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Glutamine and glucose metabolism during thymocyte proliferation*

Pathways of glutamine and glutamate metabolism

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1. Energy metabolism in proliferating cultured rat thymoyctes was compared with that of freshly prepared non-proliferating resting cells. Cultured rat thymocytes enter a proliferative cycle after stimulation by concanavalin A and Lymphocult T (interleukin-2), with maximal rates of DNA synthesis at 60h. 2. Compared with incubated resting thymocytes, glucose metabolism by incubated proliferating thymoyctes was 53-fold increased; 90% of the amount of glucose utilized was converted into lactate, whereas resting cells metabolized only 56% to lactate. However, the latter oxidized 27% of glucose to CO_2, as opposed to 1.1% by the proliferating cells. 3. Activities of hexokinase, 6-phosphofructokinase, pyruvate kinase and aldolase in proliferating thymocytes were increased 12-, 17-, 30- and 24-fold respectively, whereas the rate of pyruvate oxidation was enhanced only 3-fold. The relatively low capacity of pyruvate degradation in proliferating thymocytes might be the reason for almost complete conversion of glucose into lactate by these cells. 4. Glutamine utilization by rat thymocytes was 8-fold increased during proliferation. The major end products of glutamine metabolism are glutamate, aspartate, CO_2 and ammonia. A complete recovery of glutamine carbon and nitrogen in the products was obtained. The amount of glutamate formed by phosphate-dependent glutaminase which entered the citric acid cycle was enhanced 5-fold in the proliferating cells: 76% was converted into 2-oxoglutarate by aspartate aminotransferase, present in high activity, and the remaining 24% by glutamate dehydrogenase. With resting cells the same percentages were obtained (75 and 25). Maximal activities of glutaminase, glutamate dehydrogenase and aspartate aminotransferase were increased 3-, 12- and 6-fold respectively in proliferating cells; 32% of the glutamate metabolized in the citric acid cycle was recovered in CO_2 and 61% in aspartate. In resting cells this proportion was 41% and 59% and in mitogen-stimulated cells 39% and 65% respectively. 5. Addition of glucose (4mM) or malate (2mM) strongly decreased the rates of glutamine utilization and glutamate conversion into 2-oxoglutarate by proliferating thymocytes and also affected the pathways of further glutamate metabolism. Addition of 2mM-pyruvate did not alter the rate of glutamine utilization by proliferating thymocytes, but decreased the rate of metabolism beyond the stage of glutamate significantly. Formation of acetyl-CoA in the presence of pyruvate might explain the relatively enhanced oxidation of glutamate to CO_2 (56%) by proliferating thymocytes.

Glucose is required as major energy source for proliferating and cultured mammalian cells, and aerobic glycolysis is linked to proliferation of malignantly transformed and non-malignant cells (Warburg, 1956; Paul, 1965; Sweeney *et al.*, 1963; Burk *et al.*, 1967; Steck *et al.*, 1968; Bissell *et al.*, 1972; Morell & Froesch, 1973; Bissell, 1976; Kester *et al.*, 1977; Lang & Weber, 1978).

* Dedicated to Professor Dr. K. Decker, Freiburg, Federal Republic of Germany, on the occasion of his 60th birthday.

Increased aerobic glycolysis and enhanced glucose transport have also been reported in numerous studies with incubated mitogen-stimulated lymphocytes (Hedeskov, 1968; Roos & Loos, 1970, 1973; Culvenor & Weidemann, 1976; Whitesell *et al.*, 1977; Hume *et al.*, 1978; Brand *et al.*, 1984). Evidence has been presented that aerobic glycolysis in cultured mouse spleen lymphocytes not only is associated with cellular proliferation but more specifically is temporally related to DNA synthesis (Wang *et al.*, 1976).

However, various observations suggest that glutamine is a primary energy source for cultured HeLa cells (Reitzer *et al.*, 1979), human diploid fibroblasts (Zielke *et al.*, 1978, 1980; Submilla *et al.*, 1981), a variety of other vertebrate cells (Wice *et al.*, 1981) and ascites-tumour cells (Moreadith & Lehninger, 1984). Evidence has also been obtained that incubated lymphocytes prepared from rat mesenteric lymph nodes (Ardawi & Newsholme, 1983) or rat thymus (Brand *et al.*, 1984) utilize glutamine and respond to mitogens by increased glutamine metabolism. These observations have pointed specifically to glutamine as a major respiratory substrate for rapidly dividing cells. It may also be noted that glutamine is the most abundant amino acid both in blood plasma and in optimal culture media (Ham & McKeehan, 1979).

The main purpose of this work was to study metabolic alterations in rat thymocytes during cell proliferation. Stimulation of thymocytes by concanavalin A presumably produces *in vitro* the response that occurs to an antigenic challenge *in vivo*. Thymocytes cultured in the presence of concanavalin A and growth factor interleukin-2 are therefore a suitable tool to investigate the metabolic events associated with cell division.

In this work glucose and glutamine metabolism are compared in proliferating rat thymocytes and resting cells. A major aspect was to investigate the route(s) of entry of glutamine and glutamate into the citric acid cycle and the metabolic fate of their carbon skeletons and amino groups under various metabolic conditions.

In addition, maximal activities of enzymes involved in glucose and glutamine metabolism have been measured in freshly prepared resting and 60h-cultured proliferating rat thymocytes. Quantitative and qualitative alterations of energy metabolism during proliferation are reported.

Materials and methods

Materials

Female outbred Wistar rats (7–9 weeks old) were used for all experiments. [U-^{14}C]Glucose, [U-^{14}C]glutamine, [1-^{14}C]pyruvate and [*methyl*-^{3}H]thymidine were obtained from Amersham Buchler, Braunschweig, Germany. Concanavalin A, L-glutamine, 2-mercaptoethanol and Hepes buffer were purchased from Serva, Heidelberg, Germany; foetal-calf serum and penicillin/streptomycin were from Gibco Europe, Karlsruhe, Germany; bovine serum albumin was from Behringwerke, Frankfurt, Germany; Lymphocult-T was from Biotest, Frankfurt, Germany; RPMI 1640 medium was from Seromed, West Berlin; glutaminase and amino-oxyacetic acid were from Sigma, Tautkirchen, Germany; 2-phenethylamine was from Fluka, Neu-Ulm, Germany; L(−)-malic acid was from Roth, Karlsruhe, Germany. Aqualuma Plus and Lumasolve were products of Lumac, Schaesberg, The Netherlands. α-D-Glucose and all other chemicals were products of Merck, Darmstadt, Germany. Enzymes, coenzymes and substrates used in metabolite assays were obtained from Boehringer, Mannheim, Germany.

Methods

Incubation of thymocytes. Rat thymus lymphocytes were prepared as described by Culvenor & Weidemann (1976); viability was over 95% as judged by Trypan Blue exclusion. Incubations were performed at 37°C in 20ml plastic vials. Freshly prepared thymocytes (resting cells) were incubated at a density of 1×10^8–5×10^8 cells for 60–180min in a total volume of 3ml of incubation medium, which consisted of phosphate-buffered saline, pH7.4 (Culvenor & Weidemann, 1976), containing [U-^{14}C]glucose (4mM), [U-^{14}C]glutamine (2mM) or [1-^{14}C]pyruvate (2mM) as labelled substrate. Thymocytes cultured for 60h in the presence of concanavalin A and Lymphocult T (proliferating cells) as described below were incubated at a density of 2×10^7–8×10^7 cells for 60min in the same medium. Vials were gassed with O_2/CO_2 (19:1), then tightly stoppered with rubber caps and shaken continuously (120 oscillations/min). Incubations were initiated by the addition of the cell suspension and terminated by the addition of 1ml of 1M-$HClO_4$. Shaking was continued for a further 30min to trap all the $^{14}CO_2$ evolved in 0.5ml of 2-phenethylamine. Specific radioactivity of [U-^{14}C]glucose, [U-^{14}C]glutamine and [1-^{14}C]-pyruvate was in the range 1×10^{11}–2×10^{11} d.p.m./mol. In pilot studies it was established that substrate utilization and $^{14}CO_2$ release were linear with time for up to 4h with uncultured and up to 3h with cultured thymocytes. Samples in which 1M-$HClO_4$ was injected before the addition of radioactive substrate were used as blanks (zero time incubation). The $HClO_4$-insoluble cell pellet was washed twice with 0.1M-$HClO_4$. The combined supernatant fractions were neutralized with 1M-KOH and adjusted with buffer to a final

volume of 10ml (= neutralized cell extract). 2-Phenethylamine (0.5ml), which was placed in an Eppendorf cup during the incubation, was transferred quantitatively to scintillation vials containing 6ml of Bray's solution (Bray, 1960) and the radioactivity was determined in a LKB Rack-Beta liquid-scintillation counter. The minimal amount of $^{14}CO_2$ radioactivity in blanks stopped at zero time was subtracted from the experimental assays.

Cell culture

Rat thymocytes (1×10^8–3×10^8 cells) were grown in 70ml of RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated foetal-calf serum, 2mM-glutamine, 50μM-2-mercaptoethanol, concanavalin A (10μg/ml), 5% Lymphocult T, 15mM-Hepes, streptomycin (100 units/ml) and penicillin (200 units/ml). The cultures were incubated at 37°C in a 5%-CO_2 incubator for 60h. Cells were harvested by centrifugation for 10min at 550*g*, and washed with phosphate-buffered saline, pH7.4, containing 2% bovine serum albumin. Washing was repeated with albumin-free buffer and the cells were suspended in the same buffer. Viability of 60h-cultured cells was over 90% as judged by Trypan Blue exclusion.

Proliferation studies of thymocytes were carried out in wells of round-bottomed Falcon micro-titre culture plates; 25μl of cell suspension (2×10^5 cells) was placed in each well together with 150μl of culture medium. The cultures (12 each) were incubated at 37°C in a 5%-CO_2 incubator for 4, 24, 48, 60, 72, 96, 120 and 148h. The proliferative response was determined by measuring the uptake of [^{3}H]thymidine (1μCi/well) after a 4h pulse. Cultures without concanavalin A or without both concanavalin A and Lymphocult T were run as controls. Cultures were harvested on to glass-fibre filters by using an automated cell harvester. The dried filters were added to scintillation vials containing 2ml of Aqualuma Plus and 1% Lumasolve. Radioactivity was measured in a LKB Rack-Beta liquid-scintillation counter.

Metabolite assays

Metabolites in neutralized cell extracts were determined spectrophotometrically by using enzymic assays: glucose, glutamine and ammonia by the method of Windmueller & Spaeth (1974); glutamate by the method of Bernt & Bergmeyer (1974); aspartate by the method of Bergmeyer *et al.* (1974*b*) and alanine by the method of Grassl (1974); glucose by the coupled hexokinase/glucose-6-phosphate dehydrogenase method described by Bergmeyer *et al.* (1974*a*); lactate by the method of Gawehn & Bergmeyer (1974); and pyruvate by the method of Czok & Lamprecht (1974). The DNA content in the resting and proliferating thymocytes was assayed as described by Leyva & Kelly (1974). Protein was assayed with the biuret reaction.

Amino acids were analysed with a Biotronik amino acid analyser model LC 6000 equipped with a splitting system. Amino acid concentrations were assayed by *o*-phthalaldehyde fluorescence. Fractions (1ml each) were collected and counted for radioactivity in 6ml of Bray's solution.

Glycogen was determined as described by Keppler & Decker (1984).

Assay of enzyme activities

Cell extracts for assaying enzyme activities were obtained from freshly prepared (resting) and 60h-cultured (proliferating) rat thymocytes by freezing at −70°C for 30min and rapid thawing. Immediately after thawing the extracts were sonicated for six separate periods of 6s with a microprobe of a Branson sonifier at 40W; the extracts were maintained at 0–4°C during sonication. Enzyme assays were carried out immediately after sonication without further treatment of the extracts. The activities of all enzymes tested were stable in the undiluted extracts at 4°C for more than 6h.

Hexokinase and 6-phosphofructokinase activities were assayed essentially as described by Crane & Sols (1955) and Ling *et al.* (1955) respectively. Pyruvate kinase activity was measured as described by Fujii & Miwa (1983). Fructose-1,6-bisphosphate aldolase activity was determined as described by Willnow (1984). Glutaminase activity was measured as described by Curthoys & Lowry (1973), glutamate dehydrogenase activity as described by Schmidt & Schmidt (1983) and aspartate aminotransferase activity as described by Rej & Hørder (1983). All activities were measured at 37°C for comparison with the metabolic rates also determined at 37°C.

Expression of results

Changes in concentrations of substrates or metabolites during the incubation were determined from the net change between zero time and the time of incubation (60–180min). Rates of substrate utilization or metabolite production are expressed as μmol/h per 10^{10} cells; 10^{10} freshly prepared rat thymocytes (resting cells) are equivalent to 0.62g dry wt., and 10^{10} 60h-cultured rat thymocytes (proliferating cells) are equivalent to 1.54g dry wt. The DNA content of freshly prepared thymocytes was determined to be 67 ± 2.1 and that of proliferating cells 112 ± 6.6mg/10^{10} cells.

Results and discussion

Proliferation of concanavalin A-stimulated thymocyte cultures

In the presence of concanavalin A plus interleukin-2, [^{3}H]thymidine incorporation into thymocytes increased with time, yielding maximal proliferation rates after 60h of culture (Fig. 1). After this maximum, DNA synthesis decreased and returned to the baseline value. In the absence of mitogen, [^{3}H]thymidine incorporation after 24h of culture ceased and remained negligible throughout the culture period of 120h even in the presence of interleukin-2.

The DNA content almost doubled from 67 ± 2.1 in resting to 112 ± 6.6 mg/10^{10} cells in proliferating cells, suggesting that after 60h of culture in the presence of mitogen and T-cell growth factor almost all cells are in the S-phase of the cell cycle. The protein content increased from 448 ± 22 to 1484 ± 60 mg/10^{10} cells during proliferation. A similar proliferation kinetics have been observed by Wang *et al.* (1976) with lymphocytes from mouse spleen. They found that DNA synthesis did not appear until about 20h after concanavalin A addition and reached a peak at 50–60h after stimulation. The decline in DNA synthesis could not be prevented in their experiments by frequent changes of medium, serum replenishment or the continued presence of mitogen. Thus lymphocytes undergo a discrete proliferative response to stimulation by concanavalin A and, unlike established lines or tumour cells, retain control of their cell division. For all metabolic studies, thymocytes cultured for 60min under maximum proliferation conditions were used.

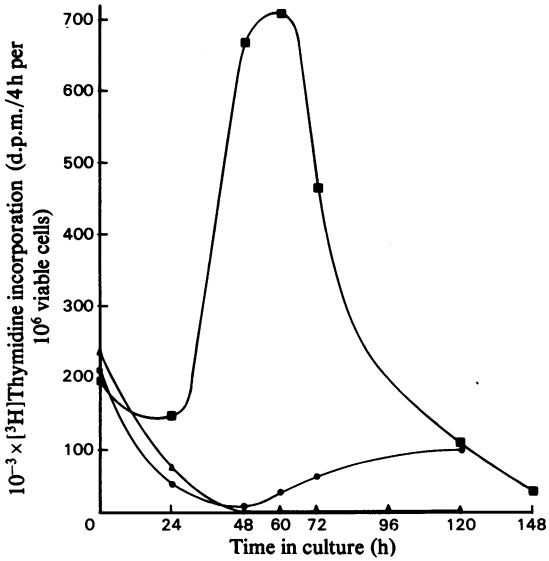

Fig. 1. *Proliferation of cultured rat thymocytes*
Proliferation studies by [^{3}H]thymidine incorporation into DNA were carried out as described in the Materials and methods section. Culture conditions were: ■, with concanavalin A plus Lymphocult T; ●, no concanavalin A, but with Lymphocult T; ▲, no concanavalin A and no Lymphocult T.

Comparison of glucose metabolism between incubated resting, mitogen-stimulated and proliferating rat thymocytes

Increased carbohydrate metabolism is necessary to meet the energy requirements of the cell in going from the dormant to the highly active dividing cell. To assess more fully the role of glucose for proliferation, we attempted to analyse the fate of [U-^{14}C]glucose in freshly prepared (resting), mitogen-stimulated and 60h-cultured (proliferating) rat thymocytes (Table 1). Glucose utilization is increased 53-fold in the proliferating cells, but glucose oxidation to CO_2 is only doubled. Some 90% of the glucose utilized by incubated proliferating thymocytes is converted into lactate, whereas incubated resting or mitogen-stimulated non-cultured cells convert only 56 or 68% respectively into lactate. Consequently 27% of the glucose utilized by resting cells is degraded to CO_2, whereas proliferating cells oxidize only 1.1% to CO_2 (Table 1). The switch from aerobic to anaerobic glucose degradation appears to be a feature not only of cultured tumour cells but also of cultured thymocytes under controlled proliferation. Mitogen-enhanced glucose metabolism of incubated thymocytes has been observed also by Culvenor & Weidemann (1976), Hume *et al.* (1978) and Brand *et al.* (1984). The new observation, however, is the qualitative change from partly aerobic to almost completely anaerobic glucose degradation and a 36-fold stimulation of glucose utilization when resting rat thymocytes go to the highly active proliferative state. No significant glycogen synthesis from [U-^{14}C]glucose could be detected in resting and proliferating rat thymocytes.

Activities of key glycolytic enzymes in resting and proliferating rat thymocytes

The activities of some of the glycolytic enzymes in rat thymocytes are given in Table 2. The maximal activities of hexokinase, 6-phosphofructokinase, pyruvate kinase and aldolase are enhanced 12-, 17-, 30- and 24-fold respectively in 60h-cultured proliferating thymocytes, compared with resting cells. Of the glycolytic enzymes whose activities were measured, that of 6-phosphofructokinase was the lowest both in resting and in proliferating cells, yet its maximal activity was about 3 times greater than the glycolytic flux (Table 1). From the glycolytic enzyme activities

Table 1. *Alterations of glucose metabolism by incubated rat thymocytes during proliferation*
Freshly prepared rat thymocytes were incubated (2×10^8–5×10^8 viable cells) in the absence (resting cells) and presence of 100 μg of concanavalin A (mitogen-stimulated cells) at 37°C in 5 ml of phosphate-buffered saline, pH 7.4, containing 4 mM-[U-^{14}C]glucose. Rat thymocytes cultured for 60 h as described in the Materials and methods section (proliferating cells) were incubated (3×10^7–8×10^7 viable cells) under the same conditions. Rates of utilization (indicated by minus sign) or production are presented as means ± S.E.M. with the numbers of separate experiments given in parentheses. Statistical significance of difference between metabolic rates of mitogen-stimulated and resting cells, and proliferating and mitogen-stimulated cells (Student's *t* test, based on difference between means), is indicated by * ($P<0.01$) or ** ($P<0.001$).

	Rates of utilization and production (μmol/h per 10^{10} cells)					
Thymocytes	Glucose	$^{14}CO_2$	Glucose converted into CO_2 (%)	Pyruvate	Lactate	Glucose converted into lactate (%)
Resting cells	−17 ± 1.2 (21)	28 ± 2.6 (5)	27	1.2 ± 0.1 (19)	19 ± 1.5 (19)	56
Mitogen-stimulated cells	−38** ± 1.3 (22)	42* ± 3.6 (5)	18	2.3* ± 0.3 (21)	52** ± 2.5 (21)	68
Proliferating cells	−893** ± 63 (13)	60** ± 6 (14)	1.1	36** ± 3.8 (15)	1612** ± 100 (13)	90

Table 2. *Alterations of enzyme activities in rat thymocytes during proliferation*
Thymocytes were extracted and enzymes assayed as described in the Materials and methods section. Activities were measured at 37°C for comparison with metabolic rates. For cell preparation and culture conditions see the Materials and methods section. Enzyme activities are presented as means ± S.E.M., with the numbers of separate experiments in parentheses. The statistical significance (Student's *t* test) of the difference in activity between resting and proliferating rat thymocytes is indicated by *$P<0.001$.

	Enzyme activities (μmol per 10^{10} cells)		
Enzymes	Resting cells	Proliferating cells	Stimulation (fold)
Phosphate-dependent glutaminase	158 ± 5.7 (4)	462* ± 31.2 (6)	3
Glutamate dehydrogenase	138 ± 25 (11)	1620* ± 232 (9)	12
Glutamate–oxaloacetate aminotransferase	3900 ± 402 (11)	22140* ± 2760 (9)	6
Hexokinase	462 ± 54 (7)	5640* ± 414 (7)	12
6-Phosphofructokinase	144 ± 30 (7)	2460* ± 492 (7)	17
Pyruvate kinase	4260 ± 504 (7)	127500 ± 6780 (7)	30
Aldolase	288 ± 30 (7)	6840* ± 336 (7)	24

measured it appears that 6-phosphofructokinase and hexokinase rather than pyruvate kinase are the potentially regulatory steps. This conclusion is supported by earlier work of Culvenor & Weidemann (1976), who performed crossover plots for glycolytic intermediates in mitogen-treated isolated thymocytes, and from measurements of enzyme activities in mesenteric and popliteal lymph nodes by Ardawi & Newsholme (1982).

The rate of pyruvate oxidation was assessed by measuring $^{14}CO_2$ liberation from [1-^{14}C]pyruvate in incubations with freshly prepared and 60 h-cultured thymocytes (Table 3). The results obtained suggest that the capacity of pyruvate degradation both in resting and in proliferating cells is rather low, 20 and 59 μmol/h per 10^{10} cells respectively. This low capacity, especially in proliferating rat thymocytes, might be the reason why glucose is almost completely metabolized to lactate and very little glucose is oxidized to CO_2.

At present, the reason for the qualitative and quantitative alterations in glucose metabolism during proliferation of rat thymocytes is not known. Whatever the explanation, aerobic glycolysis seems to be a general characteristic not only of malignantly transformed tumour cells but also of normally dividing lymphocytes.

Comparison of glutamine metabolism between incubated resting, mitogen-stimulated and proliferating rat thymocytes

Glutamine has been suggested to be a major energy source for cultured mammalian cells (Zielke *et al.*, 1984) and and for rapidly dividing cells (Krebs, 1981). In the present study, maximally proliferating (60 h cultured) and non-

Table 3. *Utilization of [1-^{14}C]pyruvate by incubated resting and proliferating rat thymocytes*

Freshly prepared (resting) and 60h-cultured (proliferating) rat thymocytes (3×10^7–8×10^7 viable cells) were incubated with 2mM-[1-^{14}C]pyruvate as described in the Materials and methods section. Rates of utilization (indicated by minus sign) or production are presented as means ± S.E.M. for *n* separate experiments. Statistical significance of differences between proliferating and resting cells (a) and between incubations with 2.5mM-glutamine and without (b) a* ($P<0.01$), a** or b** ($P<0.001$).

Thymocytes	Additions	*n*	Rates of utilization and production (μmol/h per 10^{10} cells)						
			Pyruvate	Lactate	$^{14}CO_2$	Alanine	Glutamine	Glutamate	Aspartate
Resting cells	None	6	−46 ± 3.8	13 ± 5.1	20 ± 2.2	2 ± 0.2	—	—	—
	Glutamine (2.5mM)	5	−46 ± 4.7	15 ± 2.7	18 ± 1.8	2 ± 0.2	−29 ± 5.9	19 ± 5.8	2 ± 0.8
			a**	a**	a**	a**			
Proliferating cells	None	4	−156 ± 16.1	86 ± 13.4	59 ± 4.4	13 ± 1.9	—	—	—
			a**	a**	a**	a**b**	a**	a**	a**
	Glutamine (2.5mM)	4	−177 ± 20.1	105 ± 11.8	54 ± 4.8	40 ± 1.2	−222 ± 30.9	172 ± 42.8	22 ± 6.2

proliferating (freshly prepared) rat thymocytes have been used to elucidate the importance of glutamine for cell proliferation. Metabolic-balance studies have been carried out to find out if quantitative and/or qualitative alterations of glutamine metabolism occur during the proliferative response. Glutamine utilization by incubated proliferating thymocytes was enhanced 8-fold compared with resting cells (Table 4). Addition of concanavalin A to the incubations caused a 1.4-fold stimulation of glutamine metabolism in freshly prepared resting cells, but had no effect in cultured proliferating cells. Ardawi & Newsholme (1983) reported that non-cultured mature rat lymphocytes utilize glutamine at a rate of 128 and 193 μmol/h per 10^{10} cells respectively in the absence and presence of concanavalin A. These rates are much higher than those observed in resting or mitogen-stimulated non-cultured thymocytes, but are closer to those in cultured proliferating thymocytes.

A complete or nearly complete recovery of glutamine carbon in the products glutamate, aspartate and CO_2 is obtained with resting, mitogen-stimulated and proliferating rat thymocytes (Table 4).

The rates of glutamine utilization by incubated resting and proliferating thymocytes were 20 and 56% respectively of the maximum glutaminase activities, which were 158 and 462 μmol/h per 10^{10} cells respectively (Table 2). The amount of glutamate formed by phosphate-dependent glutaminase which entered the citric acid cycle via 2-oxoglutarate (difference between the amounts of glutamine utilized and glutamate formed) was enhanced 5-fold in the proliferating cells (Table 4).

In these cells, the main routes of conversion of glutamate into 2-oxoglutarate are by two enzymes, aspartate aminotransferase and glutamate dehydrogenase. Maximum activities of these enzymes have been measured to be 3900 and 138 μmol/h per 10^{10} cells in resting and 22140 and 1620 μmol/h per 10^{10} cells respectively in proliferating thymocytes (Table 2). These enzyme activities suggest that the aminotransferase reaction is most important in the conversion of glutamate into 2-oxoglutarate. This is confirmed by measurements of metabolites. From the rates of aspartate formation, the contribution of the transamination reaction to glutamate conversion can be calculated to be 74 and 76% respectively in resting and in proliferating thymocytes (Table 4). Consequently 26 and 24% respectively of glutamate entered the citric acid cycle via the glutamate dehydrogenase reaction.

In proliferating cells 32% of the amount of glutamate converted into 2-oxoglutarate could be recovered as CO_2, and 61% as aspartate (Table 4). In resting cells 41% underwent oxidation to CO_2

Table 4. *Alterations of glutamine metabolism by incubated rat thymocytes during proliferation*

Freshly prepared rat thymocytes were incubated (2×10^8–5×10^8 viable cells) in the absence (resting cells) and presence of 100 μg of concanavalin A (mitogen-stimulated cells) at 37°C in 5ml of phosphate-buffered saline, pH 7.4, containing 2.5–4mM-[U-^{14}C]glutamine. Rat thymocytes cultured for 60h as described in the Materials and methods section (proliferating cells) were incubated (3×10^7–8×10^7 viable cells) under the same conditions. Rates of utilization (indicated by minus sign) or production are presented as means ± S.E.M. for n separate experiments. Statistical significance of difference between metabolic rates of mitogen-stimulated and resting cells, and proliferating and mitogen-stimulated cells (Student's t test, based on difference between means), is indicated by * ($P<0.01$) or ** ($P<0.001$).

Thymocytes	n	Rates of utilization or production (μmol/h per 10^{10} cells)					
		Glutamine utilized	Glutamate formed	Glutamine in aspartate	Glutamine in $^{14}CO_2$	Ammonia formed	Percentage of glutamine carbon in products
Resting cells	10	−32 ± 1.4	15 ± 2.2	10 ± 0.9	7 ± 0.4	37 ± 1.0	100
Mitogen-stimulated cells	11	−45** ± 0.8	22 ± 2.2	15* ± 0.7	9* ± 0.2	43 ± 2.7	102
Proliferating cells	17	−260** ± 17.7	169** ± 13.3	55** ± 2.5	29** ± 2.0	309** ± 27.9	97
Proliferating cells + 100 μg of concanavalin A	13	−284** ± 14	187** ± 13	56** ± 2.9	26** ± 1.8	302** ± 16.1	95

and 59% transamination to aspartate. In mitogen-stimulated thymocytes these proportions were 39% and 65% respectively (Table 4). The similar percentages obtained with all cells suggest that no significant qualitative changes of glutamine metabolism occur during proliferation. The results further indicate that glutamine in resting as well as in proliferating rat thymocytes significantly contributes to respiration. Measurements of incorporation of radioactivity into individual amino acids revealed that conversion of [U-^{14}C]glutamine into amino acids other than glutamate and aspartate can be neglected; moreover, release of ammonia and aspartate formation via transamination of oxaloacetate fully account for the nitrogen provided by glutamine and glutamate metabolism in resting and proliferating cells.

Effects of glucose, pyruvate and malate on glutamine metabolism by proliferating rat thymocytes

Addition of glucose (4mM) or malate (2mM) to the incubations strongly decreased the rates of glutamine metabolism and glutamate entry into the citric acid cycle. The pathways of glutamate metabolism, however, are affected in different ways (Table 5). In the presence of glucose, the proportion of glutamate transamination to aspartate is much decreased, and malate stimulated this proportion. Malate was used in these experiments, since Moreadith & Lehninger (1984) reported a pronounced decrease in aspartate production and a large formation of citrate and alanine when mitochondria of ascites-tumour cells were incubated with glutamine in the presence of external malate. In contrast, addition of malate to normal heart, liver or kidney mitochondria oxidizing glutamate caused a marked increase in the proportion of aspartate formation, just as in our experiments with proliferating thymoyctes (Table 5). All glutamate appears to be converted into 2-oxoglutarate, via aspartate aminotransferase when external malate is present. No accumulation of citrate could be detected, and oxidation of glutamate to CO_2 in the citric acid cycle beyond oxaloacetate was low when malate was added to the incubations (Table 5).

The low rate of aspartate formation from glutamine in the presence of glucose (8 ± 1.8 μmol/h per 10^{10} cells) determined enzymically could be confirmed by amino acid analyses measuring incorporation of radioactivity from [U-^{14}C]glutamine into aspartate (7 ± 1.1 μmol/h per 10^{10} cells). The marked decrease in aspartate formation was not compensated by a corresponding increase of glutamate oxidation to CO_2 by proliferating rat thymocytes. It therefore appears that in the presence of glucose a substantial amount of glutamate formed from glutamine is

Table 5. *Effects of glucose, pyruvate, malate and amino-oxyacetate on the metabolism of glutamine by incubated proliferating rat thymocytes*

Thymocytes cultured for 60h as described in the Materials and methods section were incubated (3×10^7–8×10^7 viable cells) for 60min in 2ml of incubation medium containing 2.5mM-[U-^{14}C]glutamine. Rates of utilization (indicated by minus sign) or production are presented as means ± S.E.M., with the numbers of separate experiments given in parentheses. Statistical significance of difference between control and experimental values (Student's *t* test, based on differences between means) is indicated by * ($P<0.01$), ** ($P<0.001$). Abbreviation: n.d. not determined.

Addition to incubations	Rates of utilization or production (μmol/h per 10^{10} cells)					
	Glutamine utilized	Glutamate formed	Aspartate formed	$^{14}CO_2$ formed	Alanine formed	Ammonia formed
None	−260 ± 17.7 (17)	169 ± 13.3 (17)	69 ± 3.1 (17)	143 ± 10 (17)	9 ± 1.6 (11)	309 ± 27.9 (17)
Glucose (4mM)	−164** ± 15.4 (13)	108** ± 8.3 (13)	8** ± 1.8 (13)	72** ± 4.8 (13)	21** ± 2.0 (12)	161** ± 14.7 (12)
Pyruvate (2mM)	−258 ± 20.9 (10)	204** ± 17.5 (10)	39** ± 8.4 (10)	150 ± 14.5 (10)	41** ± 2.2 (7)	281* ± 15.5 (10)
Malate (2mM)	−103** ± 16.6 (3)	48** ± 13.8 (3)	48** ± 4.8 (3)	100** ± 16.5 (3)	n.d.	97** ± 15.2 (3)
Amino-oxyacetate (2mM)	−127** ± 12.5 (3)	86** ± 9.9 (3)	2** ± 1.5 (3)	103** ± 10 (3)	n.d.	187** ± 27 (3)

used for other reactions than transamination to aspartate and oxidation to CO_2, e.g. nucleotide and/or protein synthesis. Consistent with this interpretation is the finding that only 86% of glutamine nitrogen could be accounted for by the production of glutamate, aspartate, alanine and NH_3. In the absence of glucose, glutamine seems to be used as energy source. When glucose is present in the incubations, glutamine in part also serves as precursor for biosynthetic processes.

The mutual effects of glucose and glutamine on their metabolic fates thus are mainly restricted to the rates of glutamine uptake and conversion into aspartate.

Amino-oxyacetate inhibited transamination of glutamate to aspartate almost completely, but did not cause complete oxidation of glutamate that entered the citric acid cycle to CO_2 (Table 5). In the presence of this inhibitor of aminotransferase nearly all glutamate has been converted into 2-oxoglutarate by glutamate dehydrogenase, as judged from the release of ammonia.

A surprising result is the finding that a relatively large amount of aspartate (69 μmol/h per 10^{10} cells) is produced from glutamine (Table 5), which has also been observed in lymphocytes by Ardawi & Newsholme (1983). The accumulation of aspartate might be the result of a high glutamate concentration, the limited availability of acetyl-CoA leading to an increase in oxaloacetate and the very high activity of glutamate–oxaloacetate aminotransferase present in both resting and proliferating thymocytes. In order to increase the supply of acetyl-CoA, pyruvate (2mM) was added to the incubations. Pyruvate addition indeed decreased the rate of aspartate formation, but, more pronounced, the rate of glutamate conversion into 2-oxoglutarate was decreased too (Table 5). As expected, the rate of glutamate oxidation to CO_2 was significantly increased in the presence of 2mM-pyruvate. However, no complete oxidative degradation of glutamate to CO_2 in the citric acid cycle could be achieved, although pyruvate decarboxylation provided sufficient acetyl-CoA (59 and 54 μmol/h per 10^{10} proliferating cells respectively in the absence and in the presence of 2mM-glutamine; Table 2) to convert all oxaloacetate derived from glutamate into citrate for further oxidation to CO_2. This observation and the fact that inhibition of transamination by amino-oxyacetate did not give complete oxidation of all glutamate that entered the citric acid cycle to CO_2 (Table 5) point to a regulation of glutamine and glutamate oxidation in rat thymocytes at the site of citrate synthase or subsequent reactions in the citric acid cycle.

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