and lacked the cristal pattern of normal mitochondria (data not shown). These mitochondria were similar to those observed in isolated Morris hepatoma mitochondria [Pedersen, 1978].

Respiration Studies

Oxygen consumption was measured polarographically at 30°C by means of a Clark oxygen electrode (Rank Brothers). Reactions were started by the addition of 100 µl mitochondrial suspension to 900 µl incubation medium (MIM) (130 mM KCl, 10 mM Mops, 2 mM $MgCl_2$ and 2 mM KH_2PO_4 at pH 7.2) in a similar method to that described [Moreadith and Lehninger, 1984]. The concentrations of added respiratory substrates are described under Results. State III and IV respiration rates and RCR (respiratory control ratio) values were calculated after the endogenous mitochondrial respiration rate had been subtracted from all subsequent rates. The solubility of dissolved oxygen in the air-saturated, temperature-equilibrated respiration medium was calculated to be 435 ng-atom O/ml at 30°C and 760 mm Hg.

Mitochondrial Incubations

Only HCM possessing RCR values greater than 3 were used in the metabolism studies as they were considered to be "intact" [Kaplan et al., 1986]. HCM were suspended at 1–2 mg protein/ml MIM (30°C and pH 7.2) and the metabolites added are listed in the results section. At the indicated times, aliquots were removed from the rapidly stirred suspension and treated as described [Moreadith and Lehninger, 1984]. The neutralised samples were stored at −20°C for up to 72 h before being assayed.

Metabolite and Protein Analysis

Amino acids in HeLa cell and mitochondrial extracts were separated using lithium buffers on a cation exchange column coupled to post-column ninhydrin derivitization on a Waters high-performance liquid chromatography (HPLC) system. Amino acid calibration tables (2 µmol/ml) were established prior to the commencement of each analytical run. α-Aminobutyric acid (20 µmol/ml) was used as an internal standard and was added to all samples prior to analysis.

Pyruvate [Czok and Lamprecht, 1974], citrate [Dagby, 1974] and malate [Gutmann and Wahlefield, 1974] recovered in the neutralised mitochondrial extracts were determined enzymatically within 24 h of completion of the experiment. Protein was measured by the method of Lowry et al. [1951] using BSA as the standard.

Calculations

The concentration of cellular amino acids was calculated using formula of Piez and Eagle [1958], assuming that water represented 80% of the HeLa cell wet weight.

RESULTS

Intracellular Amino Acid Concentrations in HeLa Cells

Table I shows the concentration of amino acids present in the cells, as compared with

TABLE I. Concentration of Free Cytoplasmic Amino Acids in HeLa Cells*

Amino acid	Metabolite concn (mM)		
	Present study	[McEvoy-Bowe, 1985]	[Kabus and Koch, 1982]
Aspartate	8.0 ± 0.4	15.0	16.0
Glutamate	22.2 ± 0.6	24.0	24.0
Glutamine	11.3 ± 0.5	12.0	25.0
Glycine	9.8 ± 0.3	5.5	5.7
Taurine	2.3 ± 0.1	2.5	—[a]
Alanine	—	—	4.7

*HeLa cells were grown in medium 199 containing 10% (v/v) FBS and upon reaching confluency, the amino acid content was measured as described under Materials and Methods. Results are expressed as mean values ± SEM, using 12 separate cultures.
[a]—, not detected.

that found in earlier studies [Kabus and Koch, 1982; McEvoy-Bowe, 1985]. There was good agreement for the concentration of glutamate for all three procedures, with the results ranging between 22 and 24 mM. The rounded off value of 20 mM was therefore chosen as the medium concentration for the measurement of glutamate respiration in HCM. It is noteworthy that the level of alanine detected in these cells was very low (<1 mM).

[^{14}C]Glutamine Uptake in HeLa Cells

When HeLa cells were exposed to 3 mM [^{14}C]glutamine for up to 40 min, 90% of the ^{14}C label was recovered in the cellular nonprotein fraction as glutamine, glutamate, and aspartate (Fig. 1), and the final relative specific radioactivities (relative to medium glutamine) of these amino acids were 1.03, 0.8, and 0.25, respectively (Fig. 2). The glutamine relative specific radioactivity showed that the glutamine cellular pool was composed entirely of exogenous glutamine, an indication that glutamine synthetase activity was either low or absent in these cells. The same result was obtained by incubating HeLa cells with $^{15}NH_4Cl$ [Street et al., 1993]. We were unable to detect alanine in these cells and therefore as such no label was recovered in this fraction. The label recovered in lactate was less than that observed in the protein fraction.

The clearance rate of [^{14}C]glutamine through the cellular compartment was 65 nmol/min per mg protein, the highest consumption rate of any of the other amino acids. Approximate estimates of the clearance through the glutamine,

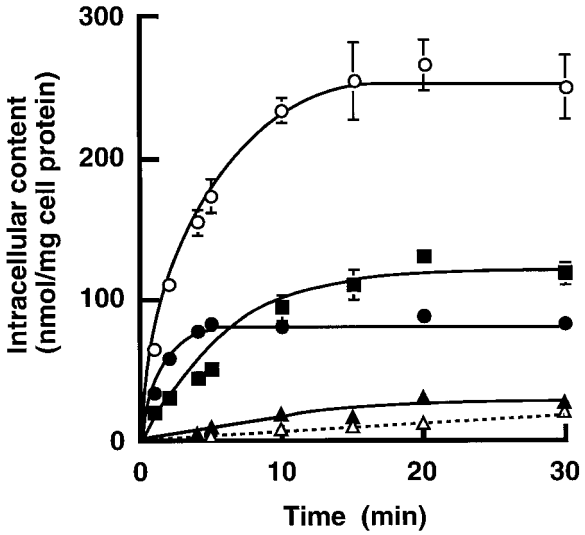

Fig. 1. Recovery of medium [^{14}C]glutamine label from cellular metabolites. Label was recovered as glutamate (■), glutamine (●), aspartate (▲), and protein (△). Glutamine uptake was represented by (○). Results expressed are the mean values ± SEM of three separate determinations.

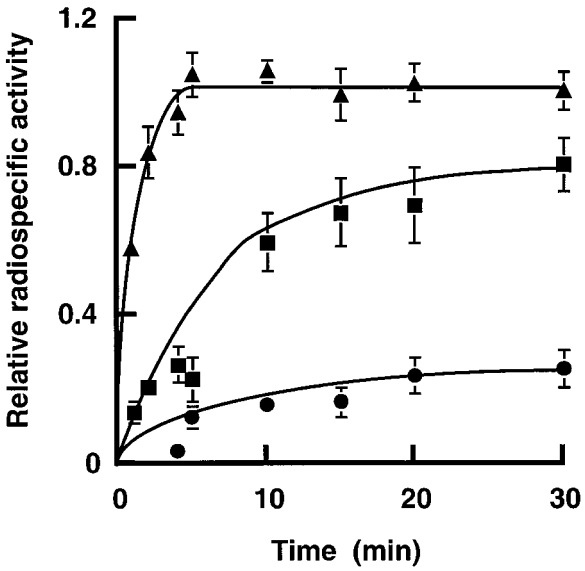

Fig. 2. Relative radiospecific activity of glutamine, glutamate and aspartate in HeLa cell extracts. Label was recovered as glutamine (▲), glutamate (■), and aspartate (●). Results are expressed as the mean values ± SEM of three separate determinations.

glutamate, and aspartate pools gave 47, 18, and 2.3 nmol/min per mg protein, respectively. These figures reflect the decline in relative specific activity, and the fact that the glutamine plus glutamate pools represent 63% of the total free amino acids (Table I). The decline in relative specific activity from glutamine to aspartate is

to be expected and was very similar to that obtained when AS-30D hepatoma cells were incubated with [2,3-^{14}C]succinate [Kelleher et al., 1987]. In the latter experiments the $HClO_4$-soluble cell fraction on addition to an anion-exchange column gave rise to a succinate peak with 83% of the total radioactivity followed by malate, aspartate and alanine in descending order.

The incorporation of ^{14}C-label into the cytoplasm over the first 4 min revealed that incorporation commenced at 28 s, and that the formation of glutamate commenced at 38 s (Fig. 3). The first result was obtained by extrapolation of the regression line for negative exponential growth ($r = 1.00$) to zero, and the second result was obtained by extrapolation of the negative exponential growth curve for glutamate ($r = 1.00$) to its intersection with the glutamine growth regression line. Since the amount of label remained at zero for the first 28 s, it was assumed that the level of nonincorporated label in the cellular extracts was insignificant.

In subsequent studies with mitochondria glutamate was used to investigate the metabolic pathway of glutamine. There were several reasons for this: (1) glutamate is predominantly derived from glutamine (80%); (2) the intracellular glutamate concentration is maintained at a high level (22 mM) and unlike glutamine this concentration is not related to the nutritional state of the cell (data not shown); and (3) glutamate can enter the mitochondria by a number of different transport pathways [Kovacevic and McGivan, 1983].

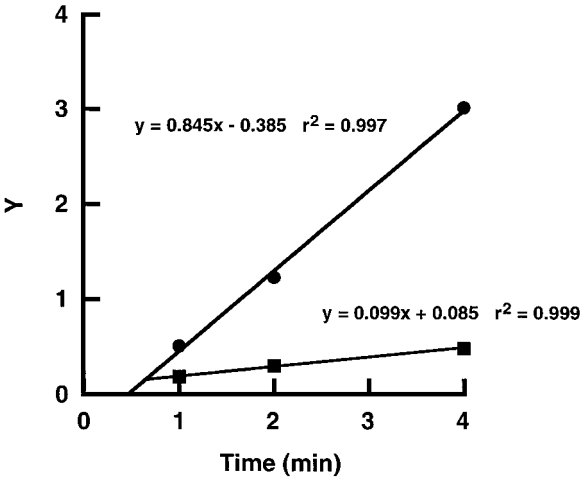

Fig. 3. Incorporation of label from [^{14}C]glutamine into intracellular metabolites. Label from [^{14}C]glutamine was recovered in the cytoplasm and the fraction present in the glutamate pool was measured as described in the Materials and Methods section. The points on the figure were calculated using the equation $y = -\ln(1 - a_n/a_0)$, where an represents the label concentration at n min and a_0 represents the label concentration when $d_a/d_t = 0$. The value for a_0 was calculated to be 82 and 120 nmol/mg protein for glutamine and glutamate, respectively. Label was recovered as glutamine (●) and glutamate (■). Results expressed are the mean values of three separate determinations.

Mitochondrial Metabolism

Malate and pyruvate have been shown to influence the flux rate and course of glutamate metabolism in tumour mitochondria [Moreadith and Lehninger, 1984; Kovacevic et al., 1991]. Table II summarizes the results obtained for HeLa cell mitochondria. In this regard the major amino acid product of glutamine oxidation in HCM was glutamate, and the major product of glutamate oxidation was aspartate with an efflux rate (16.7 nmol/min per mg protein), which was 20-fold that of alanine.

The addition of malate alone to the HCM medium resulted in the efflux of pyruvate and a lesser amount of citrate; while the addition of pyruvate alone gave rise only to citrate. When malate was added with glutamate to HCM medium there was a significant increase in aspartate output (27.3 nmol/min per mg protein), while the efflux of pyruvate and citrate remained the same as that for malate alone. The addition of pyruvate to glutamate had no effect on the rate of aspartate efflux or citrate output when compared to that of pyruvate alone, but there was an increase in the efflux of alanine. The addition of AOA to the glutamate plus malate medium resulted in complete inhibition of aspartate efflux but left the pyruvate and citrate output rates the same as they were for malate alone.

Respiration Studies

BHM were used as a control in the study of HCM respiration. State III respiration was observed in BHM when malate plus glutamate, malate plus pyruvate and succinate were the respiratory substrates (Table III).

Succinate is commonly used to estimate the RCR of tumour mitochondrial preparations [Pedersen, 1978; Kaplan et al., 1986], and in the case of HCM, it was the only substrate which induced state III respiration (Figs. 4, 5). The lack of state III respiration induced by NAD-linked substrates in HCM has not been observed in other tumour mitochondria [Moreadith and Lehninger, 1984; Gauthier et al., 1990;

TABLE II. Initial Velocities of Product Efflux in HCM*

Substrate	Products (nmol/min per mg mitochondrial protein)				
	Glu	Asp	Ala	Pyr	Cit
Malate (3)	—[a]	—	—	10.0 ± 0.4	3.8 ± 0.3
Pyruvate (3)	—	—	—		5.6 ± 0.8
Glutamine (3)	40.0 ± 2.6	15.0 ± 1.4	—	—	—
Glutamate (4)	—	16.7 ± 1.9	0.6 ± 0.2	—	—
Glu + Mal (4)	—	27.3 ± 2.5	1.5 ± 0.5	9.1 ± 0.6	4.2 ± 0.4
Glu + Mal + AOA (3)	—	—	—	10.2 ± 1.4	3.4 ± 0.5
Glu + Pyr (2)	—	16.4 ± 1.0	3.3 ± 0.4	—	5.5 ± 0.8

*HCM were incubated in the presence of glutamate (20 mM) and/or TCA intermediates (5 mM) as described in the Materials and Methods section. Results are expressed as the mean values ± SEM. Numbers of mitochondrial preparations are shown in parentheses.
[a]—, not detected.

TABLE III. Respiration Rates in BHM and HCM*

Substrates	RCR values	Oxygen uptake (ng-atom O/min per mg protein)	
		State III	State IV
BHM			
Succinate (4)	4.6 ± 0.4	93.9 ± 7.4	20.6 ± 2.0
Glutamate + malate (4)	7.4 ± 0.6	122.6 ± 11.4	16.7 ± 2.4
Malate + pyruvate (4)	5.1 ± 0.3	108.4 ± 9.2	21.4 ± 1.4
HCM			
Succinate (10)	4.2 ± 0.3	50.1 ± 5.4	11.9 ± 0.9
Glutamate + malate (6)	—[a]	—	14.5 ± 1.4
Malate + pyruvate (6)	—	—	7.0 ± 0.3

*Mitochondria (1–2 mg protein/ml) were incubated in the basic media supplemented with either glutamate (20 mM) and/or TCA cycle intermediates (5 mM) as described under Materials and Methods. Data are expressed as mean ± SEM. Numbers of mitochondrial preparations are shown in parentheses.
[a]—, not detected.

Matsuno, 1991; Dietzen and Davis, 1993]. The mean values for RCRs and state III and IV respiration are shown in Table III. The mean RCR obtained from succinate respiration in HCM (4.2) agreed with that obtained in other tumour mitochondrial preparations, and the individual values were all greater than 3 which has been proposed as a "benchmark" value for functionally intact mitochondria [Kaplan et al., 1986]. There was a delay in the onset of succinate state III respiration in HCM after the addition of ADP, a phenomenon that has been observed in other respiring tumour mitochondria [Matsuno, 1991]. The addition of ADP on its own to the HCM did not affect the mitochondrial respiration rate (data not shown).

Apart from succinate, combinations of malate plus glutamate and malate plus pyruvate were the only substrates to induce state IV respiration in HCM (Table III and Fig. 5). These results were much lower than those obtained for other tumour mitochondria [Moreadith and Lehninger, 1984; Gauthier et al., 1990; Matsuno, 1991; Dietzen and Davis, 1993].

The aminotransferase inhibitor AOA inhibited malate plus glutamate respiration in HCM (Fig. 5a). The subsequent addition of 2-oxoglutarate alone or the presence of a counter ion such as malate failed to relieve the inhibition, whereas the inhibition was relieved when 2-oxoglutarate was added to Ehrlich ascites mitochondria [Moreadith and Lehninger, 1984] and HT29 mitochondria [Gauthier et al., 1990].

DNP (10 μM) induced a state III-like respiration only in the presence of succinate (Fig. 5c), which supports the evidence that NAD-linked dehydrogenases were not coupled to respiration, and suggests that these mitochondria may have a defect in complex I of the electron transport chain.

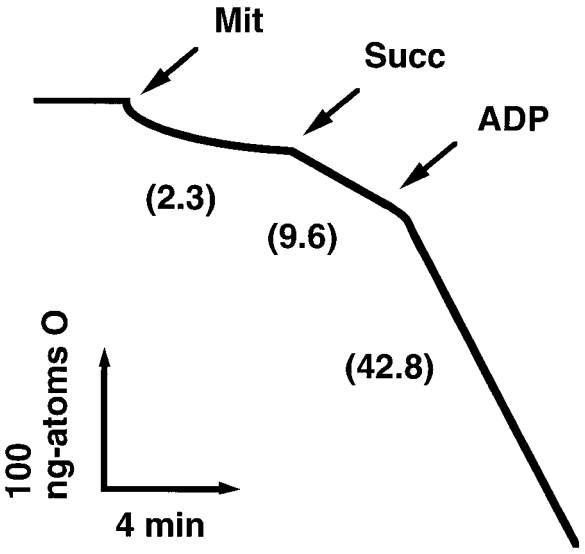

Fig. 4. Effect of 5 mM succinate on the mitochondrial respiration rate. HCM were resuspended at 1 mg/ml MIM, as described under Materials and Methods. The final concentration of added succinate and ADP was 5 mM and 0.25 mM, respectively. Numbers along the traces represent the rate of oxygen consumption expressed as ng-atom/min per mg mitochondrial protein.

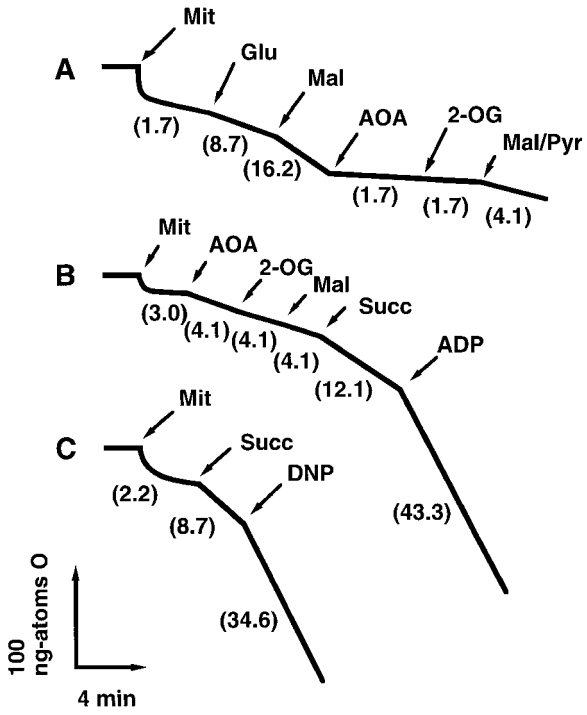

Fig. 5. Effect of various substrates on HCM respiration. HCM were resuspended at 1 mg/ml in the medium described in the Materials and Methods section. The substrates (MAL, malate; 2-OG, 2-oxoglutarate; PYR, pyruvate) were added at 5 mM final concentration except for GLU [glutamate] (20 mM), ADP (0.25 mM), AOA (2 mM) and DNP (10 μM). Numbers along the traces represent the rate of oxygen consumption expressed as ng-atom/min per mg mitochondrial protein.

DISCUSSION

Glutamine Uptake

Experiments in which HeLa cells were exposed to [^{14}C]glutamine showed that these cells display the same general metabolic features as other tumour cells [Kovacevic and McGivan, 1983; Kovacevic et al., 1991; Street et al., 1993] in that they are rapid consumers of glutamine, lack glutamine synthetase and have a very active glutaminase (Fig. 1). The only intracellular amino acids shown to be labelled were glutamine, glutamate and aspartate (Figs. 1, 2), suggesting that the oxidation of glutamate proceeded through aspartate aminotransferase, whereas Reitzer et al. [1979] had suggested that glutamate dehydrogenase was the most likely oxidation pathway.

General Respiration Studies

Respiration studies showed that HeLa cell mitochondria behaved similarly to other tumour mitochondria in regard to the mean RCR obtained with succinate respiration (4.2), but differed markedly from other tumour cells in that they lacked state III respiration induced by NAD-linked substrates (Tables II, III and Fig. 5). Since coupled respiration was observed in the presence of succinate (Fig. 4), it was concluded that HeLa cell mitochondria most likely have a defect in complex I of the electron transport chain [Pedersen, 1978].

HeLa Cells and the Truncated TCA Cycle

The evidence supporting the existence of a truncated TCA cycle based on aspartate aminotransferase can be summarised as follows: Whole cell preparations revealed that the steady state levels of exogenous glutamine carbon recovered from glutamine, glutamate and aspartate represented 90% of the total radioactivity in the protein free fraction (Fig. 1). Placing the mitochondria in a medium containing glutamine, glutamate, or glutamate plus malate resulted in the efflux of aspartate as the major metabolite (Fig. 5). The addition of the aminotransferase inhibitor AOA inhibited both respiration and the efflux of aspartate from mitochondria (Table II) incubated in the presence of glutamate and malate. The failure of 2-oxoglutarate to relieve this inhibition supports the

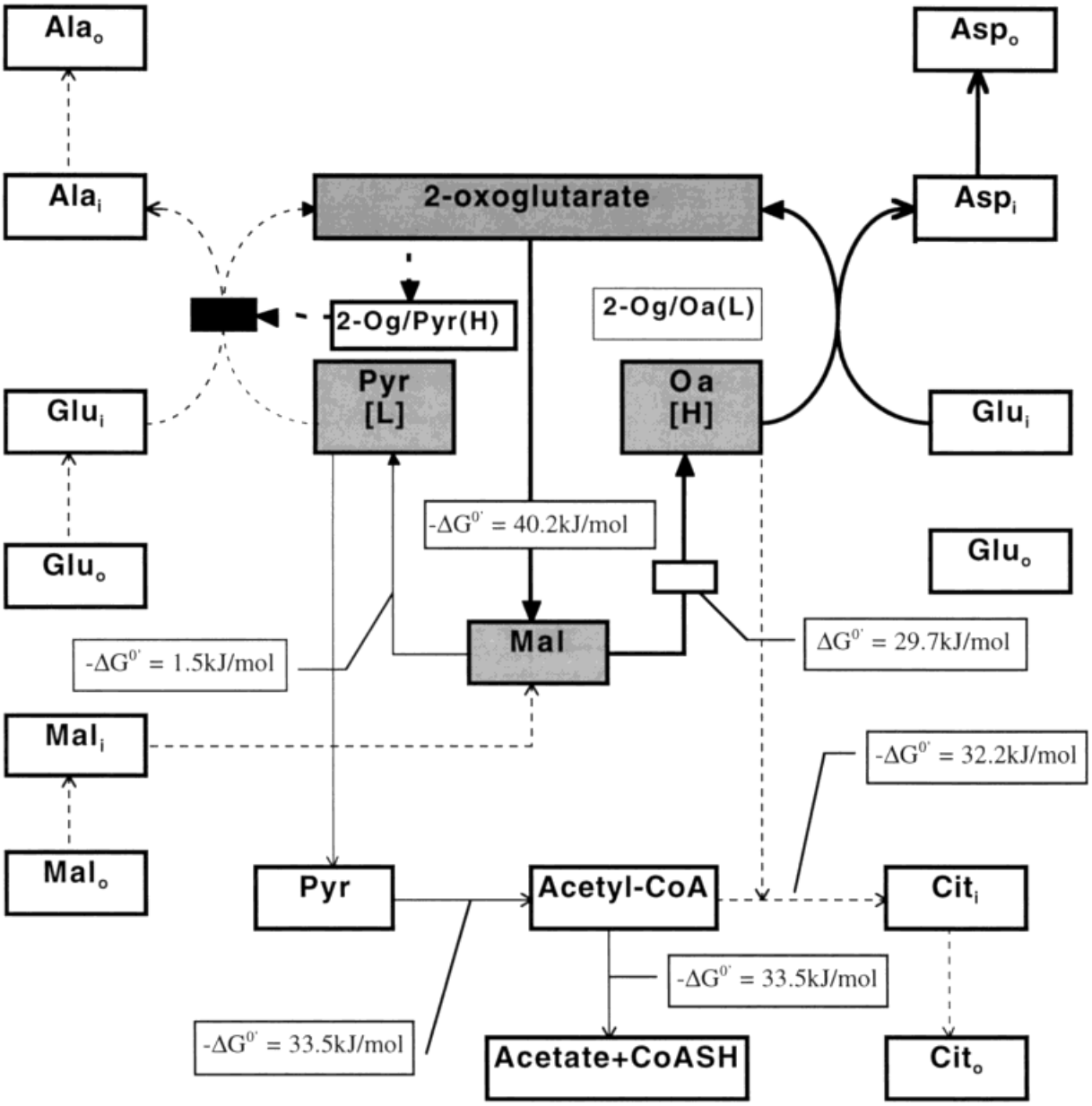

Fig. 6. Proposed regulatory mechanism in tumour cell mitochondria that determines the metabolism of glutamate when it is present as the sole metabolite. Key: High flux pathways (⟶), Low flux pathways (⟶), Zero flux pathways (⇢), Indicating inhibited reactions (– – ⤇), Truncated TCA cycles (⟶), Inhibited reactions (■), Endergonic reactions (□). Oxoacid concentration ratios—high or low—are indicated in frames.

notion that glutamate is converted to 2-oxoglutarate via aspartate aminotransferase activity. Citrate does not appear to be a component of the TCA cycle as AOA, although it inhibited respiration, did not effect the efflux of citrate from the mitochondria (Table II). Thus, the truncated TCA cycle in HeLa cells appears to be the result of both a low rate of citrate synthesis and an active citrate transporter. The addition of 2-oxoglutarate failed to relieve glutamate plus malate respiration (Fig. 5), indicating that 2-oxoglutarate is part of a well-coupled truncated cycle of which aspartate aminotransferase has been shown to be a component [Parlo and Coleman, 1984].

The metabolism observed in HCM is different to that seen in mitochondria isolated from Ehrlich ascites and AS-30D tumour cells [Moreadith and Lehninger, 1984; Kovacevic et al., 1991]. In order to help explain these differences

TABLE IV. Aspartate Mitochondrial Efflux Ratios for Different Tumour Cell Cultures*

Substrate	Aspartate flux ratios			Reference
	Ala	Cit	Asp	
HeLa cells				
Malate	0.1	0.25	2.1	This study
Pyruvate	0.2	0.3	1.0	This study
Ehrlich ascites cells				
Malate	0.7	0.7	0.2	Moreadith and Lehninger [1984]
Pyruvate[a]	0.7	0.7	0.2	Moreadith and Lehninger [1984]
Malate	0	0	1.7	Kovacevic et al. [1991]
Pyruvate	0.6	0.1	0.2	Kovacevic et al. [1991]
AS-30D cells				
Malate	0.4	0	2.4	Kovacevic et al. [1991]
Pyruvate	0.3	0	0.7	Kovacevic et al. [1991]

*The efflux values obtained from other sources have been recalculated from the data provided and are therefore approximate but well within the requirements of the table. Aspartate efflux for the various tissues in the presence of glutamate alone were: Ehrlich ascites tumour mitochondria [Moreadith and Lehninger, 1984] = 10 nmol/min per mg protein; Ehrlich ascites tumour mitochondria [Kovacevic et al., 1991] ≈ 6 nmol/min per mg protein; AS 30D hepatoma mitochondria [Kovacevic et al., 1991] ≈ 2.3 nmole/min per mg protein; and HeLa cell mitochondria = 15 nmole/min per mg protein. The aspartate efflux ratios were calculated as (amino acid flux)/(aspartate flux in the presence of glutamate alone).
[a]These results have been inferred from the malate results.

we have devised a model based on the free energies of reactions to explain the product profiles of these mitochondria when they are incubated in media containing different metabolites.

Regulation of Truncated Cycles in Tumour Cells: Possible Role of 2-Oxoglutarate as a Competitive Inhibitor of Aminotransferases

The aminotransferases operate through a nonsequential ping pong mechanism. Equation 1 represents the Lineweaver–Burk form of the reaction. The equation has been based on the following assumptions: (1) aspartate is kept at a low concentration, (2) glutamate is constant, and (3) oxaloacetate and 2-oxoglutarate are allowed to vary.

$$\frac{1}{v} = \frac{K_{G/G.Oa}}{V_F}\left\{1 + K_{G/Og} \cdot [Og] \cdot \left(1 + \frac{K_{Og/G.Og}}{[G]}\right)\right\}\frac{1}{[Oa]} + \frac{1}{V_f}\left(1 + \frac{K_{Oa/G.Oa}}{[G]}\right) \quad (1)$$

where G is glutamate, Oa is oxaloacetate, Og is 2-oxoglutarate, and $K_{G/G.Oa}$ is (CoefG/CoefG.Oa), where CoefG.Oa is the sum of the reaction pathways (rate constant vectors) specifying [G].[Oa].

The equation shows that, given the above restrictions, 2-oxoglutarate is a competitive inhibitor of aspartate aminotransferase when oxaloacetate is acting as the variable substrate, and that when glutamate and oxaloacetate are at saturating concentrations the reaction proceeds at maximum velocity. Thus mitochondria that support a high rate of flux through alanine aminotransferase can cause the inhibition of aspartate aminotransferase by increasing the 2-oxoglutarate concentration, and decreasing the oxaloacetate concentration.

It is therefore proposed that a major regulator of glutamate metabolism in tumour cells is the nonsequential ping-pong mechanism of the aspartate and alanine aminotransferases and that the direction of pathway flux through the two aminotransferases is determined by the concentration ratios 2-oxoglutarate to oxaloacetate and 2-oxoglutarate to pyruvate, respectively.

Figure 6 shows the overall scheme for glutamate carbon flux in tumour mitochondria. Flux through 2-oxoglutarate forms the essential link between the activities of the two aminotransferases, and, since the free energy difference for the two pathways favours the malic enzyme reaction, flux through the latter pathway is largely determined by the level of activity of the malic enzyme.

Figure 6 also shows the pathway for glutamate as the sole metabolite. It will be noted that the activity of the malic enzyme is irrel-

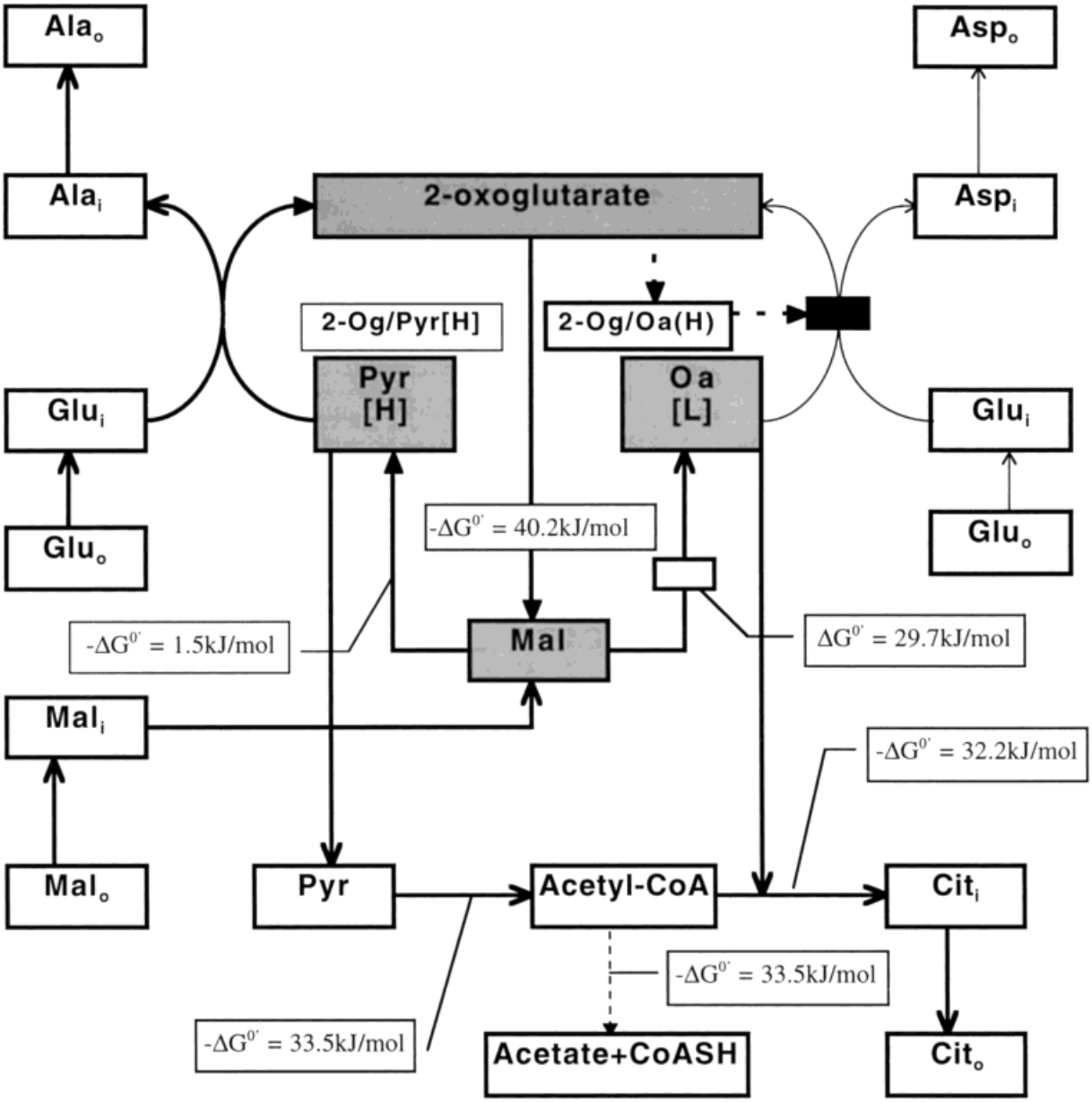

Fig. 7. Proposed metabolic regulation of glutamate plus malate in Ehrlich ascites tumour cell mitochondria [Moreadith and Lehninger, 1984]. High flux pathways (⟶); low flux pathways (⟶); zero flux pathways (⇢); indicating inhibited reactions (– – ⇛); truncated TCA cycles (⟶); inhibited reactions (■); endergonic reactions (□). Oxoacid concentration ratios (high or low) are indicated.

evant, and this happens to be the only example where this is so. The reason is that when glutamate is the sole metabolite the aspartate aminotransferase reaction exerts a higher preference for oxaloacetate over citrate synthase due to the way in which the aspartate aminotransferase reaction is coupled into the TCA cycle. In other words, cycling of the oxaloacetate in the truncated cycle is driven by a high concentration of glutamate; under conditions where the concentration of acetyl-CoA is low, citrate synthesis remains stalled. Alanine aminotransferase is also stalled due to the restricted carbon flux through malic enzyme, and the unavailability of pyruvate which is directly removed by the highly exergonic pyruvate dehydrogenase. It is for this reason that alanine aminotransferase can be active only when flux to the pyruvate pool is in excess of the flux through pyruvate dehydrogenase. Glutamate dehydrogenase has

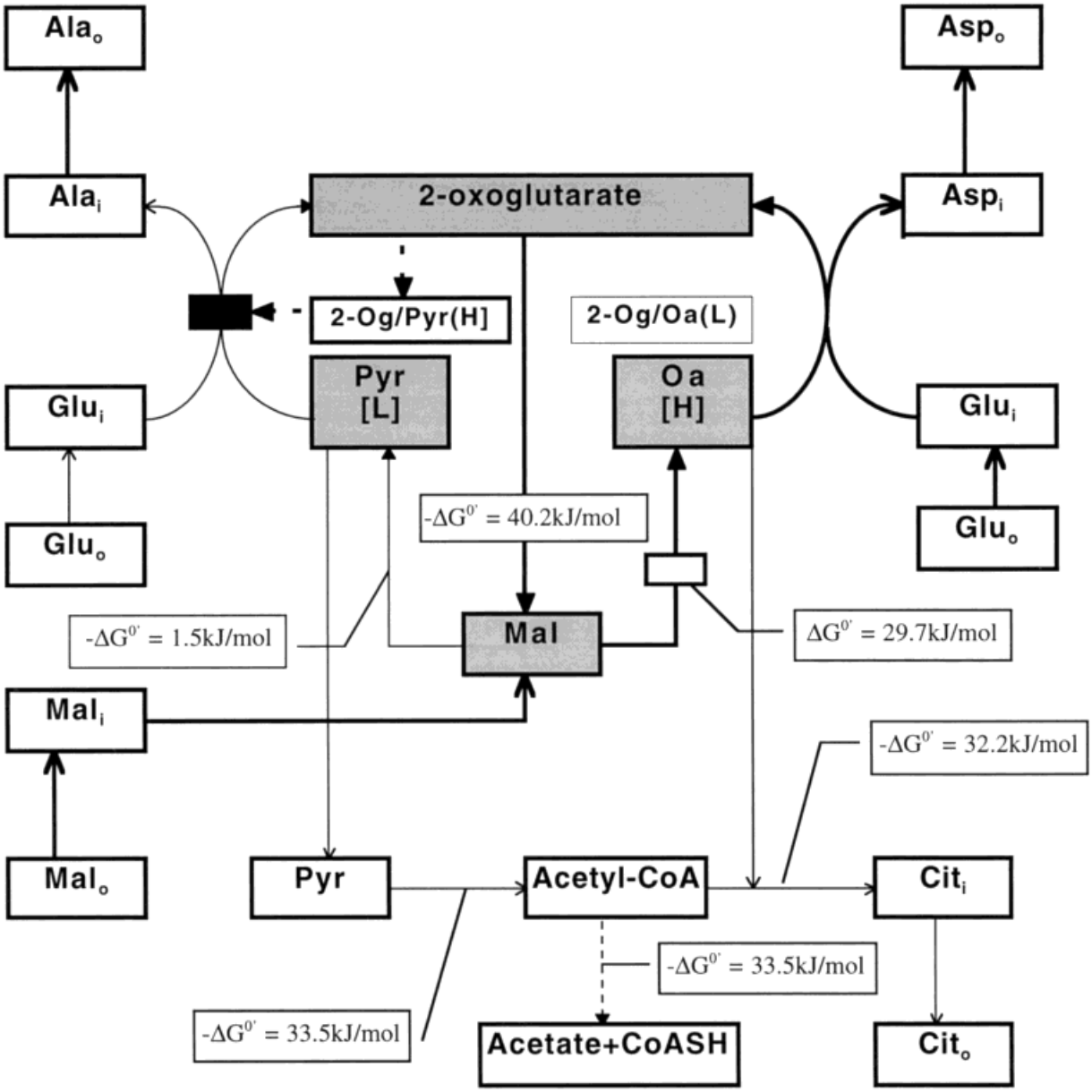

Fig. 8. Proposed metabolic regulation of glutamate plus malate in HeLa cell mitochondria. High flux pathways (⟶); low flux pathways (⟶); zero flux pathways (⇢); indicating inhibited reactions (– – ⇛); truncated TCA cycles (⟶); inhibited reactions (■); endergonic reactions (□). Oxoacid concentration ratios (high or low) are indicated.

the same anaplerotic role in the truncated cycle as in the complete TCA cycle. In other words, it promotes the flux of carbon through the cycle, and at steady state glutamate is not oxidised by this pathway.

Table IV shows that the efflux rates for alanine and citrate in Ehrlich ascites cells [Moreadith and Lehninger, 1984] were high with glutamate plus malate in the medium. Figure 7 shows the resultant pathways taken when applied to the scheme. The important result is that, in the presence of excess malate and a high malic enzyme allied to favourable free energy values, the direction of flux moves from oxaloacetate formation to pyruvate formation. Furthermore a high free energy change for citrate synthase moves oxaloacetate flux from aspartate aminotransferase to citrate synthase. The net consequence of these effects is that oxaloacetate concentration is lowered relative to that of pyruvate, resulting in the inhibition of aspartate aminotransferase. The efflux

of alanine indicates the presence of an active alanine aminotransferase, and the total pyruvate flux through alanine aminotransferase and pyruvate dehydrogenase indicates the presence of a very active malic enzyme. Thus a significant increase in the concentration of pyruvate results in a general transfer of carbon flux to the truncated cycle based on alanine aminotransferase and citrate synthesis. Moreadith and Lehninger [1984] suggested that exogenous malate had "preferred access" to a malic enzyme situated near the inner face of the malate transporter. It has been shown that malic enzyme forms a complex with pyruvate dehydrogenase that may localise it on the inner mitochondrial membrane [Teller et al., 1992]. However, the data obtained using the model (Fig. 7) suggests that in these mitochondria this "preferred access" would only be in addition to the pathway directions already imposed by the dictates of the free energy requirements. Similar conclusions were drawn by Dietzen and Davis [1993] based on the K_m and K_{eq} values of the two malic acid enzymes. Finally, it can be inferred from the above proposed mechanisms that the partitioning of the exogenous and endogenous malate is a functional partition.

In the next example, Table IV shows that the addition of malate to glutamate resulted in a large increase in the efflux of aspartate from the HeLa, Ehrlich ascites, and AS-30D cell mitochondria [Kovacevic et al., 1991]. In the presence of both malate and pyruvate efflux rates for both alanine and citrate were low in HeLa cells (Fig. 8), indicating that both alanine aminotransferase and citrate synthase were deficient. This put the main carbon flux through malic dehydrogenase to aspartate aminotransferase. The increased aspartate efflux in the presence of malate was due to the increase in oxaloacetate concentration, which not only increased directly the rate of the reaction but also reduced 2-oxoglutarate inhibition.

The addition of pyruvate to Ehrlich ascites mitochondria [Kovacevic et al., 1991] resulted in a marked increase in the efflux of alanine, indicating an active alanine aminotransferase. Associated with the increase in alanine efflux there was a marked reduction in aspartate efflux, indicating that in the presence of a high pyruvate concentration the inhibition of alanine aminotransferase by 2-oxoglutarate was considerably reduced, while the lowering of flux through malate dehydrogenase resulted in a reduction in oxaloacetate concentration and in an increased inhibition of aspartate aminotransferase. Finally, the increase in the alanine efflux in the presence of pyruvate confirmed that these mitochondria had a low malic enzyme activity and that the inhibition of aspartate aminotransferase was due mainly to the increased concentration of 2-oxoglutarate formed by the activity of the truncated cycle based on alanine aminotransferase.

ACKNOWLEDGMENTS

We thank Mr R. Jones for his help and guidance in culturing HeLa cells, Dr M.G.P. McCabe for assistance in the oxy-polagraphic studies, and Professor J.F. Williams for his critical evaluation of the manuscript. T.J. Piva gratefully acknowledges the award of a James Cook University Postgraduate Research Scholarship.

REFERENCES

Czok R, Lamprecht W (1974): Pyruvate, phosphoenolpyruvate and D-glycerate-2-phosphate. In Bergmeyer HU (ed): "Methods of Enzymatic Analysis." London: Academic Press, pp 1446–1451.

Dagby S (1974): Citrate: UV spectrophotometric determination. In Bergmeyer HU (ed): "Methods of Enzymatic Analysis." London: Academic Press, pp 1562–1565.

Dietzen DJ, Davis EJ (1993): Oxidation of pyruvate, malate, citrate, and cytosolic reducing equivalents by AS-30D hepatoma mitochondria. Arch Biochem Biophys 305: 91–102.

Gauthier T, Denis-Pouxviel C, Murat JC (1990): Respiration of mitochondria isolated from differentiated and undifferentiated HT29 colon cancer cells in the presence of various substrates and ADP generating systems. Int J Biochem 22:411–417.

Gutmann I, Wahlefield AW (1974): L-(−)-Malate: Determination with malate dehydrogenase and NAD. In Bergmeyer HU (ed): "Methods of Enzymatic Analysis." London: Academic Press, pp 1585–1589.

Kabus P, Koch G (1982): Quantitative determination of amino acids in tissue culture cells by high performance liquid chromatography. Biochem Biophys Res Commun 108:783–790.

Kaplan RS, Parlo RA, Coleman PS (1986): Measurement of citrate transport in tumor mitochondria. Methods Enzymol 125:671–691.

Kelleher JK, Bryan BM, Mallett RT, Holleran AL, Murphy AN, Fiskum G (1987): Analysis of tricarboxylic acid-cycle metabolism of hepatoma cells by comparison of $^{14}CO_2$ ratios. Biochem J 246:633–639.

Knox WE, Horowitz ML, Friedell GH (1969): The proportionality of glutaminase content to growth rate and morphology of rat neoplasms. Cancer Res 29:669–680.

Kovacevic Z, McGivan JD (1983): Mitochondrial metabolism of glutamine and glutamate and its physiological significance. Physiol Rev 63:547–605.

Kovacevic Z, Brkljac O, Bajin K (1991): Control and function of the transamination pathways of glutamine oxidation in tumour cells. Biochem J 273:271–275.

Lanks KW (1987): End products of glucose and glutamine metabolism by L929 cells. J Biol Chem 262:10093–10097.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951): Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275.

Matsuno T (1987): Bioenergetics of tumor cells: Glutamine metabolism in tumor cell mitochondria. Int J Biochem 19:303–307.

Matsuno T (1991): Pathway of glutamate oxidation and its regulation in the HuH13 line of human hepatoma cells. J Cell Physiol 148:290–294.

McEvoy-Bowe E (1985): Cooling plate for cellulose thin-layer electrophoresis and its application to amino acid analysis. J Chromatogr 437:199–208.

Medina MA, Sanchez-Jimenez F, Marquez J, Quesada AR, Nunez de Castro I (1992): Relevance of glutamine metabolism to tumor cell growth. Mol Cell Biochem 113:1–15.

Moreadith RW, Fiskum G (1984): Isolation of mitochondria from ascites tumor cells permeabilized with digitonin. Anal Biochem 137:360–367.

Moreadith RW, Lehninger AL (1984): The pathways of glutamate and glutamine oxidation by tumor cell mitochondria. Role of mitochondrial NAD(P)$^+$-dependent malic enzyme. J Biol Chem 259:6215–6221.

Newsholme EA, Crabtree B, Ardawi MSM (1985): The role of high rates of glycolysis and glutamine utilization in rapidly dividing cells. Biosci Rep 5:393–400.

Newsholme EA, Board M (1991): Application of metabolic-control logic to fuel utilization and its significance in tumor cells. Adv Enzyme Regul 31:225–246.

Parlo RA, Coleman PS (1984): Enhanced rate of citrate export from cholesterol-rich hepatoma mitochondria. The truncated Krebs cycle and other metabolic ramifications of mitochondrial membrane cholesterol. J Biol Chem 259:9997–10003.

Pedersen PL (1978): Tumor mitochondria and the bioenergetics of cancer cells. Prog Exp Tumor Res 22:190–274.

Piez KA, Eagle H (1958): The free amino acid pool of cultured human cells. J Biol Chem 231:533–545.

Piva TJ, McEvoy-Bowe E (1992): The truncated TCA cycle in HeLa cell mitochondria. Adv Exp Med Biol 316:386–391.

Reitzer LJ, Wice BM, Kennell D (1979): Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. J Biol Chem 254:2669–2676.

Rivera S, Azcon-Bieto J, Lopez-Soriano FJ, Miralpeix M, Argiles JM (1988): Amino acid metabolism in tumour-bearing mice. Biochem J 249:443–449.

Smith AL (1967): Preparation, properties, and conditions for assay of mitochondria. Slaughterhouse material, small-scale. Methods Enzymol 10:81–86.

Street JC, Delort A-M, Braddock PSH, Brindle KM (1993): A $^1H/^{15}N$ n.m.r. study of nitrogen metabolism in cultured mammalian cells. Biochem J 291:485–492.

Teller JK, Fahien LA, Davis JW (1992): Kinetics and regulation of hepatoma mitochondrial NAD(P) malic enzyme. J Biol Chem 267:10423–10432.

Zielke HR, Zielke CL, Ozand PT (1984): Glutamine: A major energy source for cultured mammalian cells. Fed Proc 43:121–125.