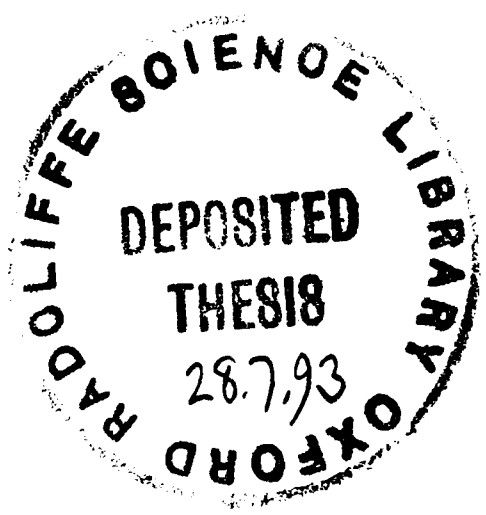
To my Mother and Father,
for all their love and support.

Studies of glutamine metabolism in skeletal muscle and other tissues

A thesis submitted to the University of Oxford in fulfilment of the requirements for the Degree of Doctor of Philosophy.

by
Elizabeth I. C. Opara

Hilary Term 1993



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Abstract

Cells of the immune system, both resting and stimulated, utilize glutamine at a high rate (see Ardawi 1983 and Newsholme et al 1985). Skeletal muscle is considered to be an important source of glutamine for cells of the immune system (see Newsholme and Parry-Billings 1990). It is proposed that the process of glutamine release from skeletal muscle is the flux-generating step for the pathway of glutamine utilization by cells of the immune system, and that, control of glutamine release from skeletal muscle influences the rate of glutamine utilization by cells of the immune sytem, and hence the function of these cells.

Studies using an *in vitro* skeletal muscle (soleus) preparation, identifying factors/conditions (e.g. branched chain amino acids, dexamethasone and hyper-thyroidism) that modify both the rate of glutamine release from muscle and muscle glutamine concentration, illustrated that glutamine release from muscle is independent of the intracellular glutamine concentration (Parry-Billings 1989). However, the rate of glutamine release from muscle over time was non-linear, which may have lead to misinterpretation of the results reported by Parry-Billings (1989). Investigation of the linearity of the rate of glutamine release from soleus muscle over time, in the present study, using the *in vitro* muscle preparation, has shown that non-linearity of the rate of glutamine release is due to the presence of an artefact in the muscle incubation medium, rather than the process of muscle glutamine release.

With the "correction" of the linearity for muscle glutamine release, a re-investigation of the effect(s) of factors (e.g. branched chain amino acids) on the rate of glutamine release from muscle (soleus and extensor digitorum longus) and muscle glutamine concentration verified that the process of glutamine release from muscle is independent of the intracellular glutamine concentration. The changes in the concentration of glutamine in muscle caused by these factors suggests that they may affect the process of muscle glutamine synthesis. Quantitative analysis of muscle glutamine release and concentration data plus a

study of the effects of factors on the maximal activity of muscle glutamine synthetase confirmed this.

Results of the study on the effect(s) of factors on muscle glutamine synthesis, release, and concentration are discussed in relation to a systematic approach used for a study of the control of metabolic pathways (see Newsholme and Crabtree 1979) and are found to be consistent with the predictions made using this systematic approach.

The possibility that other tissues, namely rat adipose tissue and immune cells of the rat, are able to provide glutamine, was investigated by determining whether these tissues possess glutamine synthetase activity and thus synthesize glutamine. The present study indicates that, although rat adipose tissue possesses a maximal glutamine synthetase activity that is approximately 6-7 times greater than that of muscle, on a whole body (200g rat) basis, rat adipose tissue may not be a significant contributor of glutamine. In contrast, a study by Frayn et al (1991) suggests that human adipose tissue may be a significant contributor of glutamine. For cells of the immune system, the present study indicates that immune cells possess glutamine synthetase activity and thus can synthesize glutamine. This ability to synthesize glutamine may be of physiological significance since it is suggested that glutamine synthetase in cells of the immune system may provide enough glutamine to maintain some immune cell function during conditions, for example following surgery, injury and during sepsis, in which the plasma glutamine concentration is decreased.

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Finally, to my Mother and Father, many thanks, from a loving daughter, and to my brothers John and Eddy – Cheers! from your crazy big sister.

Abbreviations

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
BSA	bovine serun albumin
c.p.m.	counts per minute
Con A	concanavalin A
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetic acid
HCl	hydrochloric acid
HEPES	hydroxyethylpiperazine ethanesulphonic acid
Km	Michaelis Constant
LDH	lactate dehydrogenase
LPS	lipolysaccharide
mRNA	messenger ribonucleic acid
MSO	methionine sulphoximine
NAD+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide (reduced)
NAD[P]+	nicotinamide adenine dinucleotide phosphate
PBS	phosphate-buffered saline
Pi	inorganic phosphate
PWM	pokeweed mitogen
RNA	ribonucleic acid
S-IgA	secretory immunoglobulin A
TCA	tricarboxylic acid cycle
TRIS	tris(hydroxymethyl-amino-ethane)
Vmax	maximum catalytic activity
[^{3}H]	tritium ("labelled") hydrogen

Enzymes mentioned in text and their enzyme commission (E.C.) classification numbers.

Enzyme.	E.C Number
Adenosine monophosphate deaminase	3.5.4.6
Adenosine triphosphatase.	3.6.1.3.
Alanine dehydrogenase.	1.4.1.1
Asparaginase	3.5.1.3.
Aspartate aminotransferase	2.6.1.1.
Carbamoyl-phosphate synthase	2.7.2.9.
Creatine kinase	2.7.3.2.
Glutamine 2-oxoacid amino transferase	2.6.1.15.
[Glutamine aminotransferase K]	
[Glutamine aminotransferase L]	
Glutamate dehydrogenase	1.4.1.3.
Glutaminase	3.5.1.2.
Glutamine synthetase	6.3.1.2.
Lactate dehydrogenase	1.1.1.28.
Leucine aminotranferase	2.6.1.6.
Malate dehydrogenase NAD^+ linked	1.1.1.37.
Malate dehydrogenase $NADP^+$ linked 'Malic enzyme"	1.1.1.40.
Phosphoenolpyruvate carboxykinase	4.1.1.49.
Pyruvate kinase.	2.7.1.40.
Valine aminotransferase	2.6.1.32.

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Chapter 1

General introduction.

1. A brief background of glutamine.

Since its isolation in the late 19th century, by Schulze and Bossard (1883, cited in Meister 1956), the amino acid glutamine has been subject to continued interest. Numerous studies have shown that the occurrence of glutamine in nature in the free state is widespread. The occurrence of free glutamine in certain plants has been known for many years (see Meister 1956 and Krebs 1980) and it has been suggested that glutamine may be closely involved with vital activities, such as respiration and photosynthesis, in plants (Steward and Street 1947).

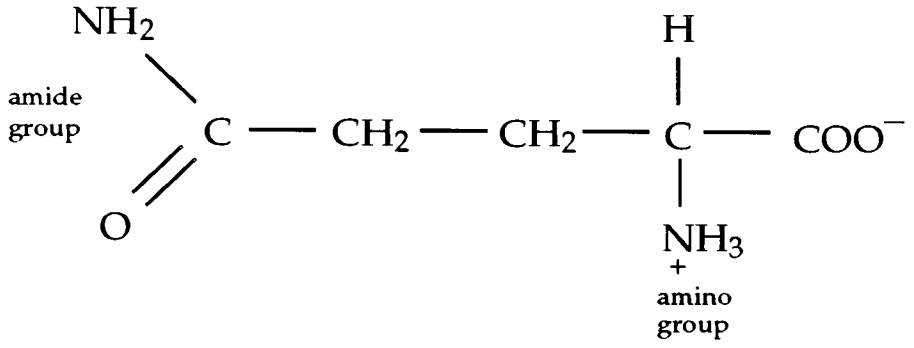
Glutamine is also distributed in animal body fluids: in human blood it is the most abundant amino acid, making up approximately 25% of the total amino acid population: its concentration approaches 1mM and this is two-fold greater than any other amino acid (see Meister 1956 and Askanazi et al 1980). Cerebrospinal fluid contains about as much glutamine as does the blood (Meister 1956): glutamine represents nearly 70% of the total free amino acids present in the cerebrospinal fluid (Alfredsson et al 1988).

Krebs et al (1949), reported that glutamine is present in liver, spleen, kidney, brain, lung, heart, pancreas, adipose, intestines and muscle of various mammals. The largest pool of glutamine exists in skeletal muscle: in skeletal muscle of rat and man, glutamine is present at concentrations of 5.5 and 20 mmoles/L intracellular water, respectively (Bergstrom et al 1974, Albina et al 1986). With the exclusion of taurine, glutamine constitutes more than 50% of total free amino acid pool in skeletal muscle (Askanazi et al 1980).

1.1.Structure and properties.

Glutamine is the amide of the acidic amino acid glutamate. It is a neutral amino acid which possesses two nitrogen-containing groups: an amino group and an amide group (see structure below). Although this amino acid is neutral it possesses a polar side chain which facilitates hydrogen bonding which increases its solubility in water.

Glutamine



Glutamine is unstable in acidic and alkali conditions (Datta and Ottaway 1969) and at high temperatures (Orekes and Kupfer 1967) and it is slowly degraded at pH 7.0 at 37°C (see Souba et al 1985).

1.2.Key enzymes of glutamine metabolism.

Glutamine can be transported across the membranes of a number of different cells including intestinal epithelial cells (Bradford and McGivan 1982), hepatocytes (Joseph et al 1978, Kilberg et al 1980), lymphocytes (Ardawi 1983), and muscle cells (Hundal et al 1987). The characteristics of glutamine transport (glutamine uptake) into intestinal epithelial cells, hepatocytes, lymphocytes and muscle cells are similar: the process of glutamine transport into these cells is an active process that utilizes the energy of the sodium gradient that exist across the cells, thus making the process of glutamine transport into these cells sodium-dependent (see Bradford and McGivan 1982, Joseph et al 1978, Kilberg et al 1980, Ardawi 1983, Hundal et al 1987). The process of glutamine transport into intestinal epithelial cells, hepatocytes and lymphocytes is pH dependent (Bradford

and McGivan 1982, Joseph et al 1978, Kilberg et al 1980, Ardawi 1983). However, that of muscle is not (Hundal et al 1987).

Cells or tissues that contain glutamine possess the principal enzymes of either glutamine synthesis i.e., glutamine synthetase or of glutamine utilization i.e., glutaminase. Glutamine synthetase was first isolated from sheep brain by Pamiljans et al (1962). It is a cytosolic enzyme that catalyses the conversion of glutamate and ammonia to glutamine (Reaction 1). Glutamine synthetase requires ATP and a divalent cation, either Mg^{2+}, Mn^{2+} or Co^{2+} for its action. This enzyme is inhibited by ADP and methionine sulphoximine.

$$\text{Glutamate} + \text{ATP} + NH_3 \longrightarrow \text{Glutamine} + \text{ADP} + \text{Pi}$$

(Reaction 1)

The reaction of glutaminase (reaction 2) was first demonstrated by Krebs (1935) in rabbit and guinea pig kidney. Glutaminase is a mitochondrial enzyme and it requires inorganic phosphate and ammonium ions for its action (Curthoys et al 1984).

$$\text{Glutamine} + H_2O \longrightarrow \text{Glutamate} + NH_3 \quad \text{(Reaction 2)}$$

Tissues or cells are either predominantly glutamine producers and so therefore have high glutamine synthetase activities, for example skeletal muscle (Rowe 1985), brain (Pamiljans 1962) and lung (Meister 1984, Ardawi 1990), or they are predominantly glutamine consumers and therefore have high glutaminase activities, for example intestine (Windmueller and Spaeth 1974, 1978, 1980, Windmueller 1982) and cells of the immune system (Ardawi and Newsholme 1982, 1983; Newsholme P. et al 1986) (see Table 1). An exception to this is the liver which can be both a significant producer or consumer of glutamine. Whether it behaves as a

producer or consumer depends upon the conditions: in healthy dogs and rats, liver consumes glutamine in the post-absorptive state (Souba and Wilmore 1983, Austgen et al 1991) however, during critical illness there is a marked change in hepatic glutamine metabolism i.e. liver releases glutamine. This change in hepatic glutamine metabolism is said to "reflect the liver's attempt to adjust glutamine heterogeneity in extra- hepatic tissues" (see Austgen et al 1991, Welbourne 1990).

Table 1: glutamine synthetase and glutaminase activities in various mammalian tissues.

	Glutamine synthetase Activity (nmoles/min/mg of prot)	Glutaminase
Tissues		
Skeletal muscle	1.8	5.67(nmoles/min/g)
Brain	13.5	-
Lung	31.7	18.7
Liver	14.3	13.4
Adipose	~10	39
Adipocytes	~20	-
Kidney	4.6	60
Intestines	-	83
Lymphocytes	-	38.4
Macrophages	-	152

Data derived from: Rowe (1985), Ardawi (1990), Cooney et al (1986) Budohoski et al (1982), Windmueller (1982), Miller (1975), Ardawi and Newsholme (1983), Newsholme P. et al (1986), Wu et al (1991).

Some tissues possess high glutamine synthetase and high glutaminase activity. Adipose tissue and adipocytes are reported to possess high glutamine synthetase (Miller 1975) and high glutaminase activities (Cooney et al 1986, Kowalchuk et al 1988) (see Table 1). However, it is unclear whether adipose tissue is predominantly a consumer or producer of glutamine.

2.The roles of glutamine in mammalian tissue: a brief review.

Glutamine is widely distributed in mammalian tissue. Krebs et al (1949) concluded that the ubiquitous presence of glutamine in a variety of mammalian tissue suggests that glutamine may have a important metabolic role in these tissues. It was already known that glutamine serves as a constituent of protein and also that it plays a role in the detoxification of phenylacetic acid (see Meister 1956). Since then, glutamine has been identified as having unique metabolic functions which are of metabolic importance.

2.1.Muliple functions of glutamine in the mammalian body.

1. Discoveries by Van Slyke et al (1943) and Pitts et al (1965) in dog, Squires et al (1976) in rat, and Owen and Robinson (1963) in man established glutamine as an important source of ammonia in acidosis. In addition studies have reported that in renal acidosis, renal uptake and utilization of glutamine are markedly increased (Hems 1972; Goldstein 1986). Thus collectively, both groups of studies have established the importance of glutamine in the regulation of acid-base balance.

2. In the late 1940s and 1950s further metabolic functions of glutamine came to light; it was found that glutamine is an amino group donor for nucleic acid precursors (Roberts and Borges 1955,

Rabinovitz et al 1956) and is also a precursor of amino sugars (see Meister 1956).

3. In the early seventies, studies by Marliss et al (1971), Krebs (1970) and Aoki et al (1972) on amino acid metabolism in skeletal muscle indicated that glutamine acts as a carrier of nitrogen between tissues.

4. Studies in the 1970s and 1980s have shown that glutamine acts as a source of energy, via its oxidation, for certain tissues. These studies also indicate that glutamine may be a source of energy for, the epithelium of the small intestines (Neptune 1965, Windmueller and Spaeth 1974, 1975, 1980), malignant cells (Lavietes et al 1974, Kovacevic and Morris 1972), fibroblasts (Donnelly and Scheffler 1976) and cells of the immune system (Ardawi and Newsholme 1982, 1983; Newsholme P. et al 1986, 1987).

5. Studies indicate that glutamine may be involved in the regulation of muscle protein metabolism. MacLennan et al (1987) and Jepson et al (1988) have demonstrated a negative correlation between muscle glutamine concentration and rates of protein degradation in muscle.

6. Studies suggest that glutamine is involved in the regulation of ammonia levels in the brain (Cooper 1988). Recently, Haussinger (1990) proposed that glutamine is involved in the regulation of ammonia levels in the liver.

The next section of this general introduction will concentrate on glutamine metabolism in cells of the immune system and skeletal muscle, since the studies described in this thesis are mainly concerned with glutamine metabolism in these tissues.

3.Glutamine metabolism in cells of the immune system.

Cells of the immune system, such as lymphocytes and macrophages, play an important role in the immune response. The functions of these cells during an immune response have been extensively characterized (see Roitt 1980). Basically, during an immune response, both lymphocytes and macrophages undergo increased rates of production, recruitment and alterations in function. Mature lymphocytes recirculate through lymphoid tissues via blood and lymph in a relatively quiescent state until stimulated to proliferate during for example, a bacterial or viral infection. Macrophages are terminally differentiated end-cells in which the ability to proliferate is gradually lost.

Relative to the amount known about the function of cells of the immune system during an immune response, little was known about the metabolism of these cells, such as the fuels used by the cells for their function, the rate of utilization of these fuels, and the fate of these fuels. In addition, little was known as to whether a change in the metabolism of cells of the immune system alters the function of these cells during an immune response. The main reason for this was that cells of the immune system were considered to be metabolically "quiescent" or "resting" and even after stimulation, when energy is required for their function, were considered to be metabolically uninteresting since they appeared to depend primarily on glucose for the formation of ATP (see Hume and Weidemann 1980).

The observation that malignant cells exhibit high rates of glucose and glutamine utilization (Warburg 1926, Roberts and Borges 1955) and the suggestion that these two processes may be important for cell growth (see McKeehan 1982), led to the investigation of the utilization of a number of fuels; glucose, glutamine, long-chain fatty acids (oleate and palmitate), and ketone bodies (3-hydroxybutyrate and acetoacetate) in lymphocytes, both

rat and human (Ardawi and Newsholme 1982, 1983; Ardawi 1983, 1988b) and mouse macrophages (Newsholme et al 1986, 1987). Investigations involved measurements of maximal activities of key enzymes involved in the metabolism of glucose, glutamine, ketone bodies and fatty acids, the rate of utilization, fates of these fuels, and the effect of these fuels on oxygen consumption by lymphocytes and macrophages. These studies indicated that both lymphocytes and macrophages utilize a number of fuels, and that of these fuels glutamine appears to be the most important.

3.1.Glutaminase activity and glutamine utilization in cells of the immune system.

The evidence that glutamine is important for cells of the immune system was initially suggested by the high activity of a key enzyme of glutamine utilization, glutaminase: Ardawi and Newsholme (1982), Ardawi (1988b) and Newsholme P. et al (1986) showed that lymphocytes, both rat and human, and mouse macrophages possess high glutaminase activity (see Table 2). In addition Ardawi and Newsholme (1982) demonstrated, that during an *in vivo* immunological response, a graft versus host reaction, rat lymphocyte glutaminase activity is increased by approximately 30%. As indicated by the high activity of glutaminase in cells of the immune system, the rate of glutamine utilization by lymphocytes and macrophages is also high (Ardawi and Newsholme 1983, Ardawi 1988b, Newsholme P. et al 1987) and is increased after an immunological challenge: stimulation of lymphocytes in culture, both rat and human, using a mitogen, conconavalin A (con A), increased the rate of glutamine utilization by rat and human lymphocytes (Ardawi and Newsholme 1983, Ardawi 1988b) (see Table 2).

Table 2: glutaminase activity and the rate of glutamine utilization in cells of the immune system.

	Glutaminase Activity	Rate of glutamine utilization (nmoles/min/mg of prot)	
Cells			
		Control	ConA
Rat Lymphocytes	39.4	3.7	5.6
Human Lymphocytes	38	3.2	4.5
Mouse Macrophages	152	1.7	-

Data derived from Ardawi (1983), Ardawi and Newsholme (1982,1983), Ardawi (1988b), Newsholme P. et al (1986,1987).

The importance of glutamine for immune cell function was highlighted by studies in which the availability of glutamine was limited. Ardawi and Newsholme (1983) demonstrated that the mitogen-stimulated proliferation of rat lymphocytes *in vitro* (as measured by [^{3}H]thymidine incorporation into DNA) is dependent on glutamine concentration, reaching a maximum at a glutamine concentration of 0.3mM, and the rate of rat lymphocyte proliferation being lowest when the culture medium is glutamine free; the addition of other amino acids or ammonia could not overcome the low rate of lymphocyte proliferation when the culture medium was glutamine free. The effect of limited availability of glutamine on immune cell function has also been demonstrated for

human lymphocyte proliferation and mouse macrophage phagocytosis *in vitro* (Parry- Billings et al 1990c).

The association of reduced glutamine levels *in vivo* with immunosuppression has been demonstrated. The concentration of glutamine available to cells of the immune system can be decreased *in vivo* by administering asparaginase, which catalyses the hydrolysis of asparagine to aspartate, but can also catalyse the hydrolysis of glutamine to glutamate (Holcenberg et al 1975). Asparagine has been shown to be essential for cells of acute lymphocyte leukaemia (Kafkewitz and Bendich 1983). Thus, asparaginase has been used in the treatment of acute lymphocyte leukaemia. However, this treatment has been shown to cause immune suppression in mice (Brambilla et al 1970): treatment of mice with asparaginase resulted in a decrease in spleen weight, a decrease in immunoglobulin bearing lymphocytes (B cells), a loss of thymus weight and a decrease in thymus cell number. In man asparaginase treatment caused a poor response of blood lymphocytes to mitogen phytohaemaglutinin (PHA), inhibited the appearance and proliferation of antibody-forming cells, and caused a delay in serum antibody production *in vivo* (Kafkewitz and Bendich 1983). Simberkoff and Thomas (1970) showed that the effect of asparaginase can be reversed by the addition of glutamine.

Malignant cells possess high glutaminase activity and utilize glutamine at a high rate (Warburg 1926, Robert and Borges 1955). In addition glutaminase activity and the rate of glutamine utilization in lymphocytes taken from sepsis-induced rats is markedly increased (Ardawi et al 1991). These high rates of glutamine utilization are reflected in decreased plasma glutamine concentrations measured in tumour bearing rats and sepsis-induced rats (Klimberg et al 1990a and Parry-Billings 1989 respectively) which may lead to immunosuppression in tumour bearing

rats (Kweon et al 1991) and during sepsis (Meakins et al 1977). The administration of glutamine to tumour bearing rats (Kweon et al 1991) and septic rats (Yoshida et al 1992) has been shown to be beneficial for macrophage function *in vivo* and lymphocyte proliferation *in vitro*, respectively. Kweon et al (1991) demonstrated that, in glutamine-fed tumour-bearing rats macrophage, phagocytosis was significantly higher than that of tumour-bearing rats not fed glutamine. Yoshida et al (1992) demonstrated that, in septic rats fed glutamine, lymphocyte proliferation was higher than for lymphocytes from septic rats not receiving glutamine.

These studies all highlight the importance of glutamine for immune cell function.

3.2.The pathway of glutamine utilization by cells of the immune system.

The pathway of glutamine utilization by cells of the immune system has been termed "glutaminolysis" because glutamine is only partially oxidized. This partial oxidation also occurs in other rapidly dividing cells such as tumour cells and fibroblasts (see McKeehan 1982). A pathway for glutamine metabolism in cells of the immune system has been proposed by Newsholme et al (1985) who measured maximal enzyme activities and end products of the utilization of both labelled and unlabelled glutamine in rat lymphocytes *in vitro*. (see Figure 1) The first reaction of glutaminolysis is the formation of glutamate and ammonia via the action of glutaminase. A small percentage of glutamate is then metabolised by transamination to 2-oxoglutarate which is converted via a span of the tricarboxylic acid (TCA) cycle to malate, which can be converted directly to pyruvate via the action of malate dehydrogenase [$NAD(P)^+$-linked], or oxaloacetate via the action of malate dehydrogenase [NAD^+-linked]. Oxaloacetate is then converted, via phosphoenolpyruvate carboxykinase,

to phosphoenolpyruvate and then to pyruvate (Ardawi and Newsholme 1983). Pyruvate (derived directly from malate, or via oxaloacetate) can be oxidized after conversion to acetyl coenzyme A however, it is mostly metabolised to lactate, or converted to oxaloacetate, via pyruvate carboxylase and subsequently to aspartate (Curi et al 1988). Glutamate is also transaminated via aspartate aminotransferase, to aspartate. Glutamate, ammonia and aspartate are major end products of glutamine utilization in lymphocytes.

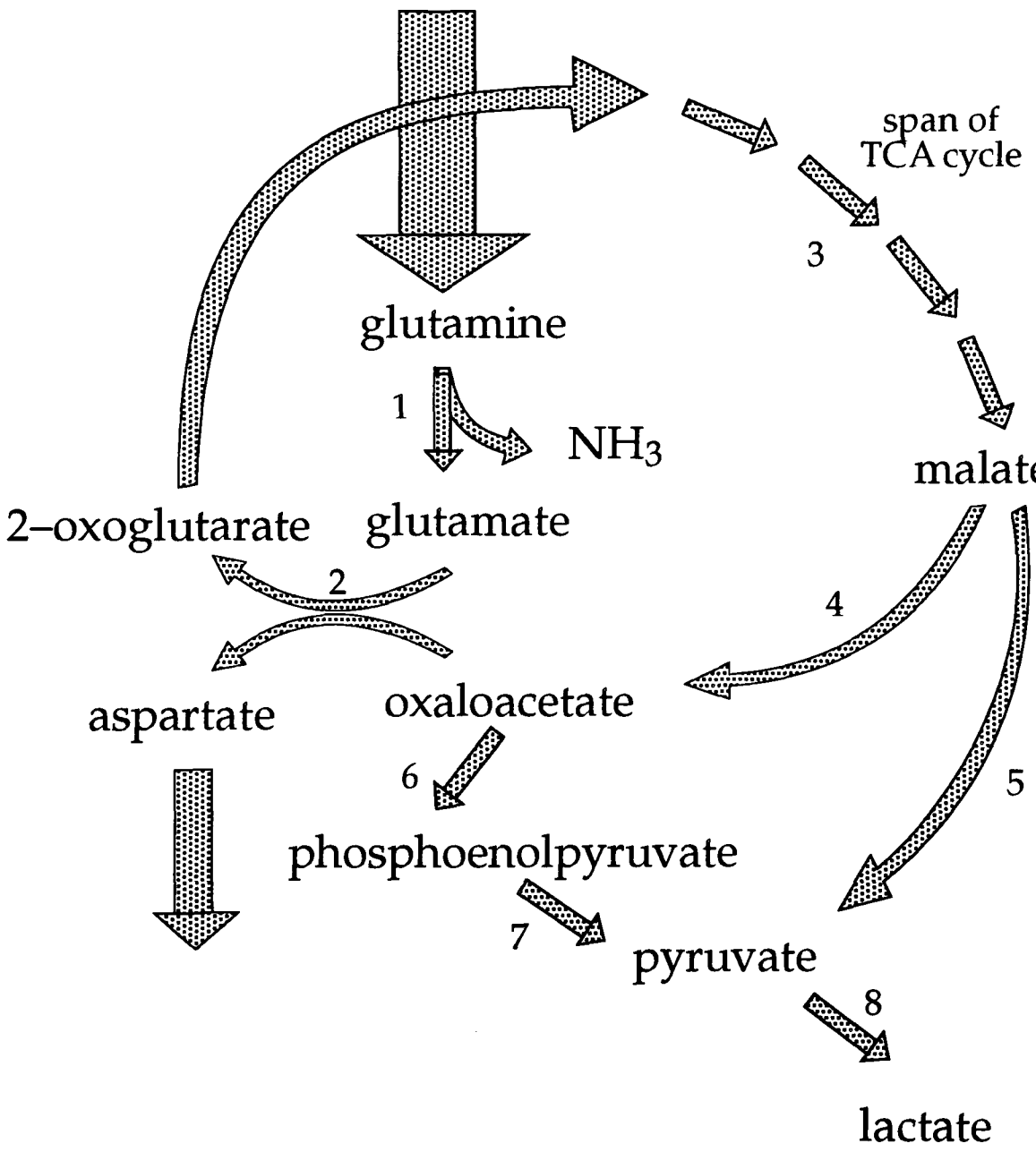


Figure 1. The proposed pathway of glutamine metabolism in cells of the immune system. The reactions are as follows; 1. glutaminase, 2. aspartate aminotransferase, 3. enzymes of the Krebs cycle converting 2-oxoglutarate to malate, 4. malate dehydrogenase (NAD+-linked), 5. malic enzyme [NAD(P)+-linked], 6. phophoenolpyruvate carboxykinase, 7. pyruvate kinase, 8. lactate dehydrogenase. (Taken from Newsholme et al 1985).

3.3.Role of glutamine metabolism in cells of the immune system.

Ardawi (1988b) showed that glutamine provides a large proportion (37%) of the energy requirement of lymphocytes, suggesting that glutamine is an important source of energy for cells of the immune system. However, the role of glutamine as an important fuel for cells of the immune system may not be the main reason for the high rate of glutaminolysis by cells of the immune system, since glutamine is only partially oxidized.

As well as providing energy for cells of the immune system, glutamine is an important source of nitrogen for a number of biosynthetic pathways including the *de novo* synthesis of purine and pyrimidine nucleotides (Tate and Meister 1973). These are required for the formation of the DNA and RNA, which are required for cell division and protein synthesis respectively. However, the rate of glutaminolysis, in cells of the immune system is greatly in excess (>100 fold) of the requirement for nucleotide biosynthesis. The maximum catalytic activity of carbamoyl-phosphate synthase 2 (CPS 2), a key enzyme in pyrimidine synthesis, and the flux through the pathway for pyrimidine synthesis, are0.8% and 0.1% respectively, of the rate of glutaminolysis by these cells, and are an even smaller fraction of maximal glutaminase activity (Szondy and Newsholme 1989) (see Table 3); Szondy and Newsholme (1991) have reported that the rate of glutaminolysis by lymphocytes is 400-fold greater than the rate of pyrimidine nucleotide synthesis in lymphocytes.

Table 3: activities of glutaminase and carbamoyl phosphate synthetase, and the rates of glutamine utilization and pyrimidine synthesis in cells of the immune system.

Cells of Immune System	Glutaminase Activity	CPS2 Activity	Rate of Glutamine utilization	Rate of Pyrimidine synthesis
	(nmoles/min/mg prot)			
Rat Lymphocytes	38	0.1	2.7	1.3×10^{-3}
Mouse Macrophages	152	0.1	1.7	-

Data derived from Ardawi and Newsholme (1982, 1983), Newsholme et al (1986, 1987) and Szondy and Newsholme (1989).

Given that glutamine is utilized at a rate which is markedly in excess of its requirement as a source nitrogen for purine and pyrimidine synthesis, and is partially oxidized, then what is the main role of the high rate of glutaminolysis in cells of the immune system? Newsholme et al (1985) proposed an hypothesis which suggests that the high rate of glutaminolysis is important to provide optimal conditions for the precise regulation of changes in the rates of synthesis of purine and pyrimidine nucleotide synthesis by enhancing the sensitivity of the pathway of purine and pyrimidine synthesis: this has been termed branched point sensitivity. Branched point sensitivity allows an increase in the rate of the biosynthetic pathways without depletion of concentration of intermediates of the glutaminolytic pathway that are precursors of purine and pyrimidine synthesis. This is very important since the concentration of glutamine may become depleted because the activity of glutaminase is markedly increased in stimulated lymphocytes, and this may limit the

concentration of glutamine despite the low Km for carbamoyl phosphate synthase 2 (CPS2) for glutamine. Thus, the high rate of glutamine utilization acts as a dynamic buffering system, allowing intermediates of the glutaminolytic pathway required for biosynthetic processes (glutamine and aspartate) to be "tapped off" at the precise rate required whenever they are required (see Appendix III for details of branched point sensitivity).

Although the role of the high rate of glutaminolysis in providing optimal conditions for the precise regulation of biosynthetic processes may at first appear wasteful, it provides a simple and effective control system for the effective functioning of cells of the immune system during an immunological challenge. This hypothesis is applicable to different cells, for example, fibroblasts, tumour cells and intestinal cells, all of which are able to divide rapidly, also endothelial cells which have the capacity to divide rapidly. The hypothesis is also applicable to cells, such as macrophages, which are not rapidly dividing but have a high protein secretory activity for which high rates of protein synthesis are required; the high rates of protein synthesis will require high rates of mRNA synthesis, for which purines and pyrimidines will be required.

The important point which needs to be highlighted from this discussion is that, glutamine must be used at a high rate by cells of the immune system, when resting and when stimulated. Thus, a continuous high rate of glutamine utilization is required. The question then raised is, what is the source of this glutamine that is important for cells of the immune system and their function? There is now considerable evidence that an important source of glutamine production is skeletal muscle. In the next section this evidence is reviewed.

4.Glutamine metabolism and production by skeletal muscle.

4.1.Glutamine utilization in skeletal muscle.

Studies have indicated that the rate of glutamine uptake by skeletal muscle is low; Hundal et al (1986a) have reported a low rate of uptake of glutamine by skeletal muscle at physiological glutamine concentrations. Glutaminase activity in skeletal muscle is reported to be low (Greenstein and Carter 1947) or absent (Rennie et al 1986, Quesada et al 1988). However, a recent study by Wu et al (1991) suggests that skeletal muscle has a high rate of glutamine utilization. In this study the rate of oxidative decarboxylation of labelled glutamine was measured and found to be high in chick and rat skeletal muscle. They also reported that glutaminase activity in chick skeletal muscle was very high, however that of rat skeletal muscle was low. Wu et al (1991) provided further evidence for glutaminase activity in chick skeletal muscle through the inhibition of glutaminase activity, by using 6-diazo-5-oxo-L-norleucine, an inhibitor of glutaminase. 6-Diazo-5-oxo-L-norleucine also inhibited oxidative decarboxylation of glutamine in chick skeletal muscle. However, it did not influence oxidative decarboxylation of glutamine in rat skeletal muscle.

Glutamine transaminates with a number of alpha-ketoacids to yield the corresponding amino acid and alpha-ketoglutaramate. There are two types of glutamine transaminases known to be present in rat tissue glutamine aminotransferase K and L. They are both cytosolic enzymes and catalyse the reaction of glutamine to phenylalanine and methionine, respectively (see reactions 4 and 5).

Phenylpyruvate + Glutamine $\xrightarrow[\text{Glutamine aminotransferase K}]{}$ Phenylalanine + 2- oxoglutaramate (Reaction 3)

thiobutyrate+ Glutamine $\xrightarrow[\text{Glutamine aminotransferase L}]{}$ Methionine + 2-oxoglutaramate (Reaction 4)

Wu et al (1991) measured the activities of glutamine aminotransferase K and L in chick and rat skeletal muscle. They reported that the activities of both these enzymes are 10-fold greater in rat than in chick skeletal muscle. From these results Wu et al (1991) concluded that skeletal muscle is able to degrade substantial amounts of glutamine.

4.2.Glutamine production in skeletal muscle.

Although the recent study by Wu et al (1991) has reported that skeletal muscle degrades substantial amounts of glutamine, it is well established that there is net production and release of glutamine by skeletal muscle. Glutamine accounts for 35% of all amino acids released from human muscle, in the post absorptive state (see review by Felig 1975). However, the proportion of glutamine in muscle protein is only 3%. This indicates that the rate of glutamine release does not reflect muscle protein degradation suggesting, therefore, that glutamine is synthesized *de novo* in skeletal muscle.

Glutamine synthetase activity in skeletal muscle has been reported (King et al 1983, Max et al 1987, 1988). The activity of glutamine synthetase in skeletal muscle is approximately 20% less than the activity in liver, brain or kidney (Lund 1980) and lung (Ardawi 1990). However, the weights of these tissues, based on % total body weight, are markedly less than that of skeletal muscle; skeletal muscle comprises 30-40% of total body weight. Thus, in terms of whole body glutamine metabolism, skeletal muscle represents a significant capacity for glutamine synthesis. (See Table 1 for glutamine synthetase activity in rat skeletal muscle.).

For the pathway of glutamine synthesis in skeletal muscle, it has been suggested that 2-oxoglutarate, an intermediate of the TCA cycle, is the carbon skeleton of glutamine since 2-oxoglutarate is the oxoacid for

glutamate, an immediate precursor of glutamine synthesis. Experiments in which the oxidation of amino acids in skeletal muscle were determined by measuring $^{14}CO_2$ production, have reported that the branched chain amino acids, (leucine, isoleucine, valine), together with asparagine and aspartate may be oxidized in skeletal muscle (Manchester 1965, Odessey and Goldberg 1972). Since the oxidation of these amino acids proceeds via 2-oxoglutarate they may be important sources of carbon for glutamine synthesis. Further evidence that the branched chain amino acids may be important sources of carbon for glutamine synthesis is provided by radiolabelled studies; in rat hindquarter perfused with ^{14}C-leucine, over 40% of the label was recovered in glutamate plus glutamine (Huston and Zaplowski 1981). As well as amino acids the carbon skeleton of glutamine may also be derived from glucose (Goldberg and Chang 1978).

Nitrogen is also required for glutamine synthesis, at two stages. The first stage, involving the transfer of nitrogen, is thought to be for the formation of glutamate from 2-oxoglutarate. An important source of nitrogen for this reaction may be the branched chain amino acids, via a transamination reaction catalysed by leucine or valine aminotransferase enzymes. Golden (1981) has demonstrated, by ^{15}N-Leucine studies *in vivo*, that branched chain amino acids can donate their nitrogen groups to glutamine.

Ammonia is required for the second stage of glutamine synthesis, for the production of glutamine from glutamate. However, the source or pathway for the provision of ammonia has not yet been determined. Garber (1980) suggests that ammonia may be taken up directly from the circulation. It has also been suggested that adenosine monophosphate may be a source of ammonia for the synthesis of glutamine from glutamate. Adenosine monophosphate is converted to inosine monophosphate, by AMP deaminase (Ruderman and Berger 1974, Snell 1980). This reaction

yields ammonia and based on its maximal activity in muscle, AMP deaminase appears to be able to supply enough ammonia for muscle glutamine synthesis (see Table 4).

Table 4: a comparison of AMP deaminase activity with glutamine synthetase activity in gastrocnemius muscle.

AMP deaminase	Glutamine synthetase
Activity (μ moles/min/g of wet wt)	
76*	0.33*

*Data from Leech (1973) and Ardawi (1990)

4.3.Glutamine release from skeletal muscle.

The rate of glutamine release from muscle has been determined *in vivo*, the measurement of arteriovenous differences has shown that glutamine is released at a rate higher than that of any other amino acid from tissues of the forearm (Marliss et al 1971, Felig 1975) and leg (Felig and Wahren 1971). Also experiments on perfused rat hindquarter (Ruderman and Berger 1974, Babij et al 1986) have shown that glutamine is released at a high rate. However, interpretation of results for the rate of glutamine release, obtained from the measurement of arteriovenous differences and perfused rat hindquarter, is a problem since as well as muscle, tissues such as adipose tissue, skin and bone, which have all been shown to release and/or take up glutamine (Ardawi1988a,Kowalchuk et al 1988, Zielke et al 1976, respectively) are present in these preparations. Thus, either technique can only provide a net balance of glutamine release across a number of different tissues. The use of *in vitro* tissue preparations, whereby tissue is incubated in a physiological medium, of skeletal muscle, skin and adipose

tissue have shown that skeletal muscle *per se* is responsible for the high rate of glutamine release from limbs (Garber 1980, Ardawi 1988a, Parry-Billings 1989).

Parry-Billings' (1989) investigation showed that the rate of release of glutamine differed in muscle whose fibre type composition differed. The rate of release of glutamine from soleus (a muscle composed of mainly type 1 fibres which have a high oxidative capacity) (see Ariano et al 1973 and Armstrong and Phelps 1984) was up to 70% higher than that of EDL (extensor digitorum longus, a muscle composed of mainly type 2b fibres which have a high glycolytic capacity). Glutamine concentration also differed between muscles composed of different fibre types: the concentration of glutamine in soleus muscle was 14% higher than in EDL.

Given the high concentration of glutamine in skeletal muscle [approximately 20mmoles/L intracellular water (Bergstrom et al 1974) in human skeletal muscle and 4mM in rat skeletal muscle (Parry-Billings 1989)], the capacity of skeletal muscle to synthesize glutamine, and the rate of glutamine release from skeletal muscle, skeletal muscle is considered to be an important source of glutamine for cells of the immune system. Thus, an understanding of the provision of glutamine from skeletal muscle to cells of the immune system is essential.

5.The regulation of the provision of glutamine from skeletal muscle to cells of the immune system.

To investigate the control of a metabolic pathway it has been proposed, by Newsholme and Crabtree (1979) that the following experimental steps be carried out:

a) Identify the equilibrium nature of the reactions in the pathway.

b) Identify the flux generating step; the flux generating step defined as the, first non-equilibrium reaction that approaches saturation with the pathway substrate.

c) Identify reactions which are regulated by external regulators,

d) Study the properties of the regulatory enzymes or systems,

e) Propose a theory of control.

f) Test and accept, modify or reject the theory of control.

A complete analysis of the control of the metabolic pathway under study; namely the pathway of glutamine synthesis, its release into the bloodstream (termed collectively as glutamine "production") and its uptake and utilization by cells of the immune system, using the procedure above, has not been completed. However, initial studies using this approach have provided some information on the control of this pathway and also on the regulation of the provision of glutamine by skeletal muscle.

From preliminary data, it appears that both the processes of glutamine synthesis and release, in skeletal muscle, are non-equilibrium; for the reaction of glutamine synthesis in skeletal muscle the free energy change (delta G) is approximately -13KJ. (Delta G was calculated by Parry-Billings (1989) using data from Lund (1970), Newsholme and Leech (1983) and Ardawi (1988a)). For glutamine release from muscle there is a marked glutamine gradient between muscle and plasma. Askanazi et al (1980) calculated that the ratio of the glutamine concentration in muscle and plasma is approximately 40:1 in man. On the basis of this concentration gradient the free energy change (delta G) for glutamine release is calculated to be approximately -10KJ/mole. The calculation for the free energy change assumes that the release process involves no other molecule or ion. (For full details of this calculation see Newsholme and Parry-Billings 1990).

The identification of the non equilibrium steps in this pathway is important, since non-equilibrium reactions give direction to a pathway and, very importantly, they are potential sites for the allosteric regulation of flux through a pathway.

Concerning the flux generating step, defined as the first non-equilibrium reaction that approaches saturation with the pathway substrate, for the metabolic pathway under investigation, there is evidence that the flux generating step for this pathway (glutamine synthesis, release into the bloodstream, and its subsequent uptake and utilization by cells of the immune system) does not reside in cells of the immune system. The Km for glutamine utilization by rat lymphocytes is 2.5mM (Ardawi 1983), which is higher than the physiological plasma glutamine concentration, of approximately 1mM. This means that, changes in the concentration of plasma glutamine would be expected to change the rate of glutamine utilization in rat lymphocytes *in vitro* (Ardawi 1983). It was suggested therefore, that the flux generating step for the metabolic pathway under investigation is present in skeletal muscle, with the flux generating step either being the process of glutamine synthesis or glutamine release, since they are both non-equilibrium processes. Parry-Billings (1989) provided evidence that the process of glutamine release from muscle is the flux generating step for the metabolic pathway under investigation. First, Parry-Billings (1989) showed that methionine sulphoximine, an inhibitor of glutamine synthetase, decreased the concentration of glutamine in skeletal muscle but had no effect on the rate of glutamine release (see Table 5). This, suggests that changes in muscle glutamine concentration would not normally cause changes in the rate of glutamine release from muscle. Second, by plotting the rates of release of glutamine from isolated incubated skeletal muscle of the rat against variations in the concentration of intracellular glutamine, an approximate Km value for glutamine

release of less than 0.3mM was provided. The concentration of glutamine in rat skeletal muscle is approximately 4mM. This indicates that the release process is approaching saturation with its substrate, muscle glutamine. On the other hand the Km of glutamine synthetase, the enzyme for glutamine synthesis, for glutamate is 5.8mM, which is higher than the intracellular concentration of glutamate (2mM). Thus, the process of glutamine synthesis is not flux generating.

The information above suggests the following:

a) The rate of synthesis of glutamine in muscle has normally no direct effect on the rate of glutamine release from muscle. So, although there is an increase in glutamine synthetase activity in conditions associated with increases in the rate of glutamine release, for example, cancer cachexia (Rivera et al 1988) and thermal injury (Ardawi 1988a) the increase in glutamine synthetase activity is not responsible directly for the increase in the rate of glutamine release, but maintains the glutamine concentration in skeletal muscle. However, during conditions where there is a marked depletion in muscle glutamine concentration, for example, during severe abdominal sepsis in man where the concentration of muscle glutamine is decreased by more than 80% (Roth et al 1982), the rate of glutamine release may no longer be flux generating and the process of release may then be controlled by the rate of glutamine synthesis, which, since it is a non-equilibrium process, may be controlled by external factors.

b) The study of the control of the rate of glutamine release from muscle must focus on the effect of possible external factors, i.e. allosteric regulators, on the process of glutamine release. Thus, the identification of external regulators of the rate of glutamine release

by skeletal muscle must focus attention on the release process rather than on glutamine synthesis.

c) The process of glutamine release is flux generating for the pathway of glutamine utilization. It follows, therefore, that the release of glutamine from muscle will act as the flux generating step for the pathway of glutamine utilization in cells of the immune system. Thus, changes in the rate of release of glutamine from skeletal muscle will influence the rate of glutamine utilization by these cells. Since, the high rate of glutamine utilization by lymphocytes and other cells provides branched point sensitivity (i.e. provides conditions for the precise regulation of changes in the rates of purine and pyrimidine synthesis) the process of glutamine release from skeletal muscle is therefore not only the point of control for the regulation of the rate of glutamine utilization by these cells, but is also important in maintaining an effective immune system during an immunological challenge. (The pathways of glutamine utilization by other tissues, namely kidney, intestine and liver, are not saturated with glutamine, thus the process of glutamine release from muscle will also act as the flux generating step for the pathways of glutamine utilization in these tissues see Figure 2).

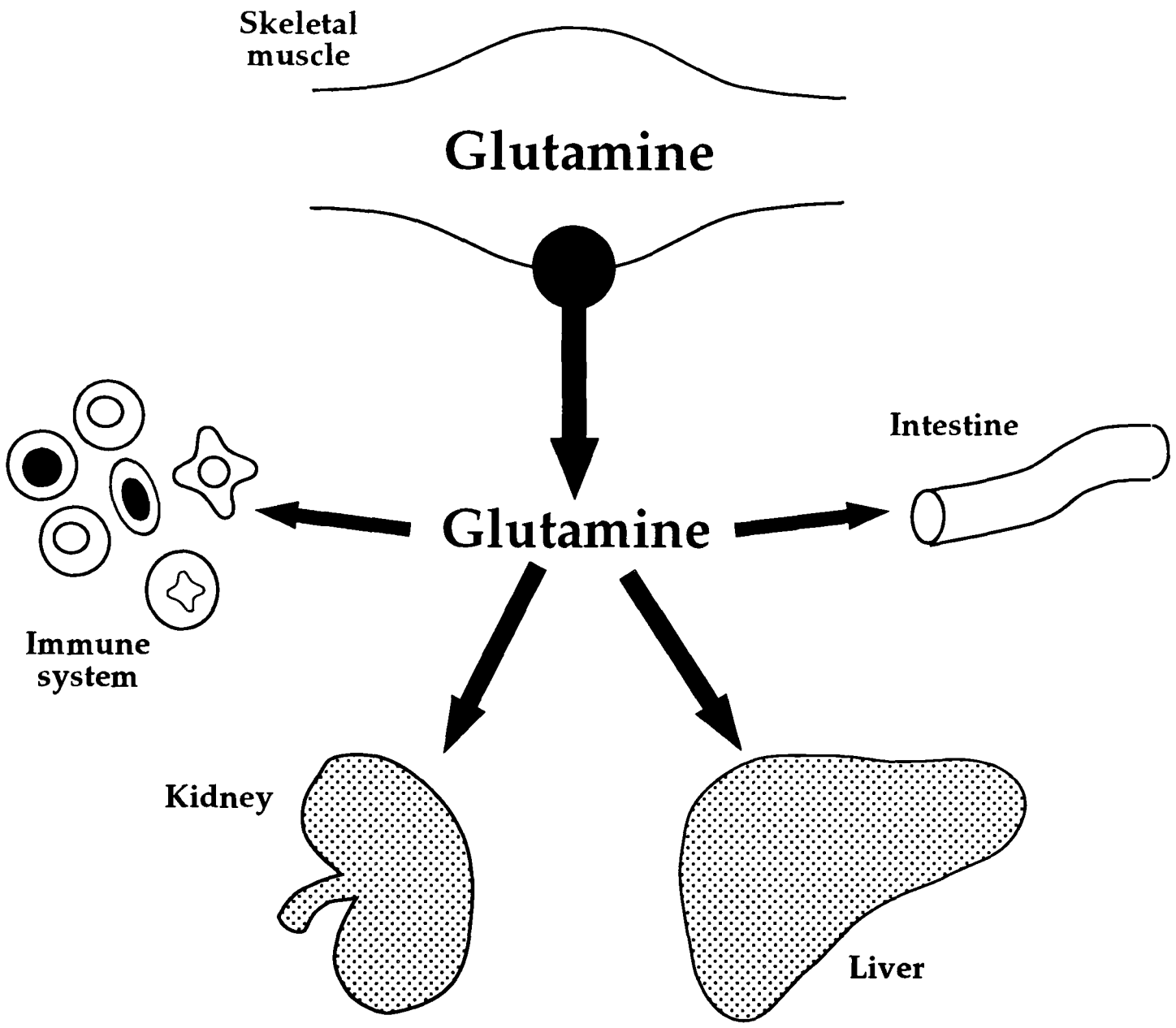


Figure 2. The release of glutamine by skeletal muscle is considered to be the flux generating step for the utilization of glutamine by a number of tissues. The arrows indicate the transport of glutamine from skeletal muscle to a number of different tissues.

In relation to suggestion **(b)**, on the identification of external regulators of the rate of glutamine release by skeletal muscle, Parry-Billings (1989) has identified hormones (glucocorticoids and thyroid hormone), metabolites, (branched chain amino acids) and conditions (sepsis, cancer cachexia and ageing) that change the rate of glutamine release from rat skeletal muscle (see Table 6).

In identifying regulators of glutamine release, Parry-Billings also noted that there is no simple relationship between changes in glutamine

concentration and glutamine release. For example, conditions and factors associated with an increase in the rate of glutamine release from skeletal muscle involved either an increase, a decrease or no change in the concentration of glutamine in muscle (see Table 6). It might have been expected that, with an increase in the rate of glutamine release, muscle glutamine concentration would be decreased. These results indicate that the processes of glutamine release and glutamine synthesis in skeletal muscle are separate and independent processes that may be linked via external "regulators".

Table 5: the effect of methionine sulphoximine (MSO) on the concentration of glutamine and the rate of glutamine release from stripped soleus muscle preparation of the rat.

The significance of the difference between groups is indicated by * ($p<0.01$).

	Incubated soleus muscle	
	Glutamine concentration (nmoles/g)	Rate of glutamine release (nmoles/min/g)
Control	3479	42.6
MSO	2455*	44.5

Data from Parry-Billings (1989).

Table 6. The relationship between changes in the rate of muscle glutamine release and muscle glutamine concentration.

	Changes in muscle glutamine concentration.		
Change in the rate of muscle glutamine release.	**Increase**	**Decrease**	**No change**
Increase	10mM valine in vitro Growth hormone, 3 days	Dexamethasone Cancer cachexia Late sepsis Severe endotoxemia	0.5mM valine in vitro Hyperthyroidism
Decrease	Long term exercise Hyperadrenalaemia	Steroid blocker Cold exposure Hypothyroidism Ageing	
No Change	Growth hormone, 10 days	Diabetes Exercise training Mild endotoxemia Late endotoxemia Early tumour bearing IGF-1, 10days	insulin, in vitro IGF-1, 1 & 3 days

Data from Parry-Billings (1989)

6.Aim of study.

Parry-Billings (1989) identified factors and conditions which regulate the process of glutamine release, and also suggested that the modification of the rate of glutamine release from muscle may "provide useful information regarding the role of glutamine release from muscle in different physiological and pathological states". These factors also affected muscle glutamine concentration, thus suggesting that external factors

affect the process of glutamine synthesis in skeletal muscle. However, in the Parry-Billings study, it was noted that the rate of release of glutamine from skeletal muscle was non-linear over time. This non-linearity may have affected the interpretation of the muscle glutamine release and muscle glutamine concentration data by Parry-Billings (1989), since it was now unclear whether the changes in the rate of glutamine release from muscle, reported by Parry-Billings (1989) were due to the factors used in the study. Thus, in the present study the linearity of the rate of glutamine release was investigated using an *in vitro* isolated muscle preparation. The effect(s) of some factors, including some used by Parry-Billings (e.g. branched chain amino acids), on the rate of glutamine release from muscle and muscle glutamine concentration were then investigated. This was so as to confirm that glutamine release from muscle is independent of the intracellular glutamine concentration.

In addition, the suggestion that external factors affect the process of glutamine synthesis in muscle lead to an investigation of the effect(s) of factors on the maximal activity of the enzyme of glutamine synthesis, glutamine synthetase, in muscle. This was so as to try and establish the relationship between muscle glutamine release and muscle glutamine synthesis.

Chapter 2.

The effect(s) of some factors on skeletal muscle glutamine release.

1. Introduction.

It has been proposed that the process of glutamine release from skeletal muscle is the flux generating step in the pathway of glutamine utilization, and is thus an important regulatory step in the pathway of glutamine utilization (see Chapter 1 section 5). Parry-Billings (1989) has demonstrated, using the isolated skeletal muscle preparation, that a number of external factors affect the process of glutamine release from skeletal muscle (see Chapter 1 section 5). Part of the work described in this chapter is an extension of the work done by Parry-Billings (1989), on the effect(s) of factors and conditions on the process of glutamine release from muscle. In association with this work, Parry-Billings (1989) observed that external factors affect muscle glutamine concentration independently of their effect on the process of muscle glutamine release (see Chapter 1, Table 6), thus indicating that there is no simple relationship between muscle glutamine release and muscle glutamine concentration.

However, Parry-Billings (1989) reported that glutamine release is not linear over 60mins. Since the suitability of an experimental technique for the study of cellular metabolite uptake or release maybe indicated by the linearity of the rate of release or uptake of the metabolite concerned (see Kilberg et al 1980, Newsholme et al 1986), the use of the isolated skeletal muscle preparation, for the investigation of glutamine release from muscle, may not be suitable for the investigation of glutamine release from muscle.

Garber et al (1976a) reported a time dependent decrease in the rate of release of glutamine and other amino acids (alanine, glutamate and aspartate) from incubated rat epitrochlearis muscle. However, the same

authors reported a linear rate of glucose uptake and concluded that the reduction in the rate of glutamine release was not attributable to reduced viability of the isolated skeletal muscle preparation.

The reason why the rate of glutamine release decreases with longer incubation times is unclear. Garber et al (1976a) showed, by performing experiments in which the incubation medium was changed periodically during the incubation period, that the decrease in the rate of glutamine release over time was not due to an accumulation of glutamine in the incubation medium. Furthermore, Parry-Billings (1989) demonstrated that muscle glutamine concentration is maintained in muscle throughout a 60mins incubation period, thus it is unlikely that the decreased rate of glutamine release could be caused by rapid depletion of intramuscular glutamine; this had been predicted by the flux generating nature of the process of muscle glutamine release (see Chapter 1 section 5). Consequently, a further investigation of the rate of glutamine release over time was performed in the present study. In addition to the glutamine release linearity study, the effects of some factors on muscle glutamine release and muscle glutamine concentration were investigated so as to determine whether the results from the present study are consistent with the suggestion that glutamine release from muscle is independent of the intracellular glutamine concentration (Parry-Billings 1989).

The factors/conditions used in this study were:

branched chain amino acids (leucine, valine and isoleucine)

lactate

2-oxoglutarate

ornithine

changes in cell volume.

1.1 Technique used in present study: isolated rat skeletal muscle preparation.

The same experimental preparation used by Parry-Billings (1989), the isolated rat skeletal muscle preparation, incubated *in vitro*, was used in the present study (skeletal muscle used were soleus or extensor digitorum longus). The isolated rat skeletal muscle preparation has been used frequently in the investigation of skeletal muscle metabolism: the incubated diaphragm was first used by Meyerhof and Himwich (1924) and has been used widely (Gemmil et al 1941, Kipnis and Cori 1957, Manchester and Young 1958, 1960). However, the diaphragm may not be representative of skeletal musculature since it contracts rhythmically throughout life. Other muscles that have been employed, and which are more representative of skeletal musculature include, extensor digitorum longus (EDL) (Maizels et al 1977; Parry-Billings 1989), epitrochlearis (Garber et al 1976a,b,c) and whole or stripped soleus (Chaudry and Gould 1969; Crettaz et al 1980; Espinal 1983; Parry-Billings 1989; Bevan 1992).

Although there has been some criticism of the isolated rat muscle preparation, this technique has been shown to be both biochemically and physiologically viable. (For a detailed discussion on the viability of the isolated muscle preparation see Appendix IV).

2.Materials and Methods.

2.1. Materials.

(Chemicals, enzymes and animals in Appendix I)

2.2. Methods.

2.2.1.Preparation of stripped and whole soleus.

Stripped and whole soleus muscles were obtained from male albino Wistar rats, weighing approximately 180g and 60g respectively. Prior to experiments using stripped soleus muscle the rats were starved for approximately 15h. For all experiments in which whole soleus muscle was used, the rats were starved for 6h. the morning of the experiment, which was carried out in the afternoon. Rats were killed by a blow to the head and cervical dislocation. It was very important that the time at which the experiments were preformed was consistent since glutamine concentration in muscle and the rate of glutamine release from muscle vary over 24 h. (Parry-Billings 1989).

Rats weighing approximately 70g were anaesthetised with a pentobarbital solution (sagatal) at a dose of 60mg/Kg body weight, and killed by cervical dislocation. This procedure minimised the muscle spasm which occur in young rats following cervical dislocation (see Appendix IV). Soleus muscles were prepared as described previously (Chaudry and Gould 1969, Crettaz et al 1980 and Espinal 1983). Whole muscles or muscle strips, not weighing more than 40mg, were removed from the animal. The muscles were then tied at the proximal and distal tendons with pre-weighed thread. The muscle and thread were weighed and the muscle was then tied under slight tension at approximately resting length. Mounted muscles were then placed immediately into flasks containing preincubation medium.

2.2.2.Preparation of extensor digitorum longus (EDL) muscle.

As with whole soleus muscle preparation, EDL muscles were isolated from rats weighing approximately 60g. Since the fibre orientation does not allow strips of EDL muscle to be taken without causing serious damage to it, small rats had to be used for experiments using this muscle, so as to obtain small and thin muscle; larger EDL muscle i.e. weighing more than 40mg, would have affected the viability of the *in vitro* muscle preparation (see Appendix IV) EDL muscles were prepared as previously described (Maizels et al 1977). They were removed from the rat, tied under tension as described above and placed in a flask containing preincubation medium. As with whole soleus, experiments with incubated EDL muscles were also performed in the afternoon; rats were starved for 6h. prior to the afternoon experiment.

2.2.3.Preparation of incubation medium.

Buffer solution.

Soleus and EDL muscles were incubated in Krebs-Henseleit bicarbonate buffer supplemented with HEPES (hydroxyethyl-piperazine ethane-sulphonic acid), 1.4% defatted bovine serum albumin (BSA) and 5.5mM glucose.

The buffer contained:

	concentration(mM)
NaCl	**120**
HEPES	**7.80**
$NaHCO_3$	**25**
KCl	**4.80**
$CaCl_2$	**1.20**
$MgSO_4$	**1.55**
KH_2PO_4	**1.55**

The buffer was gassed for 30mins with O_2:CO_2 (95:5). Then defatted BSA was added. Glucose was added to a final concentration of 5.5mM and the pH of the buffer was adjusted to 7.3 at room temperature. The buffer was then divided into the preincubation and incubation media. The preincubation medium was not supplemented unless otherwise stated. Insulin was added to the incubation medium only, at a final concentration of 10μU/ml.

Preparation of insulin.

Insulin (26.5U/mg) was dissolved to a final concentration of 10U/ml in 3mM HCl. Aliquots of insulin (100μl) were dipensed into, and stored at 20°C in Eppendorf tubes (in order to avoid repeated thawing and freezing of the stock insulin solution). Serial dilutions were carried out in plastic tubes using the Krebs-Henseleit bicarbonate buffer, and added to the incubation flasks. Insulin added to the incubation medium decreased muscle proteolysis (Fulks et al 1975), and increased muscle viability (Espinal 1983).

Preparation of bovine serum albumin.

Bovine serum albumin (BSA) was defatted using the procedure of Chen (1967), but with some modifications (Lozeman 1986). Albumin minimizes the loss of potassium from, and improves the contractility of, the incubated muscle (Pain and Manchester 1970). Although the mechanism for this is unknown, it is believed that albumin complexes metal ions such as lead and copper, trace amounts of which may be present in distilled water. (Details for the preparation of BSA are in Appendix II.)

2.2.4.The incubation procedure.

Isolated muscles (soleus and EDL) were placed immediately into stoppered 25ml Erlenmeyer flasks containing 3.2ml of preincubation medium. Muscles were 'pre-incubated' for 30mins at 37°C in a shaking water bath. The preincubation medium had been previously warmed to 37°C. Muscles were gassed throughout the preincubation period with O_2:CO_2 (95:5).

At the end of the 30mins preincubation period muscles were transferred to the incubation medium and incubated for 60mins; muscles were gassed for only 15mins. At the end of the incubation period muscles were removed, blotted using filter paper and frozen in liquid nitrogen. Muscles were stored over 'dry ice' (solid CO_2) until the end of the experiment and then stored at -70°C for later analysis. Samples of incubation medium were stored at -20°C for later analysis.

2.3.Analysis of metabolite concentrations:

2.3.1.Preparation of muscle samples for analysis.

Preparation of muscle tissues for analysis involved an acid extraction followed by neutralisation of the tissue extract. Soleus and EDL muscles were extracted in pairs (from the same treatment group). Muscle pairs were powdered in a 10ml plastic tube, placed in solid CO_2, using a spatula

cooled in liquid nitrogen. Ice cold perchloric acid (1ml of 10%) was added to the muscle powders. The acid-muscle powder mixture was then homogenised for approximately 15seconds using a Polytron homogeniser (Kinematica) set at position 4. The homogenate was then centrifuged, in a refrigerated centrifuge (4°C), at 1500g for 8mins. A sample of the supernatant (0.8ml) was neutralised with ice-cold potassium hydroxide plus 0.5M triethanolamineto pH 7-7.5 (as indicated by the addition of 15µl of universal indicator). Neutralised samples were centrifuged again, at 9000g for 2mins and the supernatant stored at -20°C for later analysis.

2.3.2.Details of metabolite assays.

Glutamine assay.

Glutamine concentration was measured in muscle extracts and samples of incubation medium using the method of Windmueller and Spaeth (1974), with some minor modifications. The enzyme used in this method, asparaginase, was dialysed over a 24-36h. period in a phosphate buffer (80mM, pH6.6) that was changed every 12h. before being used in the assay. Asparaginase was dialysed so as to remove any ammonia present in the enzyme preparation, since any excess ammonia present in the assay medium will interfere with the glutamine assay (see below for composition of assay medium).

Measurements were made using a spectrophotometer (Gilford, Stasar 3). Standard solutions of glutamine and a blank (water) were included in the assay. For the measurement of glutamine concentration in samples of incubation medium the assay medium was diluted three times and the amount of enzyme decreased four times. Also, in the analysis of samples of incubation medium a water blank and a control blank (medium incubated without muscle) were included in the assay.

Composition of assay medium.

Substance	Final concentration in a 1ml cuvette
KH_2PO_4, pH8.0 (buffer)	45mM
Glycerol	860mM
NADH	172μM
2-Oxoglutarate	3.6mM
Bovine serum albumin	0.073mM
Glutamate dehydrogenase	20units
Asparaginase	20units

Other metabolite assays.

Alanine was the other metabolite whose concentration was measured in muscle extracts and samples of incubation medium using the method of Williamson (1974). Measurements were made using a spectrophotometer (Gilford, Stasar 3), for the determination of the concentration of alanine in muscle extracts, or a fluorimeter (LS-Perkin-Elmer) for the determination of the concentration of alanine in incubation media.

Further analysis for cell volume experiments.

The measurement of the concentration of ATP in muscle, was made using the method of Lamprecht and Trautshold (1974). The measurment of the concentrations of ADP and AMP in muscle, was made using the method of Jaworek et al (1974). The ratios of the adenine nucleotide concentrations in muscle, ATP:AMP and ADP:AMP, were also determined. The intracellular water content in muscle and the osmolality of the incubation media were determined for the "changes in cell volume" experiments.

Determination of intracellular water.

Changes in intracellular water content indicated the change in muscle cell volume. To determine the volume of intracellular water, total muscle water volume and extracellular volume were determined. Total muscle water volume was measured by dry-weight analysis. The extracellular volume was determined in muscles incubated, for 60mins, in the presence of an extracellular marker [^{14}C]inulin 0.25μCi/ml. Muscles were then removed from incubation media, rinsed, blotted and frozen in liquid nitrogen. An aliquot (0.5ml) of 1M KOH was added to each muscle and left to digest for 1h. at 70°C. After neutralisation with 150μl of glacial acetic acid, the radioactivity of the digest was measured using a liquid scintillation counter (Beckman Model LS1701). By calculating the difference between the total and extracellular water volume the intracellular volume was determined.

Determination of osmolality.

The osmolality of the incubation media was determined with a vapour-pressure osmometer (Wescor 5100C).

2.3.3.Presentation of data and statistical analysis.

The concentrations of glutamine and alanine in muscle are presented in nmoles/g wet weight. The rates of glutamine and alanine release are expressed as nmoles/min /g wet weight. The concentration of ATP, ADP and AMP are expressed in μmoles/g wet weight. Total, extracellular and intracellular water are expressed in μl/g wet weight. The osmolality of incubation media is expressed in mmol/Kg. Statistical analysis was carried out using Student's t-test.

2.4.Experimental procedures.

2.4.1.Linearity of glutamine release from skeletal muscle.

(It should be noted that, in the investigation of the rate of glutamine release over time, the preincubation medium was supplemented with succinate and pyruvate, both at 5mM) .

The investigation of the rate of glutamine release over time involved the following procedure:

1) Varying the preincubation period.

The preincubation period was increased from 30mins to 50mins, since, it had been suggested that increasing the preincubation period " corrected" the non linear rate of glutamine release (Garber et al 1976a).

2) Modifications to the 'change over' procedure. It may be that the constituents (e.g succinate and pyruvate) of the preincubation medium are carried over with muscle at the point of change over, and these constituents may interfere with the process of glutamine release. Thus muscles were incubated for 5mins between the preincubation period and the incubation period, so as to wash away any preincubation constituents.

3) Manipulation of the preincubation and incubation media.

The possible 'carry-over' effect of pyruvate and succinate from the preincubation medium to the incubation medium may interfere in the linearity of glutamine release. Consequently, the rate of release of glutamine from muscle when pyruvate (5mM), pyruvate and succinate (5mM each) or succinate (5mM) were absent from preincubation media, was investigated.

The rate of glutamine release over 60mins was determined. The incubations were terminated and medium sampled over 5, 15, 30 and 60 mins. (This study was performed using stripped soleus muscle.)

2.4.2.The effect of branched chain amino acids on the rate of glutamine or alanine release from muscle, and muscle glutamine or alanine content.

Branched chain amino acids were added to flasks containing preincubation or incubation medium at a final concentration of 1mM or 10mM. Whole soleus or EDL muscles were incubated and prepared for analysis as previously described, and the concentrations of glutamine and alanine were determined. Samples of incubation media were taken as previously described, the concentrations of glutamine and alanine in incubation medium were determined. From the concentration of glutamine and alanine in the incubation medium the rates of release of glutamine and alanine were calculated.

2.4.3.The effect of branched chain ketoacids on the rate of glutamine or alanine release from muscle, and muscle glutamine or alanine content.

The three branched chain ketoacids 2-oxo-3-methylvalerate (ketoisoleucine), 2-oxoisovalerate (ketovaline), and 2-oxoisocaproate (ketoleucine) were added to the incubation media at a final concentration of 0.05, 0.25 or 1.0mM. Whole soleus and EDL were incubated as previously described. Glutamine and alanine contents and rates of release were determined as previously described.

2.4.5.The effect of lactate on the rate of glutamine release from muscle and muscle glutamine content.

Lactic acid (Li^+ salt) was dissolved in incubation media at a final concentration of 1, 5, 10, 20, 30 or 35mM. Whole soleus and EDL muscles were incubated as previously described. Glutamine and alanine contents and rates of release were determined as previously described.

2.4.6.The effect of 2-oxoglutarate on the rate of glutamine release from muscle, and muscle glutamine content.

2-Oxoglutarate (Na^+ salt) was disssolved in incubation media at a final concentration of 0.05mM or 1.0mM. Whole soleus and EDL muscles were incubated as previously described. Glutamine and alanine contents and rates of release were determined as previously described.

2.4.7.The effect of ornithine on the rate of glutamine or alanine release from muscle, and muscle glutamine or alanine content.

Ornithine was dissolved in incubation media at a final concentration of 0.6mM. Whole soleus and EDL muscles were incubated as previously descibed. Glutamine and alanine contents and rates of release were determined as previously described.

2.4.8.The effect of cell volume changes on the rate of glutamine or alanine release from muscle, and muscle glutamine or alanine content.

Stripped, whole soleus or EDL muscles were incubated as previously described in iso-osmotic (control), hypo-osmotic (120, 95 or 70mM Na^+) and hyper-osmotic (5, 10 or 50mM sucrose) media. Muscles were incubated in hypo-osmotic and hyper-osmotic media, in order to induce increases and decreases in cell volume respectively. Preincubations were always carried out in iso-osmotic (control) media. Glutamine and alanine content and rates of release were determined as previously described.

To examine the effect of the depletion of Na^+ ions from incubation media on the rate of glutamine release from muscle, stripped soleus muscle was incubated in media containing 70mM Na^+, supplemented with sucrose to maintain osmolality. Glutamine and alanine contents and rates of release were determined as previously described.

Experiments were also performed to determine if any changes in the rate of glutamine release induced by changes in cell volume were reversible. This involved preincubation of stripped soleus muscle in iso-osmotic media, followed by two consecutive incubation periods, of 50mins each, the first incubation period in hypo-osmotic media (70mM Na^+) and the second in iso-osmotic media (70mM Na^+ with added sucrose). The rate of glutamine release was determined as previously described.

To examine whether the changes in osmolarity produced a constant change in the rate of glutamine release, a time course of glutamine release in iso-osmotic medium and hypo-osmotic media was determined. All muscles (stripped soleus) were preincubated in iso-osmotic medium and then incubated in either iso-osmotic or hypo-osmotic (70mM Na^+) media for 5, 15, 30 or 60mins.

The adenine nucleotide concentrations plus the intracellular water content in muscle, and the osmolality of the incubation media, were determined as previously described.

3.Results.

3.1.Linearity of glutamine release from skeletal muscle over a 60mins incubation.

The rate of glutamine release has been shown to be non-linear with time (Figure 1). Extending the preincubation period from 30 mins to 50 mins did not 'correct' the non-linearity of glutamine release from soleus muscle (Figure 2). The incubation of muscle for 5mins., between the preincubation and the incubation period, 'corrected' the non-linear rate of release of glutamine from soleus muscle (Figure 3). The non-linear rate of glutamine release remained non linear if succinate was omitted from the preincubation media (Figure 4). However when pyruvate or pyruvate plus

succinate were omitted from the preincubation media, the rate of glutamine release was linear with time (Figure 5).

3.2.The effect of branched chain amino acid on the rate of glutamine or alanine release from muscle, and muscle glutamine or alanine content.

Isoleucine (10mM) increased the rate of glutamine release from soleus and EDL muscles, by 30 and 60% respectively. Isoleucine (1mM) also increased the rate of glutamine release from soleus and EDL muscles by 34 and 100% respectively. Valine (10mM) increased the rate of glutamine release from soleus by 20%, and EDL by 66% (Table 2.1). Valine (1mM) increased the rate of glutamine release from EDL by 33% but had no effect on the rate of glutamine release from soleus muscle. Leucine (10mM) increased the rate of glutamine release from soleus by 30% and EDL by 56%. However. leucine (1mM) had no effect on the rate of release of glutamine release from soleus and EDL (Table 2.1).

Isoleucine, valine or leucine (10mM) markedly increased the rate of release of alanine from both soleus and EDL muscles; isoleucine (10mM) increased alanine release from soleus and EDL muscles by 77 and 133% respectively, valine (10mM) increased alanine release from soleus and EDL by 25 and 69% respectively, and leucine (10mM) increased alanine release from soleus and EDL by 32 and 50% respectively (Table 2.2). Isoleucine (1mM) had no effect on the rate of alanine release from soleus muscle but increased the rate of alanine release from EDL muscle by 51%. The effects of valine or leucine (1mM) on the rate of alanine release from soleus and EDL muscle were not determined.

Isoleucine or valine (1 or 10mM) had no effect on glutamine content in soleus and EDL muscles. In contrast, leucine (10mM) increased glutamine content in both soleus and EDL muscles by 25% (Table 2.3).

Isoleucine, valine or leucine (10mM or 1mM) had no effect on alanine content in soleus or EDL muscle (Table 2.4).

3.3.The effect of the branched chain ketoacids on the rate of glutamine or alanine release from muscle, and muscle glutamine or alanine content.

2-Oxo-3-methylvalerate (1mM) increased the rate of glutamine release from soleus and EDL muscle by 18 and 29% respectively. 2-Oxo-3-methylvalerate (0.25mM) increased the rate of glutamine release from soleus muscle by 15%. There was no change in the rate of glutamine release from EDL at this concentration. There was no change in the rate of glutamine release from both soleus and EDL muscles incubated with 2-oxo-3-methylvalerate (0.05mM), 2-oxoisovalerate or 2-oxoisocaproate (0.05 or 1mM). (Table 2.5).

2-Oxo-3-methylvalerate, 2-oxoisovalerate or 2-oxoisocaproate had no effect on the rate of alanine release from both soleus and EDL muscles (Table 2.6). 2-Oxo-3-methylvalerate, 2-oxoisovalerate or 2-oxoisocaproate had no effect on glutamine content in both soleus and EDL muscles (Table 2.7). 2-Oxo-3-methylvalerate had no effect on alanine content in both soleus and EDL muscle (Table 2.8). The effect of 2-oxoisovalerate or 2-oxoisocaproate on alanine content in EDL and soleus was not determined.

3.4.The effect of lactate on the rate of glutamine or alanine release from muscle, and muscle glutamine or alanine content.

All concentrations of lactate markedly increased the rate of glutamine release from soleus and EDL muscles (Tables 2.9 and 2.12). Lactate (1, or 5mM) had no effect on the rate of alanine release from both soleus and EDL muscles (Table 2.11)*. The rate of alanine release was not determined for the higher lactate concentrations. * *See added paper*

Lactate (1 to 35mM) had no effect on glutamine content in soleus and EDL muscles (Table 2.10 and Table 2.13). Lactate (1mM) had no effect on alanine content in soleus or EDL muscles. However, lactate (5mM) increased alanine content in soleus and EDL muscles by 54 and 33% respectively (Table 2.14).

3.5.The effect of 2-oxoglutarate and ornithine on the rate of glutamine or alanine release from muscle, and muscle glutamine or alanine content.

2-Oxoglutarate (0.05mM and 1mM) had no effect on the rate of glutamine release or the rate of alanine release from soleus and EDL muscles (Table 2.15 and Table 2.16). However, at both concentrations 2-oxoglutarate increased glutamine content in EDL muscle by 30% (Table 2.17). There was no change in glutamine content in soleus muscle. Alanine content was not determined.

Ornithine (0.6mM) increased the rate of glutamine release from soleus muscle by 16% but this increase was not statistically significant (Table 2.18). There was no change in the rate of alanine release from EDL muscle (Table 2.19). Ornithine had no effect on glutamine content in soleus and EDL muscles.

3.6.The effect of cell volume changes on adenine nucleotide concentrations.

The concentrations of ATP, ADP and AMP and the ratios ATP:AMP and ADP:AMP were maintained in muscles incubated in control media and hypo-osmotic media (70mM Na+) (Table 2.20 and Table 2.21). The concentrations of ADP and AMP and the ATP:ADP concentration ratio were maintained in muscle incubated in hyper-osmotic media. The concentration of ATP and the ATP:AMP concentration ratio were decreased by 19 and 23% respectively, in muscle incubated in hyper-osmotic medium (50mM sucrose).

3.7.The effect of hypo- or hyper-osmotic medium on muscle cell volume.

The cell volume of muscle incubated in hypo-osmotic media was increased by 18%. The cell volume of muscle incubated in hyper-osmotic media was decreased by 4% (Table 2.22).

3.8.The effect(s) of cell volume on the rate of release of glutamine or alanine from muscle, and muscle glutamine or alanine content.

Increases or decreases in cell volume had no effect on glutamine or alanine content in stripped soleus muscle (Table 2.23). Increases in cell volume (hypo-osmotic media 120, 95 or 70mM Na^+) decreased the rate of glutamine release (by 18, 34 and 30% respectively) (Table 2.24). Decreases in cell volume (hyper-osmotic media 5, 10 or 50mM sucrose) increased the rate of glutamine release from stripped soleus muscle by 16, 25 and 22% respectively, although the effect with sucrose (5mM) was not statistically significant (Table 2.25). Similarly, the rate of alanine release, from muscle incubated in hypo-osmotic media or hyper-osmotic media, was decreased by 28% or increased by 35% respectively (Table 2.26).

The decreases in the rates of glutamine release in hypo-osmotic media were not the result of a decrease in the extracellular concentration of Na^+, since the rate of glutamine release from muscle incubated in medium containing 70mM Na^+, was not affected if sucrose was added so as to change the incubation media from hypo-osmotic to iso-osmotic (Table 2.24). The marked decrease in the rate of glutamine release in hypo-osmotic medium was shown to be acutely reversible in an experiment involving two consecutive incubation periods in different media; the rate of glutamine release from stripped soleus muscle was decreased during the first incubation in hypo-osmotic media but was restored to its normal rate during the second incubation period in iso-osmotic (control) media

(Table 2.27). The time courses for the rate of glutamine release from stripped soleus muscle incubated in iso-osmotic (control) and hypo-osmotic media showed that the rate of glutamine release was linear and was decreased after 30mins in the presence of hypo-osmotic media (Figure 6).

The effects of increases and decreases in cell volume on the rate of glutamine and alanine release from whole soleus and EDL muscles were similar to those from the stripped soleus muscle. For EDL muscles incubated in hypo-osmotic media (120, 95 or 70mM Na^+) the rate of glutamine release was decreased although the decrease for 120mM Na^+ was not statistically significant (Table 2.28). For soleus muscle incubated in hypo-osmotic media (120, 95 or 70mM Na^+) the rate of glutamine release was decreased (Table 2.29). The rates of glutamine release from EDL muscles incubated in hyper-osmotic media were increased: sucrose (5, 10 or 50mM) increased the rate of glutamine release (Table 2.30). However, only 50mM sucrose increased the rate of glutamine release from soleus muscle (Table 2.31).

Cell volume increases decreased the rate of alanine release from EDL and soleus muscles. The rate of alanine release from EDL muscle incubated in hypo-osmotic media (120, 95 or 70mM Na^+) was decreased although the decreases were not statistically significant (Table 2.32). The rate of alanine release from soleus muscle incubated in hypo-osmotic media (120, 95 or 70mM Na^+) was decreased (Table 2.33). The effect with 120mM Na^+ was not statistically significant. Hyper-osmotic media (5,10 or 50mM sucrose) increased the rate of alanine release from EDL muscle (Table 2.34); the effect of sucrose (5mM) was not statistically significant. Hyper-osmotic media (sucrose 50mM) increased the rate of alanine release from whole soleus muscle (Table 2.35).

Figure 1. A plot of the rate of release of glutamine from soleus muscle, against time. The rate of release of glutamine from soleus muscle was measured after 5, 15, 30 and 60mins of incubation time.

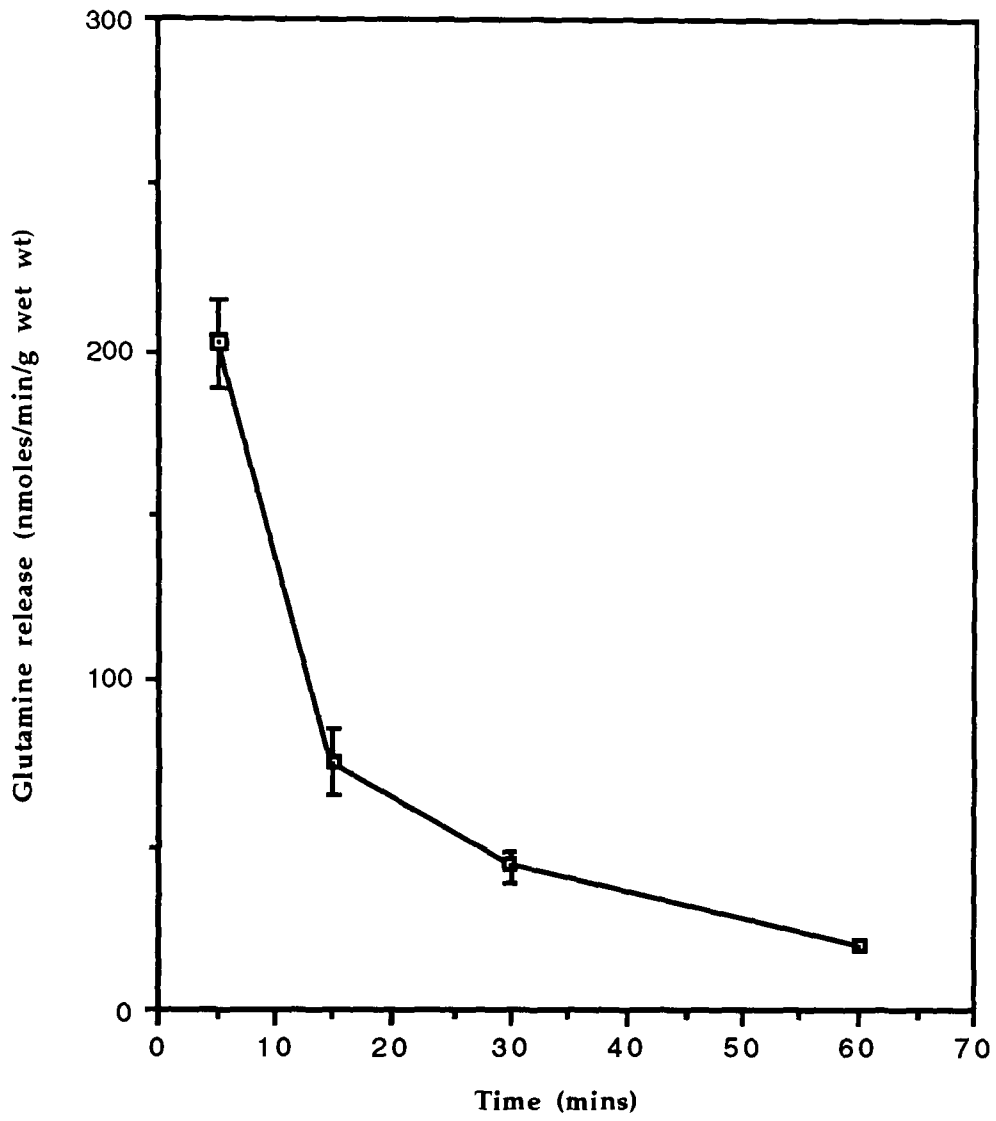


Figure 2. The effect of varying the time of pre-incubation on the rate of glutamine release from soleus muscle against time. The rate of release of glutamine from soleus muscle was measured after 5, 15, 30 and 60mins of incubation time.

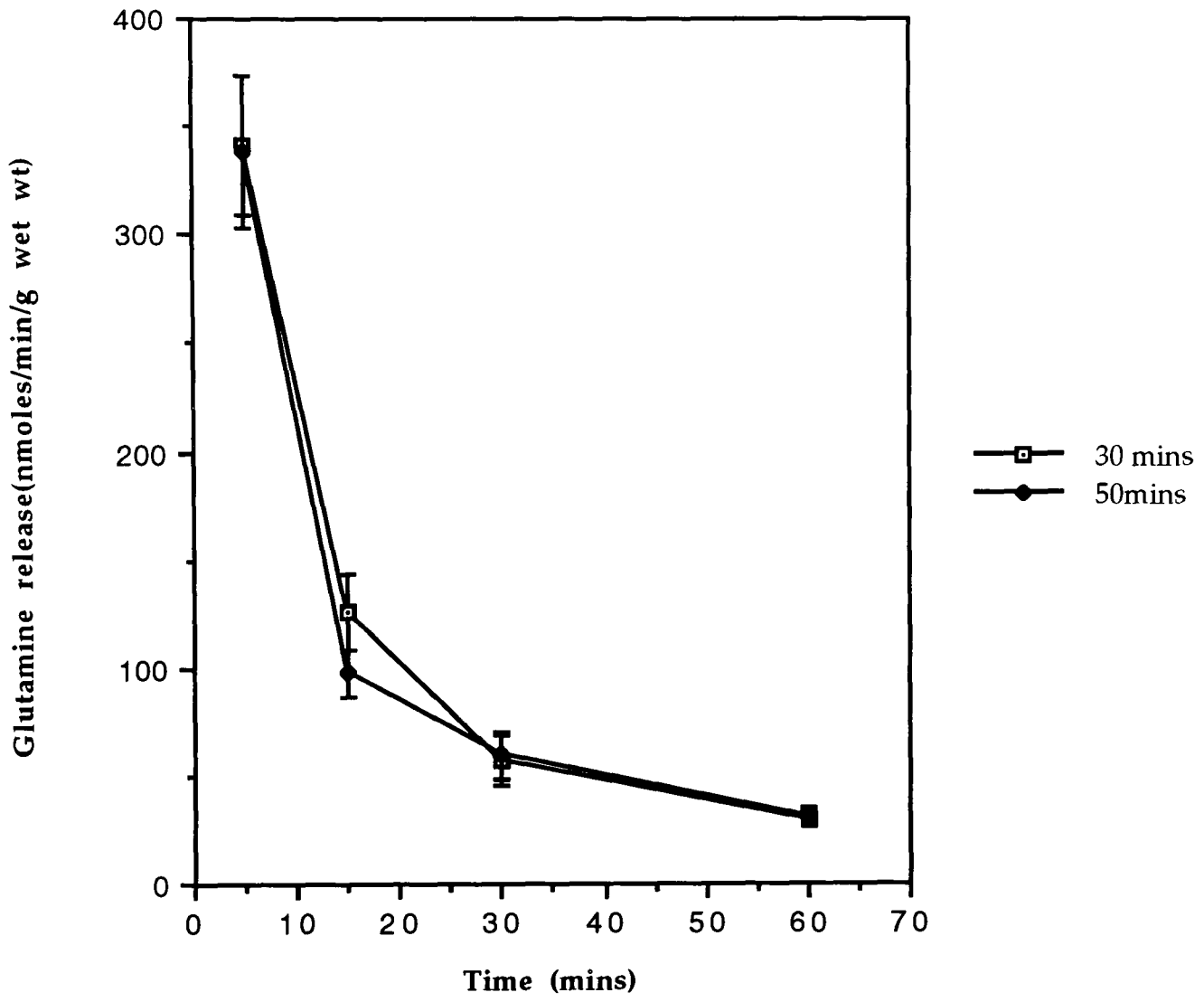


Figure 3. The effect of a 'washout' period on the rate of glutamine release from soleus muscle against time. The rate of release of glutamine from soleus muscle was measured after 5, 15, 30 and 60mins of incubation time.

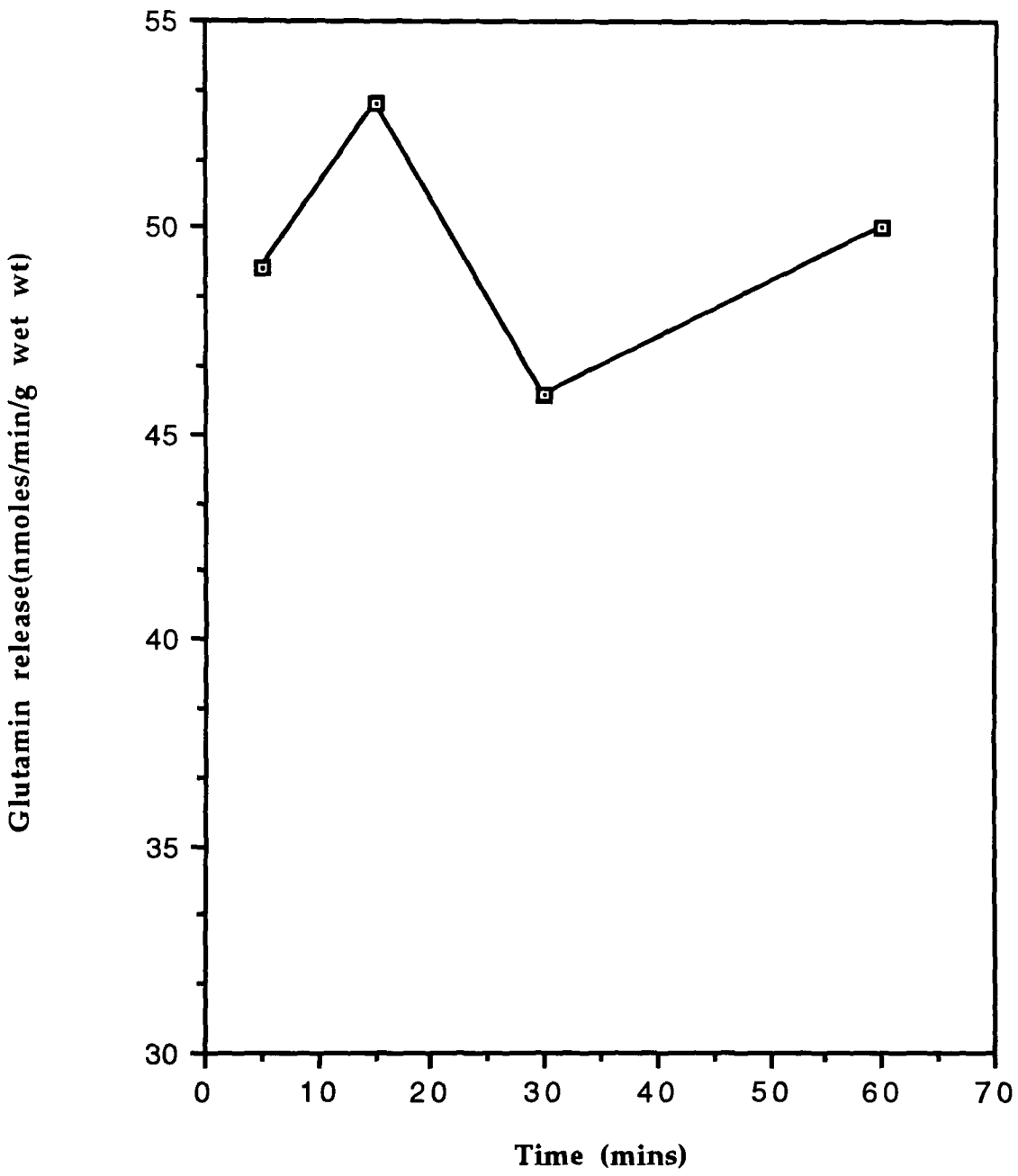


Figure 4. The effect of pre-incubation media minus pyruvate or succnate on the rate of glutamine release from soleus muscle against time.
The rate of release of glutamine from soleus muscle was measured after 5, 15, 30 and 60mins of incubation time.

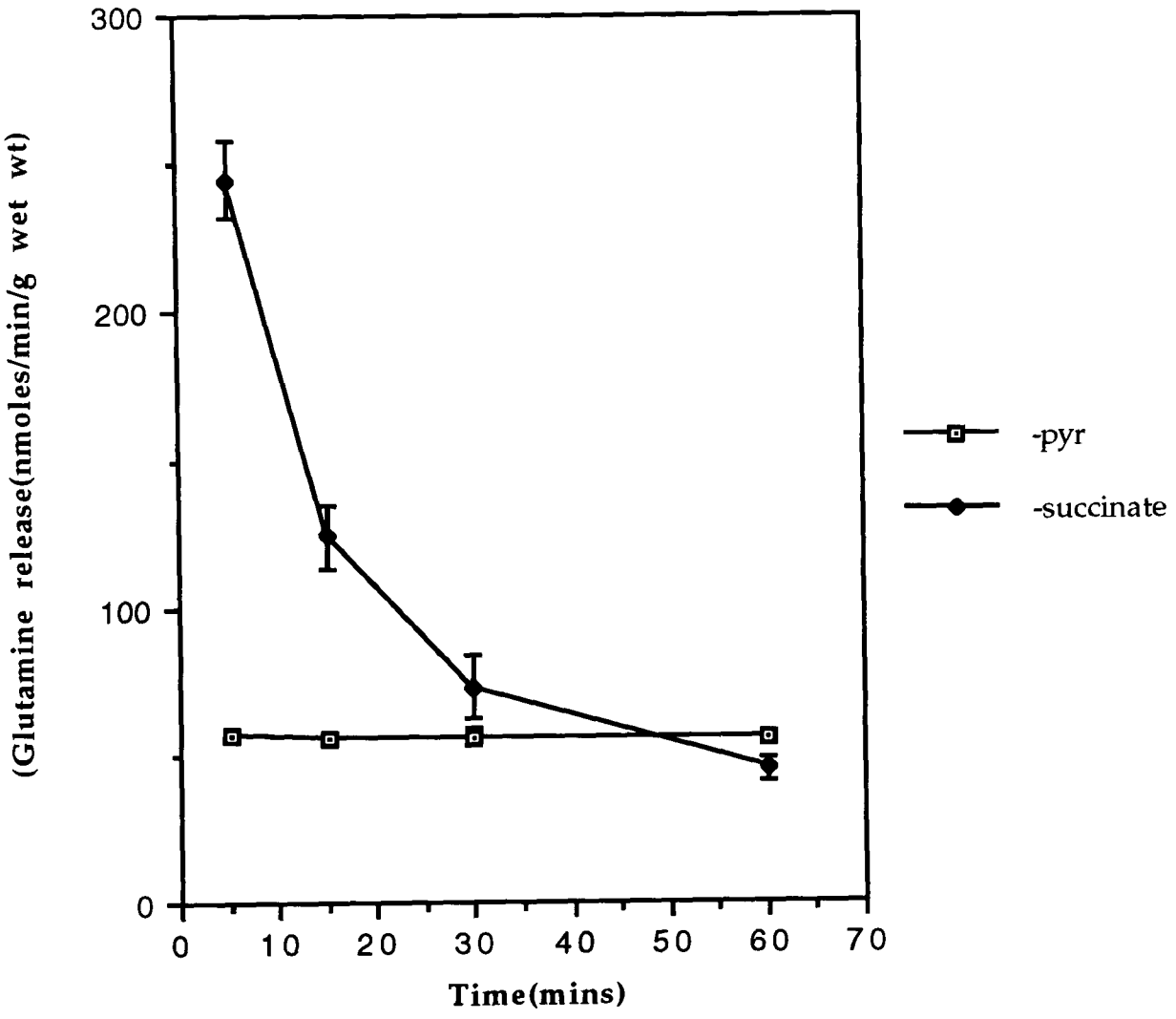


Figure 5. The effect of pre-incubation minus pyruvate and succinate on the rate of glutamine release from soleus muscle against time.
The rate of release of glutamine from soleus muscle was measured after 5, 15, 30 and 60mins of incubation time.

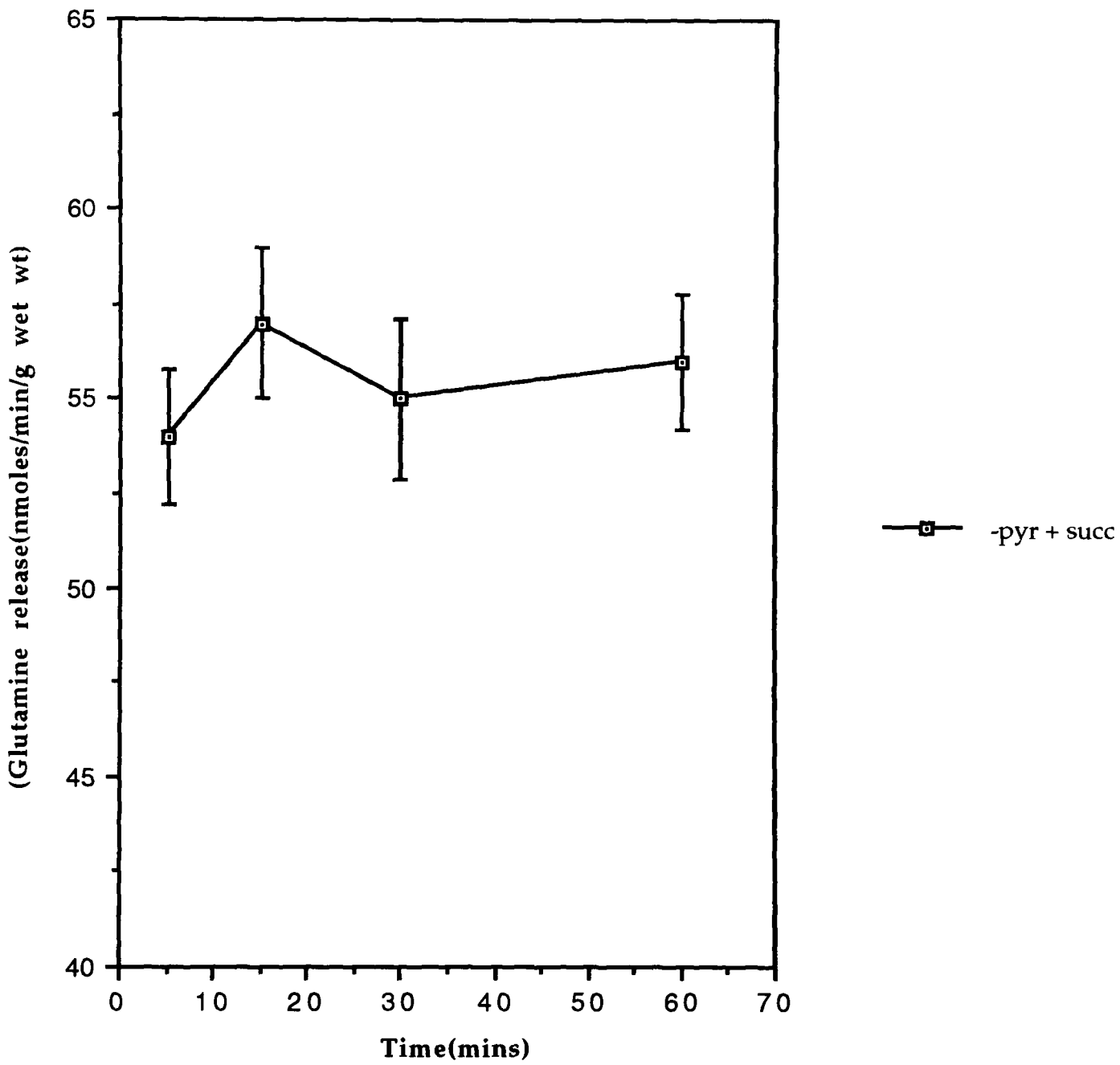


Table 2.1
The effect of isoleucine, valine or leucine on the rate of glutamine release from soleus or EDL muscle.

Addition to medium	Conc (mM)	Rate of glutamine release (nmoles/min/g wet wt)	
		soleus	EDL
Control		48±1.8 (n=15)	33.8±0.5 (n=14)
Isoleucine	10	64±2.5**** (n=16)	55±2.3**** (n=16)
Control		35.7±4.9 (n=8)	21.3±1.5 (n=7)
Isoleucine	1	47.9±2.8* (n=8)	44.6±3.0**** (n=8)
Control		40.7±2.4 (n=12)	23.3±2.5 (n=12)
Valine	10	47.4±2.1** (n=12)	38.6±4.0*** (n=14)
Control		36±1.1 (n=8)	20.4±1.8 (n=8)
Valine	1	35.9±1.1 (n=8)	27.8±2.4*** (n=8)
Control		54.7±1.6 (n=14)	39.4±2.1 (n=12)
Leucine	10	70.5±2.5**** (n=15)	61.5±2.8**** (n=16)
Control		32.8±1.2 (n=8)	20.3±1.1 (n=7)
Leucine	1	31±1.4 (n=8)	21.1±1.7 (n=8)

Values are the mean (SEM) with the number of separate experiments given in parenthesis. The statistical significance of the differences between means are denoted by *P<0.05, **P<0.02, ***P<0.01 and ****P<0.001.

Table 2.2
The effect of isoleucine, valine or leucine on the rate of alanine release from soleus or EDL muscle.

Addition to medium	Conc (mM)	Rates of alanine release (nmoles/min/g wet wt)	
		soleus	EDL
Control		15.1±0.9 (n=13)	10.5±0.6 (n=16)
Isoleucine	10	26.7±0.8**** (n=15)	24.5±0.8**** (n=16)
Control		18.5±0.7 (n=8)	11.5±1.5 (n=6)
Isoleucine	1	18.5±1.5 (n=8)	17.4±0.7**** (n=7)
Control		19.2±1.1 (n=15)	15.6±0.9 (n=15)
Valine	10	24.1±1.6** (n=16)	26.4±2.5 (n=13)
Control		15.7±0.9 (n=8)	16.1±0.4 (n=8)
Leucine	10	20.8±1.7 (n=8)	24.1±1.2**** (n=8)

Values are the mean (SEM) with the number of separate experiments given in parenthesis.
The statistical significance of the differences between means are denoted by **** P<0.001, ***P<0.01 and **P<0.02.

Table 2.3
The effect of isoleucine, valine or leucine on glutamine content in soleus or EDL muscle.

Addition to medium	Conc (mM)	Glutamine content (nmoles/g wet wt)	
		soleus	**EDL**
Control		5792±424 (n=8)	5229±271 (n=8)
Isoleucine	10	5034±201 (n=8)	5052±317 (n=8)
Control		6465±563 (n=4)	5936±631 (n=4)
Isoleucine	1	6125±188 (n=4)	5047±546 (n=4)
Control		6325±400 (n=8)	5588±384 (n=7)
Valine	10	5725±370 (n=5)	4788±250 (n=7)
Control		5358±235 (n=7)	5360±368 (n=8)
Leucine	10	6716±245*** (n=7)	6804±343** (n=7)

Values are the mean (SEM) with the number of separate experiments given in parenthesis.
The statistical significance of the differences between means are denoted by ***P<0.01 and **P<0.02.

Table 2.4
The effect of isoleucine, valine or leucine on alanine content in soleus or EDL muscle.

Addition to medium	Conc (mM)	Alanine content (nmoles/g wet wt)	
		soleus	EDL
Control		2725±814 (n=8)	2381±604 (n=8)
Isoleucine	10	2233±507 (n=8)	2847±985 (n=8)
Control		3321±570 (n=8)	2833±235 (n=8)
Valine	10	2593±108 (n=8)	2856±320 (n=8)
Control		2782±541 (n=8)	1558±325 (n=5)
Leucine	10	3028±295 (n=8)	1680±390 (n=7)

Values are the mean (SEM) with number of separate experiments given in parenthesis.

Table 2.5
The effect of 2-oxo-3-methylvalerate, 2-oxoisovalerate or 2-oxoisocaproate on the rate of glutamine release from soleus or EDL muscle.

Addition to medium	Conc (mM)	Rate of glutamine release (nmoles/min/g wet wt)	
		soleus	EDL
Control		44.3±2.7 (n=11)	33.4±1.5 (n=9)
2-oxo-3methylvalerate	0.05	47.8±4.2 (n=10)	36.1±2.4 (n=8)
	0.25	54.8±2.4** (n=9)	38.8±2.5 (n=10)
Control		42.4±2.3 (n=8)	32.7±1.8 (n=8)
2-oxo-3methylvalerate	1	50.2±2.6* (n=5)	42.2±2.0*** (n=8)
Control		48±4.7 (n=6)	37.2±3.7 (n=6)
2-oxoisovalerate	0.05	44.7±5.2 (n=5)	39.1±5 (n=5)
	1	43±1.2 (n=5)	43.0±1.2 (n=5)
Control		45.9±3.0 (n=6)	27.6±2.1 (n=6)
2-oxoisocaproate	0.05	49.8±1.2 (n=6)	32.6±2.9 (n=6)
	1	43.1±3.2 (n=5)	26.7±1.78 (n=5)

Values are means (SEM) with the number of experiments given in parenthesis. The statistical significance of the differences between means is denoted by ***P<0.01,P<0.02 and *P<0.05. .**

Table 2.6
The effect of 2-oxo-3-methylvalerate
or 2-oxoisocaproate
on the rate of alanine release from soleus or EDL muscle.

Addition to medium	Conc (mM)	Rate of alanine release (nmoles/min/g wet wt)	
		soleus	**EDL**
Control		19.2±1.8 (n=10)	11.4±1.0 (n=12)
2-oxo-3-methylvalerate	0.05	19.2±1.3 (n=9)	10.8±1.2 (n=10)
	0.25	17.7±0.7 (n=9)	12.5±1.3 (n=10)
Control		18.4±0.9 (n=7)	16.4±2.7 (n=8)
2-oxo-3-methylvalerate	1	14.9±2.2 (n=8)	14.6±4.3 (n=7)
Control		18.2±1.3 (n=6)	14.1±1.3 (n=5)
2-oxoisocaproate	0.05	20.9±2.7 (n=5)	14.1±1.7 (n=5)
	1	12.4±1.0 (n=5)	15.8±1.3 (n=5)

Values of mean (SEM) with the number of separate experiments given in parenthesis.

Table 2.7
The effect of 2-oxo-3-methylvalerate, 2-oxoisovalerate or 2-oxoisocaproate on glutamine content in soleus or EDL muscle.

Addition to medium	Conc (mM)	Glutamine content (nmoles/g wet wt)	
		soleus	**EDL**
Control		6045±357 (n=6)	6155±505 (n=5)
2-oxo-3-methyl valerate	0.05	6618±150 (n=6)	7257±189 (n=6)
	0.25	6337±353 (n=6)	7003±266 (n=6)
Control		5000±241 (n=4)	6045±432 (n=4)
2-oxo-3-methyl valerate	1	5011±217 (n=4)	5415±441 (n=4)
Control		5680±611 (n=3)	5771±326 (n=3)
2-oxoisovalerate	0.05	6561±344 (n=3)	6218±500 (n=3)
	1	5870±446 (n=3)	5358±627 (n=3)
Control		6366±255 (n=3)	6174±109 (n=3)
2-oxoisocaproate	0.05	6069±262 (n=4)	6495±484 (n=4)
	1	5538±751 (n=3)	6478±98 (n=3)

Values are the mean (SEM) with number of separate experiments given in parenthesis.

Table 2.8
The effect of 2-oxo-3-methylvalerate on alanine content in soleus or EDL muscle.

Addition to medium	Conc (mM)	Alanine content (nmoles/g wet wt)	
		soleus	EDL
Control		1536±289 (n=6)	1755±264 (n=6)
2-oxo-3-methyl valerate	0.05	2157±419 (n=6)	1177±481 (n=6)
	0.25	1959±51 (n=6)	1941±163 (n=6)

Values are the mean (SEM) with the number of separate experiments given in parenthesis.

Table 2.9
The effect of lactate on the rate of glutamine release from soleus or EDL muscle.

Concentration of Lactate (mM)	Rate of glutamine release (nmoles/min/g wet wt)	
	soleus	EDL
0	36.2±2.6 (n=60	28.3±1.5 (n=6)
1	44.1±2.4* (n=5)	36.8±2.8** (n=5)
5	50.7±3.7*** (n=5)	38.8±2.8*** (n=5)
10	61.6±3.3**** (n=5)	32.3±3.3** (n=5)
20	83.4±2.3**** (n=5)	37.0±2.0*** (n=5)

Values are the mean (SEM) with the number of separate experiments in parenthesis. The statistical significance of the differences between means are denoted by ****P<0.001, ***P<0.01, **P<0.02 and *P<0.05

Table 2.10
The effect of lactate on glutamine content in soleus or EDL muscle.

Concentration of Lactate (mM)	Glutamine content (nmoles/g wet wt)	
	soleus	EDL
0	6480±18 (n=4)	5395±232 (n=4)
1	5025±364 (n=4)	4920±282 (n=6)
5	5793±652 (n=6)	4339±62 (n=6)
10	6635±760 (n=6)	4582±297 (n=6)
20	7088±150 (n=6)	4579±530 (n=6)

Values are the mean (SEM) with the number of separate experiments given in parenthesis.

Table 2.11
The effect of lactate on the rate of alanine release from soleus or EDL muscle.

Concentration of Lactate (mM)	Rate of alanine release (nmoles/min/g wet wt)	
	soleus	EDL
0	15.9±2.1 (n=4)	10.3±0.7 (n=4)
1	25.8±1.8 (n=3)	17.5±1.1 (n=4)
5	38.2±1.6 (n=3)	27.3±4.6 (n=3)

Values are the mean (SEM) with the number of separate experiments given in parenthesis.

Table 2.12
The effect of lactate on the rate of glutamine release from soleus or EDL muscle.

Concentration of Lactate (mM)	Rate of glutamine release (nmoles/min /g wet wt)	
	soleus	EDL
0	47.3±2.3 (n=12)	35.6±3.1 (n=10)
35	78.5±2.5 **** (n=8)	53.2±3.2*** (n=10)
30	77.3±3.4 **** (n=3)	50.1±4.8*** (n=4)

Values are the mean (SEM) with the number of separate experiments given in parenthesis. The statistical significance of the differences between means are denoted by ****P<0.01 and ***P<0.01.

Table 2.13
The effect of lactate on glutamine content in soleus or EDL muscle.

Concentation of Lactate (mM)	Glutamine content (nmoles/g wet wt).	
	soleus	EDL
0	5585±174 (n=4)	5458±445 (n=6)
35	6100±300 (n=3)	6161±442 (n=6)
30	6500±795 (n=3)	6273±39 (n=3)

Values are the mean (SEM) with the number of separate experiments given in parenthesis.

Table 2.14
The effect of lactate on alanine content in soleus or EDL muscle.

Concentration of Lactate (mM)	Alanine content (nmoles/g wet wt).	
	soleus	EDL
0	2465(183) (n=6)	2491(193) (n=6)
1	2812(262) (n=6)	3026(231) (n=6)
5	3802(120)**** (n=6)	3328(165)*** (n=6)

Values are the mean (SEM) with the number of separate experiments given in parenthesis. The statistical significance of the differences between means are denoted by ****P<0.001 and ***P<0.01.

Table 2.15
The effect of 2-oxoglutarate on the rate of glutamine release from soleus or EDL muscle.

Concentration of 2-oxoglutarate (mM)	Rate of glutamine release (nmoles/min/g wet wt)	
	soleus	EDL
0	57.7±4.5 (n=11)	37.6±5.0 (n=12)
0.05	62.3±55 (n=10)	33.2±2.6 (n=10)
1	65.0±4.4 (n=10)	39.7±2.3 (n=10)

Values are the mean (SEM) with the number of separate experiments given in parenthesis.

Table 2.16
The effect of 2-oxoglutarate on the rate of alanine release from soleus or EDL muscle.

Concentration of 2-oxoglutarate (mM)	Rate of alanine release (nmoles/min/g wet wt)	
	soleus	**EDL**
0	18.7±1.0 (n=11)	14.6±1.5 (n=11)
0.05	20.5±1.4 (n=9)	10.3±3.7 (n=9)
1	18.4±0.9 (n=10)	14.5±1.2 (n=10)

Values are the mean (SEM) with the number of separate experiments given in parenthsis. .

Table 2.17
The effect of 2-oxoglutarate on glutamine content in soleus or EDL muscle.

Concentration of 2-oxoglutarate (mM)	Glutamine content (nmoles/g wet wt)	
	sol	**EDL**
0	5193±145 (n=6)	5262±424 (n=6)
0.05	4405±325 (n=6)	6781±292* (n=6)
1	5243±467 (n=6)	6904±213** (n=6)

Values are the mean (SEM) with the number of separate experiments given in parenthesis. The statistical significance of the differences between means are denoted by **P<0.02 and *P<0.05.

Table 2.18
The effect of ornithine on the rate of glutamine release from soleus or EDL muscle.

Concentration of Ornithine (mM)	Rate of glutamine release (nmoles/min/g wet wt)	
	soleus	EDL
0	53.5±3.6 (n=8)	44.3±3.9 (n=8)
0.6	62.3±5.6 (n=8)	44.8±2.6 (n=8)

Values are the mean (SEM) with the number of separate experiments given in parenthesis.

Table 2.19
The effect of ornithine on the rate of alanine release from soleus or EDL muscle.

Conc of Ornithine (mM)	Rate of alanine release (nmoles/min/g wet wt)	
	soleus	EDL
0	18.9±1.1 (n=7)	15.7±1.2 (n=8)
0.6	21.3±2.0 (n=7)	15.9±1.4 (n=8)

Values are the mean (SEM) with the number of separate experiments given in parenthesis.

Table 2.20
The effect(s) of hypo- and hyper-osmotic incubation media on soleus muscle ATP, ADP and AMP content.

Incubation conditions	Metabolite content (umoles/g wet wt)		
	ATP	ADP	AMP
Not incubated	5.98±0.11 (n=9)	0.91±0.03 (n=9)	0.087±0.001 (n=9)
Control medium	5.12±0.19 (n=9)	0.78±0.07 (n=9)	0.08±0.013 (n=9)
Hypo-osmotic medium 70mM Na^+	4.91±0.19 (n=9)	0.78±0.04 (n=9)	0.062±0.012 (n=9)
Hyper-osmotic medium 50mM sucrose	4.16±0.29* (n=9)	0.81±0.06 (n=9)	0.068±0.01 (n=9)

Values are the mean (SEM) for 9 separate experiments. The statistical significance of the differences between treated and control mean is denoted by *($p<0.02$)

Table 2.21
The effect(s) of hypo- and hyper-osmotic incubation media on soleus muscle ATP/ADP and ATP/AMP content ratios.

Incubation conditions	Adenine nucleotide content ratios	
	ATP/ADP	ATP/AMP
Not incubated	6.59±0.18 (n=9)	79±10.7 (n=9)
Control medium	6.81±0.39 (n=9)	64.5±7.1 (n=8)
Hypo-osmotic medium 70mM Na^+	6.37±0.18 (n=8)	68.4±7.1 (n=6)
Hyper-osmotic medium 50mM sucrose	5.23±0.22** (n=9)	62.4±7.6 (n=7)

Values are the mean (SEM) for 9 separate experiments. The statistical significance of the differences between treated and control means is denoted by **($p<0.01$)

Table 2.22
The effect(s) of hypo- and hyper-osmotic media on total, intracellular and extracellular water content of incubated soleus muscle.

Incubation medium composition	osmolality (mmol/kg)	Muscle water (ul/g wet wt)		
		Total	Extracellular	Intracellular
Control	298	788±6	271±13	517
Hyper-osmotic				
5mM-sucrose	303	nd	nd	nd
10mM-sucrose	308	nd	nd	nd
50mM-sucrose	350	768±5*	272±10	496
Hypo-osmotic				
120mM-Na^+	249	797±8	245±10	552
95mM-Na^+	203	nd	nd	nd
70mM-Na^+	158	814±4**	202±12**	612

Values are means (SEM); n>4 for all groups. Variations around the mean for the measurement of osmolality was <2%. The statistical significance of the differences between means is denoted by *(p<0.05) and **(p<0.01). nd, not determined.

NB Data (Tables 2.22 - 2.27 and Figure 6): Effects of changes in cell volume on the rates of glutamine and alanine release from rat skeletal muscle *in vitro*. Parry-Billings, M. Bevan , S.J., Opara, E. and Newsholme, E.A. 1991a Biochem. J. **276**: 559-561.

Table 2.23
The effects of hypo-and hyper-osmotic media on glutamine and alanine content in stripped soleus muscle.

Incubation medium composition	Content (nmoles/ml of intracellular water) Glutamine	Alanine
Control	6903±383	4340±397
Hyper-osmotic(50mM-sucrose)	6976±430	4722±322
Hypo-osmotic(70mM-Na^+)	7136±339	3992±156

Values are means (SEM) for 6 separate experiments.

Table 2.24
The effect of hypo-osmotic media on the rate of glutamine release from stripped soleus muscle.

Incubation medium composition	Glutamine release (nmoles/min/g wet wt)
Control (146mM) Na^+	33 ±1.2
Hypo-osmotic medium (120mM Na^+)	27 ±1.3***
Hypo-osmotic medium (95mM Na^+)	22 ±1.6****
Hypo-osmotic medium (70mM Na^+)	23±1.1****
Iso-osmotic medium (70mM Na^+ (+s)	34 ±1.5

Values are mean (SEM) for 6 separate experiments. Medium supplemented with sucrose to maintain isotonicity is indicated by "+s". The statistical significance of the differences between treated and controls means is denoted by *(P<0.01) and ****(P<0.001).**

Table 2.25
The effect of hyper-osmotic media on the rate of glutamine release from stripped soleus muscle.

Incubation medium composition	Glutamine release (nmoles/min/g wet wt)
Control	34 ±1.1
Hyper-osmotic medium (5mM sucrose)	37 ±1.2
Hyper-osmotic medium (10mM sucrose)	39 ±1.6*
Hyper-osmotic medium (50mM sucrose)	38 ±1.5*

Values are mean (SEM) for 6 separate experiments. The statistical significance of the differences between treated and control means is denoted by * (P<0.05).

Table 2.26
The effect of cell volume changes on the rate of alanine release from stripped soleus muscle.

Incubation medium composition	Alanine release (nmoles/min/g wet wt)
Control	24.0±1.3 (n=6)
Hyper-osmotic medium (50mM sucrose)	30.7±2.0** (n=6)
Hypo-osmotic medium (70mM Na^+)	15.5±1.5*** (n=6)

Values are means (SEM) for 6 separate experiments. The statistical significance of the differences between treated and control means is denoted by **(p<0.01) and *(p<0.001).**

Table 2.27
Changes in the rate of glutamine release from muscle, induced by incubation in hypo-osmotic media.

Rates of glutamine release (nmoles/min/g wet wt)	
Pre-incubation period Iso-osmotic (146 mM-Na^+) 33.3±1.9	Pre-incubation period Iso-osmotic (146 mM-Na^+) 37.9±2.7
First incubation period (50min.) Hypo-osmotic (70 mM-Na^+) 19.1±1.6**A**	Second incubation period (50min.) Iso-osmotic (70 mM-Na^+ with sucrose) 41.2±3.2*

Values are means (SEM) for 6 separate experiments.
The statistical significance of the difference between the first and second incubation periods is denoted by *(p<0.001), and the statistical significance of the difference between the pre-incubation and first incubation period is denoted by A(p<0.001).

Figure 6. The effect of hypo-osmotic incubation medium on the rate of glutamine release from soleus muscle against time.
The rate of release of glutamin e from soleus muscle, incubated in hypo-osmotic medium, was measured after 5, 15, 30 and 60mins of incubation time.

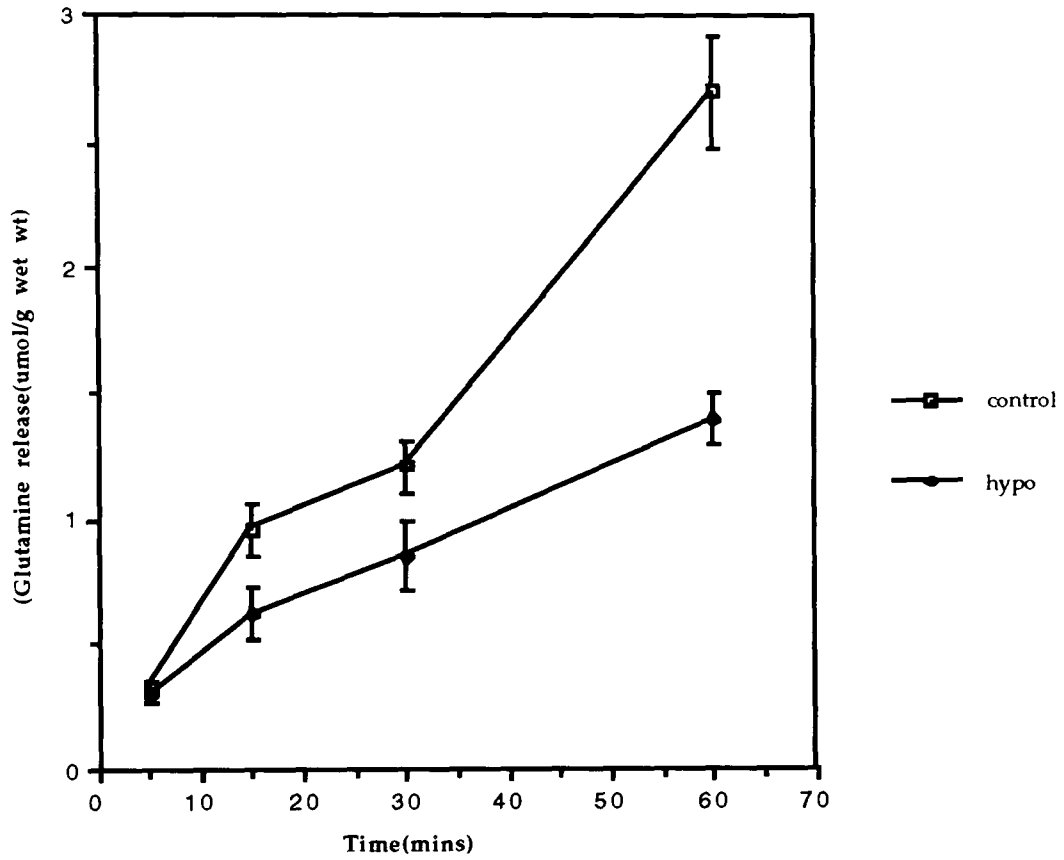


Table 2.28
The effect of hypo-osmotic media on the rate of glutamine release from EDL muscle.

Incubation medium composition	Glutamine release (nmoles/min/g wet wt)
Control (146mM Na^+)	26 ±1.1
Hypo-osmotic medium (120mM Na^+)	23 ±1.6**
Hypo-osmotic medium (95mM Na^+)	20 ±1.21***
Hypo-osmotic medium (70mM Na^+)	19 ±0.6****

Values are the mean (SEM) for 8 separate experiments. The significance of the differences between treated and control means is denoted by **(P<0.02), *(P<0.01) and ****(P<0.001).**

TABLE 2.29
The effect of hypo-osmotic media on the rate of glutamine release from whole soleus muscle.

Incubation medium composition	Glutamine release (nmoles/min/g wet wt)
Control (146mM Na^+)	41 ±1.0
Hypo-osmotic medium (120mM Na^+)	36 ±1.1**
Hypo-osmotic medium (95mM Na^+)	27 ±0.7****
Hypo-osmotic medium (70mM Na^+)	25±0.6****

Values are the mean (SEM) for 8 separate experiments .
The statistical significance of the differences between treated and control means is denoted by **(P<0.02) and **(P<0.001).**

TABLE 2.30
The effect of hyper-osmotic media on the rate of glutamine release from EDL muscle.

Incubation medium composition	Glutamine release (nmoles/min/g wet wt)
Control	21 ±1.6
Hyper-osmotic medium (5mM sucrose)	28 ±1.6***
Hyper-osmotic medium (10mM sucrose)	28 ±1.2***
Hyper-osmotic medium (50mM sucrose)	40 ±2.6****

Values are the mean (SEM) for 8 separate experiments.
The statistical significance of the differences between treated and control means is denoted by *(P<0.01) and ****(P<0.001)**

Table 2.31
The effect of hyper-osmotic media on the rate of glutamine release from whole soleus muscle.

Incubation medium composition	Glutamine release (nmoles/min/g wet wt)
Control	37 ±2.1
Hyper-osmotic medium (5mM sucrose)	44 ±3.0
Hyper-osmotic medium (10mM sucrose)	39 ±1.8
Hyper-osmotic medium (50mM sucrose)	58 ±2.7****

Values are mean (SEM) for 8 separate experiments. The statistical significance of the differences between treated and control means is denoted by **(P<0.001).**

Table 2.32
The effect of hypo-osmotic media on the rate of alanine release from EDL muscle.

Incubation medium composition	Alanine release (nmoles/min/g wet wt)
Control (146mM Na^+)	11.5 ±1.5
Hypo-osmotic medium (120mM Na^+)	10.8 ±1.5
Hypo-osmotic medium (95mM Na^+)	9.7 ±2.5
Hypo-osmotic medium (70mM Na^+)	9.5 ±0.7

Values are the mean (SEM) for 8 separate experiments.

Table 2.33
The effect of hypo-osmotic media on the rate of alanine release from whole soleus muscle.

Incubation medium composition	Alanine release (nmoles/min/g wet wt)
Control (146mM Na^+)	18 ±1.7
Hypo-osmotic medium (120mM Na^+)	15 ±2.4
Hypo-osmotic medium (95mM Na^+)	10 ±1.9**
Hypo-osmotic medium (70mM Na^+)	10 +0.7***

Values are mean (SEM) for 8 separate experiments. The statistical significance of the differences between treated and control means is denoted by **(P<0.02) and *(P<0.01).**

Table 2.34
The effect of hyper-osmotic media on the rate of alanine release from EDL muscle.

Incubation medium composition	Alanine release (nmoles/min/g wet wt)
Control	6.2 ±0.9
Hyper-osmotic medium (5mM sucrose)	8.5 ±07
Hyper-osmotic medium (10mM sucrose)	9.2 ±0.7
Hyper-osmotic medium (50mM sucrose)	11.65 ±1.1****

Values are mean (SEM) for 8 separate experiments. The statistical significance of the differences between treated and control means is denoted by **(P<0.001).**

Table 2.35
The effect of hyper-osmotic media on the rate of alanine release from whole soleus muscle.

Incubation medium composition	Alanine release (nmoles/min/g wet wt)
Control	11.2 ±0.6
Hyper-osmotic medium (5mM sucrose)	12.5 ±1.5
Hyper-osmotic medium (10mM sucrose)	1.5 ±1.1
Hyper-osmotic medium (50mM sucrose)	20 ±1.4****

Values are mean (SEM) for 8 separate experiments. The statistical significance of the differences between treated and control means is denoted by **(P<0.001).**

4.Discussion.

4.1.Investigation of the rate of glutamine release from muscle over time.

Parry-Billings (1989) reported that the rate of glutamine release decreases over time. The suitability of an experimental technique for the study of cellular uptake or release maybe indicated by the linearity of the rate of release or uptake of the metabolite concerned (see Kilberg et al 1980, Newsholme et al 1986). Thus, the possibility that the rate of release of

glutamine from skeletal muscle over time is non-linear, may lead to problems in interpreting results of studies in which the effect(s) of factors and conditions on the rate of glutamine release from muscle are investigated. Therefore it is possible that the non-linear rate of glutamine release from muscle may have affected the interpretation of data from Parry-Billings' (1989) study on the effect(s) of factors and conditions on the rate of glutamine release from muscle. This study led to the suggestion that the release of glutamine from muscle is not influenced by the intracellular concentration of glutamine. Furthermore, the non-linear rate of release of glutamine from muscle, may have led to the incorrect calculation of the approximate Km (<0.3mM) for the process of glutamine release. This calculation is the basis for the prediction that the process of glutamine release from muscle is the flux generating step for the pathways of muscle glutamine metabolism and glutamine utilization by cells of the immune system. Thus, the non-linear rate of release may raise questions as to whether the process of glutamine release from muscle, is indeed flux generating. This indicated that the investigation of the rate of glutamine release over time was essential.

It was not clear, from the study by Parry-Billings (1989), whether the process of muscle glutamine release itself is non-linear, or whether an artefact is causing release to appear non-linear. Results from the present study suggest that it is the presence of an artefact, that causes the rate of release of glutamine from muscle over time, to appear non-linear.

The 'disruption' of the linear release of glutamine from muscle was probably caused by the 'carry over' effect of pyruvate from the preincubation media to the incubation media via muscle. The question is how could pyruvate continuously alter the rate of glutamine release from muscle over a 60mins period? An experiment involving the incubation of muscle in incubation media containing pyruvate (0.5 or 5mM), and the

subsequent determination of the concentration of glutamine in the pyruvate-containing incubation media, suggests that pyruvate does not affect the linearity of glutamine release from muscle, but interferes with the assay used to determine the concentration of glutamine in incubation media.

The glutamine assay, used in the present study, involves two stages: the first stage involves the conversion of glutamine to glutamate and ammonia, which is catalysed by asparaginase (reaction 1). Ammonia generated from reaction 1 is involved in the second stage of the glutamine assay; ammonia reacts with 2-oxoglutarate, in the presence of NADH, to generate glutamate and NAD^+ (reaction 2), in a reaction catalysed by glutamate dehydrogenase. The concentration of glutamine in the incubation medium is determined by following the conversion of NADH to NAD^+, that occurs in reaction 2, spectrophotometrically.

$$\text{Glutamine} + H_2O \xrightarrow[\textbf{Asparaginase}]{} \text{Glutamate}^- + NH_4^+ \quad \text{(reaction 1)}$$

$$\text{2-Oxoglutarate}^{2-} + \text{NADH} + NH_4^+ + H^+ \xrightarrow[\textbf{Glutamate-dehydrogenase}]{} \text{Glutamate}^- + NAD^+ + H_2O \quad \text{(reaction 2)}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow[\textbf{Lactate dehydrogenase}]{} \text{Lactate} + NAD^+ \quad \text{(reaction 3)}$$

Pyruvate may interfere with the glutamine assay at the second stage of the assay since pyruvate, present in the incubation medium, may react with NADH to give lactate and NAD^+. This reaction is catalysed by lactate dehydrogenase (LDH) (reaction 3). However, studies have shown that very low amounts of lactate dehydrogenase are released from incubated muscle into the incubation medium (see Appendix IV), thus LDH may originate from another source, possibly from the asparaginase or glutamate

dehydrogenase preparations used in the assay. Therefore, the presence of pyruvate in the incubation media may be causing an 'artificial effect' on the glutamine assay. Over a period of 60mins. the level of pyruvate is decreased since, it is reacting with LDH to give lactate. Thus, the effect of pyruvate on the glutamine assay is decreased, hence the apparent reduced 'rate of glutamine release' over the 60mins incubation period.

4.2.The relationship between the effects of factors on muscle glutamine release and the effect(s) of factors on muscle glutamine concentration.

Studies by Parry-Billings (1989) were consistent with the prediction made by metabolic control logic that, changes in the concentration of muscle glutamine will not normally have an effect on the process of glutamine release (see Chapter 1, section 5). Thus, evidence was provided to suggest that the process of release may be externally regulated.

In light of the knowledge that the rate of release of glutamine from muscle over time is linear, this "non-simple" relationship between muscle glutamine release and muscle glutamine concentration, suggested by Parry-Billings (1989), was re-investigated and verified in the present study (see Table 1). Thus, data from the present study are also consistent with the prediction made by metabolic control logic (see above). In addition the calculation, by Parry-Billings (1989), of an approximate Km for the process of muscle glutamine release (< 0.3mM), which is the basis for its role as the flux generating step for the pathways of muscle glutamine metabolism and glutamine utilization for cells of the immune system, can be supported. However, it is unclear whether the concentration of glutamine in skeletal muscle determined by the method used in the present study represents a pool of free glutamine in skeletal muscle, or a combination of free and bound glutamine, for example glutamine sequestered in vesicles, in skeletal muscle. Thus, clarification of the form

in which the majority of glutamine is present in skeletal muscle, i.e. free or bound glutamine, may be helpful in addressing the question of regulation of the process of glutamine release from muscle, and also the relationship between muscle glutamine release and muscle glutamine concentration.

Table 1: relationship between changes in muscle glutamine release and changes in muscle glutamine concentration.

Factors	Change in muscle glutamine concentration	Change in release
Leucine	increase	increase
Isoleucine	no change	increase
Valine	no change	increase
2-oxo-3-methylvalerate	no change	increase
Lactate	no change	increase
Hypo-osmosis	no change	decrease
Hyper-osmosis	no change	increase

4.3.Possible mechanisms for the regulation of glutamine release from muscle.

It is generally assumed that the process of muscle glutamine release is equivalent to the rate of glutamine synthesis by muscle, so that the term "release" is synonymous with "synthesis" (see Garber et al 1976a, Ardawi 1988a and Ardawi and Majzoub 1991). For example, it is assumed that the

branched chain amino acids stimulate the rate of release of glutamine from muscle by increasing the rate glutamine synthesis in muscle, specifically via the provision of nitrogen (by the transamination of the branched chain amino acids) and carbon for the synthesis of glutamine; Golden et al (1981) found that in human subjects administered ^{15}N valine or leucine, the amide nitrogen of glutamine was greatly enriched with the ^{15}N label in skeletal muscle in the rat and Huston and Zapalowski (1981) reported that in rat hindquarter, ^{14}C of ^{14}C leucine is readily incorporated into glutamate and glutamine. Studies by Goldberg and Chang (1978) have reported that ^{14}C of ^{14}C valine is readily incorporated into glutamate and glutamine. Additionally, Yoshida et al (1991) measured the incorporation of ^{13}C from leucine C-2 of [1,2 ^{13}C] leucine, into plasma glutamine of the rat. However, the assumption that the branched chain amino acids regulate muscle glutamine release via their effect on muscle glutamine concentration is not consistent with data from the present study or the prediction made by metabolic control logic. For example, although leucine increased the concentration of glutamine in incubated muscle, as well as the rate of glutamine release from incubated muscle, isoleucine and valine increased the rate of glutamine release but this increase was not associated with an increase in muscle glutamine concentration. This relationship, i.e. an increase in the rate of glutamine release from muscle, but no change in muscle glutamine concentration, is also associated with the effect of 2-oxo-3-methylvalerate, lactate and changes in muscle cell volume.

Data from the present study suggest that the process of glutamine release from muscle may be externally regulated. This suggestion is consistent with the prediction made by metabolic control logic, on the regulation of the process of glutamine release from muscle (see Chapter 1, section 5). However, as stated previously the nature of the regulation of

glutamine release is not known. Parry-Billings (1989) proposed that external factors may modify release of glutamine by acting on the transporter for muscle glutamine release. Thus, it may be that the factors that modify the rate of glutamine release may do so by increasing the activity of the transporter, or by increasing the number of transporters in muscle.

The "changes in cell volume" experiment may provide some suggestions as to the mechanism for muscle glutamine release. Changes in extracellular osmolarity and in muscle cell volume are associated with physiological and pathological conditions. Some of these conditions are associated with changes in the rate of glutamine release from muscle. For example, prolonged exercise increases the volume of rat soleus muscle (Peeze-Binhorst et al 1990) by between 16% and 28%. The rate of glutamine release from incubated soleus muscle of the rat after prolonged exercise is decreased (Parry-Billings et al 1988), plus the rate of glutamine release from muscle is decreased in man, following marathon running (Parry-Billings 1989). On the otherhand short term intensity exercise (sprinting) may cause an increase in the rate of glutamine release from muscle: sprinting is associated with an increase in plasma glutamine concentrations (Poortmans et al 1974, Maughan and Gleeson 1988, Parry-Billings 1989). The increase in muscle cell volume, induced in the present study (18%) is of a similar magnitude to that reported by Peeze-Binhorst et al (1990) Thus, the effects of cell volume changes reported in this study could be important in the control of glutamine release from muscle *in vivo.* during different types of exercise.

The mechanism by which changes in cell volume affect the rate of release by muscle is not known. Studies have proposed that the release of glutamine from muscle is sodium-dependent (Rennie et al 1986, Hundal et al 1987, Ahmed et al 1990a, 1990b). Thus, one might have assumed that,

in the present study the decrease in the rate of glutamine release from muscle that occurred with an increase in muscle cell volume, may be due to the decrease in the concentration of sodium ions in the incubation medium. However, in the studies by Rennie et al (1986), Hundal et al (1987), and Ahmed et al (1990a, 1990b), on the dependence of muscle glutamine transport on sodium, muscle glutamine uptake was the only muscle glutamine transport process investigated. It was assumed that since the process of glutamine uptake into muscle was shown to be sodium-dependent, that the process of glutamine release from muscle was also sodium-dependent. This assumption is based on the suggestion that, glutamine transport across the muscle membrane is "symmetrical", thus implying that the process of glutamine transport is near equlibrium and that the processes of glutamine uptake and release are the same (Rennie et al 1986). This may, however, not be the case. Calculations, by Parry-Billings (1989), of the free energy change for glutamine uptake suggest that glutamine uptake may be a non-equilibrium process. In addition, metabolic control logic predicts that the process of glutamine release from muscle is flux generating. Thus, glutamine uptake and release cannot occur by the same process.

In addition to the quantitative evidence that suggests that glutamine uptake and release are separate processes, there is no evidence that the process of muscle glutamine release is sodium dependent: Parry-Billings et al (1991) (unpublished data) reported that the rate of glutamine release from skeletal muscle *in vitro* is unaffected by a number of agents (harmaline, gramicidine and amiloride) which are known to disrupt the Na^+ gradient across the membrane. In addition, the decreases in the rate of glutamine release in hypo-osmotic media, shown in the present study, were not the result of a decrease in the extracellular concentration of Na^+; the rate of glutamine release was not affected if sucrose was used to

maintain the osmolality of media containing decreased concentrations of Na^+.

Volume-regulatory processes i.e. mechanisms that regulate cell volume during osmotic changes (see Haussinger et al 1990a,b, Lang et al 1989, Graf et al 1988, Kregnow 1981) have been indicated in the regulation of hepatic glutamine metabolism. One such volume-regulatory process is the change in K^+ flux that occurs across liver cells exposed to hypo- or hyper-osmotic medium. Haussinger et al (1990a) reported that following exposure of perfused rat liver to hypo-osmotic media, liver-cell volume is restored within 8mins after initial cell swelling by the release of cellular K^+, and following exposure of perfused rat liver to hyper-osmotic media, liver-cell volume is restored after initial cell shrinkage, by K^+ reuptake.

In an investigation into the effects of cell volume changes on liver glutamine metabolism, Haussinger et al (1990b) reported that following cell swelling or shrinkage, there is an increase in the release or uptake of potassium ions by liver cells respectively. In the study by Haussinger et al (1990b), the restoration of normal liver-cell volume took place within 8mins. of the change in cell volume, yet liver glutamine metabolism still remained altered following changes in cell volume. Increases in liver cell volume led to a decrease in glutamine release from liver, and decreases in liver cell volume led to an increase in glutamine release from liver. From this study Haussinger et al (1990b) concluded that volume regulatory responses, such as changes in K^+ ion flux, may be important factors involved in the alteration of liver glutamine metabolism. Such changes may be taking place, and be involved in the regulation of glutamine release from muscle exposed to different osmotic media. However, to the author's knowledge, no evidence for volume-regulatory processes or their involvement in regulating glutamine metabolism in skeletal muscle has been reported.

Alternatively, the alterations in muscle glutamine release caused by muscle cell volume changes may involve the action of the muscle glutamine transporter. This hypothesis is based on the effect of changes in muscle cell volume on muscle glucose metabolism. The process of glucose transport in skeletal muscle involves the recruitment of glucose transporters from an intracellular membrane compartment to the plasma membrane (see Klip et al 1990). An investigation of the effect of changes in muscle cell volume on muscle glucose metabolism (Bevan 1992) showed that an increase in muscle-cell volume (incubation of muscle in hypo-osmotic medium) caused a decrease in the rate of glucose transport in skeletal muscle; a decrease in muscle-cell volume (incubation of muscle in hypo-osmotic medium) caused an increase in the rate of glucose transport in skeletal muscle. One explanation for the changes in muscle glucose transport following changes in muscle-cell volume reported by Bevan (1992), is that translocation of the glucose transporter could be affected by an increase or decrease in cell volume. Such an increase or decrease could increase or decrease the distance of translocation of the glucose transporter, from its intracellular compartment to the plasma membrane respectively.

Results from the present study, on the effect(s) of cell volume changes on muscle glutamine release, are consistent with the possibility that the transporter for muscle glutamine release may translocate from an intracellular compartment to the plasma membrane.

Lactate also modified the rate of glutamine release from muscle. Changes in muscle lactate concentration are associated with changes in muscle and plasma glutamine concentration, following short-term intense exercise; following this type of exercise, the concentration of plasma glutamine is increased (Poortmans et al 1974, Maughan and

Gleeson 1988, Parry-Billings 1989) and a concentration of between 20 and 30mM lactate in muscle, and subsequently in blood, is reached (see Newsholme and Leech 1983). In the present study, 20 or 30mM lactate markedly increased the rate of glutamine release from soleus and EDL muscle. Thus, the increase in plasma glutamine concentration that occurs following short-term intense exercise, may involve the increase in muscle and blood lactate also.

There are no obvious conclusions as to how lactate stimulates glutamine release from muscle. However, one possibility for the action of lactate on glutamine release is a pH effect. In the present study lactate, present in the incubation medium, may be taken up by muscle and oxidized. This oxidation of lactate would lead to a loss of protons and hence an increase in pH. Thus, an increase in pH may be responsible, via an effect on the transporter for glutamine release from muscle, for the increase in the rate of glutamine release from muscle.

The suggestion that, lactate may effect the rate of release of glutamine from muscle via its oxidation in muscle, may explain the quantitative difference between the effect of lactate on the rate of release of glutamine from soleus muscle and that of EDL muscle. Due to their metabolic differences, EDL has a greater glycolytic capacity than soleus, the extent to which lactate is oxidized may differ, thus, the quantitative change in pH may differ, hence leading to a quantitative difference in the effect of lactate on soleus and EDL muscle.

4.4.The possible clinical benefits of branched chain amino acids, ornithine and 2-oxoglutarate: do they occur via action on muscle glutamine release?

Studies have shown that glutamine is important for immune cell function. Ardawi and Newsholme (1983), and Parry-Billings et al (1990c), reported that the proliferation of lymphocytes in culture is decreased

when the concentration of glutamine in the culture medium is decreased. Thus, a decrease in plasma glutamine concentration may contribute to an impaired immune system.

Overtraining is a clinical condition which arises from exercise periods which are too frequent, too intense and too prolonged, thus not allowing complete recovery from each exercise period. This results in impairment in exercise performance, and a failure to adapt to exercise. Overtraining is associated with a decreased concentration of plasma glutamine, and immunosuppression (see Parry-Billings 1989). It is considered that the decrease in plasma glutamine concentration may contribute to the immunosuppression associated with overtraining. In a previous experiment, Parry-Billings (1989) reported that branched chain amino acid ingestion in man, following long-term exercise, prevents the decrease in plasma glutamine concentration, which occurs after long term exercise. The prevention in the decline of plasma glutamine may occur via the stimulation of glutamine release from muscle caused by the branched chain amino acids, as reported previously (section 3.3). Thus, the effect of branched chain amino acid ingestion on plasma glutamine levels, following long-term exercise, may be of physiological importance in the condition of overtraining. Ingestion of branched chain amino acids during this condition may increase plasma glutamine concentration, as they do following long term exercise, possibly via the stimulation of glutamine release from muscle, and in doing so may help maintain immune function in the condition of overtraining.

The administration of glutamine to man and experimental animals, post-surgery, is reported to improve nitrogen balance (Stehle et al 1989; Hammarqvist et al 1989, respectively). Numerous studies have reported that branched chain amino acids have similar effects to glutamine on nitrogen sparing: branched chain amino acids have nitrogen

sparing effects when administered to both humans (e.g. Benotti et al 1976) and experimental animals (e.g. Freund et al 1981) post-surgery. The effects of branched chain amino acids on nitrogen balance may occur via their effect on muscle glutamine release.

2-Oxoglutarate and ornithine have been used clinically as a salt complex, L(+)ornithine-2-oxoglutarate. Studies have shown that administration of the complex L(+)ornithine-2-oxoglutarate improves nitrogen balance in humans post-surgery and promotes wound healing in humans (see Table 2).

Table 2: the effects of L(+)ornithine-2-oxoglutarate on muscle glutamine concentration, nitrogen balance and wound healing in postoperative and burns patients.

Effect of L(+)ornithine-2 -oxoglutarate.	Condition	Reference
Improves nitrogen balance	Surgery	Leander et al (1985) Vesterberg et al (1987) Hammarqvist (1989) Wernerman et al (1989)
	Burns	Cynober et al (1984)
Promotes wound healing	Burns & Surgery	Cynober (1991)

As stated previously, glutamine has been shown to improve nitrogen balance. Wound healing requires amino acids for protein synthesis, and thus depends upon an improved nitrogen balance (see Blackburn et al

1977). Studies suggest that the reported effect of this complex on nitrogen balance and wound healing, are due to its effect on muscle glutamine concentration, for example Cynober (1991) suggests that, since the complex, L(+)ornithine-2-oxoglutarate, and also 2-oxoglutarate, attenuate the decrease in muscle glutamine concentration that occurs following surgery (see Hammarqvist 1989, Hammarqvist et al 1991, 1990a, and Wernerman et al 1989) it may promote wound healing via an effect of this complex on muscle glutamine synthesis. However, metabolic control logic predicts, and the data from the present study indicate, that the important process in the provision of muscle glutamine to the rest of the body is that of glutamine release and not muscle glutamine synthesis. This suggests that either the beneficial effects of L(+)ornithine-2-oxoglutarate observed are caused by an indirect effect of the complex on muscle glutamine release *in vivo* or that they do not occur via an effect on muscle glutamine metabolism. Thus, to determine the mechanism for the beneficial effect(s) of this complex, other areas, such as its effect on protein synthesis at the site of injury, or on glutamine metabolism of other tissues, require investigation.

4.5.Do these factors affect muscle glutamine synthesis?

A quantitative analysis of the effects of external factors such as, branched chain amino acids and lactate, on muscle glutamine release in relation to muscle glutamine concentration, predicts that in the presence of these factors, muscle glutamine concentration should be almost totally depleted after a 1h. incubation. However for these factors (branched chain amino acids and lactate) muscle glutamine concentration after a 1h. incubation is either maintained or increased (see Table 3).

Table 3: a quantitative analysis of the effect(s) of external factors on the rate of release of glutamine from soleus muscle in relation to soleus muscle glutamine concentration.

(N.B. for these calculation the muscle glutamine concentration for rat soleus muscle used is 4mM. Details of calculations in Appendix V)

Factor	Rate of glutamine release in presence of external factors (nmoles/min/g)	Estimated time of muscle glutamine depletion (min)	Approximate muscle glutamine concentration after 1h. incubation in presence of factor (mM)
Leucine (10mM)	70	55	6.5 (increase)
Isoleucine (10mM)	64	60	4 (unchanged)
Lactate (10mM)	61	60	4 (unchanged)
Lactate (20mM)	84	45	4 (unchanged)

The quantitative analysis summarized in Table 3 suggests that these factors may be having a stimulatory effect on the process of glutamine synthesis, possibly via an effect on the enzyme for glutamine synthesis, glutamine synthetase. As predicted by metabolic control logic, glutamine synthesis in muscle does not normally regulate the process of glutamine release from muscle. This prediction was confirmed by Parry-Billings (1989), who showed that an inhibitor for glutamine synthetase, methionine sulphoximine, had no effect on the rate of release of

glutamine from muscle. Thus, the possible stimulation of glutamine synthetase activity in muscle by these factors, would be solely for the maintenance of muscle glutamine concentration. To see if the maintenance or increase in muscle glutamine concentration reported is due to the stimulation of the process of glutamine synthesis in muscle, the effect(s) of these factors on this process were investigated and the results presented and discussed in the next chapter.

Chapter 3

An investigation of glutamine synthesis in skeletal muscle and adipose tissue.

1.Introduction.

Studies of glutamine synthesis in muscle have involved the measurement of the maximal *in vitro* enzyme activity glutamine synthetase (King et al 1983; Max et al 1987; Rivera et al 1988; Ardawi 1988a). Glutamine synthetase catalyses the reaction for the synthesis of glutamine from glutamate and ammonia coupled to the cleavage of ATP to ADP and inorganic phosphate (see reaction 1). The reaction is non-equilibrium in nature: Parry-Billings (1989) calculated the free energy change to be about -13kJ/mole, using data from Lund (1970), Newsholme and Leech (1983) and Ardawi (1988a).



The importance of the non-equilibrium reactions is that they give directionality to metabolic pathways and are potential sites for allosteric regulation of the flux through metabolic pathways.

Maximal *in vitro* activities of some key enzymes have been used to provide quantitative information about the maximum capacity of some metabolic pathways (see Crabtree and Newsholme 1970, 1972a, 1972b; Sugden and Newsholme 1973). Such information can only be obtained from the activities of enzymes that catalyze reactions of a pathway that are far-removed from equilibrium *in vivo*. This is because reactions that catalyse near equilibrium reactions possess maximum *in vitro* catalytic activities that are much greater than the maximum rate of a flux through the reactions *in vivo*.

Studies of the effects of external factors on glutamine release and content from and in skeletal muscle indicate that there is no simple relationship between muscle glutamine release and content (see Chapter 2, Table 1 and Parry-Billings 1989). For example an increase in glutamine release may be associated with an increase, a decrease, or no change in muscle glutamine content (see Table 1). These studies plus quantitative analysis of data for the effects of external factors on muscle glutamine release and concentration (see Chapter 2, Table 3), indicate that external factors may affect the process of glutamine synthesis in skeletal muscle, and thus, be potential regulators of muscle glutamine synthesis.

Table 1: the effect(s) of external factors on glutamine content and release in and from skeletal muscle.

External factors /Conditions	Effect on the rate of glutamine release	Effect on muscle glutamine content
Dexamethasone*	increase	increase
Hypothyroidism*	decrease	decrease
Hyperthyroidism*	increase	no change
Sepsis*	increase	decrease
Branched chain** amino acids	increase	no change
Lactate**	increase	no change
Cell volume changes**		
Hypo-osmotic medium**	decrease	no change
Hyper-osmotic medium**	increase	no change
Ethanol*	no change	increase

Data from * Parry-Billings (1989) Parry-Billings et al (1989, 1990a, 1990b 1991a, *~~in press~~ 1992) and ** Chapter 2 of present study.

Data from Chapter 2, on the rates of glutamine release from muscle and muscle glutamine concentrations, in the presence of different factors, can be used to provide quantitative information about the pathway of glutamine synthesis in muscle. Table 2 shows that different factors/conditions alter the estimated rate of glutamine synthesis in both

soleus and EDL muscle, and that there is great variability in the estimated rate of muscle glutamine synthesis. For example, the estimated rate of glutamine synthesis in 'control' soleus or EDL muscle is 40nmoles/min/g and 25nmoles/min/g respectively, and in 'dexamethasone' soleus or EDL muscle it is 94nmoles/min/g and 5nmoles/min/g respectively. This variability may suggest that some factors/conditions may affect muscle's capacity to synthesize glutamine. The present analysis is not able to provide definite information about the effect of the conditions, listed in Table 2, on muscle's capacity to synthesize glutamine. However, since the reaction catalysed by glutamine synthetase is non-equilibrium, an investigation of the effect(s) of these factors/conditions on the maximal activity of glutamine synthetase in skeletal muscle, *in vitro*, can provide quantitative information on whether these factors/conditions affect muscle's capacity to synthesize glutamine.

In this chapter the effect(s) of different conditions on the maximal activity of glutamine synthetase in muscle were investigated. The results of this investigation were compared with:

1) the changes in the rates of release, obtained from Chapter 2, so as to study the relationship between muscle glutamine synthesis and muscle glutamine release, and

2) the estimated rates of muscle glutamine synthesis from Table 2, to see whether changes in maximal glutamine synthetase activity in muscle, closely reflect changes in the estimated rate of glutamine synthesis in muscle.

Table 2: estimated rates of glutamine synthesis in skeletal muscle in the presence of external factors.

N.B. Control glutamine content, for soleus and EDL are averages, *signifies, no change in muscle glutamine content in the presence of external factors i.e. glutamine content in the presence of external factors is the same as for controls.

External Factors	Rate of muscle glutamine release (nmoles/min/g)	Muscle glutamine content (nmoles/g)	Estimated rate of glutamine synthesis (nmoles/min/g)
Control			
soleus	40	5000	40
EDL	25	4000	25
Leucine			
soleus	70	6716	93
EDL	61	6804	95
Isoleucine			
soleus	64	5000*	64
EDL	55	4000*	55
Lactate(10mM)			
soleus	61	5000*	61
EDL	32	4000*	32
Hypo-osmsotic			
soleus(70mM Na+)	25	5000*	25
EDL (70mM Na+)	19	4000*	19
Hyper-osmotic			
soleus(50mM sucrose)	58	5000*	58
EDL(50mM sucrose)	40	4000*	40
Dexamethasone**			
soleus	79	(3517control- 4420dex)	94
EDL	23	(3194control- 2143dex)	5
Hyper-thyroidism**			
soleus	47	(4382control- 3538hyper)	33
EDL	55	(3390control- 2700hyper)	44
Hypo-thyriodism**			
soleus	21	4392 (no change in content)	21
Endotoxin**			
soleus	76	(3064control- 1974endo)	58
Ethanol**			
soleus	40	(5290control-6700ethanol)	64
EDL	31	6390(no change in content)	31

** Data from Parry-Billings (1989) and Parry-Billings et al (1989, 1990a, 1990b, 1991, 1992). (For details of calculations see Appendix V)

The different conditions/factors used in this study were:

Dexamethasone

Hyper-thyroidism

Hypo-thyroidism

Endotoxin

Starvation

Isoleucine

Lactate

Cell volume changes

1.1.Technique used to determine glutamine synthetase activity in skeletal muscle.

The technique used to determine glutamine synthetase activity in skeletal muscle is a modification of a radiochemical technique used by King et al (1983). This radiochemical assay follows the conversion of ^{14}Cglutamate to ^{14}Cglutamine. The separation of ^{14}Cglutamine from ^{14}Cglutamate involved an ion exchange chromatographic technique based on the methods of Prusiner and Milner (1970) and Pishak and Philips (1979). (A review of techniques used to determine glutamine synthetase activity in skeletal muscle are described in detail in Appendix VI).

1.2.Glutamine synthetase activity in adipose tissue.

It has been suggested that adipose tissue is an important source of glutamine. Studies by Miller (1975) show that adipose tissue possess higher (ten-fold higher) glutamine synthetase activity (when activity is expressed per mg of protein) than skeletal muscle. In addition, adipose tissue is reported to release glutamine, 3.5nmoles/g/min and 18nmoles/mg protein/h. (Christophe and Wodon 1966 and Tischler and Goldbreg 1980 respectively). Tischler and Goldberg (1980) reported that when the rate of

glutamine release from adipose tissue is expressed, per mg of tissue weight, the values for adipose glutamine release are "comparable to those observed with skeletal muscle". Tischler and Goldberg (1980) also reported that *in vitro* the branched chain amino acids (leucine isoleucine and valine) increase the rate of release of glutamine from incubated adipose tissue. Thus, the branched chain amino acids appear to have a similar effect on glutamine release from adipose as from skeletal muscle (see Chapter 2, section 3.2, and Parry-Billings 1989). Studies by Frayn et al (1991) suggest that glutamine metabolism in adipose tissue is a significant contributor of glutamine; they reported that, as with skeletal muscle, adipose tissue releases glutamine.

The reports above, which suggest that adipose tissue is an important source of glutamine via glutamine synthetase activity and glutamine release, are in contrast to reports which suggest that adipose has the capacity to consume glutamine at a high rate. Cooney et al (1986) and Kowalchuk (1988), measured high glutaminase activity in rat adipose tissue and rat adipocytes. In relation to glutamine release, Ardawi (1988a), reported that adipose tissue releases a quantitatively insignificant amount of glutamine in relation to the amount of glutamine released from muscle. In the light of these conflicting reports as to the role of adipose tissue in relation to glutamine, it was decided to measure glutamine synthetase activity in adipose tissue and to study the effect(s) of external factors/conditions (dexamethasone, hyper- and hypo-thyroidism, sepsis and starvation) on glutamine synthetase activity in this tissue.

2.Materials and methods.

2.1.Materials.

Chemicals, enzymes and care of animals in Appendix I

2.2.Methods.

2.2.1.Preparation of homogenates:

Skeletal muscle.

Rats were killed by cervical dislocation and soleus, EDL and gastrocnemius muscles were dissected as previously described (see chapter 2, sections 2.2.1.and 2.2.2., and Parry-Billings 1989). Rats used in this study weighed between 60 and 250g (weights are specified for individual experiments). The muscles were dissected quickly, weighed and then placed in a 10ml plastic tube, which prior to use had been placed in ice. Muscle was then homogenised in 9 vol of extraction buffer (50mM Tris and 2mM EDTA pH 7.9) using a Polytron (Kinematica) homogeniser set at position 4 for 15seconds at 0°C. The homogenate was centrifuged at 200g for 14mins at 0°C. The supernatant was used for the enzyme assay.

Adipose tissue.

Peri-renal and epididymal adipose tissues were removed from rats weighing between (160-250g). and were prepared for the enzyme assay as descibed above.

2.2.2.Glutamine synthetase assay.

Glutamine synthetase activity was measured by a radiochemical assay modified from that of King et al (1983).

The reaction medium consisted of:

50mM imidazole/HCl

20mM magnesium chloride

20mM ammonium chloride

20mM sodium glutamate

15mM ATP

10mM creatine phosphate

1.2 Units creatine kinase

in 20ml of water.

The pH of the reaction medium was adjusted to 7.6 at room temperature. Then $U^{14}C$ glutamate (500µl, 50µCi/ml) was added to the reaction medium. The reaction was started by the addition of 50µl of supernatant (muscle or adipose) to 360µl of reaction media. The mixture was incubated for 30mins at 37°C. After incubation, the reaction was stopped by adding 100µl of cold 2% perchloric acid. The incubation samples were then centrifuged at 10,000g for 2 mins. Samples (360µl) were then neutralised with universal indicator (15µl), potassium hydroxide (10µl of 20%) plus triethanolamine (50µl of 0.5M). Samples were centrifuged again (10,000g for 2mins) to remove the precipitate formed during neutralisation.

$U^{14}C$ Glutamine formed in the reaction medium was separated from its precursor $U^{14}C$ glutamate by an ion exchange chromatographic technique modified from that of Pishak and Phillips (1979). Labelled glutamine and glutamate were separated on formated Dowex (Dowex-2-chloride). (The technique for formatting Dowex is given in Appendix II).

Neutralised samples (360µl) were pipetted on top of ion exchange columns which were plastic 2.5ml syringes plugged with a small amount of glass wool; the syringes contained 2ml of formated Dowex (pH 7.0). These columns were previously equilibrated with distilled water. (It is essential that the Dowex is at a neutral pH, this is so that the ion exchange resin becomes positively charged, thus the negatively charged $U^{14}C$ glutamate is retained on the column, while the neutral $U^{14}C$ glutamine, is

not retained.) The column was washed with 4x1ml distilled water and the effluent collected. A sample (1ml) of the effluent was added to 4ml of scintillant. The radioactivity was measured in a Beckman scintillation counter (model LS 1701).

2.2.3.Presentation of data and statistical analysis.

Enzyme activity is expresed as nmoles/min/g wet weight and nmoles/min/mg protein. Protein was measured by the Bio-Rad protein assay as used by Bradford 1976. Statistical analysis was performed using Student's t-test.

2.3.Experiments.

2.3.1.The effect of dexamethasone on glutamine synthetase activity in muscle and adipose tissue extracts.

Treatment: rats weighing approximately 180g were injected intraperitoneally with dexamethasone 21 acetate, at a dose of 3mg/kg body weight (for preparation of dexamethasone (see Appendix II). The injection took place at 4pm, (which is when blood glucocorticoid levels peak). The rats were treated with dexamethasone for five consecutive days. Controls were injected with HEPES or sterile saline. Rats were fasted overnight prior to experimentation, which was performed 24h. after the final injection. Rats were killed as previously described and EDL and soleus muscles, peri-renal and epididymal adipose tissues were removed, weighed and homogenised, and glutamine synthetase activity was determined as previously described.

2.3.2.The effect of hyper- or hypo-thyroidism on glutamine synthetase activity in muscle and adipose tissue extracts.

Hyper-thyroidism: Rats weighing approximately 180g were injected intraperitoneally with 3,3,5-triodothyronine at a dose of 0.75µg/g body weight per day for five days (for preparation of 3,3,5-triodothyronine see Appendix II). Control rats were injected with an alkaline solution (0.01M sodium hydroxide) or sterile saline. Confirmation that the rats were hyperthyroid, was obtained by measuring glycerolphosphate oxidase activity in the livers of these rats; the activity of this enzyme in liver is increased in hyperthyroid rats (see Lee and Lardy 1965), and it was increased in the livers of the hyperthyroid rats used in the present study (see Mitchell 1992).

Hypo-thyroidism: hypothyroidism was induced, in rats weighing approximately 200g, by administration of 6-n-propyl-2-thiouracil (PTU) for 3-4 weeks. This was dissolved in warm ethanol and added to the rat's drinking water at a final concentration of 0.5mg/g body weight. (For details see Appendix II). Ethanol was added to the drinking water of the control rats at the same final concentration (1.2%).

Hyper-and hypo-thyroid rats were fasted overnight before they were killed by cervical dislocation. EDL and soleus muscles, peri-renal and epididymal adipose tissues were removed, weighed and homogenised, and glutamine synthetase activity was determined as previously described.

2.3.4.The effect of endotoxin on glutamine synthetase activity in muscle and adipose tissue extracts.

Endotoxin (from Salmonella Typhimurium) was injected into rats weighing approximately 200g, at a dose of 5mg/g body weight, intraperitoneally. (For details see Appendix II). Control rats were injected with sterile saline. Endotoxin was injected into rats, 3, or 24h. prior to

experimentation. Rats were killed by cervical dislocation. EDL and soleus muscles, peri-renal and epididymal adipose tissues were removed, weighed and homogenised, and glutamine synthetase activity was determined as previously described.

2.3.5.The effect of starvation on glutamine synthetase in muscle and adipose tissue extracts.

Rats were starved for 15, 24 or 48h., prior to experimentation. Rats were killed, as previously described. EDL and soleus muscles, peri-renal, and epidiymal adipose tissues were removed, weighed and homogenised, and glutamine synthetase activity determined as previously described. Glutamine synthetase activities in muscle and adipose tissue extracts of starved rats was compared to those in fed rats.

2.3.6.The effect of acute ethanol administration on glutamine synthetase activity in muscle extracts.

Rats weighing approximately 160g were injected with a mixture of ethanol and saline; 21.85ml of 100% ethanol and 28.15ml of 0.9% saline. The mixture of ethanol and saline (50ml) was injected, at a dose of 1ml/100g of body weight, intraperitoneally. Control rats were injected with 0.9% saline at a dose of 1ml/100g body weight. Rats were injected early in the morning, approximately 7.30am, and killed between 9.30 and 10.00am. EDL and soleus muscles, were removed, weighed and homogenized as previously described. Glutamine synthetase activity was determined as previously described.

2.3.7.The effect(s) of lactate, BCAA(isoleucine) and changes in muscle cell volume on glutamine synthetase activity in incubated EDL and soleus muscle extracts.

Rats weighing 60g were anaesthetised and killed, as previously described. EDL and soleus were removed and incubated, for 1 h., in media containing (mM): isoleucine (10); lactate (0, 5 or 10); hypo-osmotic media (120, 95 or 70 Na^+); and hyper-osmotic media (5, 10 or 50 sucrose). Muscles were then removed, blotted and frozen in liquid nitrogen. Glutamine synthetase activity was determined as previously described.

3.Results.

3.1.Preliminary studies on the glutamine synthetase assay.

3.1.1.Glutamine synthetase activity in fresh muscle extracts, freeze-clamped muscle extracts and incubated muscle extracts.

Preliminary experiments were performed to ensure that glutamine synthetase activity can be measured in incubated muscle using the radiochemical technique employed in the present study. Muscles, both whole EDL and soleus, were removed from rats weighing 60g and incubated as previously described (see chapter 2, sections 2.2.1. and 2.2.2.). The muscles were then frozen in liquid nitrogen and then prepared for homogenisation, as previously described (see chapter 2 section 2.3.1.). The muscles were homogenised and then assayed for glutamine synthetase activity as previously described.

The activity of glutamine synthetase in extracts of incubated EDL and soleus muscle was less than the activity in extracts of fresh EDL and soleus muscle (Table 3.1.). (The glutamine synthetase activity in incubated EDL or soleus muscle was approximately 65(64)% less than that of fresh EDL or soleus muscle respectively.)

The effect of freeze-clamping of fresh muscle on glutamine synthetase activity was also investigated. Muscles, both EDL and soleus, were removed from rats weighing between 180 and 200g, and then immediately frozen in liquid nitrogen. Muscles were prepared for homogenisation as previously described (see chapter 2, section 2.3.1.). Muscles were homogenised and glutamine synthetase activity was measured as previously described. Freeze-clamping had no effect on glutamine synthetase activity in soleus or EDL muscle extracts (Table 3.1).

3.1.2.Glutamine synthetase activity in EDL and soleus muscle extracts from young and older rats.

Incubated muscles, mentioned above, were taken from young rats (approximately two weeks old) that had been anaesthetised before they were sacrificed as previously described (see chapter 2, section 2.2.4.). The age of these rats plus the fact that they had been anaesthetised may affect the activity of glutamine synthetase in muscle and therefore cause differences in activity between muscle that had been incubated and fresh muscle removed from older rats (approximately 8-10 weeks old). Young rats weighing 60g were sacrificed, as previously described. EDL and soleus muscle were homogenized and glutamine synthetase activity was determined as previously described.

Glutamine synthetase activity in extracts of EDL and soleus muscles of young rats that had been anaesthetised was the same as that in extracts of EDL and soleus muscles of older rats (Table 3.2).

3.1.2.Glutamine synthetase activity in different types of muscle.

Glutamine synthetase activity was determined, in EDL (type 2 fibre rich muscle), soleus (type 1 fibre rich muscle) and gastrocnemius (composed of mixed fibre types), as previously described.

The activity of glutamine synthetase in extracts of EDL muscle was consistently higher than that in extracts of soleus muscle (Table 3.3). These results are in agreement with Falduto et al (1992) who reported that glutamine synthetase activity is higher in rat skeletal muscle composed predominantly of type 2b fibres (white vastus lateralis muscle), than in rat skeletal muscle composed primarily of type 1 fibres (soleus muscle).

The activity of glutamine synthetase in extracts of gastrocnemius muscle (gastrocnemius muscle is composed of mixed fibre types) was also higher than that in extracts of soleus muscle (Table 3.3).

3.1.3.Glutamine synthetase activity in muscle and adipose tissue extracts.

Glutamine synthetase activity was determined in EDL and soleus muscle and peri-renal and epididymal adipose tissue as previously described .

Both extracts of peri-renal and epididymal adipose tissue had higher maximal glutamine synthetase activities, (approximately 1.5-3 times greater when expressed per g of wet weight of tissue and 6-7 times greater when expressed per mg of protein) than either extracts of EDL or soleus muscle (Table 3.4).

3.2.The effect of dexamethasone on glutamine synthtase activity in extracts of skeletal muscle and adipose tissue.

Dexamethasone caused an increase in glutamine synthetase activity in extracts of EDL and soleus muscle (Table 3.5). This result is in agreement with numerous other studies on the effect of dexamethasone on glutamine synthetase activity in rat skeletal muscle (see Max et al 1987,1988; Falduto et al 1989, 1992; Ardawi 1990). The response of glutamine synthetase to the effect of dexamethasone in EDL or soleus muscle was quantitatively similar, although studies have suggested that glucocorticoids may influence type 1 and type 2b muscle differently in

relation to glutamine metabolism. For the rate of glutamine release from muscle, Parry-Billings et al (1990a)reported that dexamethasone caused an increase in the rate of release of glutamine from soleus muscle, but had no effect on the rate of release from EDL muscle. Additionally, Max et al (1988), have reported that the response of glutamine synthetase in soleus muscle (type 1) to dexamethasone is 60-70% greater than that in plantaris muscle which is composed predominanatly of type 2a and b fibres (Armstrong and Phelps 1984).

In relation to adipose tissue, dexamethasone caused an increase in glutamine synthetase activity in extracts of peri-renal and epididymal adipose tissues.

3.3.The effect of hyper- or hypo-thyroidism on glutamine synthetase activity in extracts of skeletal muscle and adipose tissue.

Hyper-thyroidism caused an increase in glutamine synthetase activity in extracts of soleus muscle (23%). Glutamine synthetase activity in extracts of EDL was also increased by hyper-thyroidism (18%), but this increase was not significant. Hyper-thyroidism caused an increase in glutamine synthetase activity in extracts of peri-renal and epididymal adipose tissue (Table 3.6).

Hypo-thyroidism caused a decrease in glutamine synthetase activity in extracts of EDL and soleus muscle by 90 and 107% respectively. Glutamine synthetase activity in extracts of adipose tissue was unchanged (Table 3.7).

To the author's knowledge, this is the first report of the effects of hyper- and hypo-thyroidism on glutamine synthetase activity in muscle.

3.4.The effect of endotoxin on glutamine synthetase activity in extracts of muscle and adipose tissue.

Endotoxin (24h.) caused an increase in glutamine synthetase activity in extracts of both soleus and EDL muscle by 188 and 64% respectively. The activity in extracts of adipose tissue following endotoxin treatment was unchanged (Table 3.8). Endotoxin (3h.) caused an increase in glutamine synthetase activity in extracts of soleus, (30%). For extracts of EDL, glutamine synthetase activity was increased but this increase was not statistically significant. Endotoxin caused no change in glutamine synthetase activity in extracts of peri-renal or epididymal adipose tissues (Table 3.8).

3.5.The effect of starvation on glutamine synthetase activity in ex tracts of skeletal muscle and adipose tissue.

Starvation of the rat for 15, 24 and 48h. increased glutamine synthetase activity in extracts of EDL and soleus muscle (Tables 3.9 and 3.10). Starvation had no effect on glutamine synthetase activity in extracts of adipose tissue (Tables 3.9 and 3.10).

3.6.The effect(s) of acute ethanol administration, lactate, BCAA (isoleucine) and cell volume changes on glutamine synthetase activity in extracts of EDL and soleus muscle.

Acute administration of ethanol to the rat had no effect on glutamine synthetase activity in extracts of incubated EDL or soleus muscle (Table 3.11).

Lactate (5mM and 10mM), isoleucine (10mM), hypo-osmosis and hyper-osmosis had no effect on glutamine synthetase activity in extracts of incubated EDL or soleus muscle (Tables 3.12, 3.13, 3.14 and 3.15).

Table 3.1
Effect of incubation and freeze-clamping on glutamine synthetase activity in extracts of EDL or soleus muscle.

Muscle	Maximal activity (nmoles/min/g wet wt)
Fresh Edl	260±26 (n=4)
Fresh Sol	159±64 (n=4)
Incubated Edl	157±12.3*** (n=6)
Incubated Sol	97±27 (n=6)
Freeze-clamped Edl	291±7.5 (n=4)
Freeze-clamped Sol	163±7 (n=4)

Values are the mean (SEM) with the number of separate experiments in parenthesis. The statistical significance of the differences between means are denoted by ***P<0.01.
Abbreviations: EDL- Extensor digitorum longus muscle
SOL- soleus muscle.

Table 3.2
Glutamine synthetase activity in extracts of EDL and soleus muscles of young (2 week old) and older (8-10 week old) rats.

Rats	Muscle	Maximum activity (nmoles/min/g wet wt)
Young (2 wks)	EDL	250±20 (n=3)
	SOL	144±41 (n=3)
Older (8-10 wks)	EDL	260±26 (n=4)
	SOL	159±64 (n=4)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
Abbreviations:
EDL- Extensor digitorum longus muscle
SOL- Soleus muscle.

Table 3.3
Glutamine synthetase activity in extracts of EDL muscle, soleus muscle and gastrocnemius muscle.

Muscle	Maximal activity (nmoles/min/g wet wt)
Edl	261±10 (n=4)
Sol	159±17 (n=4)
Gastroc	225±9 (n=4)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
Abbreviations:
EDL- Extensor digitorum longus muscle
SOL- soleus muscle
Gastroc- Gastrocnemius muscle.

Table 3.4
Glutamine synthetase activity in extracts of EDL muscle soleus muscle, epididymal and peri-renal adipose tissues.

Tissue	Maximal activity	
	(nmoles/min/g wet wt)	(nmoles/min/mg prot)
EDL	323±29 (n=6)	1.4±0.02 (n=6)
SOL	136±16 (n=6)	1.1±0.37 (n=6)
EA	298±29 (n=6)	6.9±1.3 (n=6)
PA	335±43 (n=6)	5.6±2.1 (n=6)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
Abbreviations:
EDL- Extensor digitorum longus muscle
SOL- soleus muscle
EA- Epididymal adipose tissue
PA- Peri-renal adipose tissue.

Table 3.5
Effect of dexamethasone on glutamine synthetase activity in extracts of EDL muscle, soleus muscle, peri-renal and epididymal adipose tissues.

Tissue	Treatment	Maximal activity (nmoles/min/g wet wt)	(nmoles/min/mgprot)
EDL	control	289 ±22 (n=6)	
	dexamethasone	469±20*** (n=6)	
SOL	control	96 ±4 (n=6)	
	dexamethasone	160±16*** (n=6)	
EA	control	293 ±54 (n=4)	5.8±1.1 (n=4)
	dexamethasone	768±73*** (n=4)	21.3±5.8* (n=4)
PA	control	293 ±71 (n=4)	7.1 ±2.7 (n=4)
	dexamethasone	1147±61**** (n=4)	18.4±2.4* (n=4)

Values are the mean (SEM) with the number of separate experiments in parenthesis. The statistical significance of the differences between means denoted by ****P<0.001, ***P<0.01 and *P<0.05.
Abbreviations:
EDL- Extensor digitorum longus muscle.
SOL- Soleus muscle.
EA- Epididymal adipose tissue
PA- Peri-renal adipose Tissue.

Table 3.6
Effect of hyperthyroidism on glutamine synthetase activity in extracts of EDL muscle, soleus muscle, peri-renal and epididymal adipose tissues.

Tissue	Treatment	Maximal activity (nmoles/min/g wet wt)	Maximal activity (nmoles/min/mgprot)
EDL	control	313 ±23 (n=6)	
	Hyperthyroidism	372 ±32 (n=6)	
SOL	control	133 ±5 (n=6)	
	Hyperthyroidism	174±16** (n=6)	
EA	control	441 ±120 (n=4)	7.2 ±1 (n=4)
	Hyperthyroidism	900±18**** (n=4)	16±4** (n=4)
PA	control	364 ±61 (n=4)	5.7 ±1 (n=4)
	Hyperthyroidism	787±47*** (n=4)	17±2*** (n=4)

Values are the mean (SEM) with the number of experiments in parenthesis.
The statistical significance of the differences between means are denoted by ****P<0.001, ***P<0.01 and **P<0.02.
Abbreviations:
EDL- Extensor digitorum longus muscle.
SOL- Soleus muscle.
EA- Epididymal adipose tissue
PA- Peri-renal adipose tissue

Table 3.7
Effect of hypothyroidism on glutamine synthetase activity in extracts of EDL muscle, soleus muscle, peri-renal and epididymal adipose tissues.

Tissue	Treatment	Maximal activity (nmoles/min/g wet wt)	(nmoles/min/mgprot)
EDL	control	286 ±39 (n=5)	
	Hypothyroidism	150±13*** (n=5)	
SOL	control	193 ±44 (n=6)	
	Hypothyroidism	93±4* (n=5)	
EA	control	400 ±20 (n=4)	7.0 ±1.3 (n=2)
	Hypothyroidism	310 ±34 (n=4)	6.4 ±0.4 (n=2)
PA	control	335 ±74 (n=4)	5.7 ±1.6 (n=2)
	Hypothyroidism	331 ±12 (n=4)	8.8 ±0.3 (n=2)

Values are the mean (SEM) with the number of separate experiments in parenthesis. The statistical significance of the differences between means are denoted by ***P<0.01 and *P<0.05.
Abbreviations:
EDL- Extensor digitorum longus muscle.
SOL- Soleus muscle.
EA- Epididymal adipose tissue
PA- Peri-renal adipose tissue.

TABLE 3.8
The effects of endotoxin treatment on glutamine synthetase activity in extracts of EDL muscle, soleus muscle, epididymal and peri-renal adipose tissues.

Tissue	Treatment	Maximal activity (nmoles/min/g wet wt)
SOL	Control (24h.)	177±12.5 (n=5)
	Endotoxin (24h.)	510±28**** (n=7)
	Control (3h.)	90±4.2 (n=4)
	Endotoxin (3h.)	114±8.5* (n=4)
EDL	Control (24h.)	392±12 (n=5)
	Endotoxin (24h.)	644±37**** (n=7)
	Control (3h.)	255±19 (n=4)
	Endotoxin (3h.)	273±35 (n=4)
EA	Control (24h.)	455±84 (n=3)
	Endotoxin (24h.)	501±81 (n=3)
PA	Control (24h.)	377±70 (n=3)
	Endotoxin (24h.)	457±61 (n=3)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
The statistical significance of the differences between means are denoted by ****P<0.001 and **P<0.02.
Abbreviations: EDL- Extensor digitorum longus muscle, SOL- soleus muscle, EA- Epdidymal adipose tissue, PA- Peri-renal adipose tissue.

Table 3.9
The effect of starvation on glutamine synthetase activity in extracts of EDL muscle, soleus muscle, peri-renal and epididymal adipose tissues.

Tissue	Starvation time (h.)	Maximal activity (nmoles/min/g wet wt)
SOL	Fed	79±3 (n=9)
	15	108±6*** (n=8)
EDL	Fed	248±11 (n=9)
	15	300±11*** (n=8)
EA	Fed	404±44 (n=3)
	15	480±20 (n=3)
PA	Fed	424±63 (n=3)
	15	400±66 (n=2)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
The statistical significance of the differences between means are denoted by ***P<0.01.
Abbreviations:
EDL- Extensor digitorum longus muscle
SOL- Soleus muscle
EA- Epididymal adipose tissue
PA- Peri-renal adipose tissue.

Table 3.10
The effect of starvation on glutamine synthetase activity in extracts of EDL muscle, soleus muscle, epididymal and peri-renal adipose tissues.

Tissue	Starvation time (h.)	Maximal activity (nmoles/min/g wet wt)
SOL	Fed	79.5±3.5 (n=9)
	24	182±18**** (n=4)
	48	230±62** (n=4)
EDL	Fed	248±11.5 (n=9)
	24	404±13.5**** (n=4)
	48	464±94** (n=4)
EA	Fed	404±44 (n=3)
	24	353±47 (n=3)
	48	345±23 (n=3)
PA	Fed	424±63 (n=3)
	24	291±9 (n=3)
	48	316±51 (n=3)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
The statistical significance of the differences between means are denoted by ****P<0.001 and **P<0.02.
Abbreviations: EDL -Extensor digitorum longus muscle, SOL -soleus muscle EA-Epididymal adipose tissue, PA -peri-renal adipose tissue.

NB Data (Table 3.11): Acute ethanol administration and the metabolism of glutamine by skeletal muscle of the rat: implications of ethanol-induced reductions in protein synthesis. Parry-Billings, M., Preedy, V., Opara, E., Liu, C-T. and Newsholme, E.A. Alcohol and Alcoholism **27(6)**: 613-618.

Table 3.11
Effect of acute ethanol administration on glutamine synthetase activity in extracts of EDL and soleus muscles.

Muscle	Treatment	Maximum activity (nmoles/min/g wet wt)
Sol	Saline	86±10 (n=5)
	Ethanol	84±4 (n=5)
Edl	Saline	197±23 (n=5)
	Ethanol	192±23 (n=5)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
Abbreviations:
EDL- Extensor digitorum longus muscle
SOL- Soleus muscle

Table 3.12
The effect of lactate on glutamine synthetase activity in extracts of incubated EDL and soleus muscles.

Muscle	Lactate conc (mM)	Maximal Activity (nmoles/min/g wet wt)
EDL	0	133±10 (n=10)
	5	127±8 (n=8)
	10	121±6 (n=10)
SOL	0	60±5.5 (n=9)
	5	54±5 (n=8)
	10	52±4.5 (n=10)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
Abbreviations:
EDL- Extensor digitorum longus muscle
SOL- Soleus muscle

Table 3.13
The effect of isoleucine on glutamine synthetase activity in extracts of incubated EDL and soleus muscles.

Muscle	Isoleucine conc (mM)	Maximum Activity(nmoles/min/g wet wt)
EDL	0	174±13 (n=5)
	10	161±11.5 (n=8)
SOL	0	58±4 (n=4)
	10	62±7 (n=8)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
Abbreviations:
EDL- Extensor digitorum longus muscle
SOL- Soleus muscle

Table 3.14
Effect of hypo-osmotic media on glutamine synthetase activity in extracts of incubated EDL and soleus muscles.

Muscle	Incubation media	Maximal activity (nmoles/min/g wet wt)
EDL	Control medium (146mM Na+)	160±18 (n=6)
	Hypo-osmotic medium (120mM Na+)	143±19 (n=6)
	Hypo-osmotic medium (95mM Na+)	130±15 (n=6)
	Hypo-osmotic medium (70mM Na+)	133±28 (n=6)
SOL	Control medium (146mM Na+)	65±2.5 (n=6)
	Hypo-osmotic medium (120mM Na+)	60±8.8 (n=6)
	Hypo-osmotic medium (95mM Na+)	57±4 (n=6)
	Hypo-osmotic medium (70mM Na+)	52±3 (n=4)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
Abbreviations:
EDL- Extensor digitorum longus muscle
SOL-soleus muscle

Table 3.15
Effect of hyper-osmotic media on glutamine synthetase activity in extracts of incubated EDL and soleus muscles.

Muscle	Incubation medium	Maximal activity (nmoles/min/g wet wt)
EDL	Control medium (146mM Na+)	120±11.4 (n=6)
	Hyper-osmotic medium (5mM Sucrose)	114±10.4 (n=6)
	Hyper-osmotic medium (10mM Sucrose)	114±6.1 (n=6)
	Hyper-osmotic medium (50mM Sucrose)	106±4.6 (n=6)
SOL	Control medium (146mM Na+)	57±8 (n=6)
	Hyper-osmotic medium (5mM Sucrose)	44±7 (n=6)
	Hyper-osmotic medium (10mM Sucrose)	45±2 (n=6)
	Hyper-osmotic medium (50mM Sucrose)	38±1 (n=6)

Values are the mean (SEM) with the number of separate experiments in parenthesis.
Abbreviations:
EDL- Extensor digitorum longus muscle
SOL-soleus muscle

4.Discussion

The aims of this study were:

1) to see whether some of the factors/conditions listed in Table 2, (section 1) affect muscle's capacity to synthesize glutamine;

2) to compare the effects of these different factors/conditions on the maximal glutamine synthetase activity in muscle, with their effect(s) on the estimated rate of synthesis in muscle;

3) to compare the effect(s) of these different factors/conditions on the maximal glutamine synthetase activity in muscle, with their effect(s) on the rate of glutamine release from muscle; and

4) to determine whether the measurement of the maximal activity of glutamine synthetase gives a good quantitative indication of the flux through glutamine synthesis in skeletal muscle.

4.1.A comparison between the estimated rates of glutamine synthesis and the maximal activities of glutamine synthetase in muscle

From the estimated rates of glutamine synthesis in muscle (Table 2, section1) it was suggested that some of the factors/conditions listed in this table may affect muscle's capacity to synthesize glutamine. The present study showed that dexamethasone, endotoxin, the hypo- or hyper-thyroid states and starvation affected the maximum catalytic activity of glutamine synthetase in muscle. This indicates that these factors altered muscle's capacity to synthesize glutamine. (Possible mechanisms, specifically for the effect of dexamethasone, have been suggested. These will be discussed briefly in the General Discussion.)

The actual values of the maximal glutamine synthetase activities were in excess of the estimated rates of muscle glutamine synthesis. For example, the maximal glutamine synthetase activity in 'dexamethasone' soleus or EDL muscle is greater than the estimated rate of synthesis in 'dexamethasone' soleus or EDL muscle by 2- and 90-fold respectively. Thus, maximal glutamine synthetase activity in muscle, does not appear to be a good quantitative indicator of the rate of glutamine synthesis in muscle (see Table 3 below).

Table 3: a comparison of the estimated rates of glutamine synthesis in muscle with the maximal activities of glutamine synthetase in muscle.

Factors/ Conditions	Estimated rate of glutamine synthesis (nmoles/min/g wet wt)	Maximal glutamine synthetase activity (nmoles/min/g wet wt)
Control		
soleus	40	100
EDL	25	250
Dexamethasone		
soleus	93	160
EDL	5	450
Hyperthyroidism		
soleus	33	175
edl	44	370
Hypothyroidism		
soleus	21	90
Endotoxin		
soleus	58	500

4.2.The '*in vivo* ' muscle glutamine synthetase activity.

Miller (1975) and Rowe (1985) have determined Km values for glutamate for muscle glutamine synthetase of 4.5mM and 5.8mM respectively. The concentration of glutamate in muscle is approximately 2mM (see Parry-Billings 1989). Since the maximal activity of glutamine synthetase has been determined, it is possible to calculate the '*in vivo* ' activity of muscle glutamine synthetase assuming the enzyme obeys Michaelis-Menten kinetics:

The Michaelis-Menten equation is:

$$v = \frac{Vmax\,[S]}{[S] + Km}$$

where v = '*in vivo*' activity

Vmax = maximal activity

[S] = substrate concentration

The changes in the '*in vivo*' muscle glutamine synthetase activities could only be determined with changes in muscle glutamate concentration, and not with changes in muscle ammonia concentration. This is because the Km values determined for ammonia for glutamine synthetase are <0.1mM and 0.19mM (Miller 1975 and Rowe 1985 respectively), but the concentration of ammonia in muscle is approximately 0.3mM (data derived from Bergmeyer 1974). This indicates that muscle glutamine synthetase approaches saturation with endogenous muscle ammonia, thus changes in the concentration of ammonia in muscle will not be expected to affect the activity of muscle glutamine synthetase.

Muscle glutamate concentrations have been determined in rats treated with dexamethasone and also in hyper- or hypo-thyroid rats (see Parry-Billings 1989). Thus, with the Vmax for glutamine synthetase in muscle determined for each of these conditions, it is possible to calculate the '*in vivo*' glutamine synthetase activity in muscle from dexamethasone and hyper- or hypo-thyroid rats, using the above equation (see Table 4).

To calculate the '*in vivo*' glutamine synthetase activity in muscle from endotoxin treated rats muscle glutamate data from sepsis induced rats (see Ardawi and Majzoub 1991), were used. [Endotoxins have been implicated as a major cause of the metabolic changes in sepsis and increased blood endotoxin levels were found to be highly predictive of the

development of sepsis in patients with fever (van Deventer et al 1988). In addition endotoxin treatment has been used as a model of sepsis (Parry-Billings 1989). To the author's knowledge the effect of endotoxin on muscle glutamate concentration has not been reported.] The calculated *'in vivo '* glutamine synthetase values obtained for soleus muscle, shown in Table 4, are very similar to the estimated rates of glutamine synthesis shown in Table 2. The calculated *'in vivo'* glutamine synthetase values obtained for EDL muscle are of the same magnitude. The fact that the factors listed in Table 2 affected the maximal activity of glutamine synthetase, in the present study, suggests that they affect the *'in vivo'* glutamine synthesis not by allosteric regulation, but by altering the amount of glutamine synthetase in muscle.

Table 4: '*in vivo* ' muscle glutamine synthetase activities.

	'in vivo' glutamine synthetase activity (nmoles/min/g wet wt)	
	Km 4.5mM	Km 5.8mM
Control		
soleus	30	26
EDL	77	64
Dexamethasone		
soleus	40	33
EDL	117	96
Hyperthyroidism		
soleus	27	21
EDL	56	45
Hypothyroiism		
soleus	20	16
EDL	32	26
Sepsis		
soleus	62	52
EDL	79	66

4.3.The relationship between muscle glutamine release and synthesis.

In spite of reports that assume that the process of glutamine release is synonymous with that of glutamine synthesis (see Garber et al 1976a, Ardawi 1988a, Ardawi and Majzoub 1991) the present study is consistent with the prediction made by metabolic control logic that, changes in muscle glutamine synthesis will normally have no effect on the rate of glutamine release from muscle. The calculation of the estimated rates of glutamine synthesis (Table 2) provides evidence that changes do occur in the rate of muscle glutamine synthesis so that a steady-state concentration of muscle glutamine is maintained. For example, lactate (5mM) caused a marked increase in the rate of glutamine release from muscle (Chapter 2, section 3.4.): such an increase would be expected to deplete the muscle glutamine concentration completely in less than 1h. (see Chapter 2, section

4.5. Table 3). However, muscle glutamine concentration was unchanged. The calculation of the rate of glutamine synthesis in muscle incubated in the presence of lactate (5mM) (see Table 2) provides evidence that the maintenance of glutamine concentration in muscle incubated in the presence of lactate (5mM) was due to an increase in the rate of glutamine synthesis in muscle. The indication that the role of increases in the rate of glutamine synthesis in muscle is to maintain glutamine concentration in muscle, again highlights the process of glutamine release from muscle as important in the provision of glutamine from muscle to other tissues.

It was considered unlikely that lactate would effect the maximal activity of muscle glutamine synthetase during a 1h. incubation. This was confirmed in the present study. This suggests that lactate, and also isoleucine and changes in muscle cell volume, may affect muscle glutamine synthetase activity via allosteric regulation or via changes in muscle glutamate concentration *in vivo*. (This will be discussed briefly in the General Discussion).

4.4.Glutamine synthetase activity: a comparison between skeletal musle and adipose tissue.

As stated in the introduction for this chapter, studies on glutamine metabolism in adipose tissue indicate that it may be a predominant producer of glutamine, (Chrisophe and Wodon 1966 ; Miller 1975; Tischler and Goldberg 1980) or a predominant consumer (Cooney et al 1986; Kowlachuk et al 1988). Consistent with its suggested role as a predominant producer of glutamine, the maximal activity of glutamine synthetase determined in extracts of adipose tissue, in the present study, was greater (six-fold greater, for activity expressed per mg of protein) than that of soleus and EDL (Table 3.4). This suggests that adipose tissue, on a protein basis, has a higher capacity than muscle, to synthesize glutamine.

Miller (1975) determined the Km value for glutamate for glutamine synthetase in rat adipose tissue to be 2mM. The concentration of glutamate in rat adipose is calculated to be approximately 0.15mM (data derived from Chrisophe and Wodon 1966), and the Vmax for glutamine synthetase in extracts of peri-renal and epididymal adipose tissue in the rat, has been determined in the present study. Thus, as with glutamine synthetase activity in muscle, using the Michaelis-Menten equation, the '*in vivo*' glutamine synthetase activity in adipose tissue can be determined (see Table 5). A comparison of the '*in vivo*' glutamine synthetase activity in adipose with that in muscle shows that under 'normal' conditions muscle has a greater capacity to synthesize glutamine in the rat (see Table 5).

A comparison of the *'in vivo'* glutamine synthetase activity of adipose tissue, with that of muscle, in terms of the whole rat body, indicates that muscle synthesises more glutamine than adipose (see Table 5). (Adipose tissue, it is assumed, makes up approximately 10% of the total body weight of the rat; muscle makes up approximately 40% of the total body weight of the rat.)

It was not possible to calculate the estimated rate of glutamine synthesis in adipose tissue by the method previously used for muscle, since to the author's knowledge no data on the intracellular concentration of glutamine before and after incubation of adipose tissue has been reported. However, Ardawi (1988a) has calculated the rate of glutamine synthesis by adipose tissue. This rate, 25nmoles/min, was calculated per 100g of rat hindlimb, since Ardawi (1988a) wanted to investigate the contribution of glutamine by adipose tissue, skin and muscle present in rat hindlimb, of glutamine. The rate of glutamine synthesis calculated for adipose tissue was much less than that for muscle, 2271nmoles/min/100g. It must be stressed that in the study by Ardawi (1988a), it was assumed that the rate of glutamine synthesis and the rate of glutamine release by

adipose tissue were the same. However, as with muscle glutamine release and synthesis, glutamine synthesis and release in adipose tissue may be independent processes.

Table 5: comparison of '*in vivo*' adipose tissue glutamine synthetase activity with '*in vivo*' muscle glutamine synthetase activity.

	'in vivo' glutamine synthetase activity (nmoles/min/g wet wt)	Total 'in vivo' glutamine synthetase activity (nmoles/min/200g rat)
Tissue		
Deep adipose tissue*	23	460
Superficial adipose tissue*	21	420
Oxidative muscle*	31	2256
Non-oxidative muscle*	96	7040

* Peri-renal adipose tissue and epipididymal adipose tissues represent deep adipose and superficial adipose tissue respectively. Soleus muscle and EDL muscle represent oxidative and non-oxidative muscle respectively.

Data from the present study indicate that rat adipose tissue is not a significant contributor for glutamine. However, it is the process of release that is important in the provision of glutamine to the rest of the body. Thus, a comparison of the rate of release of glutamine from adipose tissue with that of muscle during different conditions is required, so as to determine whether the amounts released are significant.

Studies have shown that adipose tissue releases glutamine. Tischler and Goldberg (1980) have shown that, following a 48h. fast, incubated rat adipose tissue releases glutamine. Ardawi (1988a) has reported that adipose tissue from thermally-injured rats, when incubated, releases glutamine, and Ardawi and Majzoub (1991) reported that adipose tissue removed from septic rats releases glutamine. However, for the studies by Ardawi (1988a) and Ardawi and Majzoub (1991), 1mM leucine was present

in the incubation medium. Tischler and Goldberg (1980) reported that leucine (0.5mM) increases the rate of glutamine release from incubated adipose tissue by approximately 25%. Thus, the release of glutamine from adipose tissue in the studies by Ardawi (1988a) and Ardawi and Majzoub (1991) may have been augmented by leucine. However, for the purpose of a comparison of rates of glutamine release from adipose tissue with that of muscle, during different conditions, data from Ardawi (1988a) and Ardawi and Majzoub (1991) were used.

Data obtained from the studies by Tischler and Goldberg (1980), Ardawi (1988a) and Ardawi and Majzoub (1991) on adipose tissue, were compared to the rate of release of glutamine from muscle during similar conditions, it could be seen that the whole body contribution by rat muscle, of glutamine, is far greater than that by rat adipose tissue (see Table 6) with adipose tisssue contributing between approximately 1.5 and 5.2% of the glutamine contributed by rat muscle. (*Please turn over*)

Table 6: comparison of the rates of release of glutamine from adipose tissue with the rates of release of glutamine from muscle during different conditions.

Rates of glutamine release (nmoles/min/g wet wt)		*Estimated Rates of glutamine release: whole body contribution. (nmoles/min/rat body weight)
Tissue		
Adipose (Normal)	7.2	144nmoles/min/200g rat
Muscle (Normal)	40	3200nmoles/min/200g rat
Adipose (48h. fast)	7.6	87nmoles/min/115g rat
Muscle (48h. fast)	33	1650nmoles/min/125g rat
Adipose (Thermal injury)	8.9	85nmoles/min/210g rat
Adipose (Sepsis)	3.2	64nmoles/min/200g rat
Muscle (Sepsis)	59	4204nmoles/min/180g rat

Data derived from Garber et al (1976a), Tischler and Goldberg (1980), Ardawi (1988a), Parry-Billings (1989) and Ardawi and Majzoub (1991).

* In this analysis, it is assumed, as for Table 5, that adipose tissue makes up approximately 10% of total body weight of the rat; muscle makes up approximately 40% of the total body weight of the rat.

The data so far has indicated that rat adipose tissue is not, in relation to muscle, a significant contributor of glutamine. However, in man the situation appears to be different. Frayn et al (1991) reported that in whole body terms adipose tissue contributes a significant amount of glutamine, approximately one-third of that contributed by muscle, in human subjects who have fasted overnight. Frayn et al (1991) reported that the estimated whole-body tissue flux is 12μmoles/min from adipose

and 39μmol/min from muscle. If human adipose tissue is a significant contributor of glutamine, then further investigations are required to determine the provision of glutamine by adipose tissue during different conditions in man, for example injury, trauma and post-surgery. Such a study might investigate changes in glutamine release, glutamine content and glutamine synthesis during different conditions by different adipose tissue depots, since certain aspects of glutamine metabolism differ from depot to depot (see Kowalchuk et al 1988), and compare them with muscle.

Chapter 4

Glutamine synthetase in cells of the immune system.

1.Introduction.

It has been well established that cells of the immune system, lymphocytes and macrophages, utilize glutamine at a very high rate (Ardawi and Newsholme 1982,1983, Newsholme P. et al 1986, 1987). Two suggestions have been made as to the reason for the high rate of glutamine utilization by lymphocytes and macrophages: first, the pathway provides a considerable amount of ATP, so glutamine utilization might be important primarily for energy formation in these cells. However, if energy is the main reason for the high rate of glutamine utilization by lymphocytes and macrophages, then one might expect that glutamine would be fully oxidized in these cells: glutamine is only partially oxidized in lymphocytes and macrophages (Ardawi and Newsholme 1983, 1985, Newsholme P. et al 1987). Furthermore, lymphocytes and macrophages have other sources of energy, they are known to be able to oxidize fatty acids and ketone bodies, both of which can contribute to ATP generation (Ardawi and Newsholme 1982, Newsholme P. et al 1986). Second, the pathway of glutamine utilization provides intermediates for the biosynthesis of purine and pyrimidine nucleotides, by the *de novo* pathways: glutamine is a nitrogen donor for purine and pyrimidine synthesis. In addition aspartate, which is a product of glutamine utilization by cells of the immune system, is a nitrogen donor for pyrimidine synthesis. Purine and pyrimidine nucleotides are essential for the formation of DNA and mRNA, which are required for cell division and protein synthesis respectively. Thus the synthesis of purine and pyrimidine nucleotides is essential for processes involved in an immune response, for example the proliferation of lymphocytes, which requires DNA, the production of mediators of

immunity produced by lymphocytes, such as antibodies, and the production of proteins by macrophages, both of which require mRNA. However, calculations of the rate of synthesis of purine and pyrimidine nucleotides show that, in order to double the contents of the DNA and RNA in the cell, the rate is very small, approximately less than 0.1% of the rate at which glutamine is utilized by lymphocytes and macrophages (see Newsholme et al 1987). Additionally, the rate of glutamine utilization by lymphocytes is markedly in excess of the maximal rate of the pathway of pyrimidine nucleotide biosynthesis *de novo* (Szondy and Newsholme 1989). Consequently, an hypothesis of branched point sensitivity has been proposed which suggests that a high rate of glutamine utilization is important to provide optimal conditions for the regulation of changes in the rates of synthesis of purine and pyrimidine nucleotides (see Chapter 1, section 3.3., see Appendix III for details on branched point sensitivity).

As part of the investigation into the hypothesis discussed above, Szondy and Newsholme (1990) investigated whether other precursors of purine and pyrimidine synthesis could replace the requirement of glutamine for lymphocyte proliferation. They investigated the effect of nucleosides and nucleobases, which can be used for the synthesis of purine and pyrimidine nucleotides via salvage pathways (see Figure 1), on lymphocyte proliferation in the absence of glutamine. They demonstrated that the proliferation of lymphocytes (determined by measuring the incorporation of [^{3}H] thymidine into lymphocyte DNA) can be partially or totally replaced by nucleosides and nucleobases added to culture medium. This suggests that the addition of a nucleoside or nucleobase to the culture medium may supply lymphocytes with that purine and pyrimidine nucleotide which should not be able to be synthesized in the absence of glutamine. The rate of proliferation by lymphocytes cultured with nucleosides and nucleobases in the absence of added glutamine was quite

considerable (see Table 1). Purine and pyrimidine nucleotides are not interconvertible i.e. purine cannot be converted to pyrimidines and *vice versa*. Therefore, Szondy and Newsholme (1990) raised the question as to how a considerable rate of lymphocyte proliferation could be maintained in the presence of only one nucleoside or nucleobase. They suggested that lymphocytes may be able to produce glutamine and that the glutamine produced in these cells could maintain their proliferation via its provision of nitrogen for the *de novo* synthesis of the purine or pyrimidine nucleotides. This suggestion is supported by the observation, in the study by Szondy and Newsholme (1990), that addition of both a purine and a pyrimidine nucleoside, in the absence of glutamine produced a rate of incorporation, of [^{3}H] thymidine, equivalent to the rate of [^{3}H] thymidine incorporation into DNA of cultured lymphocytes when the optimum concentration of glutamine (0.3mM) is present in culture media. Thus, their interpretation of the results obtained from their study predicts that lymphocytes should possess glutamine synthetase activity.

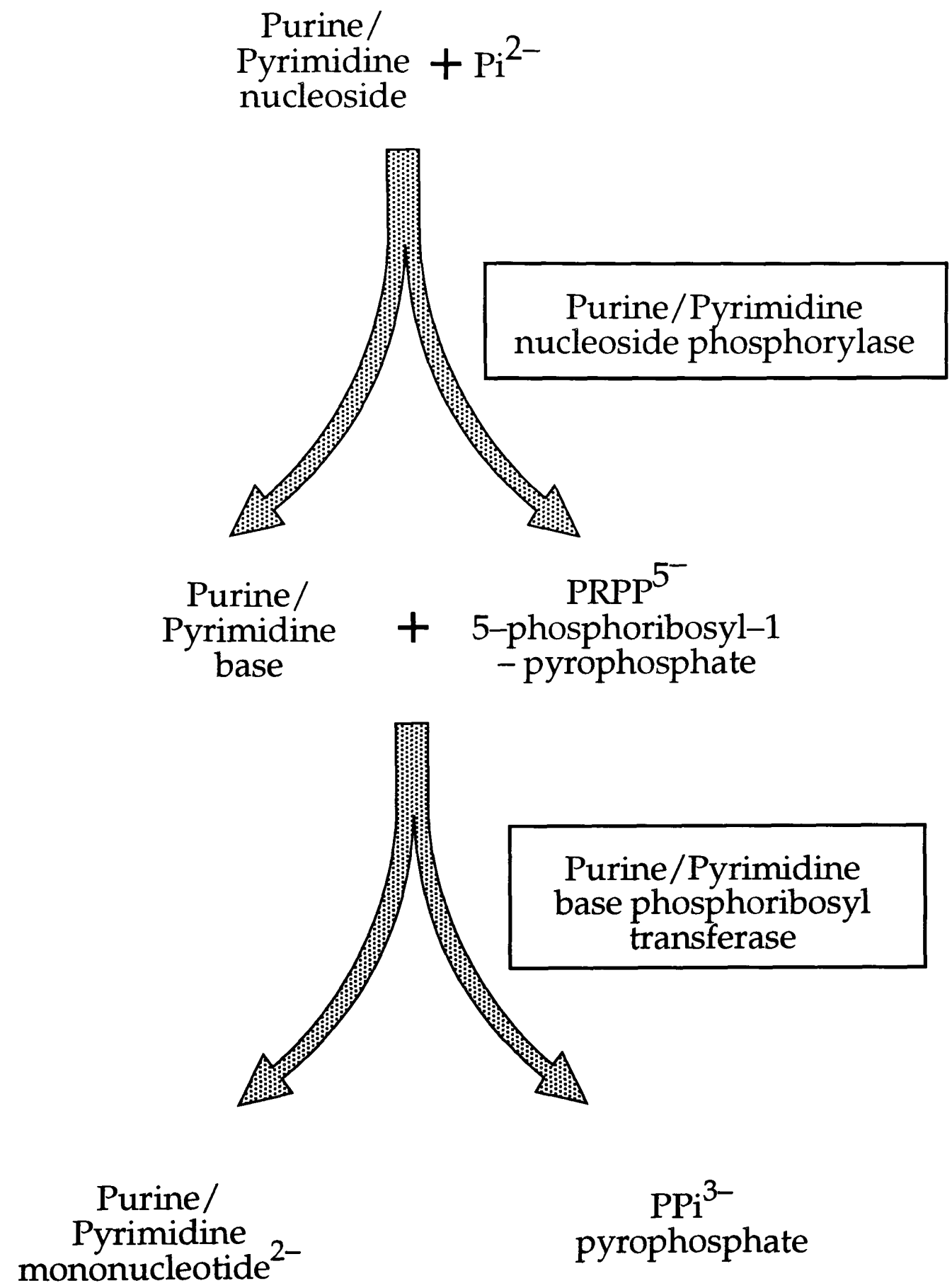


Figure 1. Salvage pathway for purine and pyrimidine nucleotide biosynthesis.

Table 1: effect of nucleobases or nucleosides on the incorporation of [^{3}H] thymidine into DNA in phytohaemagglutinin-stimulated lymphocytes in the absence of added glutamine or in the presence of 0.01mM or 0.2mM- glutamine.

	[^{3}H]thymidine incorporation (c.p.m.)		
Glutamine added to culture medium.....	None	0.01mM	0.2mM
Control (no base or nucleoside)	327	3484	8716
Adenosine	1399**	4749*	11154**
Guanosine	1033**	4216*	11481*
Uridine	1083**	4301*	11167**
Cytidine	905**	4244*	10814*

Results are represented as means for 3 or 4 experiments. Statistical significance of the differences between control and experimental results is indicated by * ($P<0.05$) or **($P<0.01$) (Student's t-test). Data derived from Szondy and Newsholme (1990).

Lymphocytes and macrophages possess very high glutaminase activities (Ardawi and Newsholme 1982, Newsholme P. et al 1986) The question that might be asked is, is it possible for cells that possess a high glutaminase activity to possess glutamine synthetase activity and thus have the capacity to synthesize glutamine? Cells that are known to possess high glutaminase activities, such as tumour cells and adipocytes (Sauer and Daunchy 1982, 1983, Kowalchuk et al 1988 respectively) are reported to possess glutamine synthetase activity (Kulka and Cohen 1973, Arad et al 1976, Matsuno and Goto 1992, Miller 1975). Thus, cells of the immune system may also possess glutamine synthetase activity and may therefore have the capacity to synthesize glutamine.

If cells of the immune system have the capacity to synthesize glutamine then a number of questions may be asked:

1) How does glutamine synthetase activity in cells of the immune system compare with their glutaminase activity?

Consequently, it was decided to measure glutamine synthetase activity in both lymphocytes and macrophages. In addition, the effect of sepsis and mitogenic stimulation on glutamine synthetase activity in lymphocytes was investigated.

2) How does the depletion of glutamine levels, in cell culture medium, affect glutamine synthetase activity in lymphocytes?

Consequently, the effect of adding decreasing glutamine concentrations to culture medium on glutamine synthetase activity in cultured lymphocytes was investigated.

3) Is glutamine synthetase activity in cells of the immune system important for immune cell function in the absence of glutamine?

Thus, the effect of an inhibitor of glutamine synthetase, methionine sulphoximine, on lymphocyte proliferation was investigated.

2.Materials and Methods.

2.1.Materials.

Details of chemicals and enzymes and care of animals are in Appendix I

2.2.Methods.

2.2.1.Preparation of lymphocytes and macrophages for the glutamine synthetase assay.

Preparation of lymphocytes.

Lymphocytes were prepared from cervical and mesenteric lymph nodes by a method modified from that of Ardawi and Newsholme (1982).

Lymphocytes were prepared from rat cervical and mesenteric lymph nodes by teasing the lymph nodes over a fine stainless steel mesh into phosphate-buffered saline at pH 7.2 and 0°C. The cells were rapidly filtered through lens tissue. The cells were then centrifuged at 0°C at 400g for 10 mins and were then resuspended in phosphate-buffered saline (2.5ml). In order to remove erythrocytes from the lymphocyte preparation, the cells were layered on to a density-gradient medium 'Lymphoprep' (density 1.077) (5ml) and centrifuged at 1000g for 20 mins at room temperature. The purified lymphocytes were then pipetted off the 'Lymphoprep', resuspended in phosphate-buffered saline and centrifuged for 10mins at 400g. Some of the supernatant was removed and the percentage viability of the cells was determined; this was done by the Trypan Blue exclusion test. Dilutions were made in 0.1% Trypan Blue and the proportion of viable cells present were determined with a haemocytometer. (The number of cells that were resuspended, were also counted.) Lymphocyte viability was 95%. Cells were then resuspended in extraction medium in preparation for the glutamine synthetase assay.

Preparation of mouse macrophages.

Mice were injected, intraperitoneally, with a thioglycollate broth so as to chemically induce peritonitis. Four days after the injection, mice were killed with CO_2. Macrophages were then removed from the peritoneal cavity, filtered using lens tissue and then centrifuged at 400g for 10 mins. The macrophage preparation was then purified by adherence to plastic petri dishes as follows: after the cell preparation was centrifuged, cells were resuspended in approximately 100ml of Modified Eagles Medium with 10% foetal calf serum (dialysed against phosphate-buffered saline in order to remove glutamine and other small molecules) and 20μg of antibiotic (gentamicin 1ml), supplemented with 2mM glutamine. Cells were then plated at a density of 2.5×10^7 cells/petri dish and incubated for 4h. at 37°C. After incubation the petri dishes were washed three times with phosphate buffered saline (15ml) to remove non-adherent cells. The cells were then gently scraped up into 2ml phosphate-buffered saline. Cells were centrifuged at 500g for 5mins and then resuspended in extraction medium in preparation for the glutamine synthetase assay.

2.2.2.The effect(s) of starvation and endotoxin treatment on glutamine synthetase activity in extracts of fresh rat lymphocytes.

Rats (200g) were fasted for 15, 24 and 48h.. They were then killed by cervical dislocation and lymphocytes were prepared as previously described. For endotoxin treatment rats (200g) were administered endotoxin as previously described (see Chapter 3, section 2.3.4.) 24 and 48h. before experimentation. Lymphocytes were prepared as previously described.

2.2.3.Preparation of lymphocytes for tissue culture.

The method for the preparation of lymphocytes for culture was the same as the preparation of fresh lymphocytes described above except that the procedure was done under sterile conditions, so as to prevent contamination of cells in culture, and only cervical lymph nodes were used.

After the first centrifugation, the cells were resuspended in sterile HEPES-buffered-RPMI medium (10ml). The resuspended cells were then layered on to sterile 'Lymphoprep' and centrifuged at 1000g for twenty mins. The purified lymphocyte preparation was removed and resuspended in sterile HEPES-buffered-RPMI (20ml). The resuspended cells were then centrifuged at 400g for 10 mins. The supernatant was removed and the cells were thoroughly resuspended in sterile HEPES-buffered-RPMI. The percentage viability of the cells was determined as before. Lymphocyte viability was 95%. The cells were then plated out (see below).

2.2.4.Procedures for cultured lymphocytes.

Lymphocytes were cultured in six well plates (maximum capacity of a well is 5ml) containing culture media which consisted of sterile HEPES-buffered-RPMI medium, sterile 10% (v/v) foetal calf serum (dialysed against phosphate buffered saline in order to remove glutamine and other small molecules), 100 units of sterile streptomycin/ml and 200 units of sterile penicillin/ml. Culture media also contained various concentrations of glutamine, mitogens [concanavalin A (Con A), a T cell mitogen, lipopolysaccharide (LPS), a B cell mitogen, and pokeweed mitogen (PWM), a T and B cell mitogen] and other factors, depending on the experiment being performed, details of which are given below. All factors contained in culture media were sterilised by sterile filtration before use. The cultures

were incubated at 37°C at an atmosphere of O_2: CO_2 (95:5) for different time periods. (Details of the preparation of factors used for tissue culture are in Appendix II).

2.2.5.Details of cell culture experiments.

Glutamine synthetase activity in extracts of lymphocytes cultured in the absence and presence of mitogens and various glutamine concentrations.

Approximately 2 x 10^7 lymphocytes in RPMI (1ml) were cultured in media composed of:

HEPES-buffered-RPMI + 10% foetal calf serum +antibiotic	2.5ml
Glutamine (0.5, 1 or 2mM)*	0.5ml
Mitogen (Con A 5µg/ml or LPS 10µg/ml or PWM 1µg/ml)*	0.5ml
RPMI	0.5ml

*RPMI (0.5ml) was used when glutamine or mitogen were absent from the culture media.

The cultures were incubated as previously described for 24 or 72h..

Glutamine synthetase activity in extracts of lymphocytes cultured in the absence and presence of methionine sulphoximine, mitogen and glutamine.

Approximately 2 x 10^7 lymphocytes in RPMI (1ml) were cultured in media composed of:

HEPES-buffered-RPMI and 10% foetal calf serum +antibiotic	2.5ml
Glutamine (2mM or 0mM)*	0.5ml
Mitogen (Con A, 5µg/ml, LPS, 10µg/ml, PWM, 1µg/ml)*	0.5ml
Methionine Sulphoximine(1mM)*	0.5ml

*RPMI (0.5ml) was used when glutamine or mitogen or methionine sulphoximine were absent from the culture media.

The cells were incubated as previously described for 24 or 72h..

All cultured lymphocytes were washed three times with phosphate buffered saline before they were prepared for the glutamine synthetase assay.

2.2.6.Preparation of homogenates.

Both fresh and cultured lymphocytes and fresh macrophages were homogenized using a glass homogeniser, which was kept on ice, in extraction buffer (50mM TRIS and 2mM EDTA pH 7.9) for the glutamine synthetase assay.

2.2.7.Glutamine synthetase assay.

Glutamine synthetase activity in lymphocytes and macrophages was measured as previously described (see Chapter 3, section 2.2.2.). However, there were some slight modifications:

1) Cell homogenate (100µl) was used in the assay, instead of 50µl which was used for the muscle glutamine synthetase assay.

2) ^{14}C Glutamate (50µl) was used, in 20ml of reaction medium, instead of 500µl which was used for the muscle glutamine synthetase assay.

3) 2-Mercaptoethanol (1mM) was present in the reaction medium.

The separation procedure was as previously described (see Chapter 3, section 2.2.2.).

Protein Content.

Protein content in lymphocyte and macrophage homogenates was measured using the Biorad assay (see chapter 3, section 2.2.3.).

2.2.8.The effect of Methionine Sulphoximine on [^{3}H]thymidine incorporation into DNA of cultured lymphocytes.

For the proliferation experiments lymphocytes, which were prepared under sterile conditions as previously described, were cultured in wells of micro titre culture plates, with a maximum capacity of 200µl. Approximately 5 x 10^5 cells in 100µl of medium of HEPES-buffered-RPMI and 10% foetal calf serum + antibiotic were present in each 200µl well also containing:

Mitogen (Con A, 5µg/ml, LPS,10µg/ml or PWM,1µg/ml)*	20µl
Glutamine (2mM)*	20µl
Methionine Sulphoximine (1mM)*	50µl
RPMI	10µl

*RPMI (20µl) was used when mitogen or glutamine were absent from culture media, and RPMI (50µl) was used when methionine sulphoximine was absent from culture media.

All factors present in the culture medium were sterile filtered before use. The cultures were incubated, as previously described, for 24 or 72h.. After each incubation period [^{3}H] thymidine (0.2µCi) was added to each 200µl well. Thymidine incorporation was measured over 18h. (which is approximately the time for a complete cell cycle in lymphocytes). After the 18h., cultures were harvested on to glass fibre filters with an automated cell harvester. These filters were washed and dried, the radioactivity on the dried filters was measured in a liquid scintillation counter (Beckman LS 1701).

2.3.Presentation of data and statistical analysis.

Activities of glutamine synthetase in lymphocytes and macrophages are expressed as nmoles/min/mg of protein. Statistical analysis was performed using Student's t-test.

3.Results.

3.1.Glutamine synthetase activity in extracts of fresh rat lymphocytes and mouse macrophages.

The assay for glutamine synthetase followed the incorporation of ^{14}C from ^{14}C glutamate to ^{14}C glutamine. Evidence to suggest that the incorporation of ^{14}C from ^{14}C glutamate to ^{14}C glutamine was due to glutamine synthetase activity present in lymphocytes and macrophages (see Table 4.1) is as follows:

1) ^{14}C incorporation is affected by changes in the concentration of the substrates for the enzyme, glutamate or ammonia, in the reaction medium (results not shown), and

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The calculation for the comparison of muscle glutamine synthetase activity with that of lymphocytes is incorrect since glutamine synthetase activity for 1ml of diluted muscle was compared to glutamine synthetase activity for 100µl of diluted cells. Since lymphocyte weight was not determined, the dilution factor for lymphocytes could not be calculated (protein content for 100µl of diluted lymphocytes was calculated, hence the specific activities). Thus, a comparison of glutamine synthetase activity per g of tissue between muscle and lymphocytes could not be done.

[redacted] (an and and Newsholme 1991). In comparison to muscle, g[redacted]tivity is very low in lymphocyte and macrophage extracts; glutamine synthetase activity in muscle extracts is approximately 30 times greater than that of glutamine synthetase activity in lymphocyte and macrophage extracts.

3.2.The effect(s) of starvation and endotoxin treatment on glutamine synthetase activity in extracts of fresh rat lymphocytes.

Starvation increased the activity of glutamine synthetase in lymphocyte extracts (Table 4.2). The effect of starvation on glutamine synthetase activity in lymphocyte extracts is similar to the effect of starvation on glutamine synthetase activity in muscle extracts(see Chapter 3, section 3.5.).

3.Results.

3.1.Glutamine synthetase activity in extracts of fresh rat lymphocytes and mouse macrophages.

The assay for glutamine synthetase followed the incorporation of ^{14}C from ^{14}C glutamate to ^{14}C glutamine. Evidence to suggest that the incorporation of ^{14}C from ^{14}C glutamate to ^{14}C glutamine was due to glutamine synthetase activity present in lymphocytes and macrophages (see Table 4.1) is as follows:

1) ^{14}C incorporation is affected by changes in the concentration of the substrates for the enzyme, glutamate or ammonia, in the reaction medium (results not shown), and

2) ^{14}C incorporation is inhibited by methionine sulphoximine (an inhibitor of glutamine synthetase activity) (Tables 4.4 and 4.5).

In comparison to the glutaminase activities of lymphocytes and macrophages, glutamine synthetase activites of both lymphocytes and macrophages are very low (see Newsholme P. et al 1986; Szondy and Newsholme 1991). In comparison to muscle, glutamine synthetase activity is very low in lymphocyte and macrophage extracts; glutamine synthetase activity in muscle extracts is approximately 30 times greater than that of glutamine synthetase activity in lymphocyte and macrophage extracts.

3.2.The effect(s) of starvation and endotoxin treatment on glutamine synthetase activity in extracts of fresh rat lymphocytes.

Starvation increased the activity of glutamine synthetase in lymphocyte extracts (Table 4.2). The effect of starvation on glutamine synthetase activity in lymphocyte extracts is similar to the effect of starvation on glutamine synthetase activity in muscle extracts(see Chapter 3, section 3.5.).

The effect of endotoxin on glutamine synthetase activity in lymphocyte extracts differed to the effect of endotoxin on glutamine synthetase activity in muscle extracts. Whereas, endotoxin increased glutamine synthetase activity in muscle extracts (see Chapter 3, section 3.4.) it markedly reduced glutamine synthetase activity in lymphocyte extracts (Table 4.3).

3.3.The effect of mitogenic stimulation and various glutamine concentrations on glutamine synthetase activity in extracts of cultured rat lymphocytes .

Mitogenic stimulation did not cause a change in glutamine synthetase activity in extracts of cultured lymphocytes. Decreases in the concentration of glutamine added to the culture medium were associated with marked increases in glutamine synthetase activity in extracts of cultured lymphocytes (Figures 1,2,3,4).

3.4.The effect of methionine sulphoximine on glutamine synthetase activity in extracts of rat lymphocytes cultured for 24 and 72h..

Methionine sulphoximine (MSO) (1mM), when added to the culture medium, inhibited glutamine synthetase activity in the extracts of lymphocytes cultured with and without mitogen or glutamine, with activities reaching non-detectable levels (Tables 4.4 and 4.5). An interesting observation from this particular study is that, the percentage inhibition of glutamine synthetase activity, in extracts of cultured lymphocytes 24h.), by MSO, was, on average, found to be greater in lymphocytes cultured with 2mM glutamine than in those cells cultured in the absence of glutamine (see Table 4.5), and for lymphocytes cultured for 72h., the situation is *vice versa*. The reason for this is not known.

3.5.The effect of methionine sulphoximine on [^{3}H] thymidine incorporation into DNA in stimulated rat lymphocytes.

MSO (1mM), when added to the culture medium, had no effect on [^{3}H] thymidine incorporation into DNA in lymphocytes cultured with glutamine. In contrast, for lymphocytes cultured in the absence of added glutamine, [^{3}H] thymidine incorporation into DNA was very low.

Table 4.1
Glutamine synthetase activity in extracts of fresh rat lymphocytes and mouse macrophages.

Cells	Maximal Activity (nmoles/min/mg prot)
Lymphocytes	2.43±0.06 n=4
Macrophages	3.25±0.23 n=4

Values are the mean (SEM) for 4 separate experiments.

Table 4.2
The effect of starvation on glutamine synthetase activity in extracts of fresh rat lymphocytes.

	Maximal Activity (nmoles/min/mg prot)
Fed	2.78±0.22 n=4
Starvation (15h.)	5.27±0.27**** n=4
Starvation (24h.)	3.98±0.16*** n=4
Starvation (48h.)	5.6±0.26**** n=4

Values are the mean (SEM) for 4 separate experiments.
The statistical significance of the difference between fed and starved rats is denoted by ****P<0.001 and .
***P<0.01**

Table 4.3
The effect of endotoxin on glutamine synthetase activity in extracts of fresh rat lymphocytes.

Treatment	Maximal Activity (nmoles/min/mg prot)
Control (24h.)	4.3 ±0.23 n=4
Endotoxin - treated (24h.)	1.4 ±0.24**** n=4
Control (48h.)	6.6 ±0.32 n=4
Endotoxin - treated (48h.)	1.9 ±0.17**** n=4

Values are the mean (SEM) for 4 separate experiments. The statistical significance of the difference between endotoxin-treated and non endotoxin-treated rats is denoted by ****P<0.001.

Figure 1. The effect of varying the concentration of glutamine added to the culture medium and the absence of added glutamine on glutamine synthetase activity in extracts of mitogen stimulated rat lymphocytes, incubated for 24h..

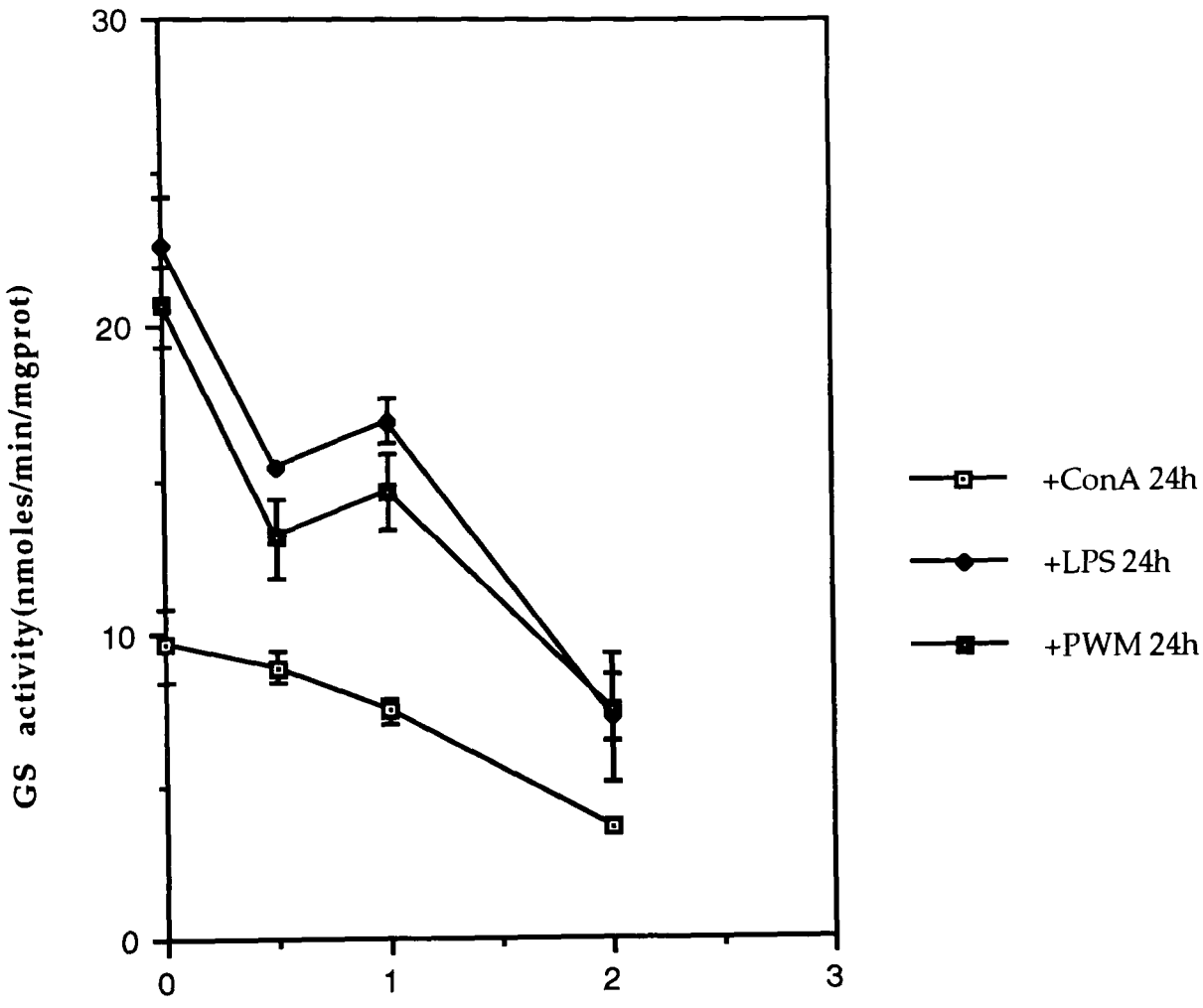

Figure 2. The effect of varying the concentration of glutamine added to the culture medium and the absence of added glutamine on glutamine synthetase activity in extracts of rat lymphocytes (- mitogens), incubated for 24h..

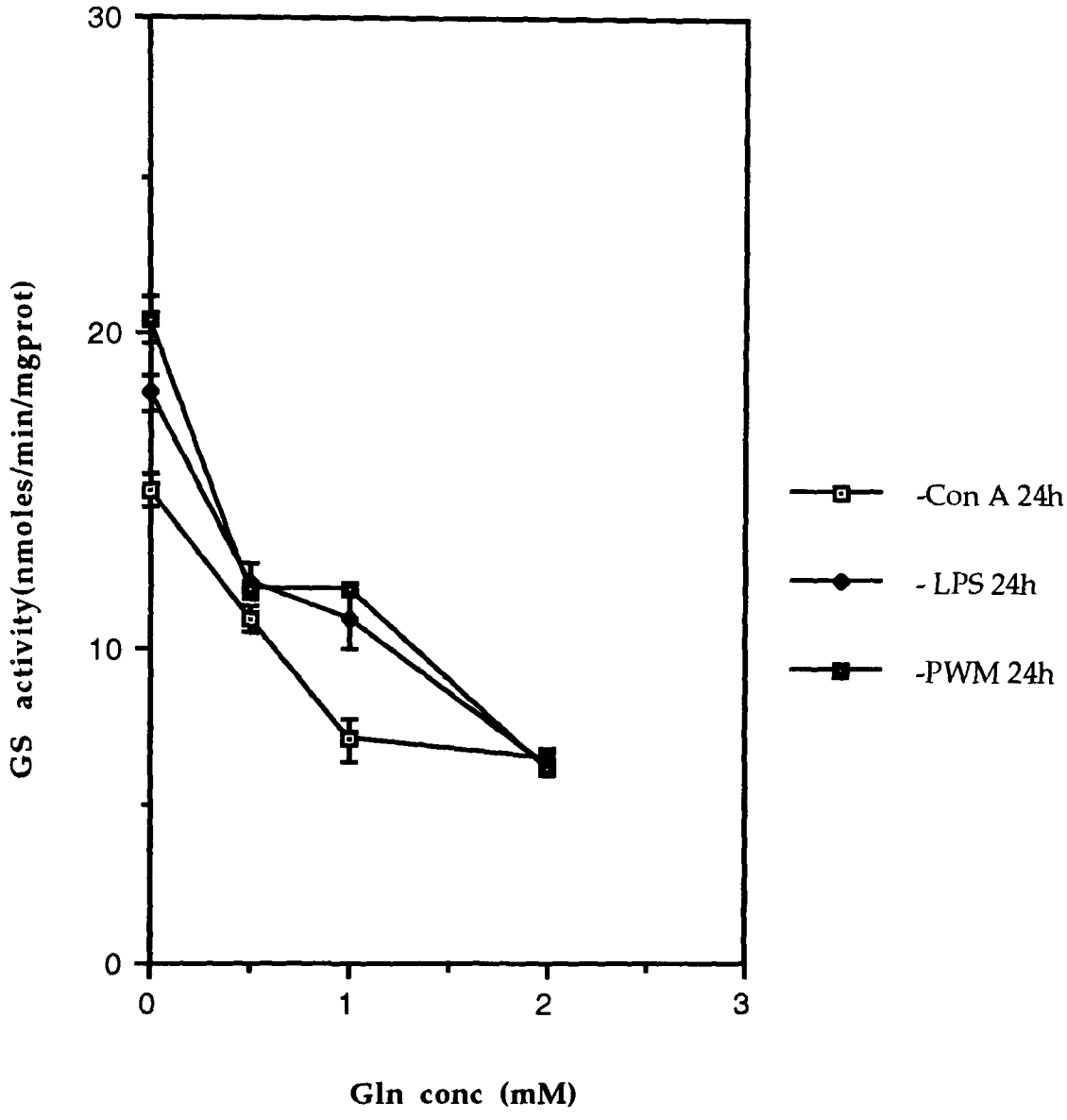

Figure 3. The effect of varying the concentration of glutamine added to the culture medium and the absence of added glutamine on glutamine synthetase activity in extracts of mitogen stimulated rat lymphocytes, incubated for 72h..

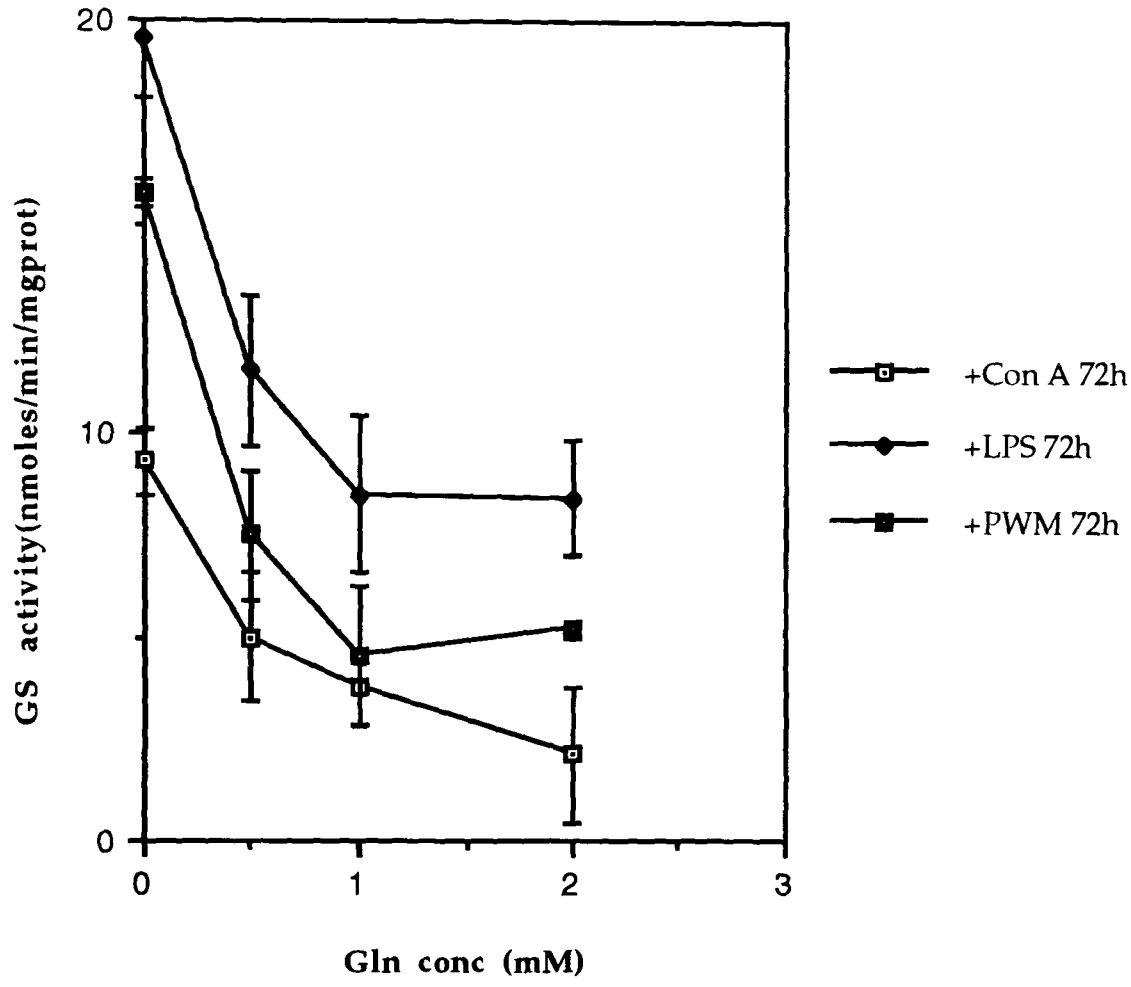

Figure 4. The effect of varying the concentration of glutamine added to the culture medium and the absence of added glutamine on glutamine synthetase activity in extracts of rat lymphocytes (- mitogen), incubated for 72h..

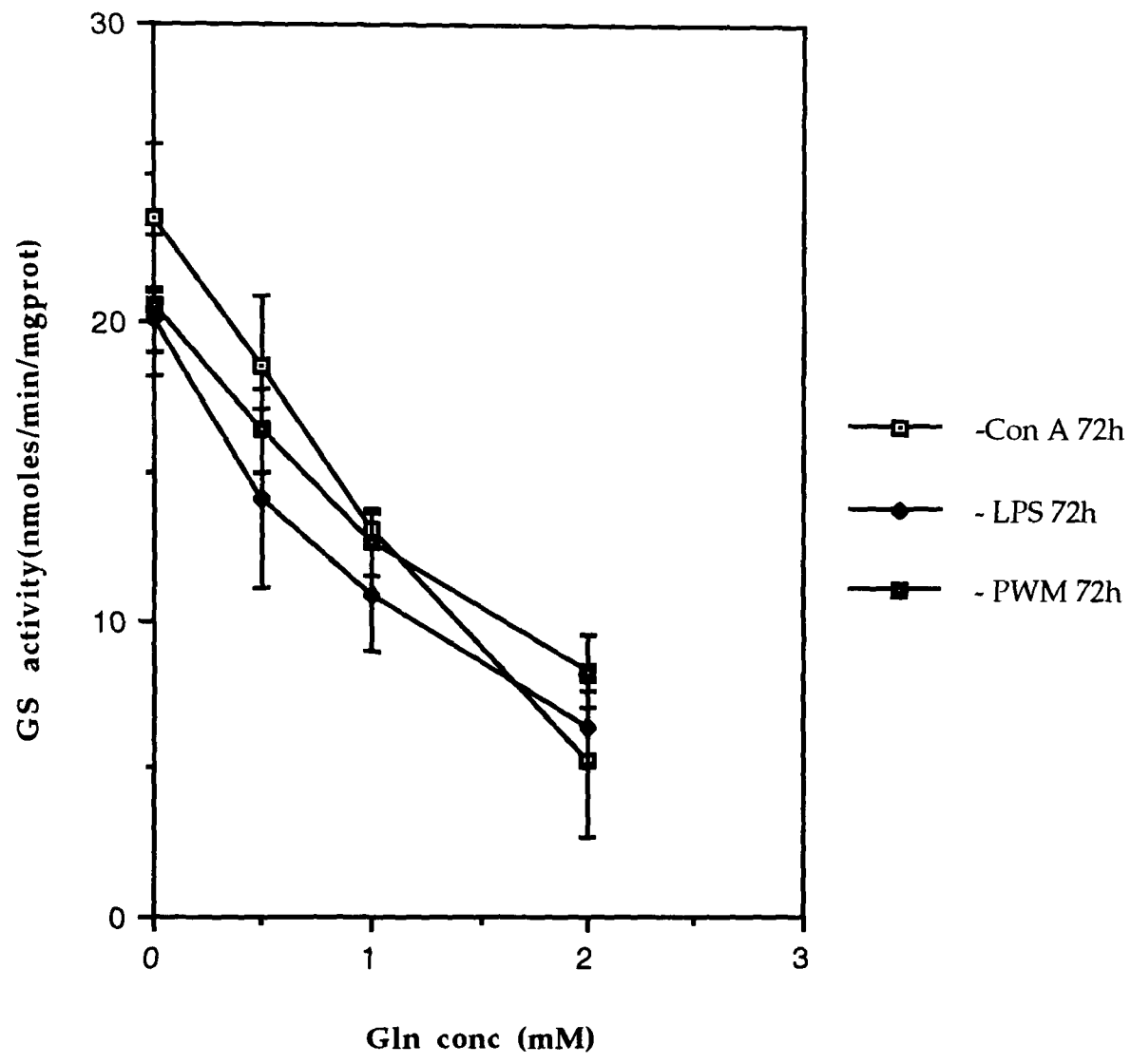

Table 4.4
The effect of methionine sulphoximine on glutamine synthetase activity in extracts of rat lymphocytes cultured for 24h.

MITOGEN	Glutamine (mM)	Glutamine synthetase activity (nmoles/min/mg of prot)		% inhibition
		(-)MSO	(+)MSO	
Con A	2	10.0	0.0	100
Con A	0	20.0	1.3	93
LPS	2	2.9	0.38	87
LPS	0	16.0	4.7	75
PWM	2	12.5	7.1	57
PWM	0	24.2	9.3	71
None	2	5.5	0.0	100
None	0	19.8	9.4	62

Table 4.5
The effect of methionine sulphoximine on glutamine synthetase activity in extracts of rat lymphocytes cultured for 72h.

MITOGEN	Glutamine (mM)	Glutamine synthetase activity (nmoles/min/mg of prot)		% inhibition
		(-)MSO	(+)MSO	
Con A	2	1.40	0.47	64
Con A	0	5.21	0.73	88
LPS	2	1.74	0.62	63
LPS	0	8.13	0.0	100
PWM	2	0.7	0.12	89
PWM	0	13.2	0.0	100
None	2	2.4	0.0	100
None	0	15.0	0.0	100

Abbreviations: MSO- Methionine Sulphoximine.
Con A-Concanavalin A
LPS- Lipopolysaccharide.
PWM- Pokeweed mitogen.

Table 4.6
The effect of methionine sulphoximine on [^{3}H] thymidine incorporation into DNA in mitogen stimulated lymphocytes in the presence of glutamine (2mM) or in the absence of added glutamine after 24 and 72h.

MITOGEN	Glutamine (mM)	[^{3}H] Thymidine incorporation			
		24h.	24h.	72h.	72h.
		(-) MSO	(+)MSO	(-)MSO	(+)MSO
Con A	0	1368± 204	47±67*	11515±453	38±8***
	2	51334±893	46973±1730	47491±969	43764±2397
LPS	0	716±42	82±11**	9560±64	33±2***
	2	11099±199	10481±302	31892±677	32134±1129
PWM	0	231±16	44±3.4	6989±246	33±2***
	2	1700±85	2414±149	20628±728	20737±2865
None	0	211±20	39±2*	5287±259	32±2***
	2	2116±140	1797±84	21021±601	19037±489

Values are the mean (SEM) for 4 separate experiments. (incubations)
The statisticalsignificance between lymphocytes cultured in the presence of MSO and lymphocytes cultured in the absence of MSO is denoted by
***P<0.05, ***P<0.02 and *** P<0.01**
Abbreviations: MSO- Methionine Sulphoximine
Con A-Concanavalin A, LPS- Lipopolysaccharide
PWM-Pokeweed mitogen

4.Discussion.

4.1.Glutamine synthetase activity in extracts of cultured lymphocytes, and the effect of various glutamine concentrations on glutamine synthetase activity in extracts of cultured lymphocytes.

The activity of glutamine synthetase in cultured lymphocytes, in the presence of added glutamine (2mM) measured in the present study (glutamine synthetase activity was between 2nmoles/min/mg of protein and 4nmoles/min/mg of protein) is of the same magnitude as that for T-lymphoblast cells cultured in the presence of added glutamine (2mM) reported by Kitoh et al (1990). Glutamine synthetase activity measured in T-lymphoblast cells was between 1nmoles/min/mg of protein and

1.2nmoles/min/mg of protein. Glutamine synthetase activity in cultured lymphocytes, in the presence of added glutamine (2mM) measured in the present study, is higher than that of B-lymphoblast cells cultured in the presence of added glutamine (2mM) (Kitoh et al 1990). Glutamine synthetase activity measured in the B lymphoblast cells was between 0.6 nmoles/min/mg of protein and 0.8nmoles/min/mg of protein.

There was a gradual decrease in glutamine synthetase activity in extracts of cultured lymphocytes associated with an increase in the concentration of glutamine added to the culture medium. A number of studies have reported decreases in glutamine synthetase activity associated with increases in glutamine concentration in culture medium in different cell types; cultured skeletal muscle cells (Smith et al 1984, Feng et al 1990), human leukaemia cell lines (Kitoh et al 1990), and hepatoma cells (Kulka et al 1973), but this has not been reported previously for lymphocytes.

The increase in glutamine synthetase activity in extracts of lymphocytes cultured in glutamine depleted media may be of physiological importance. In man, the physiological concentration of plasma glutamine is approximately 0.6mM (see Parry-Billings 1989) and, as stated previously, glutamine and its utilization are important for lymphocyte proliferation. Since, glutamine uptake by lymphocytes has been shown to be concentration dependent (Ardawi 1983), a decrease in the physiological concentration of plasma glutamine may lead to a decrease in glutamine uptake and its subsequent utilization by lymphocytes. Thus, if the concentration of plasma glutamine in man goes below the physiological concentration, then glutamine uptake into lymphocytes is decreased.

The present study, investigating the effect of changes in glutamine concentration on glutamine synthetase activity in extracts of cultured lymphocytes, suggests that with a decreased provision of glutamine to

lymphocytes, glutamine synthetase activity of lymphocytes is increased. The increase in glutamine synthetase activity may be an attempt by lymphocytes to maintain provision of glutamine for their proliferation, for example during an immunological challenge. If this is so, then the question to be asked is, does the increase in the activity of glutamine synthetase in lymphocytes cultured in the absence of added glutamine, provide sufficient glutamine for the maintainence of the proliferation of stimulated lymphocytes?

Results from the study on the effect of MSO, on [^{3}H]thymidine incorporation into DNA in mitogen-stimulated lymphocytes, may provide an answer as to whether the glutamine provided by glutamine synthetase activity is enough to permit a given rate of proliferation of lymphocytes. MSO, which inhibited markedly glutamine synthetase activity in extracts of cultured lymphocytes (see Table 4.4 and 4.5), had no effect on [^{3}H] thymidine incorporation into DNA of stimulated lymphocytes cultured in the presence of added glutamine (2mM). In contrast, MSO markedly reduced [^{3}H] thymidine incorporation into DNA of stimulated lymphocytes, cultured in the absence of added glutamine. This suggests that, in the absence of added glutamine lymphocytes may provide their own glutamine, via glutamine synthesis, for proliferation.

The synthesis of purine and pyrimidine nucleotides is required for DNA sy
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1989) shows that the activity of lymphocyte glutamine synthetase is in excess (approximately 60-fold greater) of lymphocyte carbamoyl-phosphate synthase activity. (Carbamoyl-phosphate synthase 2 is a limiting enzyme

pg 154: Activities of glutamine synthetase and CPS II in Con A stimulated lymphocytes

Glutamine synthetase activity ~4nmoles/min/mg of protein (cells incubated in culture medium + 2mM glutamine for 24h.; Figure 1).

CPS II activity = 0.15nmoles/min/mg of protein (cells incubated in culture medium + 2mM glutamine for 3h.; Szondy and Newsholme 1991).

Therefore, glutamine synthetase activity is in excess of CPS II activity by ~25fold.

lymphocytes, glutamine synthetase activity of lymphocytes is increased. The increase in glutamine synthetase activity may be an attempt by lymphocytes to maintain provision of glutamine for their proliferation, for example during an immunological challenge. If this is so, then the question to be asked is, does the increase in the activity of glutamine synthetase in lymphocytes cultured in the absence of added glutamine, provide sufficient glutamine for the maintainence of the proliferation of stimulated lymphocytes?

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The synthesis of purine and pyrimidine nucleotides is required for DNA synthesis, which in turn is required for lymphocyte proliferation. A comparison of the maximal activity of glutamine synthetase in extracts of cultured lymphocytes, 9nmoles/min/mg of protein , (see Table 4.4) with that of carbamoyl-phosphate synthase 2 in extracts of cultured lymphocytes, 0.15nmoles/min/mg of protein, (Szondy and Newsholme 1989) shows that the activity of lymphocyte glutamine synthetase is in excess (approximately 60-fold greater) of lymphocyte carbamoyl-phosphate synthase activity. (Carbamoyl-phosphate synthase 2 is a limiting enzyme

for pyrimidine nucleotide synthesis of which glutamine is a co-substrate.) This comparision suggests that glutamine synthetase activity may provide enough glutamine to maintain a rate of glutamine utilization that would provide branched point sensitivity.

A comparision of the ratio of the glutaminase/glutamine synthetase activity of lymphocytes with the ratio of lymphocyte proliferation in the presence and absence of added glutamine may provide some *prima facie* evidence as to whether glutamine synthetase activity provides enough glutamine to maintain branched point sensitivity, via its utilization by lymphocytes. A comparison of the ratio, maximal activity of glutaminase in lymphocytes cultured in the presence of added glutamine (2mM) to the maximal activity of glutamine synthetase in the extracts of lymphocytes cultured without added glutamine, with the ratio of [^{3}H]thymidine incorporation into DNA of lymphocytes cultured in the presence of added glutamine to that in the absence, has been made (see below).

[The maximal activity of glutaminase may indicate the maximum rate of glutamine utilization by lymphocytes. The maximal activity of glutamine synthetase in the extracts of lymphocytes cultured in the absence of added glutamine may indicate the maximum rate of glutamine synthesis by these lymphocytes.]

For 24h.

$$\frac{\text{Maximal activity of glutaminase}}{\text{Maximal activity of glutamine synthetase}} = \frac{228}{10} = 23$$

$$\frac{[^3\text{H}]\text{thymidine incorporation(2mM glutamine)(cpm)}}{[^3\text{H}]\text{thymidine incorporation(0mM glutamine)(cpm)}} = \frac{51334}{1368} = 37$$

For 72h.

$$\frac{\text{Maximal activity of glutaminase}}{\text{Maximal activity of glutamine synthetase}} = \frac{228}{9} = 25$$

$$\frac{[^3\text{H}]\text{thymidine incorporation(2mM glutamine)(cpm)}}{[^3\text{H}]\text{thymidine incorporation(0mM glutamine)(cpm)}} = \frac{47491}{11515} = 4$$

Data derived from Szondy and Newsholme (1991) and Fig 1 and Fig 3 of the present study. Enzyme activities are expressed as nmoles/min/mg of prot.

The ratios of glutaminase/glutamine synthetase and [^{3}H]thymidine incorporation (2mM)/[^{3}H]thymidine incorporation (0mM), after a 24h. culture period, are similar. This suggests that glutamine synthetase activity which occurs in lymphocytes cultured in the absence of added glutamine is enough to maintain a rate of glutaminolysis to satisfy a given rate of proliferation.

After a 72h. culture period, the ratios are very different, with the ratio of the enzyme activities being far greater than the ratio of [^{3}H]thymidine incorporation. However, the interpretation of the calculation at 72h. may not be justified. It is assumed, in proliferation studies, that the rate of lymphocyte proliferation increases with time. This may not be the case. Lymphocyte proliferation over a particular culture period, for example 24h., is determined by measuring the incorporation of [^{3}H]thymidine, which is added only at the 24h. time point, into the DNA of the cultured lymphocytes and incorporation is followed for a further 18h.. Thus, proliferation over a 24h. culture period is actually determined over a 42h. period. A study by Szondy et al (unpublished), investigating the kinetics of lymphocyte proliferation, found that proliferation of cultured lymphocytes, in the presence of 2mM glutamine, was not maintained over a 'long' culture period (e.g. 72h.). The study showed that, when [^{3}H]thymidine was added to approximately $5x10^5$ stimulated lymphocytes (which is the same number of lymphocytes used in the

present study) after a 24h. culture period, the maximum incorporation of [^{3}H]thymidine was not reached. (The lymphocytes in the study of Szondy et al were stimulated with Con A (5µg/ul), and cultured in the presence of 2mM glutamine.) When [^{3}H]thymidine was added to approximately the same number of lymphocytes cultured under the same conditions, after a 60h. culture period, the maximum incorporation of [^{3}H]thymidine was reached. When [^{3}H]thymidine was added to approximately the same number of lymphocytes cultured under the same conditions, after a 72h. culture period, incorporation was decreased by approximately 45%. This indicated to Szondy et al that, when [^{3}H]thymidine is added to approximately $5x10^5$ lymphocytes (stimulated with 5µg/ul of Con A and cultured in the presence of 2mM glutamine) cultured for longer than 60h., most of the proliferation of the lymphocytes would have already been completed. In light of these results, it has been suggested by Szondy et al that the number of cell divisions that the lymphocyte culture undergoes in every study done under these conditions, may be limited; this may be an effect of increasing cell concentration and exhaustion of a fuel or inhibition of proliferation by accumulation of some metabolite. Thus, if the rate of proliferation is high, for example when lymphocytes are cultured in the presence of a high concentration of glutamine (2mM), for lymphocytes cultured for 72h., most of the proliferation could have been completed by the time [^{3}H]thymidine is added to the culture medium, hence the amount of [^{3}H]thymidine that is incorporated will be low. The study by Szondy et al highlights the importance of selecting culture times carefully for lymphocyte proliferation experiments.

4.2.The effect of endotoxin-administration on glutamine synthetase activity in lymphocyte extracts.

Results from the present study indicate that following endotoxin treatment, glutamine synthetase activity is markedly decreased. This decrease in lymphocyte glutamine synthetase activity, may be associated with a reciprocal change in lymphocyte glutaminase activity: Ardawi et al (1991) demonstrated that sepsis, for which endotoxin has been implicated as a major cause of the metabolic changes that occur during this condition (see van Deventer et al 1988), causes an increase in lymphocyte glutaminase activity and also an increase in the rate of glutamine utilization by lymphocytes. Consistent with this idea, a decrease in glutamine synthetase activity which occurs in stimulated cultured lymphocytes over time (see figures 3 and 5), appears to be associated with an increase in glutaminase activity, which has been shown to occur in stimulated cultured lymphocytes over time also (see Szondy and Newsholme 1991). These observations indicate that during an immunological challenge, an increase in glutaminase activity, in cells of the immune system is associated with a decrease in glutamine synthetase activity. The mechanism involved in the decrease in glutamine synthetase activity in stimulated lymphocytes is not known.

4.3.Is a substrate cycle, for glutamate/glutamine operating between the cytosol and mitochondria of lymphocytes and macrophages?

Studies have shown that lymphocytes and macrophages possess glutaminase (a mitochondrial enzyme) activity (Ardawi and Newsholme 1982, Newsholme P. et al 1986). The present study indicates that cells of the immune system possess glutamine synthetase (a cytosolic enzyme) activity. Thus, a substrate cycle for glutamate/glutamine may be operating between cytosol and mitochondria of lymphocytes and macrophages.

There is evidence to suggest that mammalian tissues i.e. liver and skeletal muscle possess a glutamate/glutamine substrate cycle. Haussinger (1990) reported that liver possesses high activities of both glutaminase and glutamine synthetase. These activities are present within different cell populations: peri-portal hepatocytes possess glutaminase activity and peri-venous hepatocytes possess glutamine synthetase activity. Experiments which involved studying the fate of ^{15}Nglutamine in perfused rat liver (Welbourne 1987, cited in Haussinger 1990) and studies on the metabolic fate of ^{13}Nammonia in rat liver *in vivo* (Cooper et al 1987) provide evidence that there is intercellular glutamine cycling i.e. peri-portal breakdown and peri-venous re-synthesis, of glutamine in liver. Thus, in liver a glutamate/glutamine substrate cycle may be operating between these two cell populations. Skeletal muscle possesses glutamine synthetase activity and there has been a report by Wu et al (1991) that skeletal muscle also possesses glutaminase activity. Thus, Parry-Billings and Newsholme (1991) proposed that a glutamate/glutamine substrate cycle may be operating between cytosols and mitochondria of muscle cells.

Substrate cycles may improve sensitivity in metabolic control (Newsholme and Crabtree 1976). Thus, the advantage of the operation of such a cycle in cells of the immune system is that, it may enhance the sensitivity in the metabolic control of glutamine utilization by these cells, and would thus, help to maintain immune cell function, particularly during an immunological challenge.

4.4.Possible differences in glutamine synthetase activity between different lymphocyte populations.

Glutamine synthetase activity in lymphocytes may vary depending on lymphocyte type ie T-lymphocyte and B-lymphocyte. Kitoh et al (1990), investigating glutamine synthetase activity in T-and B-leukaemic cells,

reported that B leukaemic cells are highly dependent on glutamine for growth and that T-leukaemic cells can grow in glutamine free medium. (This was demonstrated by comparing growth of cells cultured with medium containing 2mM glutamine with growth of cells cultured in glutamine free medium. The growth of cells cultured in the presence or absence of glutamine, was determined by counting the number of viable cells present. The growth of cells cultured in glutamine free medium was expressed as a percentage of that in medium containing glutamine.) Kitoh et al (1990) reported that T-leukaemic cells showed growth, in glutamine free medium, of more than 50% of control cultures (control cultures being T- leukaemic cells grown in the presence of 2mM glutamine) and that glutamine synthetase activity for the T cell line is greater than that of the B cell line. Thus, the study by Kitoh et al (1990) suggests that there is an inverse relationship between glutamine dependency and the activity of glutamine synthetase.

Whether there are differences in glutamine synthetase activity between T-and B-lymphocytes is not known since the present study was performed on a mixed T-and B-lymphocyte population. However, it is possible to separate T-lymphocytes from B-lymphocytes (see Hunt 1987, Szondy and Newsholme 1991) thus, further investigations on glutamine synthetase activity in different lymphocyte types should be carried out.

Chapter 5

Effect of glutamine feeding on muscle glutamine metabolism and on immune cell function.

1.Introduction.

Muscle is considered to be an important source of glutamine for cells of the immune system (see Chapter 1 section 5). During a number of conditions in experimental animals or man, muscle glutamine concentration is markedly decreased, for example during injury or sepsis in rats (Ardawi 1988a, Parry-Billings et al 1989, Ardawi and Majzoub 1991), following surgery or sepsis in dogs (Kapadia et al 1982, Karner et al 1989) and in man following major surgery, major injury, burns, with severe abdominal sepsis, or in malnourished man with or without sepsis (see Vinnars et al 1975; Furst et al 1979; Stjernstrom et al 1986; Askanazi et al 1980; Roth et al 1982; Milewsky et al 1982; Stinnet et al 1982, Kapadia et al 1985).

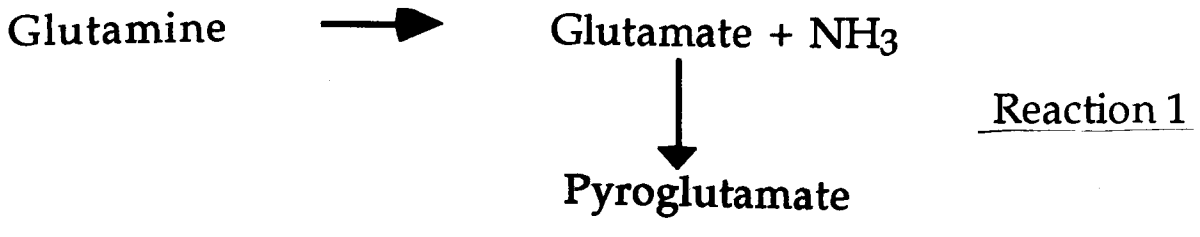
Some of the studies mentioned above, specifically those on septic rats, post surgery or septic dogs (Ardawi and Majzoub 1991 Parry-Billings et al 1989, Kapadia et al 1976 and Karner et al 1989), and on man following major injury, surgery, or during abdominal sepsis (Askanazi et al 1980, Roth et al 1982, Stinnet 1982, Kapadia et al 1985, Stjernstrom et al 1986), have reported that decreases in muscle glutamine concentration, which occur during the conditions stated, are associated with decreases in plasma glutamine concentration. A decrease in the level of plasma glutamine may be of significance for the function of cells of the immune system. Studies have shown that glutamine is used at a high rate by some cells of the immune system, and hence it is considered to be important for immune cell function (see Chapter 1, sections 3.1 and 3.3, Chapter 4, section1). In addition, studies have shown that, during conditions associated with reduced plasma glutamine levels, for example sepsis,

surgery and burns, immune function is depressed in man (Baker et al 1980, Green and Faist 1988). Thus, a reduction in plasma glutamine concentration may contribute to immunosuppression.

Since a decrease in the provision of glutamine from an important source, such as skeletal muscle, may lead to a decrease in plasma glutamine levels, a study was set up using rats, to investigate the effects of an exogenous supply of glutamine, i.e glutamine feeding, on muscle and plasma glutamine levels, and on immune cell function.

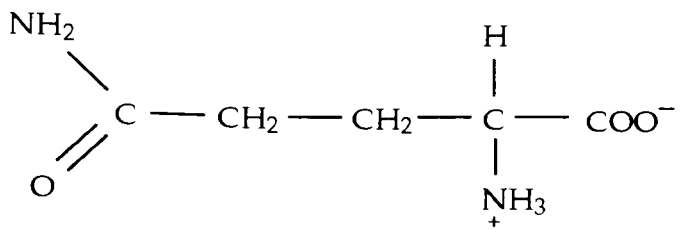
1.1.Glutamine administration.

Early literature, from the 1980s, on glutamine feeding indicated that, since glutamine is rapidly metabolised in the intestine (see Windmueller and Spaeth 1974, 1978) to ammonia and glutamate, the feeding of free glutamine is not effective in increasing glutamine levels in muscle or plasma. In addition, there was the question of stability and safety in the administration of free glutamine. Glutamine degrades slowly at 37°C (see Souba et al 1985) and solutions that are intravenously administered to patients require heat sterilisation prior to use. Thus, during heat sterilisation, glutamine would degrade to form ammonia, which when present in excess amounts is toxic (see Newsholme and Leech 1983), and pyroglutamate (see reaction 1), which is a neurotoxic compound when present in excess amounts (see Stehle and Furst 1987). Pyroglutamate is a cyclic derivative of glutamate (see below). In recent years it has been possible to carry out cold sterilisation using ultrafiltration, thus the problem of glutamine degradation at high temperatures has been abolished.

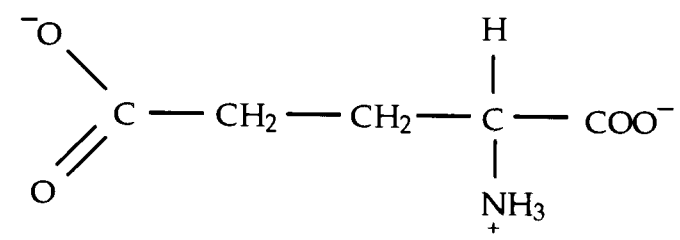


Reaction 1

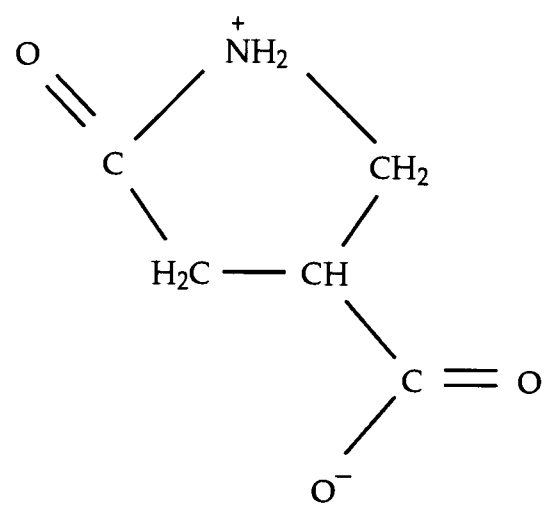
Glutamine



Glutamate



Pyroglutamate



As an alternative to glutamine, glutamine-containing dipeptides, alanyl-glutamine and glycyl-glutamine, have been used. *In vivo* studies in man and experimental animals provided evidence that alanyl-glutamine is readily utilized and that, following the intravenous administration of alanyl-glutamine, the levels of the free amino acids of the dipeptide, alanine and glutamine, increased in muscle and plasma (see Furst et al 1987, 1989a, 1989b). Studies have also shown that alanyl-glutamine is a safe source of glutamine in total parenteral nutrition (TPN), in rat and man. TPN is a method used for the administration, into a vein, of a solution containing essential nutrients. Furst et al (1987) studied the effect of heat sterilization on a solution of TPN, which

contained alanyl-glutamine, and reported that ammonia, pyroglutamate or glutamate occurred were not formed.

Whether alanyl-glutamine could maintain muscle glutamine, during conditions in which muscle glutamine levels are decreased, has been studied using TPN with and without the inclusion of alanyl-glutamine in injured rats (Furst et al 1987). It was reported, that muscle glutamine levels remained decreased in injured rats receiving TPN without alanyl-glutamine supplementation. In contrast, with the addition of alanyl-glutamine to TPN, muscle glutamine levels were increased. Furst et al (1987) and Hammarqvist et al (1990b) have studied the effects of alanyl-glutamine supplementation on muscle glutamine levels in patients after surgery. Both studies reported that muscle glutamine levels are maintained in patients receiving TPN containing alanyl-glutamine. Thus, these studies indicate that alanyl-glutamine is effective in maintaining muscle glutamine levels.

Glycyl-glutamine has also been used as an alternative to free glutamine. Animal studies have established that glycyl-glutamine is quite stable and is also a suitable source of free glutamine (Abumrad et al 1989, Lochs et al 1992). It has been suggested that glycyl-glutamine may be superior to alanyl-glutamine. This suggestion is based on the assumption that glycyl-glutamine exhibits a more prolonged half life than alanyl-glutamine and thus a greater fraction of the infused glycyl-glutamine could reach tissues in intact form (see Abumrad et al 1989). However, Karner et al (1989) reported that glycyl-glutamine, administered to dogs which became septic after surgery, did not increase muscle glutamine levels.

Studies in man on the safety of glutamine administration (Wilmore 1990, Lowe et al 1990, Khan and Elia 1991 and Khan et al 1991), have indicated that glutamine is both safe, with very little glutamine

being degraded to ammonia, glutamate or pyroglutamate, and effective in increasing plasma glutamine levels. For details of these studies see Appendix VII.

1.2.Method of administering glutamine.

In addition to the administration of nutrition to patients via the parenteral route, i.e. the infusion of nutrients directly into the blood stream, nutrition can be administered via the enteral route. This involves nutrients being taken either by mouth or through a nasogastric tube passed via the nose to the stomach or jejunum, and thus via the gastrointestinal tract.

Since, glutamine administered enterally is absorbed by the small intestine, which utilizes glutamine at a high rate (Windmueller and Spaeth 1974, 1978) there has been some debate as to whether enteral feeding of glutamine is effective in increasing glutamine levels in plasma and muscle. Ziegler et al (1989a) demonstrated that there are differences between the metabolism of enterally administered glutamine, by oral means, and parenterally administered glutamine in healthy humans. Three consecutive experiments were carried out, by Ziegler et al (1989a), in which glutamine was administered enterally or parenterally (with each consecutive study increasing amounts of glutamine were used). The study showed that very little of the enterally administered glutamine (less than 15%) appeared in the blood stream and the levels of citrulline and arginine (known end products of glutamine metabolism in the intestine) rose in proportion to the amount of glutamine administered. In contrast, much more of the glutamine administered parenterally was present in the blood stream (approximately 85%). From this study Ziegler et al (1989a) concluded the following: that most of the enterally administered glutamine is taken up by the intestine; that the metabolism of glutamine

by the intestine may lead to the differences observed in the level of blood glutamine; and that differences in the route of glutamine administration may have implications in the role of glutamine as a component of nutritional care.

Although the study by Ziegler et al (1989a), indicated that enteral feeding of glutamine is not as effective in increasing blood glutamine levels as parenteral feeding, studies on humans by Dechelotte et al (1991), and on the rat (tumour bearing) by Souba et al (1990a) and Klimberg et al (1990a,b) have shown that enteral administration of glutamine, (nasogastrically in the study by Dechelotte et al (1991) and orally in the rat studies), is effective in increasing plasma and muscle glutamine concentrations. Thus, it was decided to set up a study to investigate the effects of oral glutamine administration in rats. Muscle glutamine synthesis, the rate of glutamine release from muscle, immune cell function, lymph node metabolite levels, and muscle and plasma glutamine concentrations were investigated.

1.3.Summary of study.

1) To investigate the effect of orally administered glutamine on muscle and plasma glutamine levels in normal rats and endotoxin treated rats.
2) To investigate the effect of orally administered glutamine on immune cell function of the rat, *in vitro*, and on rat lymph node metabolite levels
3) To investigate the effect of orally administered glutamine on muscle glutamine synthetase activity and the rate of glutamine release by isolated incubated muscle of the rat.

The advantage of using rats was that it enabled *in vitro* studies to be undertaken on the capacity of muscle to synthesize and release glutamine.

2.Materials and methods.

2.1.Materials.

Chemicals, enzymes and care of animals in Appendix 1.

2.2.Methods.

2.2.1.Glutamine feeding regimen.

Male Wistar rats (100g) were divided into three groups:

1) Controls - fed standard chow

2) Alanine-fed - fed standard chow plus alanine, 0.25gN/Kg/day. (The effect(s) of alanine feeding was also investigated so as to ensure that the effect(s) of glutamine feeding were not due to the presence of nitrogen.)

3) Glutamine-fed - fed standard chow plus glutamine, 0.25gN/Kg/day.

Rats consumed these diets for two weeks. They were then sacrificed by cervical dislocation and tissues were prepared for analysis (see below).

The endotoxin-treated rats were on the diets for two weeks as above and then received endotoxin as previously described (see chapter 3, section 2.3.4.) for 48h. prior to sacrifice.

Muscle and plasma glutamine concentration and lymph node metabolite levels were determined in normal rats and endotoxin-treated rats. Glutamine release and glutamine synthetase activity were determined in normal rats only. Normal rats were used for the lymphocyte proliferation study.

2.2.2.Preparation of tissues.

Preparation of muscles for analysis: glutamine synthetase assay, glutamine content, and glutamine release.

Soleus and EDL muscles were prepared for measuring glutamine content and glutamine synthetase activity as previously described (Chapter 2, section 2.3.1. and Chapter 3, section 2.2.1. respectively). The isolated and

incubated muscle preparation was used to measure the rate of glutamine release from muscle as previously described (Chapter 2, section 2.2.4.).

Preparation of plasma for analysis: glutamine content.

Immediately after death, blood samples were taken from the heart, into heparinised 5ml syringes. Blood was placed in previously cooled Eppendorf tubes on ice. Less than 60mins after collection of the first sample, all samples were centrifuged at 9000g for 2mins. Plasma was extracted in ice-cold perchloric acid (5%) and the supernatant, after centrifugation, was neutralised with ice-cold potassium hydroxide (3.5M, plus 7.7mM triethanolamine). The neutralised samples were centrifuged at 9000g for 1.5mins and the supernatant collected and stored at -20°C. Since glutamine breaks down rapidly in acidic conditions, all procedures were carried out in previously cooled Eppendorf tubes on ice and only two samples were extracted and neutralised at one time.

Preparation of lymph nodes for analysis and preparation of lymphocytes for proliferation experiment.

Lymph nodes and lymphocytes were removed from rats as previously described (see chapter 4, section 2.2.1.). Lymph nodes were then freeze clamped in liquid nitrogen and were stored at -70°C prior to analysis. When removed from liquid nitrogen the lymph nodes were resuspended in 1ml phosphate buffered saline and homogenized using a Polytron homogeniser (Kinematica) at setting 6 for 10 seconds. An aliquot (40µl) was taken for protein determination. The remaining sample was deproteinized using perchloric acid and the extract neutralised with the addition of potassium hydrogen phosphate. The neutralised volume was made up to 2ml with water, and samples taken for metabolite determinations. Lymph nodes from normal and septic rats were analysed.

The method for measuring lymphocyte proliferation was as previously described (see chapter 4 section 2.2.8.). However, contents of culture medium differed.

2.2.3.Composition of culture medium:

5x 10^5 cells in HEPES-buffered-RPMI + 10% foetal calf serum + antibiotics (100µl).

Con A 20µl (5µg/ml).

Glutamine (2, 1, 0.5 or 0.05mM).

RPMI - the proportions of glutamine solution to HEPES-buffered-RPMI were variable, depending on the final concentration of glutamine in the culture medium, but the total volume was always 80µl (see below).

Composition of culture medium.

Concentration of glutamine in culture medium (mM)	**volume of glutamine added to culture medium**	**volume of RPMI added to culture medium (µl)**
2	20ul of a 20mM Gln standard	60
1	10ul of a 20mM Gln standard	70
0.5	50ul of a 2mM Gln standard	30
0.05	50ul of a 0.2mM Gln standard	30

The rates of lymphocyte proliferation were determined following 24 and 48h. in culture. Proliferation was determined in lymphocytes from normal rats only.

2.2.4.Metabolite analysis.

The concentrations of glutamine in muscle, incubation media, plasma and lymph node extracts were determined using an enzymatic assay previously described (see chapter 2, section 2.3.2.). The concentrations of glutamate and aspartate in lymph node extracts were determined using enzymatic assays previously described (Lund 1974 and Bergmeyer 1974 respectively). All measurements were recorded at 340nm using an Aminco DW-2 spectrophotometer. Concentrations of alanine in lymph node extracts were determined using the method of Williamson (1974).

Glutamine synthetase assay.

Methods were as previously described (see Chapter 3, section 2.2.2.).

Protein determination.

The protein content of the lymph nodes was determined by the method of Lowry et al (1951) using bovine albumin as a standard.

2.3.Presentation of data and statistical analysis.

Glutamine concentration in muscle and plasma is expressed as nmoles/g wet weight and nmoles/ml respectively. The concentrations of glutamine, glutamate and aspartate are expressed as mM. Glutamine synthetase activity is expressed as nmoles/min/g wet weight. The rate of glutamine release from muscle is expressed as nmoles/min/g wet weight. Lymphocyte proliferation is expressed as counts per minute (c.p.m.) in terms of [^{3}H]thymidine incorporation. Statistical analysis was determined using the Student's t-test.

3.Results .

3.1.The effect(s) of glutamine or alanine feeding on plasma and muscle glutamine levels in normal and endotoxin- treated rats.

Glutamine feeding increased muscle and plasma glutamine levels in normal rats. Alanine feeding had no effect on muscle and plasma glutamine levels (Table 5.1 and 5.2). Glutamine feeding increased plasma glutamine levels in endotoxin-treated rats, however this increase was not statistically significant. Muscle glutamine levels remained decreased in glutamine-fed endotoxin-treated rats. Alanine feeding had no effect on muscle and plasma glutamine levels of endotoxin-treated rats (Tables 5.3 and 5.4).

3.2.The effect(s) of glutamine or alanine feeding on the rate of glutamine release from rat skeletal muscle and on glutamine synthetase activity in extracts of rat soleus and EDL muscles.

Glutamine or alanine feeding had no effect on the maximal activity of glutamine synthetase in extracts of soleus and EDL muscles. Glutamine or alanine feeding had no effect on the rate of glutamine release from isolated incubated soleus muscle.

3.3.The effect(s) of glutamine or alanine feeding on [^{3}H] thymidine incorporation into DNA of cultured lymphocytes.

There was an increase in [^{3}H] thymidine incorporation into DNA of lymphocytes, removed from:

1) glutamine fed rats, and cultured for 48h.

2) alanine fed rats, and cultured for 24h. or 48h. (Table 5.7 and 5.8).

3.4.The effect(s) of glutamine or alanine feeding on metabolite levels in lymph nodes from non-endotoxin treated rats and endotoxin-t

Glutamine feeding had no effect on glutamine, glutamate c levels in lymph nodes of non-endotoxin-treated rats. Howe endotoxin treated rats, the glutamine levels were decrease nodes after alanine feeding (Table 5.9). Lymph node gluta were markedly decreased in rats treated with endotoxin while standard diet. In contrast, lymph node glutamine levels were endotoxin-treated rats when alanine or glutamine was presen In endotoxin-treated rats fed with glutamine the lymph no level, compared to that of endotoxin-treated control-fed or rats, was decreased.

Glutamine or alanine feeding increased lymph node a in non-endotoxin-treated rats. Alanine feeding increased l alanine levels in endotoxin-treated rats.

Table 5.1
The effect of glutamine or alanine feeding on glutamine levels in plasma

Diet	Plasma Glutamine levels (nmoles/ml)
Control	1045±28 n=8
Alanine-fed	1036±28 n=8
Glutamine-fed	1132±32* n=7

Values are the mean (SEM) for 4 separate experiments. The statistical significance between plasma glutamine levels for control and glutamine fed rats is denoted by *P<0.05.

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Effect of glutamine or alanine feeding on metabolite concentrations.
The increase in alanine concentration for endotoxin-treated rats fed th statistically significant when alanine concentration for endotoxin-trea alanine diet is compared to that of endotoxin-treated rats fed the changes in the concentrations of the other metabolites discussed (with aspartate; see Table 5.9) were not statistically significant, however t changes were between 40-75% (see Table 5.9)

3.4.The effect(s) of glutamine or alanine feeding on metabolite levels in lymph nodes from non-endotoxin treated rats and endotoxin-treated rats.

Glutamine feeding had no effect on glutamine, glutamate or aspartate levels in lymph nodes of non-endotoxin-treated rats. However, in non-endotoxin treated rats, the glutamine levels were decreased in lymph nodes after alanine feeding (Table 5.9). Lymph node glutamine levels were markedly decreased in rats treated with endotoxin while receiving a standard diet. In contrast, lymph node glutamine levels were increased in endotoxin-treated rats when alanine or glutamine was present in the diet. In endotoxin-treated rats fed with glutamine the lymph node aspartate level, compared to that of endotoxin-treated control-fed or alanine-fed rats, was decreased.

Glutamine or alanine feeding increased lymph node alanine levels in non-endotoxin-treated rats. Alanine feeding increased lymph node alanine levels in endotoxin-treated rats.

Table 5.1
The effect of glutamine or alanine feeding on glutamine levels in plasma

Diet	Plasma Glutamine levels (nmoles/ml)
Control	1045±28 n=8
Alanine-fed	1036±28 n=8
Glutamine-fed	1132±32* n=7

Values are the mean (SEM) for 4 separate experiments. The statistical significance between plasma glutamine levels for control and glutamine fed rats is denoted by *P<0.05.

Table 5.2
The effect of glutamine or alanine feeding on glutamine levels in skeletal muscle.

Diet	Glutamine levels (nmoles/g wet wt)	
	Soleus	EDL
Control	7018±278 n=8	6504±263 n=8
Alanine-fed	7426±240 n=8	6922±242 n=7
Glutamine-fed	7709±276 n=7	7337±363* n=7

Values are the mean (SEM) for 4 separate experiments. The statistical significance beteween muscle glutamine in control and glutamine fed rats is denoted by *P<0.05.

Table 5.3
The effect of glutamine or alanine feeding on glutamine levels in plasma following endotoxin administration.

Diet	Plasma Glutamine levels (nmoles/ml)
Control	845±14 n=4
Alanine-fed	903±113 n=4
Glutamine-fed	963±94 n=4

Values are the mean (SEM) for 4 separate experiments

Table 5.4
The effect of glutamine or alanine feeding on glutamine levels in skeletal muscle following endotoxin administration.

Diet	Glutamine levels (nmoles/g wet wt)	
	Soleus	EDL
Control	5294±37 n=4	4907±354 n=4
Alanine-fed	3892±150 n=4	3628±386 n=4
Glutamine-fed	4176±246 n=4	3975±588 n=4

Values are the mean (SEM) for 4 separate experiments

Table 5.5
The effect of glutamine or alanine feeding on the rate of glutamine release from soleus muscle.

Diet	Rate of Glutamine release (nmoles/min/g wet weight)
Control	37±1 n=13
Alanine-fed	36±1 n=14
Glutamine-fed	38±1 n=24

Values are the mean (SEM) for 6 separate experiments.

Table 5.6
The effect of glutamine or alanine feeding on glutamine synthetase activity in soleus and EDL muscles.

Diet	Glutamine synthetase activity (nmoles/min/g wet wt)	
	Soleus	**EDL**
Control	94±10 n=6	247±16 n=6
Alanine-fed	104±6 n=6	238±15 n=6
Glutamine-fed	95±4 n=8	250±16 n=8

Values are the mean (SEM) for 6 separate experiments.

Table 5.7
The effect of glutamine or alanine feeding on [^{3}H] thymidine incorporation into DNA of lymphocytes cultured for 24h.

	[^{3}H] thymidine incorporation (cpm)				
[Glutamine]	.....2mM	1mM	0.5mM	0.05mM	0mM
Diet					
Control-fed	37910±5046 n=4	4257±3091 n=4	4201±3810 n=4	14889±1628 n=4	1291±139 n=4
Glutamine-fed	28766±2936 n=4	33143±6929 n=4	23861±3148 n=4	11569±1755 n=4	1375±64 n=4
Alanine-fed	21365±6178 n=4	33478±4374 n=4	38962±3214 n=4	17224±2415 n=4	2505±51 D n=4

Values are the mean (SEM) for 4 separate experiments.

The statistical significance between [^{3}H] thymidine incorporation into DNA of cultured rat lymphocytes from control-fed rats and alanine-fed rats is denoted by D $P<0.001$.

Table 5.8
The effect of glutamine or alanine feeding on [^{3}H] thymidine incorporation into DNA of lymphocytes cultured for 48h.

	[^{3}H] thymidine incorporation (cpm)				
[Glutamine]	.....2mM	1mM	0.5mM	0.05mM	0mM
Diet Control-fed	39449±3723 n=4	39092±2946 n=4	32036±3644 n=4	16160±1116 n=4	1591±228 n=4
Glutamine-fed	54209±2932A n=4	58435±2760C n=4	50511±1774C n=4	25955±332D n=4	2229±162 n=4
Alanine-fed	62427±2067C n=4	56896±1211C n=4	56590±1986C n=4	32887±520D n=4	5066±605C n=4

Values are the mean (SEM) for 4 separate experiments.

The statistical significance between [^{3}H] thymidine incorporation into DNA of cultured rat lymphocytes from control- fed rats and alanine- fed rats and between control- fed rats and alanine-fed rats is denoted by A P<0.05, B P<0.02, C P<0.01 and D P<0.001.

Table 5.9
The effect of glutamine or alanine feeding

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The increase in [ALA] for endotoxin-treated rats fed the alanine diet is statistically significant when compared to [ALA] for endotoxin-treated rats fed the control diet; **(A= P=0.05)**. In addition, the difference in [ALA] between endotoxin- and non-endotoxin-treated rats is statistically significant; ***=P<0.01.**

	...treated rats				Endotoxin-treated rats			
	GLN	GLU	ASP	ALA	GLN	GLU	ASP	ALA
Control	5.2±3	24.8±3	12.2±3	3.5±1.5	3.2±3	24.1±3	13.7±5	25.1±1.5
Alanine-fed	2.5±2C	23.6±5	11.7±2	6.1±3D	5.4±2B*	24.1±1	14.1±5	30.7±2A
Glutamine-fed	5.4±1	23.9±7	11.4±5	5.6±0.9D	5.2±2A	24.1±1	10.6±B	26.1±0.7

Values are the mean (SEM) for 3 separate experiments.

The significance of the diets in each treatment is denoted by A P<0.05, B P<0.02, C P<0.01 and D P<0.001.

The significance between non-endotoxin treated rats and endotoxin treated rats is denoted by*P<0.001.

Abbreviations GLN-glutamine, GLN-glutamate, ASP-aspartate, ALA-alanine

Table 5.8
The effect of glutamine or alanine feeding on [^{3}H] thymidine incorporation into DNA of lymphocytes cultured for 48h.

	[^{3}H] thymidine incorporation (cpm)				
[Glutamine]	.....2mM	1mM	0.5mM	0.05mM	0mM
Diet	39449±3723	39092±2946	32036±3644	16160±1116	1591±228
Control-fed	n=4	n=4	n=4	n=4	n=4
Glutamine-fed	54209±2932A	58435±2760C	50511±1774C	25955±332D	2229±162
	n=4	n=4	n=4	n=4	n=4
Alanine-fed	62427±2067C	56896±1211C	56590±1986C	32887±520D	5066±605C
	n=4	n=4	n=4	n=4	n=4

Values are the mean (SEM) for 4 separate experiments.
The statistical significance between [^{3}H] thymidine incorporation into DNA of cultured rat lymphocytes from control- fed rats and alanine- fed rats and between control- fed rats and alanine-fed rats is denoted by A P<0.05, B P<0.02, C P<0.01 and D P<0.001.

Table 5.9
The effect of glutamine or alanine feeding on glutamine, glutamate, aspartate and alanine concentrations in lymph nodes removed from non-endotoxin and endotoxin treated rats.

	Metabolite concentrations (mM)							
	Non-endotoxin treated rats				Endotoxin-treated rats			
Diets	GLN	GLU	ASP	ALA	GLN	GLU	ASP	ALA
Control	5.2±3	24.8±3	12.2±3	3.5±1.5	3.2±3	24.1±3	13.7±5	25.1±1.5
Alanine-fed	2.5±2C	23.6±5	11.7±2	6.1±3D	5.4±2B*	24.1±1	14.1±5	30.7±2A
Glutamine-fed	5.4±1	23.9±7	11.4±5	5.6±0.9D	5.2±2A	24.1±1	10.6±B	26.1±0.7

Values are the mean (SEM) for 3 separate experiments.
The significance of the diets in each treatment is denoted by A P<0.05, B P<0.02, C P<0.01 and D P<0.001.
The significance between non-endotoxin treated rats and endotoxin treated rats is denoted by*P<0.001.
Abbreviations GLN-glutamine, GLN-glutamate, ASP-aspartate, ALA-alanine

4.Discussion.

A large number of glutamine feeding studies involving rats and humans, have been performed over the last three or four years. However, to the author's knowledge, the majority of glutamine feeding studies on rats have investigated the effect(s) of parenterally and enterally administered glutamine on gut function. These include Alverdy et al (1992) Burke et al (1989) Flynn et al (1992), Fox et al (1988), Klimberg et al (1989, 1990b), and see Souba et al (1990b). Only three rat studies (Ziegler et al 1989b, Souba et al 1990a and Klimberg et al 1990a) have investigated the effect(s) of enteral glutamine feeding (by nasogastric or oral means) on plasma and/or muscle glutamine levels. The majority of glutamine feeding studies in humans have involved investigating the effect(s) of glutamine feeding on plasma and muscle glutamine levels. These include studies by Hammarqvist et al (1989), Scheltinga et al (1991) and Ziegler et al (1992). Only four human studies by Dechelotte et al (1991), Matthews (1990), Ziegler et al (1989a), and Ziegler et al (1990) have studied the effect(s) of enteral glutamine feeding (by nasogastric or oral means) on plasma and/or muscle glutamine levels, and these have been in healthy humans.

To the author's knowledge, the current study on the effects of enteral feeding of glutamine, by oral means, on the capacity to synthesize and release glutamine by muscle, on the function of immune cells, and on muscle and plasma glutamine levels in normal and endotoxin-treated rats, is the first to be reported.

4.1.The effect(s) of glutamine feeding on plasma and muscle glutamine levels in normal and endotoxin-treated rats.

The feeding of glutamine increased both muscle and plasma glutamine levels in normal rats. This indicates that the enteral feeding of glutamine,

by oral means, is effective in increasing muscle and plasma glutamine levels, in spite of the study by Ziegler et al (1989a) which suggests that, due to enterally administered glutamine being taken up and metabolized by the intestine, it is not an effective means of increasing plasma glutamine levels. Results from this present study are consistent with studies on the effect of enteral glutamine feeding on plasma glutamine levels in healthy humans: Dechelotte et al (1991) reported that, with the enteral administration of increasing amounts of glutamine to healthy adults, there is an increase in plasma glutamine levels; Matthews (1990) reported that, in healthy humans receiving tracer levels of ^{15}N glutamine enterally, the tracer enrichment in the blood reached approximately 50% of that observed when the same amount is infused intravenously. The latter study is important because it shows that, in comparison with intravenously administered glutamine, enterally administered glutamine contributes a significant amount of glutamine to the blood

For the investigation of the effect(s) of glutamine feeding on muscle and plasma glutamine levels of rats given endotoxin, rats were fed glutamine before they were administered endotoxin. Since, in non-endotoxin-treated rats, muscle and plasma glutamine levels were increased (see Tables 5.1 and 5.2), one can assume that their muscle and plasma glutamine levels were increased prior to the endotoxin treatment. Results from the endotoxin study, therefore indicate that endotoxin treatment abolishes this increase in muscle and plasma glutamine levels.

There is evidence, both *in vivo* and *in vitro,* to suggest that the marked decrease in muscle glutamine levels that occurred following endotoxin treatment was due to a marked increase in the rate of glutamine release from muscle. Ardawi and Majzoub (1991) observed, by measuring the femoral arterio-venous difference for glutamine in rats with sepsis, that the rate of glutamine release from muscle is markedly

increased. (As stated previously, endotoxin has been implicated as a major cause of the metabolic changes that occur during this condition (see Chapter 3, section 4.2.). Following sepsis or endotoxin treatment Parry-Billings et al (1989) showed that the rate of glutamine release from incubated muscle of septic or endotoxin-treated rats is markedly increased. This increase in the rate of glutamine release from muscle was associated with a marked decrease in muscle glutamine levels. Thus, the decrease in muscle glutamine levels following endotoxin administration appears to be due to an increase in the rate of glutamine release from muscle.

For the decrease in plasma glutamine concentration that occured in rats administered endotoxin, an increase in the rate of glutamine utilization by cells of the immune system is proposed. Ardawi et al (1991) demonstrated that, during sepsis, in rats, glutaminase activity and the rate of glutamine utilization by lymphocytes isolated from septic rats are markedly increased. Thus, an increase in the rate of glutamine utilization by cells of the immune system, following endotoxin administration, may have caused the decrease in the plasma glutamine concentration shown.

The possibility that the feeding of larger amounts of glutamine to the endotoxin-treated rats, might be more successful in maintaining muscle and plasma glutamine levels was considered, however this was not investigated.

4.2.The effect(s) of glutamine feeding on the rate of glutamine release from rat soleus muscle and on glutamine synthetase activity in extracts of rat soleus and EDL muscles.

It was considered that the increase in plasma glutamine concentration caused by glutamine feeding may "down regulate" the process of glutamine release from muscle. Since muscle glutamine release is a key process in the provision of glutamine to cells of the immune system

(Chapter 1, section 5), a down regulation of this process would ultimately lead to impairment of immune cell function. In relation to the use of glutamine as a component of nutritional therapy for patients, it is unlikely that such an effect would occur after short-term administration of glutamine. However, after long-term administration of glutamine, the likelihood of a down regulation of glutamine release from muscle and the subsequent impairment of immune cell function, would be increased, thus decreasing any beneficial effect that long-term glutamine feeding may have on the immune function of patients. The measurement of the rate of release of glutamine from soleus muscle of glutamine-fed rats in the present study, although *in vitro*, using the isolated muscle preparation suggests that glutamine feeding does not decrease muscle's ability to release glutamine.

It has been shown that the activity of glutamine synthetase in cultured muscle cells is decreased with increasing glutamine concentration in the culture medium: the maximal glutamine synthetase activity in cultured muscle cells, was 3- to 4-fold lower when cells were cultured with glutamine, than that of cells cultured in the absence of added glutamine (Smith et al 1984, Feng et al 1990a). In the light of these studies it was suggested that the increase in muscle glutamine concentration (a 9% increase in soleus muscle and an 11% increase in EDL muscle) caused by enteral glutamine feeding, could decrease the capacity of muscle to synthesize glutamine, via a decrease in the level of glutamine synthetase in muscle. However, in the present study, the increase in muscle glutamine concentration had no effect on the maximal glutamine synthetase in muscle. Although it is not known what effect an increase in the muscle glutamine level is having on the actual activity of glutamine synthetase activity in muscle, *in vivo*, this study indicates that

glutamine feeding does not decrease muscle's maximum capacity to synthesize glutamine.

The results of the present study, i.e. for the effect(s) of glutamine feeding on muscle glutamine release and glutamine synthetase activity, highlight the advantages of glutamine feeding in that with the increase in muscle and plasma glutamine levels, the maximum capacities of muscle glutamine synthesis and release are not affected.

4.3.The effect(s) of glutamine or alanine feeding on [^{3}H] thymidine incorporation into DNA of cultured lymphocytes.

In the present study there was an increase in [^{3}H] thymidine incorporation into the DNA of lymphocytes, removed from rats fed with:

1) glutamine, and cultured for 48h.;

or

2) alanine, and cultured for 24h. and 48h.,

in comparison with [^{3}H]thymidine incorporation into the DNA of cultured lymphocytes removed from control-fed rats. The rats used in this proliferation study were normal, thus, their immune system had not been stimulated *in vivo*. Yet, when the lymphocytes of these animals were cultured, and stimulated with the mitogen concanavalin A, their rate of proliferation was increased in comparison with that of lymphocytes cultured under the same conditions and which were taken from control-fed rats. The question asked is, why are the rates of proliferation of lymphocytes removed from rats fed glutamine or alanine higher than those of lymphocytes removed from control-fed rats?

It is known that glutamine is necessary for lymphocyte proliferation. In the absence of glutamine the rate of lymphocyte proliferation *in vitro* is very low (see Chapter 1, sections 3.1. and 3.3., Chapter 4, section 1.). It is not known how lymphocytes 'know' that

glutamine is present in the culture medium. It is possible that the lymphocyte is able to 'assess' the level of glutamine, perhaps via the presence of a glutamine receptor. However, in the present proliferation study, all lymphocytes, whether from control, alanine or glutamine-fed rats, were cultured in the same concentration of glutamine, i.e. 2, 1, 0.5 or 0.05mM glutamine. Thus, for example, lymphocytes from rats fed either the glutamine, alanine or control diets cultured for 48h., in medium containing 1mM glutamine, would have 'assessed' the same level of glutamine, and thus one may have expected that the rates of proliferation would have been similar. For the 48h. culture period, the rates of proliferation of lymphocytes removed from glutamine or alanine-fed rats, and cultured with 2, 1, 0.5 or 0.05mM glutamine were between 1.5- to 3-fold higher than those of lymphocytes removed from control-fed rats, cultured under the same conditions. These results suggest that lymphocyte proliferation, *in vitro,* may not just be dependent on the levels of glutamine *per se* present in culture media, and that proliferation may involve, or be dependent upon the levels of other amino acids in addition to those of glutamine.

Ardawi (1983) showed that, for incubated lymphocytes a decrease in the concentration of glutamine present in the incubation medium causes a decrease in the rate of glutamine uptake by the incubated lymphocytes. This suggests that, *in vivo,* the rate of uptake of glutamine by lymphocytes is dependent on plasma glutamine levels. In the present study glutamine feeding increased plasma glutamine levels in normal rats. This increase may be associated with an increase in the rate of glutamine uptake by lymphocytes which may lead to an increase in lymphocyte glutamine levels. A similar situation may apply to alanine-fed rats: although the data is not shown, alanine feeding increased plasma alanine levels by approximately 8%; this increase was not statistically significant. Thus,

alanine feeding may ultimately lead to an increase in lymphocyte alanine levels. If lymphocytes are responding to the increased levels of glutamine and/or alanine, how is this achieved? It could be achieved via the level of a specific peptide whose concentration is dependent, via peptide synthesis, on the level of these amino acids, and the level of this peptide would probably be assessed via a specific receptor. The synthesis of this peptide could of course involve amino acids other than glutamine and alanine. It could be that this peptide belongs to the group found in the intestine, and known to be present in the brain possibly acting as neurotransmitters, for example bradykinin and bombesin (see Smith et al 1976; Hughes et al 1977; Synder 1980; and Kreiger and Martin 1981). If so, then these peptides may be present in the immune cells of the intestine.

4.4.The effect(s) of glutamine feeding on metabolite levels in lymph node extracts from non-endotoxin and endotoxin-treated rats.

In this particular study the effect(s) of glutamine feeding on levels of glutamine and on levels of major products of lymphocyte glutamine metabolism, glutamate and aspartate (Ardawi and Newsholme 1983), were determined in the extracts of lymph nodes from non-endotoxin-treated (control) and endotoxin-treated rats. This was in order to try and give an indication of the effect of glutamine feeding on glutamine utilization by lymphocytes during normal conditions and during conditions where plasma glutamine levels are depleted. However, it was considered that the concentration of these metabolites in lymph nodes could only provide suggestions as to what is happening in these feeding studies in terms of glutamine utilization by lymphocytes grouped collectively in lymph nodes *in vivo* rather than by isolated lymphocytes *in vitro*. A comparison of the levels of glutamine, glutamate and aspartate in lymph nodes with those of isolated lymphocytes shows that the

concentrations of these metabolites in lymph nodes are very similar to those in isolated lymphocytes (see Table 1). This comparison is important because it indicates that the glutamine metabolism in lymphocytes *in vivo* may be similar to that *in vitro* and that changes in glutamine utilization by lymphocytes *in vitro* may well reflect changes occurring *in vivo*.

In the light of the mechanism proposed for the stimulation of lymphocyte proliferation, the results for the levels of glutamine and alanine in lymph nodes taken from glutamine- or alanine-fed non-endotoxin-treated rats may be of significance. Glutamine or alanine feeding increased alanine levels in lymph nodes removed from non-endotoxin-treated rats. This increase is consistent with the suggestion that glutamine or alanine feeding, by causing increases in glutamine and/or alanine levels in lymphocytes, may, via an increase in the level of a specific putative peptide regulator, stimulate their rate of proliferation. The level of glutamine in lymph nodes taken from glutamine- or alanine-fed endotoxin-treated rats, and the alanine level in lymph nodes taken from alanine-fed endotoxin-treated rats, were also increased. Although the proliferation of lymphocytes removed from glutamine- or alanine-fed endotoxin-treated rats was not determined in the present study, the increases in glutamine and alanine levels in lymph nodes removed from endotoxin-treated rats may suggest that not only glutamine but also alanine feeding may maintain immune cell function during an immunological challenge.

Table 1. A comparison of metabolite levels/concentrations in lymph nodes with those in lymphocytes.

	Metabolite concentrations(mM)				
	Gln	Glu	Asp	G-6-P	PEP
Lymph nodes* (non-endotoxin-treated rats)	5.2	24.8	12.0	0.9	-
Lymphocytes** (non-stimulated)	6.7	13.9	13.3	0.2	0.70
Lymph nodes* (endotoxin-treated)	3.2	24.1	13.7	0.9	-
Lymphocytes (stimulated with Con A)	7.7	16.3	11.4	0.5	0.5

*Lymph node data taken from Table 5.9 of current chapter.
**Lymphocytes were incubated in medium containing 0.5mM glucose and 2mM glutamine for 60mins.

An interesting point about the concentrations of glutamate and aspartate in lymph nodes and isolated lymphocytes is that they are very high in comparison with the concentrations of glycolytic intermediates, glucose-6-phosphate and phosphoenolpyruvate (see Table 1). The reason for the differences in the concentrations of these intermediates of these two metabolic pathways, in lymphocytes, is not known. In addition, it is unclear whether the concentrations of the glutaminolytic intermediates measured are free intermediates or whether thay are bound, for example sequestered in vesicles. An investigation of the differences between the concentration of glutaminolytic and glycolytic intermediates may prove to be an interesting study for the future.

Chapter 6

General discussion.

1.Introduction.

Metabolic control logic predicts that the process of glutamine release from skeletal muscle, rather than the process of glutamine synthesis in muscle, is the flux generating step for the pathway of glutamine release from muscle into the blood and its subsequent uptake and utilization by cells of the immune system (see Chapter 1, section 5). (The flux generating step is defined as the first non-equilibrium process of a metabolic pathway that approaches saturation with its substrate.)

The reasons for this prediction are as follows:

1) A plot of the rates of release of glutamine from isolated incubated rat soleus muscle against variations in the concentration of muscle glutamine provides an approximate Km value for the release process of less than 0.3mM glutamine. In the soleus muscle of the rat the concentration of glutamine is normally >4mM (see Chapter 1, section 5),

2) Inhibition of glutamine synthetase by methionine sulphoximine results in a decrease in the concentration of glutamine in muscle, but has no effect on the rate of glutamine release from muscle (see Chapter 1, section 5).

This interpretation suggests the following:

1) That the rate of glutamine synthesis in muscle will normally have no effect on the rate of glutamine release from muscle,

2) That factors that control the rate of glutamine release from muscle are affecting the flux generating step. Thus, this process requires an in depth study to identify factors that affect glutamine release from muscle (see Chapter 2 and Parry-Billings 1989),

3) That, since the process of glutamine utilization by cells of the immune system in the rat does not approach saturation with glutamine at the normal plasma levels (which is approximately 1mM in the rat), the control of the rate of release of glutamine from muscle will, via changes in the level of plasma glutamine, influence the rate of glutamine utilization and hence could influence the function of these cells.

2.The relationship between glutamine uptake and release by skeletal muscle.

Muscle, as well as releasing glutamine, takes it up. A comparison of some of the properties of glutamine uptake with those of glutamine release suggest that these processes are different. First, it is reported that the process of glutamine uptake into muscle has a Vmax of 1020nmoles/min/g of muscle, and a Km for glutamine of 7.1mM (Hundal et al 1986a). The Vmax for the process of glutamine release from muscle is approximately 100nmoles/min/g and the Km for muscle glutamine release is <0.3mM (see Chapter 1, section 5). Secondly, it has been shown that the process of glutamine uptake by muscle is sodium dependent; kinetic analysis has suggested that the co-transport of glutamine and the sodium ion occurs with a stoichiometry of 1:1 (Hundal et al 1986b). However, studies have shown that the process of glutamine release from muscle is not sodium dependent (Chapter 2, section 3.8, Parry-Billings 1989). Thirdly, the effects of certain factors on muscle glutamine uptake differ to their effect in muscle glutamine release: glutamine uptake is shown to be sensitive to insulin (Watt et al 1985): however, Parry-Billings (1989) showed that the rate of release of glutamine from muscle is not affected by insulin. In addition, branched chain amino acids and dexamethasone inhibit muscle glutamine uptake (Hundal et al 1986b), but Babij et al 1986

stimulate muscle glutamine release (Chapter 2, section 3.2 and Parry-Billings et al 1990a respectively). However, in spite of these differences it has been assumed that the processes of glutamine uptake and release are the same (Rennie et al 1986). It has been suggested that glutamine transport across muscle membrane is "symmetrical", thus implying that both processes are near equilibrium. This may not be the case, since the free energy change for each process suggests that they are non-equilibrium (Parry-Billings 1989). Thus, if both processes are non-equilibrium, transport across muscle membrane cannot be "symmetrical". Consequently, it is proposed that the process of glutamine release from muscle, and the process of glutamine uptake into muscle are different.

Since these two processes are considered to be separate, and since they both transport glutamine but in opposite directions, if they are simultaneously active then a translocation cycle exists between extra-cellular and intra-cellular glutamine pools. A translocation cycle is identical to a substrate cycle, which is produced when a non-equilibrium reaction in the reverse direction of a pathway opposes a non- equilibrium reaction in the forward direction of the pathway.

So what is the significance of this proposed translocation cycle for glutamine in muscle? Substrate cycles may improve sensitivity in metabolic control (Newsholme and Crabtree 1976). In the light of the importance of the process of glutamine release from muscle as the key step in the provision of glutamine, the presence of the proposed translocation/substrate cycle may be important in enhancing the sensitivity of control of glutamine release from muscle. For example, during conditions in which the plasma glutamine concentration is decreased, a decrease in the rate of glutamine uptake by muscle would be expected. This decrease would have the effect of a net increase in the rate of glutamine release despite the fact that the activity of the outward

transporter had not been affected. On the otherhand, an increase in the level of plasma glutamine would be expected to increase the rate of glutamine uptake into muscle and thus have the effect of a net decrease on the rate of release of glutamine from muscle. In this way the translocation cycle would enable the plasma concentration of glutamine to influence the rate of release; the cycle would provide a simple mechanism for the feedback regulation of the plasma glutamine concentration via changes in the plasma glutamine level. The overall effect of the cycle would be to maintain a constant plasma glutamine level.

In the present study, the long-term feeding (2 weeks) of glutamine to rats caused an increase in plasma glutamine levels. Considering the role of a substrate cycle between glutamine uptake and release in muscle in maintaining a constant level of plasma glutamine, an increase would lead to a decrease in the net rate of glutamine release from muscle. This effect would be a rapid and transient effect depending on the plasma glutamine level. However, it is possible that this transient effect could have a long-term down-regulation effect on the process of glutamine release. Since the process of glutamine release from muscle is important for the provision of glutamine to cells of the immune system, a decreased capacity for release might influence the rate of release once glutamine feeding had ceased. This could therefore ultimately lead to impairment of immune cell function. This is an important point that needs considering if long-term feeding of glutamine is to be used in the nutritional therapy of patients. However, the study of the effect of long-term glutamine feeding, in rats, on the rate of glutamine release from muscle *in vitro* (Chapter 5, section 3.2), suggests that the net rate of glutamine release from muscle is not affected by long-term glutamine feeding.

3.Can muscle satisfy the demand for glutamine by cells of the immune system, both resting and stimulated?

Quantitative analysis of data for the rates of glutamine release and utilization from muscle (during normal conditions and sepsis) and lymphocytes (resting and stimulated) of the rat respectively was used to determine whether muscle can satisfy the demand for glutamine by cells of the immune system (see Table 1).

Table 1: a comparison of the rates of glutamine release during normal conditons and sepsis with the rates of glutamine utilization by cells of the immune system(lymphocytes) when rested and stimulated, in a 200g rat. Units are μmoles/h.

	Rate of glutamine release from muscle	Rate of glutamine utilization by cells of the immune system (lymphocytes)
Normal	192	20 - 52
Sepsis	295	45 - 115

(See Appendix VIII for details of calculations).

The table above indicates that during septic as well as under normal conditions, rat muscle is able to cope with the demands of glutamine by cells of the immune system. If this is so, then why is sepsis in the rat associated with a decrease in rat plasma glutamine concentration (Parry-Billings et al 1989)? As Table 1 indicates, lymphocytes were the cells of the immune system used in the calculation. This suggests that other cells, for example macrophages, contribute to the decrease in plasma glutamine concentration during sepsis. Both, intestine and macrophages utilize glutamine at a high rate (Windmueller and Spaeth 1974, 1978 and Newsholme et al 1986, 1987). Thus, it appears that during sepsis in the rat,

muscle, via release, is not able to satisfy the demand for glutamine by cells of the immune system, i.e. there is too high a rate of utilization and too low a rate of production of glutamine. The "inability" of muscle to cope with the demand for glutamine is also reflected by decreases in plasma glutamine concentration that occur in man following surgery, burns and injury (Askanazi et al 1980, Kapadia et al 1985,Stjernstromet al 1986).

The "too low a rate of production of glutamine" indicates that glutamine synthesis and/or glutamine release in muscle are/is limiting during conditions such as sepsis. The present study indicates that muscle's capacity to synthesize glutamine during normal and septic conditions (based on the Vmax for glutamine synthetase in muscle, see Chapter 3, section 3.1.1. and 3.4.) is greater than its capacity to release glutamine (see Chapter 2, section 3 and Parry-Billings et al 1989). This suggests that the process of glutamine release from muscle is limiting in the provision of glutamine to cells of the immune system. Thus, glutamine release needs to be well controlled so that it can be stimulated or inhibited when increased or decreased amounts of glutamine are required. Since glutamine release from muscle is flux generating, pharmacological agents could be used to regulate the process of glutamine release from muscle. Such agents could be used as an alternative to the use of glutamine as a component for nutritional therapy for patients with conditions associated with an impaired immune system. This represents a novel approach to the treatment of patients with 'hypoglutamine' and may have advantages over nutritional provision of glutamine for the immune system; that is, it may be more physiological.

There are conditions in which the process of glutamine synthesis in muscle may become limiting. During conditions in which the concentration of glutamine in muscle is markedly decreased for example, by 80% in patients with severe abdominal sepsis (Roth et al 1982), there

could be a decrease in the rate of release of glutamine from muscle which could result in a decrease in plasma glutamine concentration. In such a situation the process of glutamine release from muscle may no longer be flux generating, and consequently the process of glutamine synthesis in muscle may control glutamine release and the pathway of glutamine utilization by cells of the immune system. If this is the case, then an important question is, can muscle glutamine synthesis cope with the demand for glutamine or does it seriously limit the provision of glutamine from muscle during severe abdominal sepsis? The concentration of glutamine in plasma of patients with this condition could give an indication as to whether or not muscle glutamine synthesis is seriously limiting in the provision of glutamine to cells of the immune system. It might be expected that with an 80% decrease in the concentration of muscle glutamine the plasma glutamine concentration of these patients would also be markedly decreased. However, Roth et al (1982) reported that for plasma glutamine concentrations, there was no statistically significant difference between controls and surviving or non-surviving patients, and no difference between surviving and non-surviving patients (see Table 2).

Table 2: a comparison of plasma and muscle glutamine concentrations between normal subjects, surviving and non-surviving severe abdominal sepsis patients

	Plasma glutamine concentration µmoles/l	Muscle glutamine concentration mmoles/l intracellular water
Normal subjects	526	19
Surviving patients	326	6.7
Non-surviving patients.	384	2.7

Data from Roth et al (1982)

One reason why a marked decrease in plasma glutamine concentration was not observed may be due to the technique used to measure amino acid levels in the Roth et al (1982) study. Automated amino acid analysis was used in this study. This analysis, which is routinely performed, involves the adjusting of samples to pH 2.2. Since glutamine is unstable in acidic conditions (see Chapter 1, section 1.1) the adjusting of samples to such a low pH may cause significant breakdown of glutamine. Thus, this technique may lead to the masking of changes in plasma glutamine concentration.

If there was a marked decrease in the plasma glutamine concentration of patients with severe abdominal sepsis, this would suggest that the process of glutamine synthesis is seriously limiting the provision of glutamine from muscle. Thus, stimulation of this process would decrease this limitation. Glutamine synthetase catalyses the non-equilibrium process of glutamine synthesis, thus it is possible to affect glutamine synthesis by affecting the activity of glutamine synthetase by external factors (see Chapter 3). Some mechanisms have been proposed for

the regulation of glutamine synthetase in rat muscle by external factors, these include:

1) *Altering the concentration of glutamine synthetase in muscle.* Glutamine and dexamethasone are factors that have been shown to decrease and increase the concentration of this enzyme in muscle respectively. Glutamine causes a decrease possibly via an increase in the rate of degradation of this enzyme (see Smith et al 1984 and Feng et al 1990a), and dexamethasone causes an increase by increasing the levels of glutamine synthetase mRNA via the binding of dexamethasone to a glucocorticoid receptor (Max et al 1987).

2) *Allosteric regulation.* King et al (1983) reported that, when branched chain amino acids are added to the muscle glutamine synthetase assay medium, the activity of the enzyme is increased. From this observation King et al (1983) suggest that glutamine synthetase may be regulated allosterically by the branched chain amino acids.

3) *Covalent modification.* Phosphorylation/dephosphorylation is another mechanism which has been proposed for the regulation of muscle glutamine synthetase (see Konagaya et al 1986, 1987; Feng et al 1990b). However, identification and elucidation of the control of the interconverting enzyme that would be involved in covalently modifing glutamine synthetase, to the author's knowledge, has not been reported.

4) *Changes in substrate concentration.* As well as external regulation, glutamine synthetase in muscle may be regulated by its substrate glutamate. A comparison of the Km values for glutamate and ammonia for muscle glutamine synthetase in the rat, with their

concentrations in rat muscle indicate that only changes in glutamate will cause changes in glutamine synthetase activity in muscle (see Chapter 3, section 4.2.).

Changes in the concentration of ATP, which is also required for glutamine synthetase activity (See Chapter 3, section 1) will normally have no effect on glutamine synthetase activity in muscle; the Km value of ATP for glutamine synthetase is 1.1mM (Rowe 1985), the concentration of ATP in muscle is approximately 5mM (see Parry-Billings 1989, Ardawi 1990).

Thus, it is possible that precursors of glutamate synthesis in muscle could be beneficial, via the stimulation of glutamine synthetase activity, in conditions in which muscle glutamine synthesis is flux generating. These precursors include, branched chain amino acids, which are considered to be sources of nitrogen for glutamate, and 2-oxoglutarate which is the carbon skeleton for glutamate (see Chapter 1, section 4.2.). Branched chain amino acids may be clinically beneficial since they have been shown to improve nitrogen balance in some patients after surgery (Benotti et al 1976). Since they stimulate the rate of release of glutamine from muscle (see Chapter 2, section 3.2.), it is suggested that their effect on nitrogen balance occurs via this stimulation of release. In the light of the importance of the process of glutamine synthesis during conditions where the concentration of glutamine in muscle is markedly decreased for example, severe abdominal sepsis, it appears that branched chain amino acids may also be beneficial via the stimulation of the process of glutamine synthesis in muscle.

2-Oxoglutarate improves nitrogen balance and promotes wound healing in man when administered as the complex L(+)ornithine-2-oxoglutarate (Leander et al 1985, Vesterberg et al 1987, Hammarqvist 1989, Wenerman et al 1989, Cynober 1991). It increases the concentration of glutamine in muscle but unlike the branched chain amino acids it has no

effect on the rate of release of glutamine from muscle (Chapter 2, section 3.3.), thus, it is suggested that the beneficial effects of 2-oxoglutarate do not occur via muscle glutamine metabolism (Chapter 2, section 4.4.). However, 2-oxoglutarate may be beneficial via muscle glutamine metabolism, during conditions, such as severe abdominal sepsis, associated with a marked decrease in the concentration of glutamine in muscle.

None of the mechanisms discussed above were investigated in this thesis: however, a detailed study of these mechanisms may prove to be interesting work for the future.

4.Adipose tissue, does it have a role in the provision of glutamine?

In the present study quantitative analysis indicated that rat adipose tissue, in relation to rat muscle, is not a significant contributor of glutamine (Chapter 3, section 4.3.). In contrast a study on human adipose tissue suggests that it is a significant contributor of glutamine (Frayn et al 1991). Arterio-venous differences were used to determine glutamine release across adipose tissue of subjects fasted overnight. It may be possible to use this technique to determine adipose tissue glutamine release in man during sepsis, following injury or surgery. Assuming that the release of glutamine from adipose tissue is the important process in the provision of glutamine from adipose tissue, a study of adipose tissue glutamine release during different conditions would give an idea of whether or not human adipose tissue is a significant contributor of glutamine under various conditions. It could be that adipose tissue acts as a support for muscle when the demand for glutamine is increased, and the significance of adipose tissue in terms of provision of glutamine, may vary with age, sex and also different physiological conditions, such as starvation and exercise: starvation, between 2 days and 1 week, has been shown to decrease the

amount of adipose tissue in the rat by approximately 40 and 80% respectively (Tischler and Goldberg 1980 and Hirsch and Han 1969 respectively). Exercise has been shown to decrease the amount of adipose tissue in man, a comparison of adipose tissue between athletes and non-athletes showed that the amount of adipose tissue in the former was approximately 50% less than that of the latter (Bjorntorp et al 1972). The provision of glutamine by adipose tissue may also be affected by its locality; Kowalchuk et al (1988) reported that glutaminase activity varied considerably between several rat adipose tissue-sites. Thus, it is possible that the rates of glutamine synthesis and release may differ between different adipose tissue-sites in man.

Rat adipose tissue has a high capacity to synthesize glutamine (Chapter 3, section 3.1.3.). Thus, human adipose may have a high capacity as well. Although it is assumed above that, as with muscle, glutamine release is important in the provision of glutamine by adipose tissue, it could be that glutamine synthesis is the important process in the provision of glutamine. This lack of knowledge of the key process/es of the control of glutamine metabolism in adipose tissue, coupled with the possibility that adipose tissue may be a support for muscle in relation to the provision of glutamine, highlights the importance of understanding the regulation of the provision of glutamine by adipose tissue. This could be achieved using metabolic control logic (Chapter 1, section 5).

5.Glutamine synthesis in cells of the immune system.

It has been established that glutamine and its subsequent utilization are important for cells of the immune system and that skeletal muscle is an important source of glutamine (see Chapter 1, section 3.1.). In this thesis it was shown that cells of the immune system possess glutamine synthetase activity (Chapter 4, section 3.1). Two important observations from this

study suggest that glutamine synthetase activity may be important in providing glutamine to these cells when the levels of exogenous glutamine are low, they are as follows:

1) When lymphocytes were cultured in the absence of glutamine, glutamine synthetase activity of these cells is increased (Chapter 4, section 3.4.);

2) When glutamine synthetase activity in lymphocytes cultured in the absence of glutamine was inhibited by methionine sulphoximine, proliferation of these cells was not maintained (Chapter 4, section 3.5).

These observations may suggest that, during conditions associated with immunosuppression, and in which plasma glutamine concentration is markedly decreased, for example sepsis, burns, injury (Baker et al 1980, Green and Faist 1988), and overtraining (Parry-Billings 1989), cells of the immune system may be able to provide themselves with glutamine so as to maintain branched point sensitivity and thus maintain some function during such conditions. In the light of the possible significance of glutamine synthetase activity in cells of the immune system, an understanding of the regulation of the concentration of this enzyme in these cells may be of significance.

The suggestion that an increase in glutamine synthetase activity in lymphocytes may help to maintain some proliferation of these cells during conditions in which plasma glutamine concentration is decreased, may be of significance when considering proliferation of tumour cells. Glutamine is the principal amino acid consumed by rapidly proliferating tumour cells, and may serve as their principal fuel (McKeehan 1982). Several studies in rats have demonstrated that the depletion of plasma glutamine concentration is a characteristic feature of malignant disease (see Yamamoto et al 1974, Souba et al 1988, Klimberg et al 1990a, Parry-

Billings et al 1991b, and Austgen et al 1992). Further it is likely that, at sites of rapid proliferation in solid tumours the local concentration of glutamine could be very low. This may cause a severe decrease in lymphocyte proliferation and activity. It has been shown that tumour cells possess glutamine synthetase activity (Kulka et al 1972, Kulka and Cohen 1973, Arad et al 1976, Arad and Kulka 1978 and Matsuno and Goto 1992). The activity of glutamine synthetase in tumour cells, specifically hepatoma cells, cultured for 24h. in the absence of glutamine is reported to be between 4 and 20 nmoles/min/mg of protein (Kulka et al 1972 and Kulka and Cohen 1973). The activity of glutamine synthetase in lymphocytes cultured for 24h. in the absence of glutamine, determined in this thesis, is reported to be between 9 and 22nmoles/min/mg of protein, depending on the mitogen used (Chapter 4, section 3.4., Figures 1 and 2). Thus, in the light of the effect of decreases in the concentration of glutamine, *in vitro*, on proliferation of lymphocytes, could it be that hepatoma cells, and other tumour cells, are able to continue proliferating when plasma glutamine levels are markedly depleted because of their glutamine synthetase activity?

An increase in glutamine synthetase activity of cells of the immune system may therefore be significant with regard to the anti-tumour activity of immune cells. One of the approaches to the treatment of cancer has been to identify immune cells that can mediate, directly or indirectly, anti-tumour effects (Rosenberg 1991 and Rosenberg and Lotze 1986). Several types of cells have been identified that can be used in the regression of cancer in some patients. These cells include the lymphokine activated killer (LAK) cell which is capable of lysing fresh tumours but not fresh normal cells in culture, and the tumour infiltrating lymphocyte (TIL) which is capable of recognizing unique antigens on tumour cells (see Rosenberg 1991). Recently numerous methods have been used to try and

increase the anti-tumour activity of TIL. These methods include: enhancing the *in vivo* effectiveness of TIL by combining them with cytokines such as interleukin-2 (IL-2) and interleukin-6 (IL-6), and genetic modification of TIL, which has involved the expression of cytokine genes for example tumour necrosis factor (TNF) in TIL (see Rosenberg 1991). IL-2, IL-6 and TNF have been shown to cause regression of a variety of cancers in experimental animals and man (see Rosenberg 1991, Rosenberg et al 1989 and Mule et al 1990). As an alternative to the methods mentioned above, it could be possible to increase the anti-tumour activity of these immune cells by increasing their rate of proliferation by increasing their glutamine synthetase activity. This could be achieved by activation of the promoter for this enzyme in selective immune cells in cancer patients, which could result in more immune cells and thus more immune activity at the site of the tumour.

6.The benefits of glutamine feeding.

Studies have reported that glutamine feeding improved the recovery rates of patients following surgery (Ziegler et al 1992), and also of rats with sepsis (Ardawi 1991, 1992). The question that is asked is, how does glutamine work to improve recovery of the subjects mentioned above? The effect of glutamine on immune function has been suggested by the reports cited above. This thesis has shown that the rate of proliferation of lymphocytes removed from rats fed glutamine is higher than that of lymphocytes removed from control-fed rats (Chapter 5, section 3.3). However, the increase in the rate of proliferation of lymphocytes removed from alanine-fed rats was also higher than that of lymphocytes removed from control-fed rats (Chapter 5, section 3.3). Thus, it is speculated that lymphocyte proliferation is dependent on other amino acids as well as glutamine, and that the mechanism for proliferation involves an increase

in the concentration of a specific peptide whose synthesis depends on the presence of certain specific amino acids, which may include glutamine and alanine.

It is also speculated that this specific peptide is similar to peptides present in the intestine (Chapter 5, section 4.3.). Such a possibility may be significant when considering the beneficial effect(s) of glutamine feeding on the intestine and its immune system. The intestine, specifically the intestinal mucosa, acts as a protective barrier against the absorption of bacteria and other toxins. The impermeability of the cellular membranes and the tight junctions between the mucosal cells, play an essential role in this function of the intestinal mucosa (Saadia et al 1990). The immune system of the intestine also plays a role in preventing the absorption of bacteria. An intestinal secretory immunoglobulin, S-IgA, prevents the attachment of bacteria to the mucosal cells, a step which is thought to be the crucial initiating step in the invasion of bacteria. B- and T-lymphocytes and macrophages within the intestine, are involved in the synthesis of S-IgA. B-lymphocytes under the influence of maturation factors develop into polymeric S-IgA producing plasma cells. The development of B-lymphocytes into these plasma cells is regulated by cytokines released by T-lymphocytes and macrophages (see Alverdy et al 1992).

In the light of the results of the feeding study in this thesis, there is a possibility that other amino acids, as well as glutamine, could play a role in the stimulation of lymphocyte proliferation and indirectly be involved in the maintenance of the intestine as an effective barrier against bacteria. This would open up a new area in the investigation of the mechanism of lymphocyte proliferation, and might provide the basis of alternative nutritional care for patients.

As well as stimulating immune cell function it has been suggested that glutamine feeding maintains the levels of the antioxidant GSH (γ-

glutamyl-cysteinyl-glycine). GSH is released by the liver, which contributes to the maintenance of circulating GSH in the plasma (see Sies and Graf 1985 and Lash and Jones 1985). Antioxidants are compounds that protect against the toxic agents known as free radicals. These are highly reactive reduced products of oxygen, for example superoxide and hydrogen peroxide, that attack and damage unsaturated lipids, proteins and DNA (see Newsholme and Leech 1983). Lash et al (1986) have shown that exogenous GSH provides rat intestinal epithelial cells with protection against injury induced by generators of free radicals, menadione, a quinone which, via its reduction, generates superoxide and t-butyl hydroperoxide, which generates hydrogen peroxide. They also tested whether protection of the epithelial cells of the intestine was due to the supply of amino acid precursors (glutamate, glycine and cysteine) of GSH, and showed that very little protection was provided by these amino acids. Lash et al concluded that the synthesis of GSH within epithelial cells could not account for the protection of these cells by GSH. In addition Martensson et al (1990), using electron microscopy, showed that GSH deficiency in mice leads to severe degeneration of the epithelial cells of the intestine, and that following the oral administration of GSH or a GSH monoester there was an almost complete absence of intestinal tissue degeneration.

It is suggested that glutamine, via its hydrolysis to glutamate, maintains GSH levels (Augsten et al 1992). In this thesis it is reported that the concentrations of lymph node glutaminolytic intermediates (glutamate and aspartate) are much higher than those of the glycolytic intermediates (glucose-6-phosphate and phosphoenolpyruvate) (Chapter 5, section 4.4). Perhaps the high concentration of glutamate in lymph nodes plays a role in maintaining GSH levels in lymph nodes, thus providing lymphocytes with protection against free radical injury. Since

lymphocytes are present in the intestine, the high concentration of glutamate may also provide protection, against free radical injury, in intestinal cells via GSH synthesis. It has been suggested that GSH synthesis within intestinal cells could not account for protection of intestinal cells by GSH (Lash et al 1986, see above). However, the concentration of glutamate used in this study was 1mM, the concentration of lymph node glutamate measured in the present study was between 13 and 24mM (see Chapter 5, section 4.4). Some free radical protection of intestinal cells may therfore be provided by GSH synthesized in the immune system of the intestine.

Thus, as well as maintaining an effective immune system and intestine via the stimulation of immune cell function, glutamine feeding may be beneficial to both tissues by providing protection against free radical injury, via the maintenance of the high concentration of immune cell glutamate. This newly recognized role of glutamine further highlights the importance of glutamine for cells of the immune system, and the investigation of a possible role of glutamine and glutamate in the protection of tissue from oxidative injury may prove fruitful.

APPENDICES

Appendix I

1. Animals: source and care

Male albino Wistars and Sprague-Dawley rats were obtained from Harlane-Olac Ltd, Bicester, U.K.
Mice (PO strain) for the glutamine synthetase in cells study (Chapter 4) were obtained from the William Dunn School of Pathology, Oxford.
Animals were kept in the Department's animal house for at least 2 days prior to experimentation. They were housed at approximately 23°C, under a 12h. light-dark cycle (lights on 0800-2000h.) Animals, excluding glutamine- or alanine-fed rats, were given free access to water and food (rat and mouse number 1 beta diet, B.P. Nutrition Ltd, Witham, Essex).

2. Chemicals and enzymes

The following chemicals were obtained from:

a) Sigma Chemical CO., Poole, Dorset.
HEPES, triethanolamine, insulin, Dowex-2-chloride, charcoal, mercaptoethanol, tri-iodothyronine, propylthiouracil, leucine, valine, isoleucine, dexamethasone acetate, lactic acid, endotoxin (from salmonella thyphimurium) concanavalin A, pokeweed mitogen, lipopolysaccharide, imidazole/hydrochloride and methionine sulphoximine.

b) BDH, Poole, Dorset.
KH_2PO_4, $NaHCO_3$, KOH, KCl, $MgCl_2$, HCl, glutamine, glycerol, hydrazine hydrate, perchloric acid, formic acid, sucrose and universal indicator.

c)Fisons Scientific Equipment, Loughborough, Leics.
Sodium chloride, calcium chloride, D-glucose.

d) Boeringer Mannheim, Lewes, Sussex.
ATP, NADH, NAD+, EDTA (Na salt), bovine serum albumin (fraction V) and creatine phosphate.

e) East Anglia Chemicals, Hadleigh, Ipswich, Suffolk.
Ammonium chloride and magnesium sulphate.

f)Amersham Int., Bucks.
[^{3}H] inulin, ^{3}H2O, U^{14}C Glutamate, [^{3}H] thymidine.

g) Hayman Ltd., Witham, Essex.
Ethanol.

h) May and Baker Ltd, Dagenham, Essex.
Sagatal (pentobarbitone).

i) Difco, Laboratories, Detroit U.S.A.
Thioglycollate broth.

j) Flow Laboratories, Irvine, Scotland.
HEPES-buffered-RPMI (1640) glutamine free culture medium, foetal calf serum, penicillin, streptomycin, 5ml well culture plates and round-bottomed microtitre culture plates.

k) Oxoid Ltd, Basingstoke, Hants, U.K.
Phosphate buffered saline tablets.

l) Gelman Sciences, Ann Arbor, Michigan, U.S.A.
sterile filters ("acrodiscs", pore size 0.2um).

m) Nycomed Ltd., Birmingham.
Density gradient medium ("lymphoprep").

n) National Diagnostics Ltd., Aylesbury, Bucks.
Liquiscint.

o) SDS Ltd, Witham, Essex.
SDS rat food Nº 1 containing 1.44g glutamine per 100g of food.
SDS rat food Nº 1 containing 1.76g alanine per 100g of food.

The following enzymes were obtained from Boehringer Mannheim, Lewes, East Sussex.
Creatine kinase, asparaginase, alanine dehydrogenase, glutamate dehydrogenase.

Appendix II

Preparation and care of reagents.

1) Defatting of Bovine Serum Albumin

Bovine serum albumin (BSA fraction V) (60g) was layered on to distilled water (400ml) and left to dissolve. Washed charcoal (30g) was added to the BSA. The pH of the charcoal/BSA mixture was altered to 3 by the addition of concentrated HCl (11M) and stirred on ice for 1h. These conditions promoted the dissociation of free fatty acids from albumin and their adsorption on to charcoal. The charcoal/BSA mixture was filtered through a Whatman number 1 filter in a Buchner funnel, under suction. The filtrate was further cleaned by a series of filtrations using nitrocellular filters (twice through 0.45μM filters and three times through 0.2μM filters).The pH of the filtrate was then adjusted to 7 by addition of concentrated NaOH (10M). The defatted BSA was dialysed overnight at 4°C in 7L of NaCl (0.9%). Following dialysis the fitrate was made up to 600ml (10% w/v) dispensed as 20ml aliquots, and stored at -20°C.

2) Preparation of Dowex Formate

Dowex-2-chloride (Dry Mesh 200-400, approximately 250g) was washed with water until the supernatant was clear (first wash was done using distilled water, further washes were done using deionized water). The resin was filtered (Whatman number 1) using a Buchner funnel, under suction, then added to a flask containing 4L of 1M NaOH, and mixed for 30mins to generate the hydroxylated form. The resin was allowed to settle and the NaOH decanted off and replaced with 4L of distilled water for further washing. This procedure was repeated a further 4 times to ensure the suspending medium was free of alkali. The hydroxylated Dowex was fitered again through a Buchner funnel. The funnel was then placed in a fume cupboard and filled with formic acid solution (98%, approximately 250ml) which was left to percolate through the Dowex into a flask beneath. The formated Dowex was washed with distilled water (formated Dowex was washed approximately 3 times with 1.5L of distilled water), and stored, soaking in excess water, at 4°C until use.

3) Preparation of dexamethasone, 3,3,5-tri-iodothyronine, propylthiouracil (6-n-propyl 2 -thiouracil) and endotoxin (Salmonella thyphimurium)

Dexamethasone (3mg) was dissolved in 3ml HEPES and stored at 4°C over the 5 day treatment.
3,3,5-Tri-iodothyronine was dissolved in 1ml NaOH (0.01M) and stored at 4°C over the 5 day treatment.
For the preparation of propylthiouracil, ethanol (5ml) was added to 200mg propylthiouracil. The ethanol/propylthiouracil mixture was dissolved in

boiling water. Tap water (400ml) was then added to the solution. The solution was stored at 4°C before use.
Endotoxin (from salmonella thyphimurium) (1g) was dissolved in 1ml HEPES and stored at 4°C before use.

4) Reagents for tissue culture
The preparation of reagents was performed using sterile equipment and in a sterile hood. Reagents were sterile filtered after preparation and prior to use.

a) **Phosphate buffered saline (PBS)** PBS tablets (100) were dissolved in distilled water (1L) and the solution autoclaved. PBS was stored at 4°C.

b) **Foetal calf serum (FCS)** FCS was dialysed for 4 days against phosphate buffered saline (previously autoclaved). The phosphate buffered saline was changed twice a day.

c) **Preparation of mitogens** Stock solutions of concanavalin A and pokeweed mitogen, at concentrations of 500µg/ml and 100µg/ml respectively, were prepared by adding HEPES-buffered-RPMI (1640) medium (100ml) to 50mg or 10mg of concanavalin A or pokeweed mitogen respectively. These solutions were stored at -20°C. Suspensions of lipopolysacchride, at a concentration of 1mg/10ml, were prepared by adding lipopolysaccharide (10mg) to HEPES-buffered-RPMI (1640) medium (100ml). The suspension of lipopolysaccharide was not stored but used immediately after its preparation. It was not possible to sterile filter the lipopolysaccharide preparation since it was in suspension.

d) **Glutamine**. Stock solutions of glutamine (200mM) were prepared by dissolving glutamine (2.92g) in sterile PBS (100ml). The stock glutamine solution was stored at -20°C.

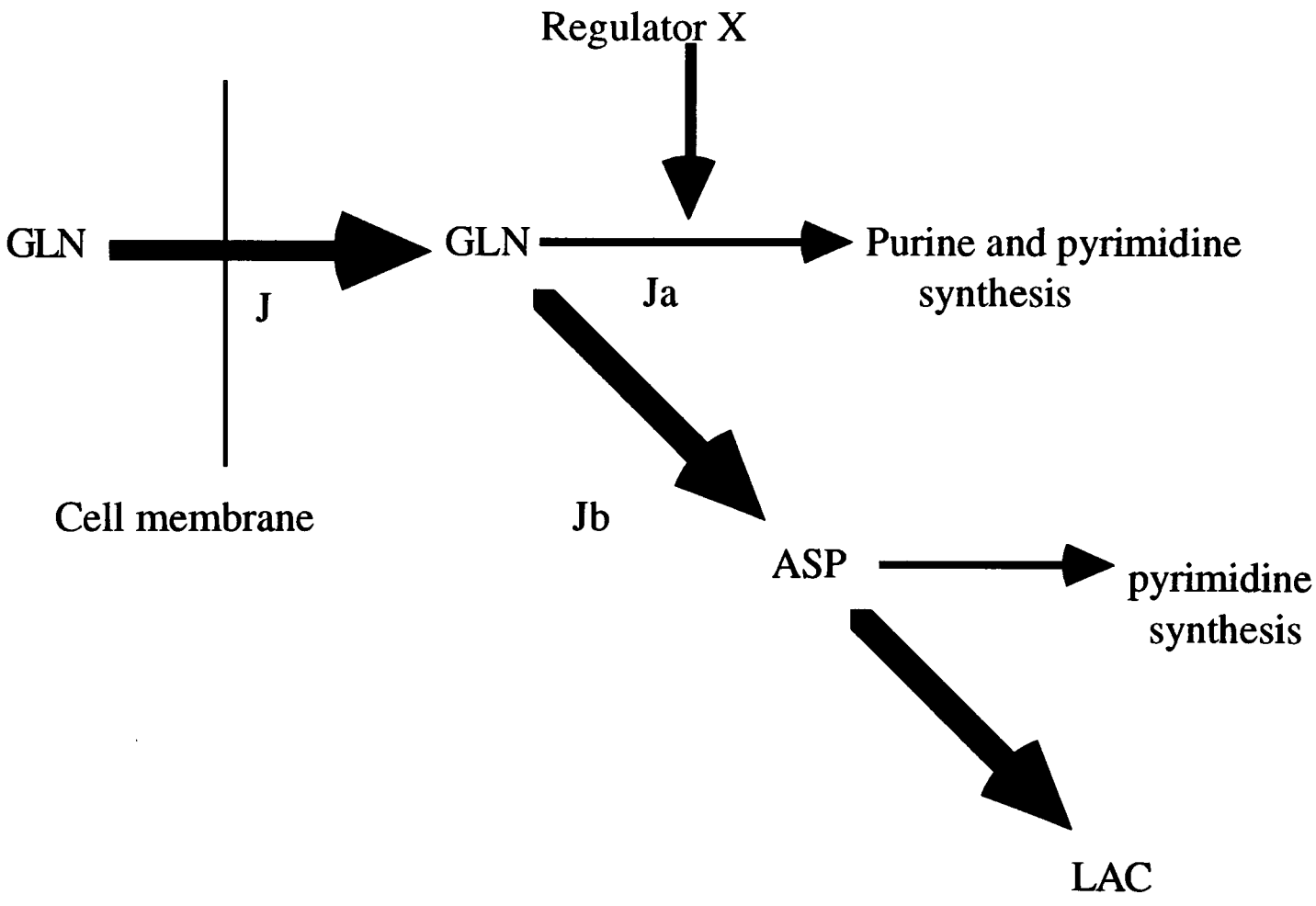
e) **Methionine sulphoximine (MSO)**. Stock solutions of MSO (11mg/15ml) were prepared with HEPES-buffered-RPMI (1640) medium and were used immediately after preparation.

f) **Antibiotic solution**. Streptomycin (1g) was dissolved in PBS (5ml). Penicillin (600g) was dissolved in PBS (1.2ml). The final antibiotic solution contained the following: streptomycin solution (2.5ml), penicillin solution (1ml) and PBS (6.5ml). The final concentration of both streptomycin and penicillin was 50mg/ml.

Appendix III

Branched point sensitivity

It can be shown that if a metabolic influx, J (see figure below), divides into two fluxes Ja and Jb, and Ja is regulated by factor X, the highest sensitivity of flux Ja to changes in the regulator X is achieved when Jb >> Ja. The highest sensitivity is achieved because the rate of the pathway, Ja, can be increased markedly without decreasing significantly the concentration of the metabolic intermediate(s) of the main pathway (B). The glutaminolytic pathway (B) exhibits a high rate and the pathway provides precursors for biosynthetic pathways, purine and pyrimidine synthesis, but the fluxes through the pathways of purine and pyrimidine biosynthesis (Ja) are very small in comparison to the flux through glutaminolysis (Jb). However, the flux through the biosynthetic pathways will increase considerably during the synthesis of DNA and RNA. Since provision of these intermediates, for cell proliferation, is required at a precise time, the sensitivity of the biosynthetic processes to their regulator/s (X) would need to be very high.



The regulation of flux through a branched pathway. GLN denotes glutamine, ASP denotes aspartate and LAC denotes lactate. J, Ja and Jb represents metabolic fluxes. (Figure taken from Newsholme et al 1988).

Appendix IV

Studies to assess the viability of the isolated muscle preparation

In assessing the viability of the isolated muscle both biochemical and physiological properties of the *in vitro* preparation have been studied. Biochemical assessment of the muscle preparation has been used to determine its metabolic characteristics. In the isolated skeletal muscle, the ability to store and maintain high energy phosphate levels e.g. phosphocreatine and the adenine nucleotides, comparable to that *in vivo*, is considered to be a sensitive indice of tissue integrity (see Garber et al 1976a and Crettaz et al 1980). In the author's laboratory, adenine nucleotide levels have been shown to be maintained in incubated muscle (see Parry-Billings 1989). The ratios of the concentration of the adenine nucleotides, ATP:AMP and ADP:AMP, in incubated muscle, which are indicators of the muscles energy status (Newsholme and Start 1973) and therefore important in terms of the metabolic regulation of various processes in muscle, have been shown to be very similar to muscle *in situ* (see Parry-Billings 1989). A normal lactate:pyruvate concentration ratio, which is indicative of the NADH:NAD ratio (see Espinal 1983) and a low rate of glycogenolysis (Newsholme et al 1986) have also been used as indices of viability. Viability may be reduced by an inadequate supply of oxygen to the muscle. A high rate of glycogenolysis (indicated by an absence of glycogen in the muscle core) is taken as evidence of hypoxia in the muscle core (Segal and Faulkner 1985, Maltin and Harris 1985). This problem may be caused by the use of large and thick muscle preparations, the size of which may limit the rate of diffusion of oxygen into the interior of the muscle preparation, causing hypoxia. The problem of hypoxia may be prevented by the use of small and thin muscle preparations. However, it is interesting to note that in a study by Maltin and Harris, muscles were obtained from rats that weighed between 40-70g (the muscles probably weighed approximately 25-35mg) yet hypoxia still occured. In the author's work, if rats of this size are used, excessive muscular spasms are observed, on sacrifice by cervical dislocation. This 'twitching' may be responsible for high rates of glycogen breakdown. For this reason, small rats are anaesthetized with pentobarbitone prior to cervical dislocation. Maltin and Harris did not indicate whether they used this procedure in their method.

Glucose metabolism i.e the rates of glucose incoporation into glycogen, glycolysis and glucose transport have also been used to assess the viability of incubated muscle (Crettaz et al 1980; Newsholme et al 1986). These rates are reported to be linear over a 120 mins incubation (Crettaz et al 1980) and over a 60 mins incubation (see Newsholme et al 1986).

Measurement of lactate dehydrogenase (LDH) activity in the incubation medium can also be used to assess the viability of incubated muscle since it is a measure of tissue damage to the plasma membrane.

Garber et al (1976a) and Crettaz et al (1980) reported that only a small percentage of muscle's lactate dehydrogenase activity, 4.3% and 2.6% respectively, was measured in the incubation medium.

Physiological assessment of the viability of incubated muscle has focused on the contractile properties of the muscle (peak twitch tension, maximum tetanic tension and the rate of tension development; see Segal and Faulkner 1985). Additionally, the incubation of muscle at resting tension is very important since, flaccid muscle tends to swell and gain weight, the swelling and weight gain lead to poor oxygenation (see Pain and Manchester 1970). It has also been reported that keeping the muscle at resting tension limits the increase of the rate of protein degrdation which occurs in flaccid incubated muscle (Barcacos and Goldberg 1986).

Another indication of viability is electron microscopy of incubated muscle. This indicates normal mitochondrial morphology. If muscle was hypoxic then mitochondria would expect to show changes in their ultrastructure (see Newsholme et al 1986).

Parry-Billings (1989) examined the viability of the soleus muscle preparation used in the present study for the investigation of glutamine metabolism by measuring the concentration of glutamine in muscle before and after a 60 mins incubation. Parry-Billings (1989) demonstrated that glutamine concentration is similar before and after a 60mins incubation. Such a result is very important since it indicates that the incubation system used in the present study does not alter the concentration of glutamine in incubated muscle. Parry-Billings (1989) also demonstrated that the rate of lactate release from incubated soleus muscle is linear over time.

Appendix V

Quantitative analysis.

a) Calculation of depletion time of glutamine concentration in soleus muscle following 60mins incubation, assuming no glutamine is synthesized by soleus muscle during the 60mins

Example, Lactate (10mM):
rate of release of glutamine from soleus muscle in the presence of lactate (10mM) is 61.6 nmoles/min/g of muscle.

Concentration of glutamine in soleus muscle =

5mM = 5000nmoles/g of muscle.

In 60mins, in the presence of lactate, soleus muscle releases

61.6x60nmoles/g = 3696nmoles/g.

Thus, assuming no glutamine synthesized by soleus muscle, then after a 60mins incubation the concentration of glutamine in soleus muscle

= 5000 - 3696 = 1304nmoles/g.

Thus, soleus muscle glutamine concentration completely depleted in

$$60\text{mins} + \frac{1304}{61.6}\ \text{mins} = 81\text{mins}.$$

b) Calculation of rate of glutamine synthesis in soleus muscle from muscle glutamine release and concentration data

Example, Lactate (10mM):
Rate of release of glutamine from soleus muscle in the presence of lactate (10mM) is 61.6 nmoles/min/g of muscle.

After 60mins incubation soleus muscle in the presence of lactate (10mM)

= 3696 nmoles/g of muscle.

If soleus muscle does not synthesize any glutamine, then after a 60mins incubation soleus muscle glutamine concentration

= 1304 nmoles/g of muscle.

However, after 60mins incubation concentration of glutamine in soleus muscle incubated in the presence of lactate (10mM) approximately 5mM
= 5000 nmoles/g of muscle.

Thus, soleus muscle synthesizes
(5000 - 1304) = 3696nmoles glutamine per g of soleus muscle in 60mins

Thus the rate of glutamine synthesis by soleus muscle incubated with lactate (10mM) = $\frac{3696}{60}$ = 61.6 nmoles/min/g of muscle

Appendix VI

Techniques used to determine glutamine synthetase activity in skeletal muscle

Colorimetric assays

Colorimetric assays modified from the method of Elliot (1951), who measured glutamine synthetase activity in bacterial, plant, brain and kidney extracts in the prescence of ATP, L-glutamate, hydroxylamine and Mg^{2+}, were generally used to measure glutamine synthetase activity. The assay is based on the principal that hydroxylamine can replace ammonia and that the γ- glutamyl hydroxamic acid (see reaction below) forms a brown colour with acidic ferric chloride that absorbs in the region of 550nm (Pamiljans et al 1962, Rowe et al 1970).

Glutamate + hydroxylamine + ATP ⟶ γ- glutamyl hydroxamate + ADP +Pi

This method has been used in monitoring the purification of glutamine synthetase in sheep brain (Palmijans et al 1962, Ronzio et al 1969) and also to measure glutamine synthetase in various preparations, liver and heart (Wu 1963); brain (Berl 1966); and kidney (Reiner and Hudson 1953, Iqbal and Ottaway 1970). Although this method is reliable and convenient, it is only adequate for enzyme preparations contaning little or no adenosine triphosphatase (ATPase) activity. This method was found to be unsatisfactory when applied to crude homogenates of rat tissues, such as muscle, where glutamine synthetase activity compared with adenosine triphosphatase (ATPase) activity is relatively low, with the result that ADP, which is a powerful inhibitor of glutamine synthetase, accumulates and thus prevents the maximum activity of glutamine synthetase from being measured (see Elliot 1951, Lund 1970, Iqbal and Ottaway 1970). To overcome this problem Iqbal and Ottaway (1970) developed a method based on the coupling of ADP production, as the reaction proceeds, to the oxidation of NADH, by adding pyruvate kinase, phosphoenol pyruvate and lactate dehydrogenase to the system. This new method allowed a correction to be made for the activity of other ADP-producing reactions in the tissue extract. However this method is not specific for glutamine synthetase, which was measured by the increase in the rate of NADH disappearance on the addition of glutamate, since the rate of NADH oxidation caused by the hydrolysis of ATP, by ATPase, was usually very great before the glutamate was added and as a conseqence only small amounts of tissue extracts could be added.

Herzfeld (1973) took a similar approach using a pyruvate kinase and phosphoenol pyruvate ATP-regenerating system, developed by Knox et al (1971). However with this method the pyruvate generated from pyruvate kinase reaction reacts with hydroxylamine to yield an oxime that produces a brown colour with acidic ferric chloride used to generate the colour change. Thus, muscle with high ATPase activity yield artificially high values of glutamine synthetase activity if the appropriate blank (ie glutamate) is not included (see Vorhaben et al 1973). Thus, due to the possibility of endogenous glutamate in the blank, it was preferable not to use the pyruvate kinase-regeneration system for the colourimetric assay for glutamine synthetase. Liemieux et al (1976) used a creatine phosphate/creatine kinase ATP regenerating system for the glutamine synthetase assay. This ATP regenerating system was previously used by Robinson (1967) and Crabtree (1969) for the measurement of hexokinase in muscle and adipose. This system avoids the generation of pyruvate, and thus prevents interference with the colourimetric assay for glutamine synthetase activity.

Radiochemical assays for the measurement for glutamine synthetase activity in muscle

In light of the problems experienced using the colourimetric assay technique to measure glutamine synthetase activity, Lund (1970) developed a radiochemical assay, in which the inhibition of glutamine synthetase activity by ADP was minimized by incorporating an ATP-regenerating system, using phosphoenol pyruvate and pyruvate kinase. (There was no danger of this ATP-regenerating system interfering with the radiochemical method since the assay was not based on colour changes.) Shorter incubation times were also used, 6 mins as compared to 30 mins for the Iqbal and Ottaway (1970) method and 20 mins for the Herzfeld (1973) method. This assay method also used ammonia, the natural substrate for glutamine synthetase, instead of hydroxylamine. The assay method of Lund (1970) is suitable for crude tissue extracts that contain high ATPase activity, such as muscle.

The assay method of Lund (1970) involves the incubation of Mg^{2+}, ammonia, ATP, phosphoenol pyruvate, pyruvate kinase, a small amount of glutamine, cycloserine, water, Tris-HCL buffer and $U^{14}C$ glutamate with tissue extract. The non-labelled glutamine is included to dilute the $U^{14}C$ glutamine formed and thus minimize the loss of label as a result of glutaminase activity. The $U^{14}C$ glutamine produced from this assay is then separated from $U^{14}C$glutamate by thin layer chromatography, the non-labelled glutamine provides a marker for $U^{14}C$ glutamine in the thin layer chromatography. Cycloserine is present in the incubation medium to prevent the reaction of $U^{14}C$ glutamate and alanine aminotransferase, which is present in muscle, to form an oxo-^{14}C glutarate, which overlaps with glutamine on the chromatograph. This technique has been used to measure glutamine synthetase activity in muscle (Lund 1970, Hundal et al 1991). However the separation technique (ie thin layer chromatography)

used for the separation of U^{14}Cglutamine from U^{14}C glutamate is not rapid.

King et al (1983) have also used a radiochemical assay to measure glutamine synthetase activity in skeletal muscle. The radiochemical assay used by King et al (1983), is modified from that of Tiemeier and Milman (1972). The incubation medium for this technique is similar to that used by Lund (1970) except for the following: excess unlabelled glutamate is present in the incubation so as to ensure conditions for the measurement of the maximal activity of glutamine synthetase *in vitro*. In addition, the phosphocreatine/creatine kinase ATP regenerating system was used. The method of separation also differed from the method of Lund (1970): ion exchange chromatography was used to separate labelled glutamine from labelled glutamate. This separation technique is based on methods used by Prusiner and Milner (1970) and Pishak and Phillips (1979), and it provides a very high percentage (90%) recovery of labelled glutamine formed (see Prusiner and Milner 1970, Pishak and Phillips 1979, King et al 1983). That the radiochemical assay measured glutamine synthetase activity was confirmed by showing the dependency of the assay on glutamate, ammonia and ATP (Prusiner and Milner 1970). That the product of the assay was indeed glutamine was verfied by Prusiner and Milner (1970) and Pishak and Philips (1979) using thin layer chromatography and radioautography.

The radiochemical assay used by King et al (1983) is reported to be at least 200 fold more sensitive than the γ-glutamyl hydroxamate assay (see Tiemeier and Milman 1972). It is also suitable for multiple determinations (see Pishak and Phllips 1979). Many of the radiochemical assays used to determine glutamine synthetase activity in skeletal muscle and also the effect of different factors and conditions on glutamine synthetase activity in muscle, (Muhlbacher et al 1984; Max et al 1988,1987; Ardarwi 1988, 1990; Feng 1990a, b) are modifications of the method used by King et al (1983). This technique was used in the present study.

Appendix VII

Evaluation of the safety of glutamine administration in man

Early literature suggests that the administration of free glutamine is neither effective or safe (see Furst et al 1987). More recent studies on the administration of free glutamine in man have shown that this does not seem to be the case. Wilmore (1990) evaluated the effectiveness and safety of short term glutamine administration in man: glutamine was administered orally or via the intravenous route to normal subjects. Plasma glutamine levels increased and subjects experienced no subjective effects following oral or intravenous glutamine administration. Vital signs (temperature, blood pressure, pulse rate, and respiratory rate) were normal and there were no changes in hepatic function, which indicated no associated signs of toxicity. In addition, there was no significant change in the levels of ammonia and glutamate.

Lowe et al (1990) investigated the safety of long term glutamine administration in normal human subjects; Wilmore (1990) investigated the safety of long term glutamine administration in both normal human subjects and patients after surgery. Lowe et al (1990) reported that plasma glutamine levels increased with glutamine administration. Wilmore (1990) showed that plasma glutamine levels in normal subjects increased, with those of patients increasing slightly. Both studies reported that glutamate and ammonia levels did not change with glutamine administration. Detailed evaluation of subjects, performed during these studies, did not detect any alterations in central nervous system function throughout the study. Both studies showed that there were no associated signs of toxicity in both normal subjects and patients.

Khan and Elia (1991) and Khan et al (1991) investigated the stability of L-glutamine in solution and factors that may affect its stability. The effects of temperature, pH, light and oxygen on the degradation of L-glutamine, in water and various buffer and TPN solutions were investigated. They reported that at room temperature the rate of degradation of glutamine depended on the type of solution used and on the pH of the solution. For a TPN solution at pH 6.4 the rate of glutamine degradation was 0.8%/day. The degradation rate of glutamine was essentially unaffected by light or O_2. In relation to temperature, they reported that the degradation rate of L-glutamine in TPN solution was less than 0.15%/day at 4°C, minimal at -20°C and undetectable at -80°C. They also reported that glutamine degradation resulted in the formation of ammonia but not enough to cause clinical problems. In this same study, there was no glutamate formation and they presumed that since glutamine degradation did not result in the formation of glutamate, it resulted in the formation of pyroglutamate. However, since the rate of glutamine degradation was low, it was assumed that the amount of the pyroglutamate formed was small. Khan and Elia (1991) reported that it was unlikely that the pyroglutamate formed would have toxic effects partly because mammalian tissues are normally exposed to small

concentrations of pyroglutamate; pyroglutamate is involved in amino acid metabolic pathways and in the transport of amino acids into cells. via the g-glutamyl cycle which has been proposed by Meister (1981), and it is present in protein: pyroglutamate is present in a conjugate form as the N-terminus of proteins including a number of biological active peptides such as gonadotrophin releasing hormones (see Abraham and Podell 1981).

From their studies, Khan and Elia (1991) concluded that: glutamine degradation in solution is variable due to the effect of physico-chemical factors (temperature and pH), and that glutamine degradation in TPN solution is sufficiently slow, especially during storage at 4°C or below, to consider its inclusion in TPN solutions for clinical use.

Appendix VIII

Quantitative analysis to indicate whether glutamine released from skeletal muscle can supply the demand of glutamine by cells of the immune system, when rested and stimulated (for example during sepsis), in a 200g rat

During normal conditions

The whole body rate, for a 200g rat, of glutamine release from muscle.
The rate of glutamine release from muscle = 40nmoles/min/g of muscle.
Muscle makes up 40% of the total body weight of a rat.
The rate of glutamine release from muscle for a 200g rat = 3200nmoles/min=192μmoles/h.

The whole body rate, for a 200g rat, of glutamine utilization by lymphocytes.
The concentration of glutamine in rat plasma = 0.9-1mM however, there are no data for the rate of glutamine utilization by lymphocytes incubated in medium containing between 0.9-1mM glutamine.
There are data for rates of glutamine utilization by lymphocytes incubated in medium containing 0.5mM or 2mM glutamine (Ardawi 1983 and Ardawi and Newsholme 1983 respectively). These have been used in this quantitative analysis.

At 0.5mM glutamine the rate of glutamine utilization by $2x10^7$ lymphocytes incubated for 60mins = 61.4μmoles/h/g dry wt.
Since $1x10^{10}$ lymphocytes = 0.815g of dry wt of lymphocytes then 61.4μmoles/h/g of dry wt for $2x10^7$ lymphocytes = 0.1μmoles/h.
There are approximately $4x10^9$ lymphocytes in a 200g rat (Ardawi and Newsholme 1985).
Therefore the whole body rate, for a 200g rat, of glutamine utilization by lymphocytes = $2x10^7/4x10^9$ x 0.1= 20μmoles/h.

At 2mM glutamine the rate of glutamine utilization by $5x10^7$ lymphocytes incubated for 60mins = 160μmoles/h/g of dry wt.
The whole body rate, for a 200g rat, of glutamine utilization by lymphocytes = 52μmoles/h. (for a 200g rat)

Thus, during normal conditions the whole body rate, for a 200g rat, of glutamine utilization by lymphocytes is between 20 and 52μmoles/h..

During sepsis

The whole body rate, for a 200g rat, of glutamine release from muscle (for a 200g rat).

The whole body rate, for a 200g rat, of glutamine release from muscle = 295µmoles/h.

The whole body rate, for a 200g rat, of glutamine utilization by lymphocytes.
For stimulated lymphocytes the rate of glutamine utilization by rat lymphocytes is increased by 51% following mitogenic stimulation (Ardawi and Newsholme 1983). It is difficult to determine the increase in the number of lymphocytes during an immunological challenge. However, on the basis of accounts of cellular proliferation following antigenic challenge (Roitt 1984), it may be suggested that the number of cells may be increased by approximately 50%.

At 0.5mM glutamine the rate of glutamine utilization by stimulated lymphocytes = 92.7µmoles/h/g of dry wt. Since the number of lymphocytes when stimulated is increased by 50%, this rate of glutamine utilization is for $3x10^7$ lymphocytes.
In a 200g rat the number of lymphocytes when stimulated would increase from $4x10^9$ to $6x10^9$. With $1x10^{10}$ lymphocytes = 0.815g of dry wt of lymphocytes.
The whole body rate, for a 200g rat, of glutamine utilization by lymphocytes = 55µmoles/h..

At 2mM glutamine the rate of glutamine utilization by stimulated lymphocytes = 237µmoles/h/g of dry wt for $7.5x10^7$ lymphocytes.
The whole body rate, for a 200g rat, of glutamine utilization by stimulated lymphocytes = 115µmoles/h..

Thus, during an immunological challenge the whole body rate, for a 200g rat, of glutamine utilization by lymphocytes is between 55 and 115 µmoles/h..

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