Biochem. J. (1989) 261, 979–983 (Printed in Great Britain)

The effect of glutamine concentration on the activity of carbamoyl-phosphate synthase II and on the incorporation of [^{3}H]thymidine into DNA in rat mesenteric lymphocytes stimulated by phytohaemagglutinin

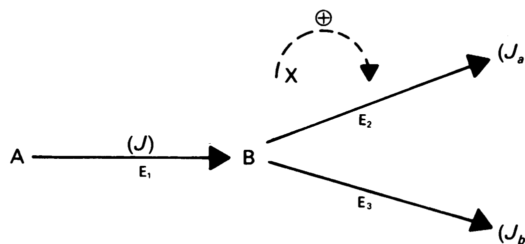
Zsussa SZONDY and Eric A. NEWSHOLME
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

The maximum catalytic activities of carbamoyl-phosphate synthase II, a limiting enzyme for pyrimidine nucleotide synthesis, are very much less than those of glutaminase, a limiting enzyme for glutamine utilization, in lymphocytes and macrophages; and the flux through the pathway for pyrimidine formation *de novo* is only about 0.4% of the rate of glutamine utilization by lymphocytes. The K_m of synthase II for glutamine is about 16 μM and the concentration of glutamine necessary to stimulate lymphocyte proliferation half-maximally is about 21 μM. This agreement suggests that the importance of glutamine for these cells is provision of nitrogen for biosynthesis of pyrimidine nucleotides (and probably purine nucleotides). However, the glutamine concentration necessary for half-maximal stimulation of glutamine utilization (glutaminolysis) by the lymphocytes is 2.5 mM. The fact that the rate of glutamine utilization by lymphocytes is markedly in excess of the rate of the pathway for pyrimidine nucleotide synthesis *de novo* and that the K_m and 'half-maximal concentration' values are so different, suggests that the glutaminolytic pathway is independent of the use of glutamine nitrogen for pyrimidine synthesis.

INTRODUCTION

Previous work from this laboratory has shown that the rate of glutamine utilization by isolated incubated mesenteric lymphocytes is very high and that the oxidation is only partial (Ardawi & Newsholme, 1983, 1985). The significance of a high rate of glutaminolysis has been considered previously for rapidly dividing cells, including tumour cells (see Krebs, 1980; McKeehan, 1982; Kovacevic & McGivan, 1983). Two suggestions have been made: first, the pathway provides a considerable amount of ATP, so that glutaminolysis might be primarily important for energy formation in these cells; second, the pathway provides intermediates for the biosynthesis of purine and pyrimidine nucleotides, which are essential for the cell cycle. However, there are difficulties with both these explanations. First, if energy generation in itself were important, it might be expected that more of the carbon of the glutamine would be fully oxidized (e.g. via the complete tricarboxylic acid cycle). Second, calculation of the rate of the synthesis of purines and pyrimidine nucleotides, in order to double the contents of the DNA and RNA in the cell, indicates that this rate is very small, perhaps $< 0.1\%$ of the rate at which glutamine is utilized by these cells (see Newsholme *et al.*, 1987). Consequently, a new hypothesis has been proposed which suggests that a high rate of glutaminolysis is important to provide optimal conditions for the regulation of the rates of synthesis of purine and pyrimidine nucleotides at specific times during the cell cycle.

It can be shown that if a metabolic influx, J, divides into two fluxes J_a and J_b, and J_a is regulated by factor X, the highest sensitivity of flux J_a to changes in the regulator X is achieved when $J_b \gg J_a$ (see Scheme 1). [In non-mathematical terms, high sensitivity is achieved because the rate of the biosynthetic pathway (that is, J_a) can be increased markedly without decreasing significantly the concentration of the metabolic intermediate(s) of the main pathway (B), which would 'oppose' the stimulation of the biosynthetic pathway.] The glutaminolytic pathway exhibits a high rate and the pathway provides precursors for biosynthetic pathways (see above), but the fluxes through these biosynthetic pathways are very small in comparison to the flux through glutaminolysis. However, the flux through the biosynthetic pathways will



Scheme 1. Regulation of a flux through a branch in a branched pathway

The overall response of J_a to X is most effective when J_b is much greater than J_a: that is, when J_a is much smaller than the total flux, J. Under these conditions the flux does not become seriously limited by changes in precursor concentrations during the 'deflection' of flux from J_b to J_a and is therefore most sensitive to action of regulator X (see Newsholme *et al.*, 1985*a*,*b*; Crabtree & Newsholme, 1985).

Abbreviation used: CPS II, carbamoyl-phosphate synthase II.

increase considerably during the synthesis of DNA and RNA. Since provision of these intermediates at a precise time of the cell cycle is very important, the sensitivity of the biosynthetic processes to their specific regulators would need to be very high. To understand the full advantage of this mechanism, it is important to appreciate that the source of the glutamine for use by these cells is muscle (Newsholme *et al.*, 1988). This means that, for an increased activity of the immune system, more glutamine is required by these cells (see Ardawi & Newsholme, 1985), so that muscle will need to form and release glutamine at a higher rate. How would the muscle 'know' that the immune system had increased its functional activity? The simplest means of information transfer between the two tissues is a feedback-control mechanism. However, even if a sensitive feedback mechanism were developed to link the immune-system cells with the muscle, it could only operate by providing a higher concentration of glutamine in the bloodstream, and this would increase the rate of utilization of this compound by other tissues.

Although at first sight this may appear to be a wasteful mechanism, it provides a simple and effective control system that can permit macromolecular synthesis, and hence cell division, to occur whenever required for essential purposes in the animal: cell division in lymphocytes is an essential component of the immune system.

The hypothesis predicts that the K_m of the key regulatory enzyme for pyrimidine synthesis would be low, and probably lower than that of glutamine utilization, to ensure that any small variations in the glutamine concentration would not affect markedly the rate of pyrimidine synthesis. Secondly, if glutamine utilization is important to provide optimal conditions for proliferation, it would be expected that rate of proliferation would be dependent on the glutamine concentration, and the 'K_m' for proliferation might be expected to be similar to that of the key biosynthetic enzymes.

Consequently, two kinds of experiments have been carried out. First, the kinetic properties of the key regulatory enzyme in the pathway of pyrimidine synthesis *de novo*, carbamoyl-phosphate synthase II (CPS II; EC 6.3.5.5), have been measured in extracts of lymphocytes. Second, the concentration of glutamine necessary for the half-maximal stimulation of the rate of DNA synthesis in phytohaemagglutin-stimulated lymphocytes has been studied by monitoring the incorporation of [^{3}H]thymidine into DNA. The concentration of glutamine required for half-maximal stimulation of DNA synthesis can then be compared with the K_m of CPS II for glutamine.

MATERIALS AND METHODS

Animals

Male Wistar albino rats (200–220 g) were obtained from Batin and Kingman, Grimston, Hull, U.K.

Chemicals and enzymes

All chemicals and enzymes were obtained from Boehringer Corp. (London), U.K., except for the following: glycerol, scintillants and all inorganic compounds were obtained from Fisons Scientific Apparatus, Loughborough, Leics, U.K.; phytohaemagglutinin, bovine serum albumin, ornithine carbamoyltransferase, $MgCl_2$ solution and amino acids were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Hepes-buffered RPMI solution that did not contain glutamine and the standard glutamine solution were obtained from Flow Laboratories, Irvine, Scotland, U.K. All radiochemical compounds were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.: [^{3}H]thymidine, 2 Ci/mmol; $NaH^{14}CO_3$, 20 mCi/mmol.

Preparation of lymphocytes and macrophages

Lymphocytes were prepared from rat mesenteric lymph nodes as described by Ardawi & Newsholme (1983). Cells were suspended in phosphate-buffered saline (Culvenor & Weidemann, 1976) that had been oxygenated for 30 min with 100% O_2. For cell-culture experiments, the cells were prepared under sterile conditions and suspended in a Hepes-buffered RPMI solution which did not contain glutamine (5×10^5 cells/200 μl well). Elicited peritoneal macrophages were obtained from 12–16-week-old female mice of TC C57 BL/C strain bred in the Sir William Dunn School of Pathology, Oxford.

Incubation procedures

Incubations were performed at 37 °C in 10 ml silicone-treated Erlenmeyer flasks in a total volume of 1.0 ml of phosphate-buffered saline (Ardawi & Newsholme, 1983) which had been oxygenated with 100% O_2 for 30 min, and which was supplemented with 5% (w/v) defatted bovine serum albumin at pH 7.2. The incubation medium contained 2 mM-glutamine when glutamine utilization was to be measured, or 2 mM-glutamine, 15 mM-$KHCO_3$ and 40 μCi of $NaH^{14}CO_3$ when the rate of pyrimidine synthesis was to be measured. Each incubation contained 5×10^7 cells and the incubation time was 60 min; the gas phase was 100% O_2. The reaction was stopped by addition of 200 μl of ice-cold 25% (w/v) $HClO_4$.

Determination of rates of glutamine utilization and glutamate plus aspartate production

The concentrations of metabolites in the medium were determined spectrophotometrically by enzymic methods, by measuring the differences in the absorption of NAD^+/NADH in a 1.0 cm light-path cell containing the reaction mixture. Glutamine was measured by the method of Windmueller & Spaeth (1974).

Determination of the rate of pyrimidine synthesis

The rate of pyrimidine synthesis was measured by monitoring the rate of ^{14}C incorporation from $H^{14}CO_3$ into uridine nucleotides. Uridine nucleotides from the acid-soluble cell fraction were converted into UMP by heating to 100 °C for 10 min in acid (Crandall *et al.*, 1978); this nucleotide was isolated by the crystallization technique described by Crandall *et al.* (1978), and the radioactivity was measured in a liquid-scintillation counter (Beckman LS 7500).

Cell culture

Lymphocytes were cultured in wells of micro-titre culture plates (approx. 5×10^5 cells in 100 μl of medium were present in each 200 μl well) in a Hepes-buffered RPMI medium containing 10% (v/v) foetal-calf serum, 100 units of streptomycin/ml, 200 units of penicillin/ml and various concentrations of glutamine plus 10 μg of phytohaemagglutin/well. (The foetal-calf serum was

Table 1. Values of $V_{max.}$ and K_m for CPS II from lymphocytes, bone marrow, brain, liver and macrophages

Results are presented as means ± S.D. for tissue obtained from at least four separate animals, or four separate pools of tissue from a greater number of donor animals.

		Properties of CPS II				
		$V_{max.}$ (nmol/h per mg of protein)	K_m (μM)			
Animal	Tissue		Glutamine	NH_3	MgATP	HCO_3^-
Rat	Lymph node	6.3 ± 0.64	16.5 ± 0.3	17200	1300	10200
	Bone marrow	4.0 ± 0.34	16.5 ± 0.3	11500	1200	2100
	Brain	0.8 ± 0.13	18.0 ± 0.5	14300	1300	2100
Mouse	Macrophage	6.9 ± 0.45	–	–	–	–

dialysed against phosphate-buffered saline, to remove glutamine and other small molecules.) The cultures were incubated at 37 °C in an atmosphere of 5% CO_2 in air. [^{3}H]Thymidine (0.2 μCi) was added to each 200 μl well at various time points of the culture (see the Figures), and the proliferation response was measured from the incorporation over a period of 18 h. Cultures were then harvested on to glass-fibre filters with an automated cell harvester. The filters were washed and dried: the radioactivity on the dried filters was measured in a liquid-scintillation counter (Beckman LS 7500).

CPS II assay

Lymph node, bone marrow, brain or macrophages (Newsholme *et al.*, 1986) were homogenized in 4 vol. of 150 mM-potassium phosphate containing 30% (v/v) dimethyl sulphoxide, 5% (w/v) glycerol and 1 mM-dithiothreitol, pH 7.4, with a 1 ml hand-held Potter–Elvehjem-type homogenizer. The enzyme activity was determined in the 100000 ***g*** supernatant fraction as described by Aoki *et al.* (1982). For studying the properties, the supernatant was filtered on a Sephadex G-25 column. For liver, CPS II was separated from CPS I as described by Aoki *et al.* (1982). Protein was determined by the method of Bradford (1976) with globulin as a standard.

Expression of results

For the incorporation of [^{3}H]thymidine into DNA in Table 2 and Figs. 1, 2 and 3, incorporation is expressed as c.p.m. incorporated per micro-titre well. Preliminary experiments established that this rate of incorporation was directly and linearly related to the increase in cell number over 72 h of culture.

Table 2. Effect of glutamine concentration on the incorporation of ^{3}H from [^{3}H]thymidine into DNA in phytohaemagglutinin-stimulated rat mesenteric lymphocytes

Lymphocytes were cultured at various concentrations of glutamine: after 48 h [^{3}H]thymidine was added to culture medium and incorporation into DNA was measured 18 h later. Lymphocytes (5×10^5) were cultured in the presence of different concentrations of glutamine for 48 h. Results are presented as means ± S.D. for three or four separate incubations.

Glutamine concn. (mM)	[^{3}H]Thymidine incorporation (c.p.m.)
0	586 ± 206
0.005	1660 ± 1135
0.01	2032 ± 355
0.03	3733 ± 674
0.05	4886 ± 837
0.07	6172 ± 325
0.09	7351 ± 1820
0.1	7804 ± 1740
0.3	9608 ± 998
0.5	5566 ± 2480
0.7	6643 ± 1335
0.9	5300 ± 1092
1.0	6355 ± 2610
3.0	4041 ± 377
5.0	4129 ± 294
7.0	2950 ± 805

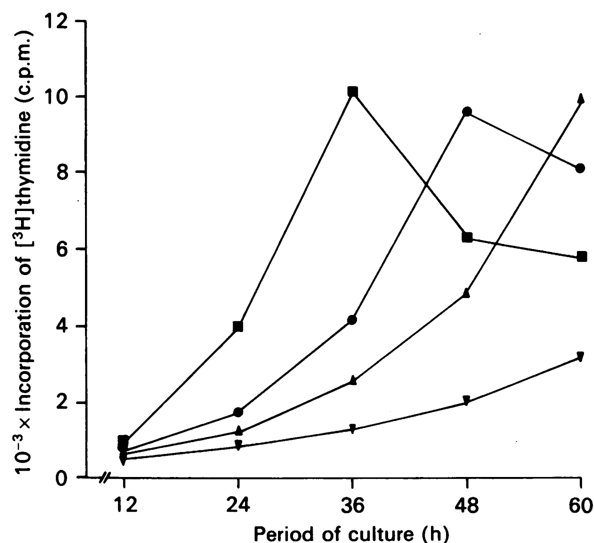


Fig. 1. Incorporation of [^{3}H]thymidine into the DNA of phytohaemagglutinin-stimulated lymphocytes at different concentrations of glutamine at various times of culture

Lymphocytes (approx. 5×10^5 cells) were cultured in wells of micro-titre culture plates. After the times indicated, [^{3}H]thymidine was added and the ^{3}H incorporated into DNA was measured after a further 18 h in culture. The concentrations of glutamine present at the beginning of the culture were as follows: ▼, 0.01 mM; ▲, 0.05 mM; ●, 0.3 mM; ■, 1.0 mM.

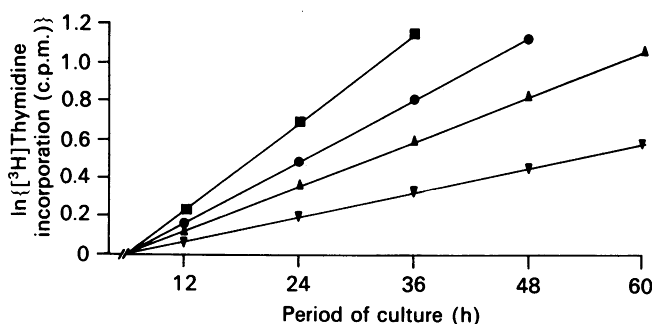


Fig. 2. Plot of logarithm of [^{3}H]thymidine incorporation into DNA of phytohaemagglutinin-stimulated lymphocytes against period of culture at different concentrations of glutamine

The data in Fig. 1 are plotted as logarithm of incorporation against period of culture [the values presented are log (c.p.m. for glutamine added) minus log (c.p.m. when no glutamine added)].

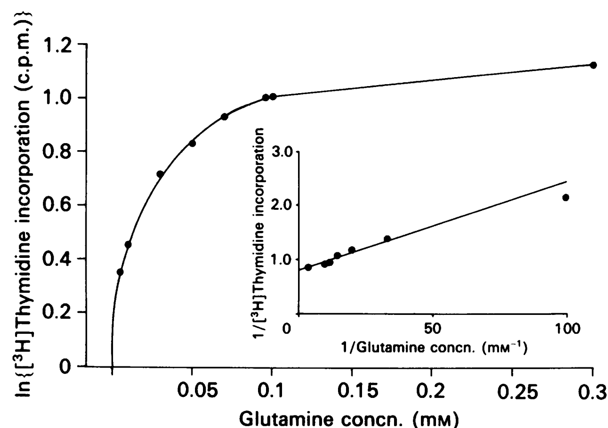


Fig. 3. Plot of logarithm of rate of incorporation of [^{3}H]thymidine into DNA in phytohaemagglutinin-stimulated lymphocytes against concentration of glutamine in culture medium, and (inset) double-reciprocal plot of logarithm of rate of incorporation against glutamine concentration

Conditions and experiments as described in the legend to Fig. 1, except that lymphocytes were cultured for 48 h before addition of [^{3}H]thymidine. The values presented are log (c.p.m. for glutamine added) minus log (c.p.m. when no glutamine added).

RESULTS AND DISCUSSION

Enzyme activities and rate of synthesis of pyrimidine nucleotides

The pathway of pyrimidine biosynthesis *de novo* provides nucleotides for both DNA and RNA synthesis, and is particularly important during cell division. In mammalian tissues, CPS II is considered to catalyse a key regulatory step for the pathway for pyrimidine synthesis *de novo* (Ito & Uchino, 1973; Jones, 1980). The activity of this enzyme has been reported for human, but not rat, lymphocytes (Ito & Uchino, 1973). In the present work, the maximum activity of the enzyme in rat lymphocytes was found to be 6.3 nmol/h per mg of protein; similar values were found in bone marrow, liver and macrophages, but the activity in rat brain was only about 10% of this value (Table 1). These activities are very much lower than the activities of glutaminase in these tissues [0.2% and $<0.1\%$ for lymphocytes and macrophages respectively; see Newsholme *et al.* (1986) for the latter activities].

To compare these enzyme activities with the flux in the pyrimidine nucleotide biosynthetic pathway, the rate of incorporation of ^{14}C from $H^{14}CO_3^-$ into uridine nucleotides was measured in incubated lymphocytes: it was found to be 0.08 ± 0.005 nmol/h per mg of protein. This is only about 1.5% of the maximum activity of CPS II; it is also only about 0.04% of the rate of glutamine utilization for cells under similar conditions, which in the present work was found to be similar to that reported by Ardawi & Newsholme (1983), i.e. about 223 nmol/h per mg of protein.

These findings confirm the view that the rate of glutamine utilization by resting lymphocytes is greatly in excess of either the rate or the capacity of the pathway for pyrimidine synthesis *de novo*.

Values of K_m for CPS II and concentration of glutamine which stimulates DNA synthesis half-maximally

CPS II utilizes glutamine as a co-substrate for the provision of nitrogen for the formation of carbamoyl phosphate. The K_m values for glutamine, and also for HCO_3^-, ammonia and MgATP, for the enzyme from several tissues are given in Table 1. The K_m value for glutamine for the enzyme from lymphocytes is 16 μM, which is similar to the values for the enzyme from bone marrow, brain and liver. It is noteworthy that values of K_m for MgATP, NH_3 and HCO_3^- are approx. 100-fold greater (Table 1). Since the concentration of glutamine in lymphocytes is normally similar to that of ATP or HCO_3^- (i.e in the mM range), the low K_m may be of some significance in relation to pyrimidine nucleotide synthesis, since the enzyme would normally be expected to approach saturation with its co-substrate glutamine, so that small variations in the glutamine concentration would not cause major changes in the enzyme activity.

The effect of glutamine concentration in the culture medium on the rate of incorporation of [^{3}H]thymidine into DNA after stimulation of phytohaemagglutin in culture over a 48 h period was studied. The results confirm those reported by Ardawi & Newsholme (1983), that is, there is an optimum concentration of about 0.3 mM-glutamine over this time period (Table 2). A plot of the rate of incorporation of [^{3}H]thymidine into DNA versus glutamine concentration is sigmoid, and the double-reciprocal plot is concave upwards (results not shown). From a Hill plot [(log of incorporation)/(log of maximum incorporation minus log of incorporation) versus glutamine concentration], the concentration of glutamine that gives 50% stimulation of incorporation ($s_{0.5}$), under these conditions, is approx. 55 μM.

Effect of glutamine concentration on rate of [^{3}H]thymidine incorporation over different time periods

To study this problem further, a time course (up to 60 h) of the effect of the concentration of glutamine in the culture medium on the rate of incorporation of [^{3}H]thymidine into DNA was investigated. The results are presented in Fig. 1: at each concentration of glutamine, the incorporation against time was non-linear (exponential). However, the plot was linear when the logarithm of incorporation was plotted against time at each glutamine concentration (Fig. 2). This is charac-

teristic of cells that are growing at a constant rate: the time between cell divisions is constant, and hence the incorporation is exponential; the linearity with time when plotted as a logarithm is expected from these characteristics of growth and division of cells (see Wilkinson, 1972). The results presented in both plots demonstrate that the rate of incorporation of thymidine into DNA is increased markedly as the concentration of glutamine is increased over the range 0.01–1 mM.

When the linear rates of [^{3}H]thymidine incorporation are plotted against glutamine concentration as a double-reciprocal plot (Fig. 3), the plot is linear and the K_m value for glutamine is 21 μM, which is almost identical with the K_m for CPS II for glutamine (16 μM; Table 1). This agreement supports the view that one important role for the glutamine-utilization pathway in lymphocytes is provision of nitrogen for the '*de novo*' pyrimidine pathway.

However, the K_m of the glutamine-utilization pathway for extracellular glutamine in intact lymphocytes during incubation is about 100-fold greater (it is about 2.5 mM; Ardawi & Newsholme, 1983). This difference suggests that the process of glutamine utilization is separate from, and apparently independent of, the role of glutamine in nitrogen provision for purine and pyrimidine synthesis. This independent yet inter-related function of glutaminolysis is totally consistent with the proposed branched-chain sensitivity role for the pathway (see the Introduction), since it only requires that the rate of glutaminolysis is well in excess of the rate of biosynthesis of purine and pyrimidine. The greater the rate of glutaminolysis, the greater the sensitivity of the biosynthetic processes, but the branch-point sensitivity is unrelated to the K_m of utilization. It is also consistent with the proposed role of glutaminolysis in provision of ATP for these cells. However, the latter role for glutaminolysis appears to be unlikely, since these cells are known to be able to oxidize fatty acids, ketone bodies, pyruvate and glucose, all of which could contribute to ATP generation.

If the K_m for the role of glutamine in provision of nitrogen for pyrimidine, and hence DNA and RNA, synthesis is about 20 μM, then, to obtain a rate of DNA synthesis approaching maximal, the concentration of glutamine would need to be greater than 200 μM (a concentration of substrate 10-fold higher than the K_m provides 91% of the maximum if the response is hyperbolic). This value is similar to the optimal concentration required for incorporation of [^{3}H]thymidine into DNA (300 μM; Table 2). The concentration of plasma glutamine in normal animals is about 0.6 mM, which will ensure that sufficient glutamine is available to provide for near-maximal activity of pyrimidine synthesis if required. However, it suggests that a marked decrease in the plasma glutamine concentration (as occurs in injury, sepsis or burns in man; see Askanazi *et al.*, 1980) could interfere in the response of the immune system to a challenge, since this response depends, in part, on the proliferation of lymphocytes. This suggestion is further supported by the observation that the rate of incorporation of [^{3}H]thymidine into DNA in lymphocytes is lower if the concentration of glutamine in the culture medium is below 1 mM (Fig. 2). The rate of response of the lymphocytes to an infection, for example, could be of importance in controlling the extent of an infection and therefore the survival of the host. The fact that the plasma concentration of glutamine is the highest of all the amino acids and that skeletal muscle, a major site of production of glutamine, responds to infection by increasing the rate of release of glutamine (Stjernstrom *et al.*, 1986; Ardawi, 1988; Parry-Billings *et al.*, 1989) is consistent with the above findings and conclusions concerning the important role of this amino acid in the response of lymphocytes to an immune challenge (Newsholme *et al.*, 1987).

We thank Dr. Simon Hunt for generous provision of laboratory facilities for this work. Z.S. gratefully acknowledges a Scholarship from the Soros Foundation.

REFERENCES

Aoki, T., Morris, H. P. & Weber, G. (1982) J. Biol. Chem. **257**, 432–438

Ardawi, M. S. M. (1988) Clin. Sci. **74**, 165–172

Ardawi, M. S. M. & Newsholme, E. A. (1983) Biochem. J. **212**, 835–842

Ardawi, M. S. M. & Newsholme, E. A. (1985) Essays Biochem. **21**, 1–44

Askanazi, J., Carpentier, Y. A., Michelsen, O. V., Elwyn, D. H., Furst, P., Kantrowitz, L. R., Gump, F. E. & Kinney, J. M. (1980) Ann. Surg. **182**, 78–85

Bernt, E. & Bergmeyer, H.-U. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.), 2nd edn., pp. 1704–1708, Academic Press, London and New York

Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254

Crabtree, B. & Newsholme, E. A. (1985) Curr. Top. Cell. Regul. **25**, 21–76

Crandall, D. E., Lovatt, C. J. & Tremblay, G. C. (1978) Arch. Biochem. Biophys. **188**, 194–206

Culvenor, S. G. & Weidemann, M. J. (1976) Biochim. Biophys. Acta **437**, 354–363

Ito, K. & Uchino, H. (1973) J. Biol. Chem. **248**, 389–392

Jones, M. E. (1980) Annu. Rev. Biochem. **49**, 253–279

Kovacevic, Z. & McGivan, J. D. (1983) Physiol. Rev. **63**, 547–605

Krebs, H. A. (1980) in Glutamine: Metabolism, Enzymology and Regulation (Mora, J. & Palacios, R., eds.), pp. 319–329, Academic Press, New York

McKeehan, E. L. (1982) Cell Biol. Int. Rep. **6**, 635–650

Newsholme, E. A., Crabtree, B. & Ardawi, M. S. M. (1985*a*) Biosci. Rep. **4**, 393–400

Newsholme, E. A., Crabtree, B. & Ardawi, M. S. M. (1985*b*) Q. J. Exp. Physiol. **70**, 473–489

Newsholme, P., Curi, R. & Newsholme, E. A. (1986) Biochem. J. **239**, 121–125

Newsholme, E. A., Newsholme, P. & Curi, R. (1987) Biochem. Soc. Symp. **54**, 145–162

Newsholme, E. A., Newsholme, P., Curi, R., Challoner, E. & Ardawi, M. S. M. (1988) Nutrition **4**, 261–268

Parry-Billings, M., Leighton, B., Dimitriadis, G., De Vasconcelos, P. R. L. & Newsholme, E. A. (1989) Int. J. Biochem. **21**, 419–423

Stjernstrom, H., Lund, J., Wiklund, L., Bergholme, U., Vinnars, E., Hamgerger, B. & Jorfeldt, L. (1986) Clin. Nutr. **5**, 123–131

Wilkinson, J. F. (1972) Introduction to Microbiology, pp. 47–50, Blackwell Scientific Publications, Oxford

Windmueller, H. G. & Spaeth, A. E. (1974) J. Biol. Chem. **249**, 5070–5071

Received 20 December 1988/22 March 1989; accepted 21 April 1989