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Rates of utilization and fates of glucose, glutamine, pyruvate, fatty acids and ketone bodies by mouse macrophages

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1. The concentrations of ATP and the ATP/AMP concentration ratios were maintained in thioglycollate-elicited mouse peritoneal macrophages incubated *in vitro* for 90 min in the presence or absence of added substrate: rates of glycolysis, lactate formation and glutamine utilization were approximately linear with time for at least 60 min of incubation. 2. The rate of oxygen consumption by macrophages was only increased above the basal rate (i.e. that in the absence of added substrate) by addition of succinate or pyruvate, or by addition of the uncoupling agent carboxyl cyanide *m*-chlorophenylhydrazone ('CCCP'); it was decreased by 75% by the addition of KCN. These findings suggest that metabolism of endogenous substrate can provide most, if not all, of the energy requirement of these cells, at least for a short period. 3. The rates of glucose and glutamine utilization by incubated macrophages were approx. 300 and 100 nmol/min per mg of protein respectively. A large proportion of the glutamine that is utilized is converted into glutamate and aspartate, and very little (perhaps < 10%) is oxidized. Similarly almost all of the glucose that is utilized is converted into lactate and very little is oxidized. This characteristic is similar to that of resting lymphocytes and rapidly dividing cells; in non-proliferating macrophages it may be a mechanism to provide precision in control of the rate of biosynthetic processes that utilize intermediates of these pathways, e.g. purines and pyrimidines for mRNA for the synthesis of secretory proteins and glycerol 3-phosphate for phospholipid synthesis for membrane recycling. 4. No utilization of acetoacetate or 3-hydroxybutyrate by macrophages was detected. In contrast, both butyrate and oleate were oxidized. The rate of [^{14}C]oleate conversion into $^{14}CO_2$ (1.3 nmol/h per mg of protein) could account for most of the oxygen consumption by incubated macrophages, suggesting that long-chain fatty acids might provide an important fuel *in situ*. This may be one explanation for the secretion of lipoprotein lipase by these cells, to provide fatty acids for oxidation from the degradation of local triacylglycerol.

INTRODUCTION

A detailed investigation of the maximum activities of key enzymes of some metabolic pathways in mouse macrophages demonstrated that the capacities for utilization of glucose, glutamine and fatty acids were relatively high, as high if not higher than those of rat lymphocytes (Newsholme *et al.*, 1986). The conclusion was that, in general, the metabolism of the macrophage may be similar to that of the lymphocyte. In view of the importance of these cells in the immune response, it was considered important to study the rates of utilization of glucose, glutamine, fatty acids, ketone bodies and the fates of these fuels in mouse macrophages.

MATERIALS AND METHODS

Animals

Elicited peritoneal macrophages were obtained from 12–16-week-old female mice of the C57 BL/6 strain, bred in the Sir William Dunn School of Pathology, Oxford.

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Mannheim, Lewes, Sussex, U.K., except for the following: asparaginase, L-alanine, acetoacetyl-CoA, and nigrosine were obtained from Sigma Chemical Co., Poole, Dorset, U.K.; scintillant was obtained from National Diagnostics, Somerville, NJ, U.S.A; hydrazine hydrate was obtained from BDH Chemicals, Poole, Dorset, U.K.; all inorganic reagents were obtained from Fisons Scientific Apparatus, Loughborough, Leics., U.K.; Repelcote was obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K.; and all radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Preparation of macrophages

Macrophages were prepared as previously described (Newsholme *et al.*, 1986). After removal from culture, the cells were resuspended in a small volume of chilled oxygenated phosphate-buffered saline, before addition to incubation media in a 10 ml Erlenmeyer flask.

Incubation of macrophages

Macrophages were incubated and rates of fuel utilization and end-product formation measured as described by Ardawi & Newsholme (1983) for lymphocytes, except for the following points: macrophages were preincubated in the absence of substrate for 15–20 min at 37 °C before addition of substrate; metabolites were determined with a Gilford Stasar III spectrophotometer; glutamine was determined as described by Windmueller & Spaeth (1974), except that asparaginase was dialysed for 48 h against four changes of potassium phosphate buffer, pH 6.6; lactate was determined by the method of Eagle & Jones (1978); $^{14}CO_2$ was collected as described

Table 1. Concentrations of adenine nucleotides in incubated peritoneal mouse macrophages

Macrophages were incubated for the times indicated at a density of approx. 3 mg of protein/ml: incubations were terminated as described in the Materials and methods section. Values are presented as means ± S.E.M.

Substrate added to incubation medium	Incubation time (min)	Concn. (nmol/mg of protein) ATP	ADP	AMP	Total adenine nucleotide	Ratio [ATP]/[AMP]
None	0	4.5±1.0	1.8±0.3	1.1±0.2	7.4	4.1
	30	5.6±0.1	2.1±0.4	1.0±0.2	8.7	5.4
	60	6.7±0.2	1.2±0.1	0.6±0.4	8.5	11.4
	90	6.9±0.1	1.3±0.4	0.7±0.5	8.9	9.8
2 mM-glutamine	0	4.5±1.0	1.8±0.3	1.1±0.2	7.4	4.1
	30	6.3±0.3	1.7±0.3	0.9±0.2	9.0	6.7
	60	7.3±0.3	1.8±0.5	0.8±0.2	9.9	9.2
	90	7.5±0.2	1.3±0.3	0.7±0.2	9.5	10.7

by Leighton *et al.* (1985); and ATP was determined by the method of Stanley & Williams (1969).

Expression of results

Rates of substrate utilization or product formation are expressed as nmol/min per mg of protein. Protein was assayed as described by Bradford (1976).

RESULTS

The concentrations of adenine nucleotides were measured in macrophages immediately after preparation for incubation and after 30, 60 and 90 min of incubation in the presence of no added substrate or 2 mM-glutamine. The concentrations of ATP and the ATP/AMP concentration ratios were rather low at zero time (immediately after preparation), but were increased at 30 and especially by 60 min of incubation (Table 1). These ATP/AMP concentration ratios were similar to those reported for incubated lymphocytes (Ardawi & Newsholme, 1983) and some other tissues, including mouse brain and rat liver (see Beis & Newsholme, 1975). This finding suggests that the incubated macrophages are biochemically viable: this is also supported by the findings that the rates of uptake of glucose, pyruvate and glutamine, under some conditions, are linear with time for 60 min incubation (see below).

Macrophages that had been cultured in air/CO_2 (19:1) utilized oxygen at a rate of almost 50 nmol/h per mg of protein in the absence of any added substrate, and this was decreased by about 75% in the presence of KCN (Table 2), indicating that most of the oxygen consumed required the participation of cytochrome oxidase and was not involved in production of toxic oxygen products. This is consistent with the finding that macrophages that are cultured at a high density lose some of their capacity to secrete toxic oxygen products (Johnston *et al.*, 1978). The addition of an uncoupling

Table 2. Oxygen consumption by isolated peritoneal mouse macrophages cultured in air and oxygen

Macrophages were cultured and prepared as described in the Materials and methods section. Culture in air was air/CO_2 (19:1), and culture in oxygen was O_2/CO_2 (19:1). O_2 consumption was measured polarographically by means of a Clark-type oxygen electrode. The basal rate was determined for every measurement before the addition of substrate, inhibitor or uncoupler. Macrophages at a density of 4–5 mg of protein/1.2 ml of buffer (pH 7.2–7.4) were incubated at 37 °C in a glass-jacketted vial, which was rapidly stirred for 15–20 min, during which time O_2 consumption was linear. Values of O_2 consumption are presented as means ± S.E.M., with the numbers of separate determinations given in parentheses. Significant difference (Student's *t* test) from basal O_2 consumption is indicated by: $*P < 0.05$, $\dagger P < 0.01$, $\dagger\dagger P < 0.001$. Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Additions to the incubations	Rate of O_2 consumption (nmol/h per mg of protein) Culture in air	Culture in oxygen
None	48.6±6.15 (12)	20.5±3.05 (8)
10 mM-succinate	71.8±9.1 (7)*	60.0±6.5 (4)††
10 mM-succinate, 2 mM-glutamate	75.3±10.2 (7)*	61.4±11.2 (4)†
10 mM-succinate, 2 mM-glutamine	–	71.7±13.3 (3)†
1 mM-pyruvate	71.0±8.1 (7)*	40.9±7.0 (4)*
2 mM-KCN	12.7±1.6 (4)††	–
8 μM-CCCP, 10 mM-succinate	95.8±12.5 (4)†	157±23.4 (4)††

Table 3. Rates of utilization of glucose and glutamine and of production of lactate, glutamate and aspartate by isolated incubated mouse peritoneal macrophages

Macrophages were incubated as described in the Materials and methods section for 60 min at a density of 3–6 mg of protein/ml. Rates are given as means ± S.E.M.; the number of separate experiments was at least four. A negative sign indicates utilization; differences in rates between incubations with glucose and glucose plus glutamine for glucose and glutamine utilization and aspartate production that are statistically significant (Student's t test) are indicated by $*P < 0.05$ or $\dagger P < 0.01$. Rates of lactate, glutamate and aspartate production in the presence of glucose, glutamine or both substrates have been corrected for endogenous (i.e. blank) rates of production.

Addition to incubation medium	Rates (nmol/h per mg of protein)				
	Glucose	Glutamine	Lactate	Glutamate	Aspartate
None	−0.1	−0.1	51.9±0.9	2.5±1.3	2.3±2.0
Glucose (5 mM)	−339±15.8	−14.0±5.75	634±22.2	25.8±1.80	22.4±10.9
Glutamine (2 mM)	−0.1	−102±5.0	62.4±5.4	99.0±9.5	33.9±1.9
Glucose (5 mM) plus glutamine	−285±14.8*	−62.3±11.5†	665±18.9	81.0±3.9	17.8±2.1†

agent (carbonyl cyanide *m*-chlorophenylhydrazone) doubled the rate of oxygen consumption (Table 2); even this latter rate is only 4% of that which would be expected if oxoglutarate dehydrogenase provided a quantitative index of the maximum flux through the tricarboxylic acid cycle, as it does for muscle tissue (see Newsholme & Paul, 1983). Of the various substrates that were added to the incubated macrophages [glucose (5 mM), pyruvate (1 mM), succinate (10 mM), glutamine (2 mM), glutamate (2 mM), alanine (2 mM), acetoacetate (3 mM), 3-hydroxybutyrate (3 mM), oleate (0.5 mM) and butyrate (0.5 mM)], only succinate or pyruvate significantly increased the rate of oxygen uptake, and addition of succinate plus glutamate or succinate plus glutamine did not increase the rate above that in the presence of succinate alone (Table 2). This suggests that metabolism of endogenous substrates can provide much, if not all, of the energy required by these cells, at least for a short period of incubation (15 min). Similar findings were obtained for macrophages that had been cultured in O_2/CO_2 (19:1), except that the rate of oxygen consumption in the absence of added substrate was lower (20.5 nmol/h per mg of protein) but was increased to a higher rate (157 nmol/h per mg of protein) by the addition of carbonyl cyanide *m*-chlorophenylhydrazone plus succinate (Table 2).

At an initial glucose concentration of 5 mM, glucose is used at a rate of more that 300 nmol/min per mg of protein by macrophages, and almost all of this glucose utilization can be accounted for as lactate (Table 3). This is considerably greater than the rate of glucose utilization by rat lymphocytes (48.5±4.6 nmol/h per mg of protein). The rates of glucose utilization and lactate production are approximately linear with time (Fig. 1). Glutamine is utilized at a rate of approx. 100 nmol/min per mg of protein by macrophages (Table 3). The rates of production of glutamate, lactate and aspartate are high; indeed, the total rate of formation of all three end-products is considerably higher than the rate of utilization of glutamine. This problem is discussed further (see below). In the presence of both glucose and glutamine, the rates of utilization of both substrates were decreased, in comparison with the rates with either substrate alone; this is in contrast with the findings with the lymphocytes, when both rates were higher in the presence of both substrates (see Ardawi & Newsholme, 1983). Also in contrast with lymphocytes, there were no detectable rates of utilization of either 3-hydroxybutyrate or acetoacetate (Table 3), and, not surprisingly therefore, there was no effect of acetoacetate on the rates of glucose utilization or lactate production (results not shown).

Since there is evidence that macrophages can take up protein from the medium (endocytosis) and that the fate of this protein is probably lysosomal digestion and subsequent utilization of the amino acids, it was decided to investigate the rates of utilization of glucose and glutamine in the absence of albumin in the incubation

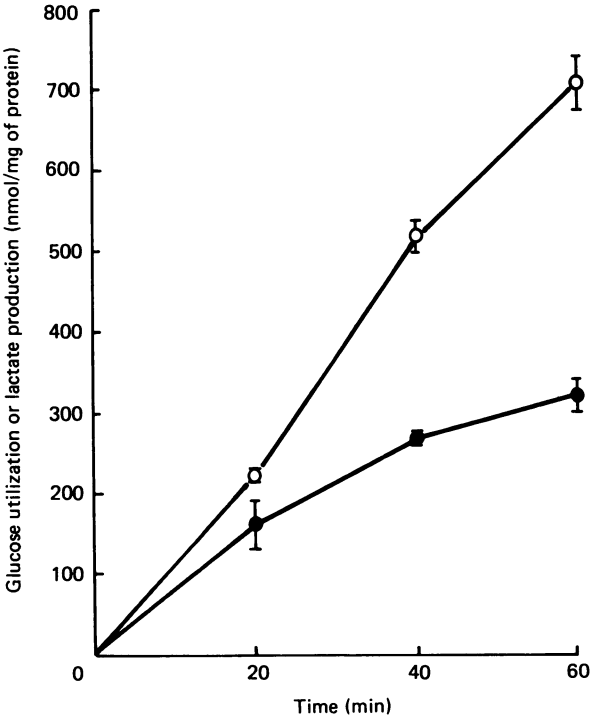

Fig. 1. Time course of glucose utilization (●) and lactate production (○) by incubated mouse macrophages

Incubations of macrophages were terminated at the times indicated, and glucose and lactate concentrations were measured. Initial concentration of glucose was 5 mM. Results are presented as means of four separate experiments: bars represent ± S.E.M. Cells were incubated at a density of 3–6 mg of protein/ml.

Table 4. Rates of utilization of glucose and glutamine and of production of lactate, glutamate and aspartate by mouse macrophages incubated with or without albumin

Macrophages were incubated as described in the Materials and methods section for 60 min at a density of 3–6 mg/ml. Rates are given as means ± S.E.M.; the numbers of experiments are given in parentheses. A negative sign indicates utilization; differences in rates between incubations without and with albumin (1.5%) that are statistically significant (Student's *t* test) are indicated by $*P < 0.05$ or $\dagger P < 0.01$.

Addition to incubation medium		Rates (nmol/h per mg of protein)				
Fuel	Albumin (1.5%)	Glucose	Glutamine	Lactate	Glutamate	Aspartate
Glucose (5 mM)	–	−355 ± 25.8 (4)	–	632 ± 17 (4)	–	–
	+	−325 ± 19.6 (4)	–	637 ± 48 (4)	–	–
Glutamine (2 mM)	–	–	−186 ± 8.2 (7)†	33.0 ± 1.3 (7)†	137 ± 6.4 (7)†	25.5 ± 2.8 (7)*
	+	–	−102 ± 5.0 (7)	62.4 ± 5.4 (7)	99 ± 9.5 (7)	34.0 ± 1.9 (7)
Glucose (5 mM) plus glutamine (2 mM)	–	−288 ± 18.4 (4)	−130 ± 28.0 (4)	659 ± 10.0 (4)	104 ± 5.2 (4)*	18.5 ± 3.5 (4)
	+	−282 ± 26.1 (4)	−65 ± 11.5 (4)	671 ± 38.2 (4)	81 ± 3.9 (4)	17.8 ± 2.1 (4)

medium. This manipulation had no effect on the rates of glucose utilization or lactate formation, but it changed glutamine metabolism. Thus, in the absence of albumin, the rate of glutamine utilization was almost 100% higher (Table 4), and there were small differences in the rates of formation of glutamate and aspartate in comparison with those in the presence of albumin. Furthermore, in the absence of albumin, the rate of glutamine utilization was almost linear with time for 60 min, whereas, in the presence of albumin, it reached a plateau after 40 min (Fig. 2): it is likely that, at 40 min, amino acid production from albumin degradation is making a quantitatively significant contribution to metabolism. Interestingly, the rate of glutamate production is higher in the absence of albumin, but there was little difference in the rate of aspartate production (Fig. 2).

The rate of oxidation of [^{14}C]oleate was measured by monitoring the production of $^{14}CO_2$ by incubated macrophages. The effect of concentration of oleate on the rate of oxidation is shown in Fig. 3: at a concentration of albumin of 2.5% (w/v), the optimal concentration of oleate was found to be 0.3 mM and, in contrast with lymphocytes and other tissues (see Ardawi & Newsholme, 1985), the rate of oxidation decreased dramatically as the concentration of oleate increased to 1 mM. The rate of oxidation at 0.3 mM-oleate was 1.3 nmol/h per mg of protein (Table 5). Since 1 nmol of oleate requires 26 nmol of O_2 for complete oxidation, this suggests that fatty acid could account for the basal rate of O_2 consumption (see Table 2), and therefore long-chain fatty acids might represent a major fuel for macrophages *in vivo*. This finding might provide an explanation for the ability of macrophages to secrete large quantities of lipoprotein lipase (Khoe *et al.*, 1981), since, in the absence of other fuels such as glucose or glutamine, the release of fatty acids from any triacylglycerol in the medium surrounding the macrophage could provide an immediate fuel for these cells. It should be noted that the rate of uptake of oleate by macrophages (Lokesh & Wrann, 1984) is considerably higher than that reported to be oxidized in the present work: much of that taken up by the cells is incorporated into cellular lipids.

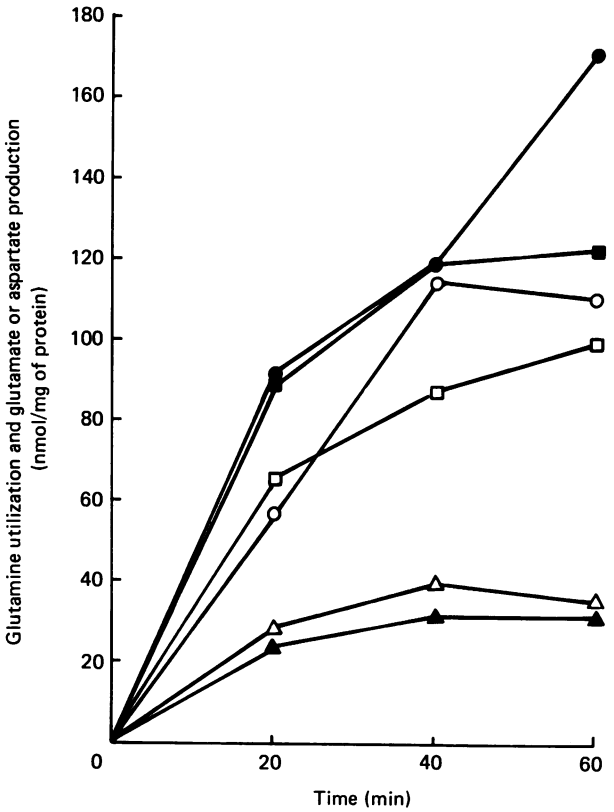

Fig. 2. Time course of glutamine utilization (○, ●) and glutamate (□, ■) and aspartate (△, ▲) production by incubated mouse macrophages

Incubations of macrophages were terminated at the times indicated, and glutamine, glutamate and aspartate concentrations were measured as described in the Materials and methods section. Initial concentration of glutamine was 2 mM; ○, △, □, incubations in the presence of 1.5% (w/v) albumin in the incubation medium; ●, ▲, ■, incubations in the absence of albumin. Cells were incubated at a density of 3–6 mg of protein/ml. Results are present as the means of four separate experiments: S.E.M. values are not given, but they are less than 10% of means.

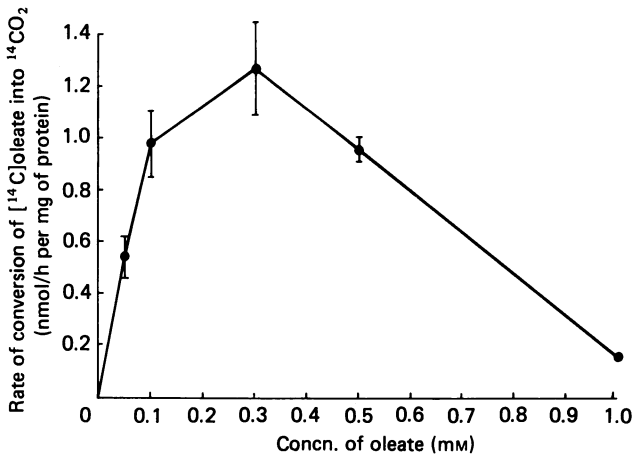

Fig. 3. Effect of concentration of [1-^{14}C]oleate on rate of $^{14}CO_2$ production by incubated mouse macrophages

Macrophages were incubated and $^{14}CO_2$ was collected and measured as described in the Materials and methods section. Results are means of at least four separate experiments: bars represent ± S.E.M. Cells were incubated at a density of 3–6 mg of protein/ml.

The rates of oxidation of glucose, glutamine, pyruvate and butyrate have been investigated by measuring the production of $^{14}CO_2$ from ^{14}C-labelled substrates. The results suggest that, of the glucose or glutamine utilized by the cells, only a small proportion is oxidized (about 3% of the glucose and 10% of glutamine) (Table 5). Stubbs *et al.* (1973) have shown that, for the $^{14}CO_2$ produced from [U-^{14}C]glucose, very little was obtained via the tricarboxylic acid cycle. Since conversion of glutamine into aspartate involves the oxoglutarate dehydrogenase reaction, which releases CO_2, this suggests that very little, if any, of the glutamine carbon enters the tricarboxylic acid cycle via acetyl-CoA; that is, the metabolism of glutamine is only partial. A similar conclusion has been made regarding the metabolism of lymphocytes (see Ardawi & Newsholme, 1985). In contrast, for butyrate, a high proportion of that which is utilized is oxidized (Table 5). An interesting observation was made concerning the rates of oxidation of [1-^{14}C]- and [3-^{14}C]-pyruvate: almost 60% of the [1-^{14}C]pyruvate that was utilized appeared as $^{14}CO_2$, whereas for the [3-^{14}C]pyruvate only about 15% of that utilized appeared as $^{14}CO_2$. Since [1-^{14}C]pyruvate loses its carbon as $^{14}CO_2$ in the pyruvate dehydrogenase reaction, whereas [3-^{14}C]pyruvate loses its carbon as $^{14}CO_2$ in the tricarboxylic acid cycle, this finding is consistent with the suggestion of a low rate of utilization of pyruvate via the cycle. We cannot rule out decarboxylation of labelled oxaloacetate via phosphoenolpyruvate carboxykinase, giving rise to $^{14}CO_2$ (via pyruvate carboxylase and randomization of label in the tricarboxylic acid cycle), but this process will occur irrespective of the original position of ^{14}C in the pyruvate used in these experiments; consequently the marked difference between the rates of $^{14}CO_2$ production from [1-^{14}C]pyruvate and [3-^{14}C]pyruvate suggests a fate for acetyl-CoA produced from pyruvate other than oxidation in the tricarboxylic acid cycle. This fate remains to be discovered.

Table 5. Rates of utilization and oxidation of glucose, glutamine, pyruvate, butyrate and oleate by isolated incubated mouse peritoneal macrophages

Macrophages were incubated for 60 min at a density of 3–6 mg/ml, and rates of utilization and oxidation were measured as described in the Materials and methods section. Results are presented as means ± S.E.M. for at least four separate experiments.

Substrate	Concn. (mM)	Rates (nmol/h per mg of protein) Utilization	Oxidation
[U^{14}C]Glucose	5	339 ± 15.8	10.7 ± 1.5
[U-^{14}C]Glutamine	2	102 ± 5.0	9.4 ± 0.45
[1-^{14}C]Pyruvate	1	73.4 ± 0.8	42.7 ± 2.7
[3-^{14}C]Pyruvate	1	73.4 ± 0.8	11.2 ± 2.6
[1-^{14}C]Butyrate	0.5	3.4 ± 1.60	1.5 ± 0.70
[1-^{14}C]Butyrate	0.25	1.0 ± 0.39	1.3 ± 0.52
[1-^{14}C]Oleate	0.3	–	1.3 ± 0.18

DISCUSSION

It has been known for many years that rapidly dividing cells convert glucose into lactate at a high rate, and that very little of the glucose is oxidized (see Warburg, 1956*a*,*b*). A similar finding was observed for resting lymphocytes (which have the potential for rapid cell division) (Roos & Loos, 1973; Hume *et al.*, 1978; Ardawi & Newsholme, 1983). More recently it has been found that rapidly dividing cells, including tumour cells (see Lund, 1980; Krebs, 1980; Kovacevic & McGivan, 1983) and resting lymphocytes (Ardawi & Newsholme, 1983), utilize glutamine at a high rate and, since the glutamine oxidation is also only partial, the process has been termed glutaminolysis (McKeehan, 1982). A hypothesis to explain the high rates of both glycolysis and glutaminolysis in rapidly dividing cells, including tumour cells, fibroblasts and enterocytes, and in those cells with the potential for rapid cell division such as lymphocytes, has been put forward (Newsholme *et al.*, 1985*a*,*b*). The hypothesis proposes that the high rates of these pathways provide ideal conditions for precise regulation of the rates of processes that utilize intermediates of these pathways for biosynthesis; these intermediates include glucose 6-phosphate (for ribose phosphate formation) and glycerol 3-phosphate (for phospholipid synthesis) in glycolysis, and glutamine (for purine and pyrimidine synthesis) and aspartate (for purine and pyrimidine synthesis) in glutaminolysis. The present work has shown similar high rates of glycolysis and glutaminolysis in mouse peritoneal elicited macrophages, which are terminally differentiated and have little or no ability to proliferate. Nonetheless, we believe that the above hypothesis still applies to macrophages. These cells are characterized by high rates of protein secretion and membrane recycling, for which high rates of protein and lipid biosynthesis will be required. The high rate of protein synthesis will require high rates of mRNA synthesis, for which purines, pyrimidines and ribose phosphate will be required, and the lipid synthesis will require glycerol 3-phosphate. Thus it is concluded that high rates of glycolysis and glutaminolysis will characterize not only proliferating cells and those which have the

potential for rapid proliferation, but also cells which are characterized by high rates of mRNA synthesis and/or phospholipid synthesis. This extension of the hypothesis may also explain the high rate of glutamine utilization by enterocytes: although all of these cells have usually been included in the classification of rapidly dividing, Hanson & Parsons (1987) have pointed out that it is only a small proportion of the enterocytes, those in the crypts of the villi, that are so characterized. However, most, if not all, of the enterocytes will synthesize and secrete various proteins (e.g. digestive enzymes, mucin) so that, similarly to macrophages, high rates of mRNA synthesis may also be a characteristic of these cells. This would require precision in the control of the rates of purine and pyrimidine synthesis and hence, according to the hypothesis, high rates of glutaminolysis.

It is of considerable interest that high rates of glutamate (and also aspartate) production occurs in the macrophages in the absence of added glutamine; this suggests that albumin, which is present in the incubation medium, may be taken up by these cells by endocytosis, and the amino acids produced by the degradation of this protein may well be transaminated to produce glutamate from 2-oxoglutarate. If glutamine is an essential compound for the protein-secretory activity of macrophages (in a similar manner to the requirement of glutamine for proliferation of lymphocytes; Ardawi & Newsholme, 1983), it may be of considerable importance to the functioning of the immune system that these cells can utilize protein in the surrounding medium, to obtain glutamine and other amino acids, if and when these amino acids are decreased to very low concentrations, for example in localized areas of infection.

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