STUDIES ON METABOLISM

IN MACROPHAGES

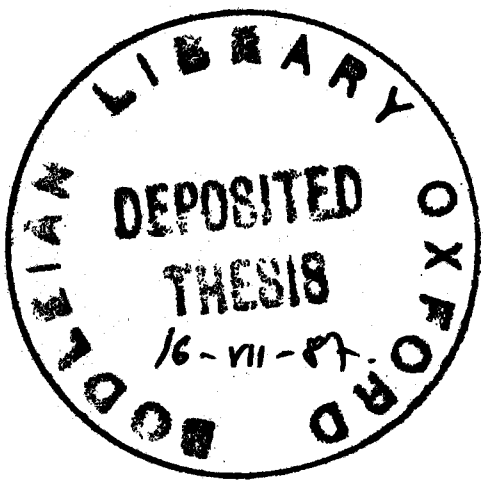
A Thesis submitted to the University of Oxford

for the Degree of Doctor of Philosophy

PHILIP NEWSHOLME

Wolfson College

May, 1987



To Annette and my parents

STUDIES ON METABOLISM IN MACROPHAGES

Thesis submitted to the University of Oxford
for the degree of Doctor of Philosophy

Philip Newsholme,
Wolfson College,
Oxford.

May, 1987.

ABSTRACT

A general metabolic profile of macrophages was established by measurement of maximum catalytic activities of enzymes in energy-producing pathways and rates of utilisation of glucose, glutamine, fatty acids and ketone bodies under various conditions. It was found that glucose, glutamine and fatty acids can be used to satisfy the energy requirement of the cell. Although a significant proportion of utilised glutamine or fatty acid was converted to CO_2 by the macrophage, most glucose was not oxidised and was converted, almost stoichiometrically, to lactate. Utilised fatty acids were not only oxidised by the macrophage, but were incorporated into cellular lipid (mainly triacylglycerol and phospholipid). The triacylglycerol rich macrophage was shown to be able to release fatty acids into the culture medium.

The importance of glutamine in macrophages was indicated from the high activity of phosphate-dependent glutaminase. Glutamine is probably metabolised by the following enzymes in macrophages: phosphate-dependent glutaminase, aspartate aminotransferase (or other amino acid aminotransferases), oxoglutarate dehydrogenase followed by enzymes of the TCA cycle and metabolism of oxaloacetate by phosphoenolpyruvate carboxykinase. Pyruvate derived via this pathway may be metabolised via pyruvate dehydrogenase or pyruvate carboxylase. A study of the sub-cellular distribution of some of these enzymes suggested that phosphate-dependent glutaminase has a cytosolic as well as a mitochondrial localisation. Further characterisation suggested that the non-mitochondrial activity could be associated with the plasma membrane. To the author's knowledge, this is the first report of a non-mitochondrial localisation for phosphate-dependent glutaminase. Glutaminase was shown to be activated by phosphate and inhibited by glutamate and 2-oxoglutarate. Significant inhibition of glutaminase occurred only at high concentrations of these compounds.

Glucose and glutamine were utilised at very high rates by the macrophage, but were not fully oxidised even though the cells were incubated in aerobic conditions. The significance of these high rates of utilisation to the macrophage is discussed.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, and father, Dr. Eric Newsholme for his sound advice, his patience and his encouragement at all times.

I would also like to thank Dr. Eva Blomstrand for measuring 5'-nucleotidase activity and fatty acid concentrations; Mr. Tony Gascoyne for performing amino acid autoanalysis; Dr. Steven Rabinowitz for help and advice with cell culture techniques; Dr. David Hayes for help and advice in the measurement of oxygen consumption; Mr. Peter Fisher for help and advice with lipid analysis and Mr. Matthew Kaser for his help with part of the art work.

I would like to thank Dr. Paul Crocker, Dr. Hugh Rosen and Dr. Brendan Leighton for helpful comments and stimulating discussions, plus Dr. Siamon Gordon for making the facilities of his laboratory available to me.

I would especially like to thank Dr. Rui Curi for his friendship, advice, help and encouragement throughout this work. Thanks are also due to Claudia Curi for her help in the preparation of some tables and figures.

I am grateful to the late Professor R. R. Porter, F.R.S. and I thank Professor E. M. Southern, F.R.S. and Professor H. Harris, F.R.S. for making the facilities of the Department of Biochemistry and the Sir William Dunn School of Pathology available to me. Thanks to Andrea Bates and especially to Shirley Greenslade and Jackie Hewitt for typing this thesis at such short notice.

During the course of this work I was a recipient of a S.E.R.C. Training Award which I gratefully acknowledge.

Last but not least I would like to thank my fiancee Annette and my parents for their patience and support. Without their help and encouragement this work would never have been accomplished.

PUBLICATIONS

Metabolism of glucose, glutamine, long-chain fatty acids and ketone bodies by murine macrophages.
Philip Newsholme, Rui Curi, Siamon Gordon and Eric A. Newsholme (1986). Biochem J., 239, 121-125.

Rates of utilisation and fates of glucose, glutamine, pyruvate, fatty acids and ketone bodies by murine macrophages.
Philip Newsholme, Siamon Gordon and Eric A. Newsholme (1987). Biochem. J., 242, 631-636.

Localization and characterization of glutaminase activity in the murine macrophage.
Philip Newsholme, Rui Curi, Eva Blomstrand, Siamon Gordon and Eric A. Newsholme (1987). Biochem. Soc. Trans. (in press).

Abbreviations

Acetyl CoA	acetyl coenzyme A
ADP	adenosine 5'-diphosphate
Ala	alanine
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
Ca^{2+}	calcium ion
cAMP	cyclic-3',5'-adenosine monophosphate
CCCP	carbonyl cyanide m-chlorophenyl-hydrazone
CN^-	cyanide ion
CoA	coenzyme A (reduced)
CTP	cytidine 5'-triphosphate
2-DG	2-deoxyglucose
DNA	deoxyribonucleic acid
DNP	dinitrophenol
ΔG	Gibbs free energy change
ΔG^o	Gibbs standard free energy change
DTNB	5',5'-dithiobis-(2-nitrobenzoic acid)
EDTA	diaminoethanetetra-acetic acid
F^-	fluoride ion
FCS	foetal calf serum
Gln	glutamine
Glu	glutamate
GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
Gly	glycine

H^+	hydrogen ion (proton)
$HClO_4$	perchloric acid
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
IAA	iodoacetic acid
Ig	immunoglobulin
K^+	potassium ion
K_{eq}	equilibrium constant
K_1	inhibitor constant
KJ	kilo joule
K_m	Michaelis constant
MEM	Eagle's minimum essential medium
mRNA	messenger RNA
Na^+	sodium ion
OAA	oxaloacetate
2-OG	2-oxoglutarate
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxykinase
Pyr	pyruvate
R	universal gas constant (8.314 joules/mol-degree)
RNA	ribonucleic acid
TCA cycle	tricarboxylic acid cycle
T.L.C.	thin layer chromatography
Tris	Tris(hydroxymethyl) aminomethane
V_f	rate of forward reaction <u>in vivo</u>
V_{max}	maximum catalytic activity <u>in vitro</u>
Vol	volume
V_r	rate of reverse reaction <u>in vivo</u>

Enzymes mentioned in the text and their Enzyme Commission (E.C.) classification numbers.

Enzyme	E.C. Number
Acetoacety CoA thiolase	2.3.2.9.
Acetyl CoA carboxylase	6.4.1.2.
Aconitase	4.2.1.3.
Alanine aminotransferase	2.6.1.2.
Aldolase	4.1.2.13.
Asparaginase	3.5.1.1.
Aspartate aminotransferase	2.6.1.1.
Carnitine palmitoyltransferase	2.3.1.2.1.
Carbamyl phosphate synthetase	2.7.2.9.
ATP-citrate lyase	4.1.3.8.
Citrate synthase	4.1.3.7.
Glucose-6-phosphate dehydrogenase	1.1.1.49.
Glutamate dehydrogenase	1.4.1.2.
(Phosphate-dependent) glutaminase	3.5.1.2.
Glutamine synthetase	6.3.1.2.
Hexokinase	2.7.1.1.
HMG-CoA reductase	1.1.1.88.
3-Hydroxybutyrate dehydrogenase	1.1.1.31.
Isocitrate dehydrogenase (NAD^+-linked)	1.1.1.41.
Isocitrate dehydrogenase ($NADP^+$-linked)	1.1.1.42.
Lactate dehydrogenase	1.1.1.27.
Lipoprotein lipase	3.1.1.34.
Malate dehydrogenase	1.1.1.37.
Malate dehydrogenase ($NADP^+$-linked)	1.1.1.40.
5'-nucleotidase	3.1.3.5.
Oxaloacetate decarboxylase	4.1.1.3.
3-Oxo-acid CoA-transferase	2.8.3.5.
Oxoglutarate dehydrogenase	1.2.4.2.
Phosphoenolpyruvate carboxykinase	4.1.1.49.
6-Phosphofructokinase	2.7.1.11.
6-Phosphogluconate dehydrogenase	1.1.1.44.
Phosphorylase	2.4.1.1.
Pyruvate carboxylase	6.4.1.1.
Pyruvate dehydrogenase	1.2.4.1.
Pyruvate kinase	2.7.1.40.
Succinate dehydrogenase	1.3.99.1.
Triglyceride lipase	3.1.1.3.1.

TABLE OF CONTENTS

CHAPTER 1:

General Introduction

CHAPTER 1

1.1 General Introduction

The immune system is extremely complex probably because it plays a variety of roles in maintaining health and combating disease. Similarly to the endocrine system, it exerts its effects within the body by virtue of circulating components capable of acting at sites far-removed from their point of origin.

There are two types of immunity which protect the body. First, there is the cell-mediated immune process, which combats fungi and viruses and initiates the rejection of tumours and foreign tissues such as transplants. Secondly, there is the humoral immune process, which is effective against bacterial infection and viral re-infection. However, these processes are not separate but represent an interrelated and intercommunicating process for the protection of the body against foreign invasion.

The complexity of the immune system derives, at least in part, from the intricate communication network which is capable of exerting multiple effects based on relatively few distinct cell types. The major cellular components of the immune system are the macrophages and lymphocytes (Fig. 1.1); other cell types which are present at various states of differentiation include plasma cells, mast cells, neutrophils, eosinophils and basophils. The leucocytes, which include macrophages, lymphocytes, neutrophils, eosinophils and basophils, are derived from the haematopoetic tissues (i.e. the bone marrow in mammals): they are wandering cells, leaving the blood-stream to patrol the tissues, or making a massive invasion of a particular area during inflammation. The types of leucocytes found

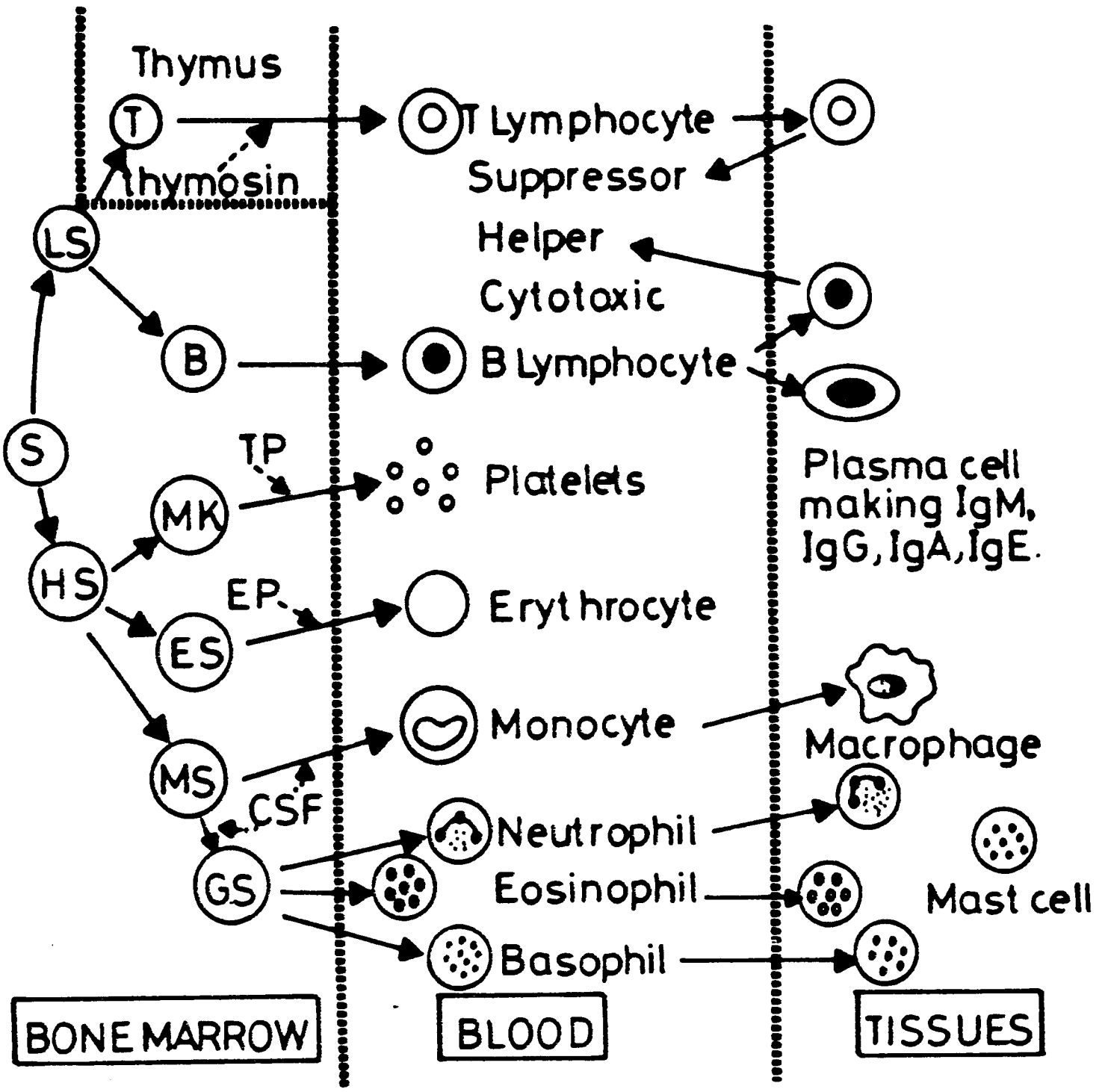


Figure 1.1 Cells involved in immunity and their origin

Stem cell (S); haemopoietic stem cell (HS); lymphoid stem cell (LS); megakaryocyte (MK); erythroid stem cell (ES); myeloid stem cell (MS); granulocyte stem cell (GS); thrombopoietin (TP); erythropoietin (EP); colony stimulating factor(s) (CSF).

Adapted from Playfair (1979).

in mammals are described as follows.

Firstly the mononuclear cells, in which the nucleus is discretely single;

(i) The macrophage [so called as it is large and can phagocytose, (i.e. eat) cells and particulate matter] is the principal resident phagocyte of the tissues and serous cavities. It has a nucleus that is generally indented and surrounded by clear cytoplasm. The cytoplasm is characterized by an abundance of lysosomes containing hydrolytic enzymes. The endocytic properties of the macrophage not only play a part in killing foreign organisms by causing their ingestion, but they also play an important role in initiating the immune response. Phagocytosed insoluble antigens and pinocytosed soluble antigens are ingested and may be enzymatically degraded into small, perhaps less complex, fragments. These fragments can be packaged and transported back to the plasma membrane for presentation to lymphocytes in the required form: this process also leads to concentration of the antigen at the cell surface. An absolute requirement for the response to most antigens involves physical contact between macrophages and lymphocytes during which antigen comes into contact with recognition receptors on the lymphocyte.

Macrophages are not only characterized by a high endocytic activity, they also have the capacity to secrete a large number of compounds including enzymes, plasma proteins, reactive metabolites of oxygen, prostaglandins and cell regulatory factors (see next section).

Macrophages also play a key role in processes unconnected

with the immune system, such as wound-healing, organ modelling during development and the destruction of dead and dying cells both in tissues and in the vascular and lymphoid sinuses (see Gordon, 1986).

(ii) The lymphocyte is a small cell with a discrete, compact and round nucleus and very little cytoplasm. It circulates in blood and lymphatic vessels, patrols through tissues and is mobilised in the secondary lymphoid organs (e.g. spleen, lymph nodes). Although indistinguishable in appearance, lymphocytes comprise several distinguishable types of cell, the T- and B-cells and their sub-sets. They contain genetically defined cell membrane antigens, some of which are associated with the histocompatibility (or tissue) uniqueness of the individuals within a species (e.g. Ia antigens), others are characteristic for the lymphocyte set or sub-set on which they occur. Lymphocytes can also recognise (via specific receptors) antigen epitopes, each cell containing receptors for one specific epitope. The lymphocyte remains in a quiescent form until activated upon contact with an antigen carrying an epitope for which it has a recognition receptor. B-cells upon activation will secrete antibody, while T-cells may secrete lymphokines, may help B-cells respond to T-dependent antigens, or suppress the ability of B-cell to respond to antigen, depending on the sub-set of T-lymphocyte that has been activated.

(iii) The null cell is similar to lymphocytes but lacks the surface markers and functional properties of T- or B-cells.

(iv) The natural killer cell (NK cell) expresses cytotoxicity against certain target cells such as tumour or

virally-infected cells. This activity is enhanced by interferon.

Secondly, the polymorphonuclear cells (also termed granulocytes), which are characterized by granules in the cytoplasm are described as follows.

(v) The eosinophil contains large granules, is phagocytic and perhaps cytotoxic for some larger parasites.

(vi) The basophil contains large granules which contain heparin and vasoactive amines; this cell is important in the inflammatory response.

(vii) The neutrophil is the commonest leucocyte of the blood. It contains smaller granules which contain bactericidal substances. It is a scavenging cell and is phagocytic.

The humoral (i.e. non-cellular) components of the immune system comprise mainly immunoglobulins, which recognise the epitopes of foreign antigens, and complement components, which are involved in foreign cell lysis and the mobilisation (by chemotactic attraction) of cells of the immune system.

For further general details on the subject of immunology see Klein (1979) or Roitt (1980).

1.2 The Macrophages

It was a century ago that Metchnikoff discovered that during the inflammatory response certain leukocytes engulf micro-organisms by a process he termed 'phagocytosis' (Metchnikoff, 1901), phago meaning to eat, and cyte meaning blood cell.

Mononuclear phagocytes arise in the bone marrow from a pluripotential stem cell common to all of the haematopoietic cells, including erythrocytes, megqkaryocytes, granulocytes and mononuclear

phagocytes (see Figure 1.1). As the stem cell becomes more committed through progressive cell divisions, the mononuclear phagocytes and the granulocytes continue to share a common committed stem cell. The first progenitor cell identifiable as specific for the mononuclear phagocyte system is the monoblast. In the mouse, each monoblast divides once, giving rise to the promonocytes (Table 1.1). The promonocytes mature into monocytes which are released into the blood from the bone marrow. The monocytes are circulating mononuclear phagocytic cells, and have a half-life in the blood of approximately 22 hours in the mouse (Table 1.2). The monocytes migrate from the blood into the tissues and serous cavities (Tables 1.1 and 1.2) where they will mature into macrophages. In general, monocytes do not re-enter the bloodstream. During inflammation, the rate of monocyte formation in the bone marrow is increased (Table 1.2). Macrophages are found in most tissues [Table 1.1; Lee et al., (1985)] but the tissues that possess large numbers of macrophages include the liver (Kupffer cells), lung (alveolar macrophages) and the serous cavities (peritoneal and pleural macrophages), (Table 1.2). During inflammation, both the influx of blood monocytes from the blood and the local rate of proliferation of immature macrophages increase markedly.

Macrophages are one of the first cells of the immune system to be produced during development of the foetus, but the major role of the macrophage, at this time, is not related to immunity. Tissue resorption, as illustrated by the disposal of senescent neurones and axons following programmed cell death during embryonic development, may be the important function for macrophages

Table 1.1 Cells of the mononuclear phagocyte system in normal and inflamed tissues

Adapted from Van Furth (1980), & Werb (1984)

Cells	Localisation
Stem cells	Bone marrow
↓	
Monoblasts	Bone marrow
↓	
Promonocytes	Bone Marrow
↓	
Monocytes	Bone Marrow/Blood
↓	
Macrophages	Tissues
Normal State	
Histiocytes	Connective tissues
Alveolar macrophages	Lung
Kupffer cells	Liver
Pleural and peritoneal macrophages	Serous cavities
Osteoclasts	Bone
Microglial cells	Nervous system
Synovial type A cells	Joints
Free and fixed tissue macrophages	Spleen, lymph nodes, bone marrow and other tissues
Inflammation	
Exudate macrophages	Any tissue
Activated macrophages	Any tissue
Elicited macrophages	Any tissue

Table 1.2 Production and fate of mononuclear phagocytes of the mouse

Adapted from Werb (1984).

Cell	Property	
Monoblast	Pool size	2.5×10^5.Kg
	Cell cycle time	12 h
Promonocyte	Pool size	5×10^5/Kg
	Cell cycle time	16 h
	Percentage of nucleated marrow cells	0.25%
Monocyte	Pool size in marrow	2.5×10^6
	Production rate, basal	0.6×10^5/h/Kg
	Production rate, inflammation	1×10^5/h/Kg
	Time in marrow	2 h
	Pool size in blood	1×10^6/ml
	Half-time in blood	22 h
	Pool size in the capillaries	3-4 times Circulating pool
Tissue macrophages		
Liver	Steady-state distribution from blood (as percentage of monocyte's steady state)	56
Alveoli		15
Peritoneum		8
Other		21
	Time in tissue	8-60 days

derived from early haematopoetic activity. Recent work has indicated that bone-marrow stromal macrophages may be involved in the process of stimulating proliferation and differentiation of some intermediate-stage cells within the marrow, which are derived from haematopoietic progenitor cells. These recently-proposed functions of the macrophage have been reviewed by Gordon (1986).

In discussing the macrophage as a member of the immune system the major areas for discussion are as follows: endocytosis, secretory activity, resident, inflammatory and activated macrophages; metabolism; and the role of the macrophage in the immune response. These will be covered in separate sections.

1.2.1 Endocytosis

Endocytosis (phagocytosis and pinocytosis, see below) is one of the most important properties of the macrophage. Pinocytosis (pino: to drink) is the ingestion of both solute and solvent from interstitial fluids by the formation of micropinocytic vesicles, which are usually 0.2 μM in diameter, or by the formation of macropinocytic vesicles, which are 1-2 μM in diameter. Micropinocytosis appears to occur continuously in culture, whereas macropinocytosis is more intermittent.

Phagocytosis is stimulated when insoluble particles are bound to specific receptors (e.g. receptors that can bind the Fc portion of immunoglobulin, or C3b, produced from the complement component C3 via activation of the complement pathway by bacteria etc.) or non-specific receptors on the plasma membrane. Antibody-coated sheep erythrocytes are phagocytosed via the specific receptor or the opsonin-dependent pathway, whereas polystyrene or

latex particles are phagocytosed via the non-specific receptor or opsonin-independent pathway. The binding of a particle to the macrophage, and its ingestion, are distinct processes, so that binding does not necessarily lead to subsequent phagocytosis. For ingestion to occur, the particle is circumferentially surrounded by the membrane of the phagocyte, in a process termed zippering. The phagocytic vesicle so formed is generally >0.4 μM in diameter. The process of phagocytosis has been reviewed by Silverstein and Loike (1980). Phagocytic vesicles fuse with lysosomes, discharging their contents into the newly formed phagolysosome for digestion. Membrane proteins may be recycled back to the plasma membrane after the formation of the phagolysosome (see Muller et al., 1980). However, it is unclear how much of the plasma membrane lipids utilised for the formation of the phagocytic vesicle are returned to the plasma membrane intact after fusion with the lysosome, or are subsequently digested and resynthesised (or newly synthesised) elsewhere, and then incorporated into the plasma membrane.

1.2.2 Secretory activity

Although the phagocytic activity of macrophages has been known for a century, only over the last 10-15 years has the secretory activity of macrophages been recognised. More than 50 secretory products have now been identified (Table 1.3). Of these only lysozyme is produced in large amounts and only lysozyme and complement components appear to be secreted continuously. The secretion of other products such as prostaglandins, acid hydrolases and neutral proteinases is stimulated via specific cell surface receptors, by endocytosis, or by exposure of macrophages to

Table 1.3 Secreted products of macrophages

Adapted from Werb (1984)

Enzymes

Neutral proteinases

- Plasminogen activator
- Elastase
- Collagenases
- Cytolytic proteinase

Arginase
Lysozyme
Lipoprotein lipase
Angiotensin-converting enzyme
Acid hydrolases
- Proteinases and peptidases
- Glycosidases
- Phosphatases
- Lipases
- Others

Plasma Proteins

α_2-Macroglobulin
Fibronectin
Transcobalamin II
Apolipoprotein E
Coagulation proteins
- Tissue thromboplastin
- Factor V
- Factor VII
- Factor IX
- Factor X

Plasma proteins (cont.)

Complement components

- C1
- C2
- C3
- C4
- C5

Reactive metabolites of oxygen

- Superoxide anion
- Hydrogen peroxide
- Others

Bioactive lipids

Prostaglandin E_2
6-ketoprostaglandin $F_{1\alpha}$
Thromboxane B_2
Leukotriene C
12-hydroxyeicosatetranoic acid

Nucleotides

cAMP
Thymidine
Uracil
Uric acid

Factors regulating cellular functions

Interleukin-1
Angiogenesis factor
Interferon
Erythropoietin

endotoxin or drugs that are known to act on the cell membrane (e.g. ionophores). Secretory activity can also be stimulated by activated lymphocytes, changes in tissue pH or by changes in oxygen tension.

The secreted acid hydrolases and neutral proteinases have inflammatory functions; lysozyme and arginase have antimicrobial functions; complement components have both antimicrobial and inflammatory functions; coagulation factors are utilised for blood coagulation; interferon has antiviral activity; interleukin-1 is immunoregulatory (see later); superoxide ($O_2^{-\cdot}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$) and singlet oxygen ($^1O_2$) all have antimicrobial and antitumour functions; and prostaglandins have inflammatory and immunoregulatory functions.

The compounds secreted by macrophages and their physiological functions have been reviewed by Takemura and Werb (1984).

1.2.3. Resident, inflammatory and activated macrophages

Resident macrophages are tissue macrophages that have not met foreign materials and have low functional activities e.g. low rates of secretion of neutral proteinases or production of reactive metabolites of oxygen. When resident macrophages are exposed to lymphokines (substances released by activated lymphocytes) they show enhanced antimicrobial activity, increased capacity to kill tumours, they spread faster on glass, ruffle their membranes more prominently, increase their size, increase their content of lysosomes and show a higher rate of phagocytosis. These macrophages have been termed activated. Macrophages that are obtained from animals which have been treated with non-microbial inflammatory

stimuli which do not stimulate lymphocytes to secrete lymphokines (such as Brewer's thioglycollate broth, caseinate or peptone solutions) display a similar but not identical pattern of changes (Table 1.4). They show many of the same metabolic, phagocytic, plasma membrane enzyme and lysosomal changes as described for activated macrophages. However, they lack enhanced antimicrobial and antitumour activity. These macrophages are referred to as inflammatory or elicited.

Activation of macrophages occurs as a result of their interaction with mediators from antigen- or mitogen-stimulated lymphocytes (known as lymphokines), with the products of activation of complement components or with interferon. Activation of macrophages may also occur by direct interaction with agents such as endotoxin. In contrast to the immunologic activation of T and B lymphocytes, the activation of macrophages is not specific to the primary infecting organism

Activated or elicited macrophages can be obtained by injecting suitable material (live or dead microbial organisms for activated, non-specific inflammatory agents such as thioglycollate broth for elicited macrophages) into a tissue or serous cavity (e.g. peritoneal cavity) and, after 1-7 days, digesting the tissue with collagenase, or washing out cells from the serous cavity, then purifying the macrophages by adherence to plastic cell culture dishes (see Cohn, 1974).

For reviews on changes occurring upon macrophage activation, see Cohn, 1978; Karnovsky and Lazdins, 1978; North, 1978.

Table 1.4 Activity of inflammatory macrophages compared to that of quiescent residence macrophages

Adapted from Werb (1984).

Non-specific inflammatory events	
Increased size	
Increased rate of spreading	
Increased adherence to glass	
Increased rate of phagocytosis	
Increased rate of fluid phase pinocytosis	
Increased rate of secretion of:	
Elastase	
Collagenase	
Plasminogen activator	
O_2^-	
Increased cellular ATP concentration	
Increased rate of utilisation of oxygen	
Increased rate of prostaglandin release	
Decreased rate of leukotriene C release	
Lymphokine-mediated events	
Increased H_2O_2 release	
Increased microbicidal acitivity	
Increased ability to kill tumours.	

1.2.4. Metabolism

Almost all metabolic studies concerning the macrophage have focussed on events associated with phagocytosis or the respiratory burst. The respiratory burst is characterised by activation of a membrane-associated oxidase and an increase in oxygen consumption. This oxidase is dependent upon NADPH as an electron donor. Rossi et al. (1985) have reviewed the properties and localisation of NADPH oxidase. The oxidase system reduces molecular oxygen to superoxide anion, which dismutates to hydrogen peroxide. Superoxide and hydrogen peroxide can interact to give rise to the hydroxyl radical and possibly singlet oxygen. The reactive metabolites of oxygen that are generated at or near the cell surface and within the phagocytic vacuole exert antimicrobial and antitumour effects (see Nathan, 1980, for review concerning the toxicity of H_2O_2). The macrophage itself is protected from the toxicity of reactive oxygen metabolites by glutathione peroxidase and catalase (Rossi et al., 1980) glutathione peroxidase activity results in the oxidation of reduced glutathione. Oxidised glutathione is reduced by the action of glutathione reductase, utilising NADPH as a source of reducing equivalents: NADPH formation is therefore important in these cells.

NADPH is generated in the pentose phosphate pathway, which is stimulated either by the process of phagocytosis accompanied by the respiratory burst or the respiratory burst itself (see Rossi et al., 1980). However, the respiratory burst, although intimately associated with phagocytosis is not an essential accompaniment to phagocytosis. Many soluble agents including antigen-antibody

complexes, C5a (a protein of the complement pathway) and ionophores, can trigger the respiratory burst without phagocytosis. Phagocytosis can also proceed without the respiratory burst. The release of superoxide from macrophages in different states of stimulation has been measured accurately by Johnston et al. (1978). Karnovsky et al. (1970), using guinea pig elicited macrophages reported that, when phagocytosis was stimulated, approximately 20% of the total oxygen consumption was utilised for the generation of reactive species of oxygen; and also phagocytosis was not inhibited by mitochondrial respiration inhibitors such as CN^- or dinitrophenol but it was inhibited by glycolytic inhibitors such as F^-, iodoacetic acid and, as reported by Michl et al. (1976a), 2-deoxyglucose. From these results it was concluded that glycolysis, but not mitochondrial respiration, was essential for phagocytosis. However, 2-deoxyglucose and F^- were later shown to inhibit phagocytosis by mechanism(s) unrelated to their effects on glycolysis (Michl et al., 1976b; Sung and Silverstein, 1985) so that the fuel used to support phagocytosis is still not known.

Of the possible fuels available to the macrophage, the metabolism and fate of only glucose has been investigated. The metabolism of the fuel during phagocytosis has been shown to differ from that in the resting cell. Karnovsky et al. (1970) report an approximately 10-fold increase in rates of conversion of [1-^{14}C]- and [6-^{14}C]-glucose to $^{14}CO_2$ during phagocytosis in guinea pig elicited macrophages, whereas the rate of production of lactate did not change. This suggests either that the flux through the overall pathway of glucose utilisation plus the TCA cycle has been increased

by phagocytosis or that the increased flux through the TCA cycle is small compared to the overall flux through the glycolytic pathway so that the small change in rate of lactate production would not be easily detected. Karnovsky et al. (1975), using either murine resident, activated or elicited peritoneal macrophages, reported increases in rates of oxygen consumption (approximately 2-fold), conversion of [1-^{14}C]-glucose to $^{14}CO_2$ (approximately 4-fold) and conversion of [6-^{14}C]-glucose to $^{14}CO_2$ (approximately 4-fold) by phagocytosing compared with resting cells. Similar results concerning glucose oxidation were reported by Stubbs et al. (1973) for murine peritoneal resident and activated macrophages.

Loike et al. (1979) have shown that macrophages contain creatine phosphate and that the concentration of this compound decreased during phagocytosis in both resident and elicited macrophages. They suggested that this compound provided considerable energy for this process

An important metabolic study, unrelated to phagocytosis, measured the catalytic activities of three enzymes, 6-phosphofructokinase, pyruvate kinase, and cytochrome oxidase in alveolar and peritoneal macrophages (Simon et al., 1977). The activity of cytochrome oxidase was higher in alveolar macrophages but those of 6-phosphofructokinase and pyruvate kinase were lower, which suggests that these macrophages depend more on aerobic metabolism. The alveolar macrophage is situated in areas of high oxygen tension while the peritoneal macrophage is situated in an area of low oxygen tension. When the alveolar macrophages were cultured in anaerobic conditions for 96 h, these activities changed

such that they resembled those in the peritoneal macrophages. Thus it appears that the tissue macrophages, which have a common precursor cell, the monocyte, can adapt metabolically to their particular environment, and, the enzymatic adaptions of a macrophage to its environment may be reversible.

1.2.5. The role of the macrophage in the immune response

Macrophages play an important role in the initiation and regulation of the immune response. Macrophages can process and present antigen to lymphocytes. This function requires that the T cell and macrophage display the same major histocompatibility-encoded determinants (that is Ia antigens). However, it should be noted that not all macrophages express Ia antigen.

Macrophages may stimulate or suppress the proliferation of lymphocytes depending on the products secreted. Interleukin-1 is produced and secreted by macrophages (Durum et al., 1985; Kampschmidt, 1985), and this compound is required for the expansion of an activated T cell pool. A number of secreted products of macrophages may suppress the proliferation of lymphocytes e.g. interferon (Lucas and Epstein, 1984), prostaglandin E (Stenson and Parker, 1980) and arginase (Kung et al., 1977), but the mechanism(s) of suppression is complex and rarely involve direct action of the above compounds on proliferating lymphocytes.

1.3. The type of macrophage used for the experiments reported in this thesis

Study of substrate utilization and end-product formation requires a large number of cells to be used for many incubations.

These cells should be from a homogeneous population. Thioglycollate-elicited peritoneal macrophages from mice were used since they can be obtained fairly easily in reasonable yields (approximately 5-10-fold in excess of the resident population) and they are a homogeneous, stable and well characterised cell population (Cohn, 1978). The viability of the cultured cells was found to be high (see Chapter 4) and if removed from culture, maintained a high degree of viability (both biological, as measured by trypan blue exclusion, and biochemical, as measured by ATP, ADP and AMP concentrations -see Chapter 3).

The employment of cell culture methods in purifying and maintaining a population of macrophages was ideal for the work reported in this thesis. Eagle (1959) has referred to the advantages of cell culture for metabolic studies as follows: "...the opportunity is presented for the study of metabolism at the cellular level ..... The cells and the medium can be separately analysed, balance experiments can be set up, metabolic processes can be examined qualitatively and quantitatively under controlled conditions, and the corresponding enzymic activities can be explored in cell-free extracts."

Since the macrophage is purified simply and easily and is readilly maintained with a high degree of viability with the use of cell culture techniques it was logical to utilise this system for the study of macrophage energy metabolism. The aim of the work carried out for this thesis was to study systematically the rates of utilisation and fates of a number of possible fuels for the macrophage. The ideas proposed by Eagle (1959) and quoted above,

were made use of in this work. Fuels that may be available *in vivo* and a brief introduction to energy metabolism are considered below.

1.4. Energy supply and fuel utilisation in cells

Energy is required by cells for a large number of processes e.g. the maintenance of cellular structure and ion gradients, for biosynthetic reactions and the regulation of metabolic process (all of which occur in resting cells). The formation of biologically useful energy in cells occurs through the ATP/ADP cycle. Energy released during the oxidation of certain substances, which, by analogy with man-made machines, can be termed fuels (e.g. glucose, fatty acids), is transferred to ATP by phosphorylation of ADP in specific reactions. The hydrolysis of ATP to ADP then releases this energy which can be coupled to ion transport, biosynthetic reactions, etc. In this way energy yielding and energy-requiring processes are energetically linked, and the link in the transfer of chemical energy is the ATP/ADP coupled system (Figure 1.2).

To maintain the steady-state concentration of ATP, the rate of utilisation must equal the rate of synthesis. That this occurs is demonstrated by the many observations that the ATP concentration remains remarkably constant in various tissues (e.g. muscle) despite dramatic variations in metabolic activity (see Newsholme and Start, 1973). These observations strongly suggest the existence of specific mechanisms that regulate the rate of ATP formation to that of its utilisation. The various types of metabolic mechanisms that exist for provision of sensitive regulation include the following: effects of substrates and

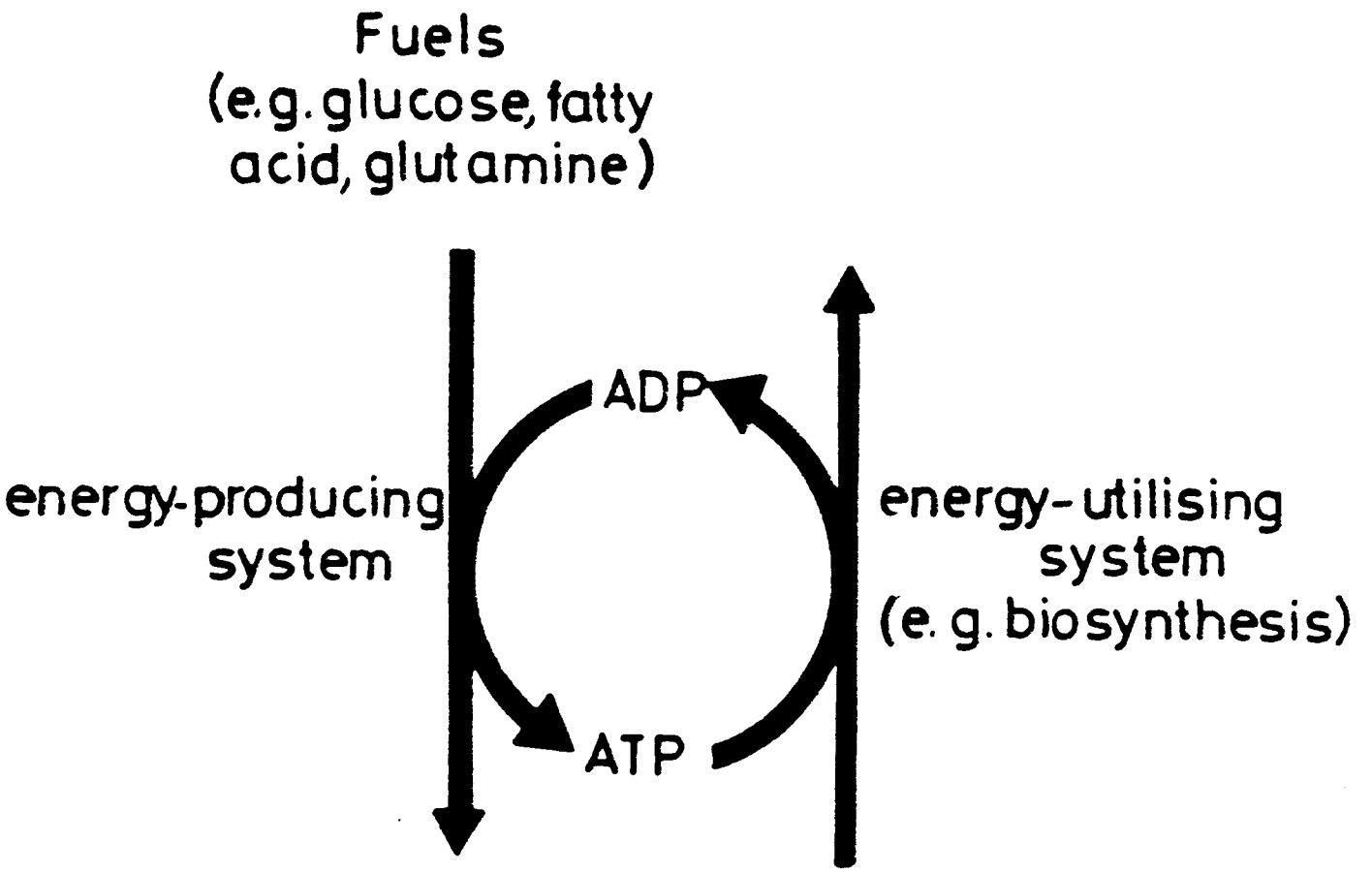


Figure 1.2 ATP as an energy transfer compound

products on enzymes that catalyse near-equilibrium reactions; effects of substrates or allosteric effectors on enzymes that catalyse non-equilibrium reactions either directly or indirectly via substrate or interconversion cycles (for reviews see Newsholme and Crabtree, 1973; 1976; 1979; Crabtree and Newsholme, 1975; 1978; Newsholme, 1977; 1978). Near- and non-equilibrium enzymes are further discussed in Appendix A.

In general, tissues of the body are able to utilise a variety of fuels for the production of biological energy in the form of ATP . These include glucose, ketone-bodies, fatty acids and some amino acids (see Table 1.5). They are obtained either from the diet or from storage molecules such as glycogen, tracylglycerol and protein. The most important metabolic pathways through which these fuels are degraded to yield ATP are shown in Figures 1.3, 1.4 and 1.5. It is evident that the TCA-cycle plus electron transport chain is responsible for the complete oxidation of all the fuels shown. Carbohydrates, fats and proteins must first be partially degraded before they enter the TCA cycle. The fuels that circulate in the blood and are therefore available for all cells of the body are mainly glucose, triacylglycerol, fatty acids, ketone bodies and amino acids. Each of these fuels can be converted into acetyl CoA within the cell (via specific metabolic pathways) for oxidation in the TCA cycle.

Muscle is used to illustrate the utilisation and metabolism of such fuels since most work has been done on this tissue.

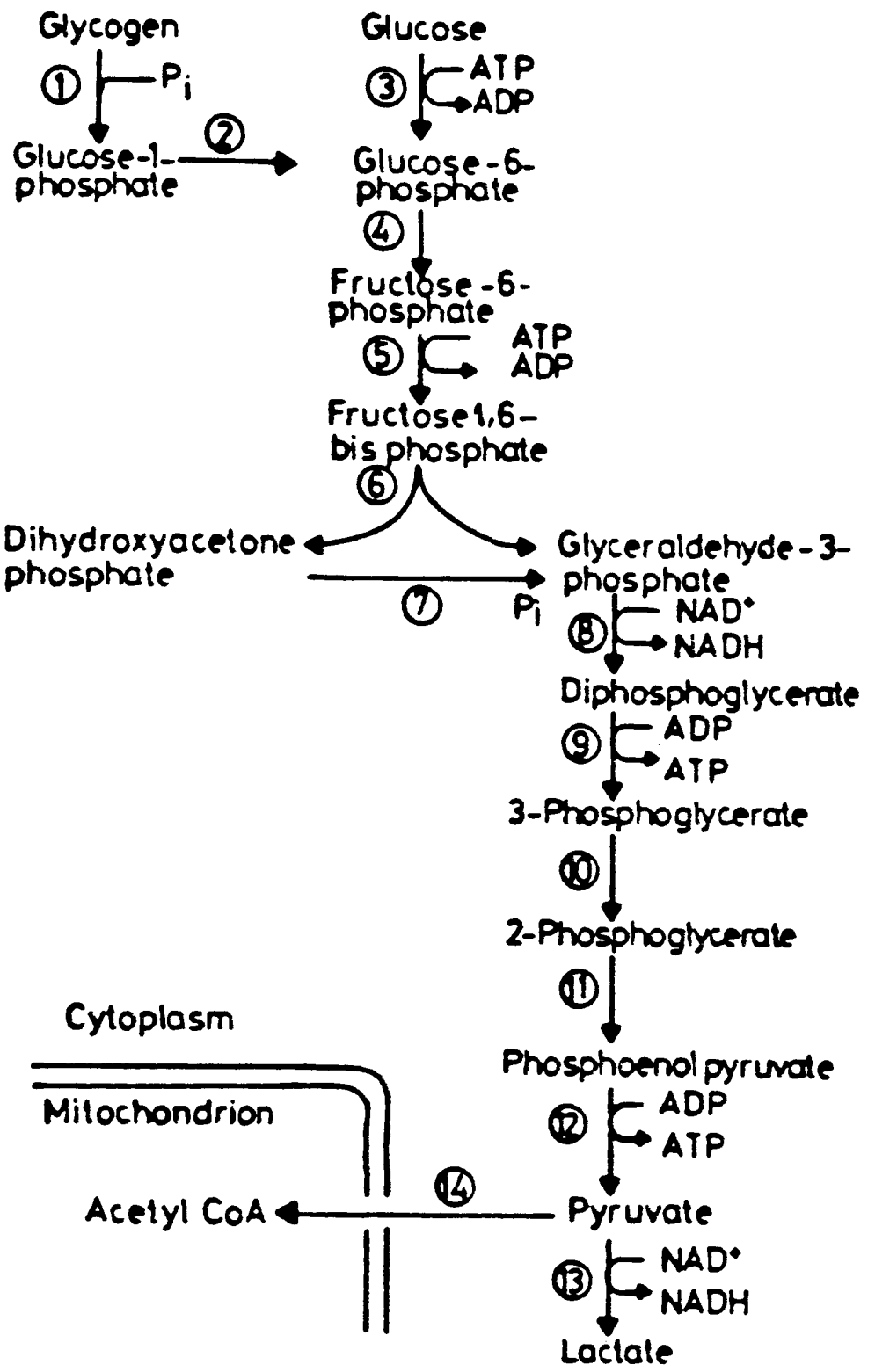


Fig. 1.3 The glycolytic pathway

1. Phosphorylase; 2. phosphoglucomutase; 3. hexokinase; 4. phosphoglucoisomerase; 5. phosphofructokinase; 6. aldolase; 7. triosephosphate isomerase; 8. glyceraldehyde phosphate dehydrogenase; 9. phosphoglycerate kinase; 10. phosphoglycerate mutase; 11. enolase; 12. pyruvate kinase; 13. lactate dehydrogenase; 14. pyruvate dehydrogenase.

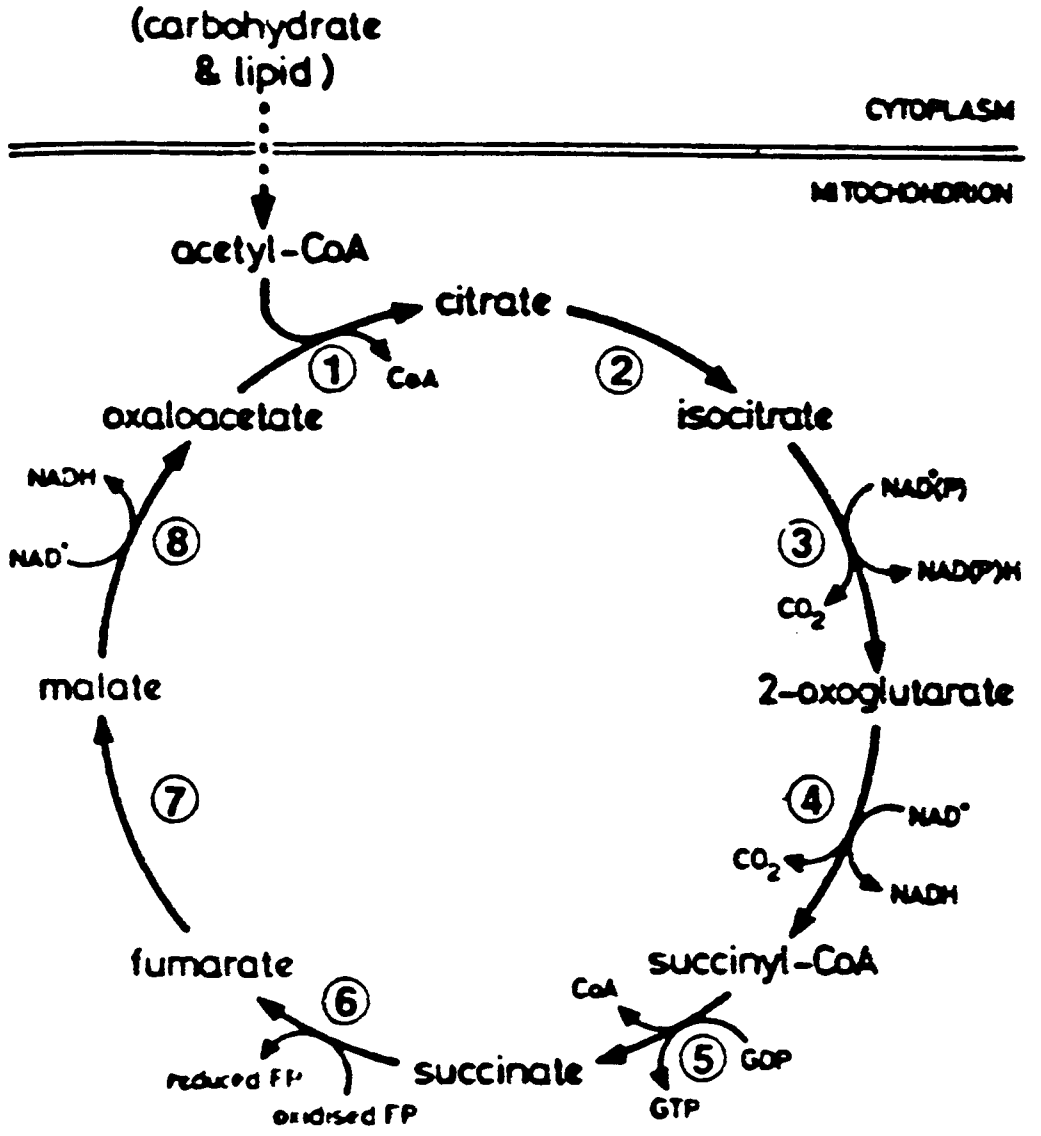


Fig. 1.4 The tricarboxylic acid cycle

1. Citrate synthase, 2. aconitase; 3. isocitrate dehydrogenase;
4. oxoglutarate dehydrogenase; 5. succinyl thiokinase;
6. succinate dehydrogenase; 7. fumarase; 8. malate dehydrogenase.

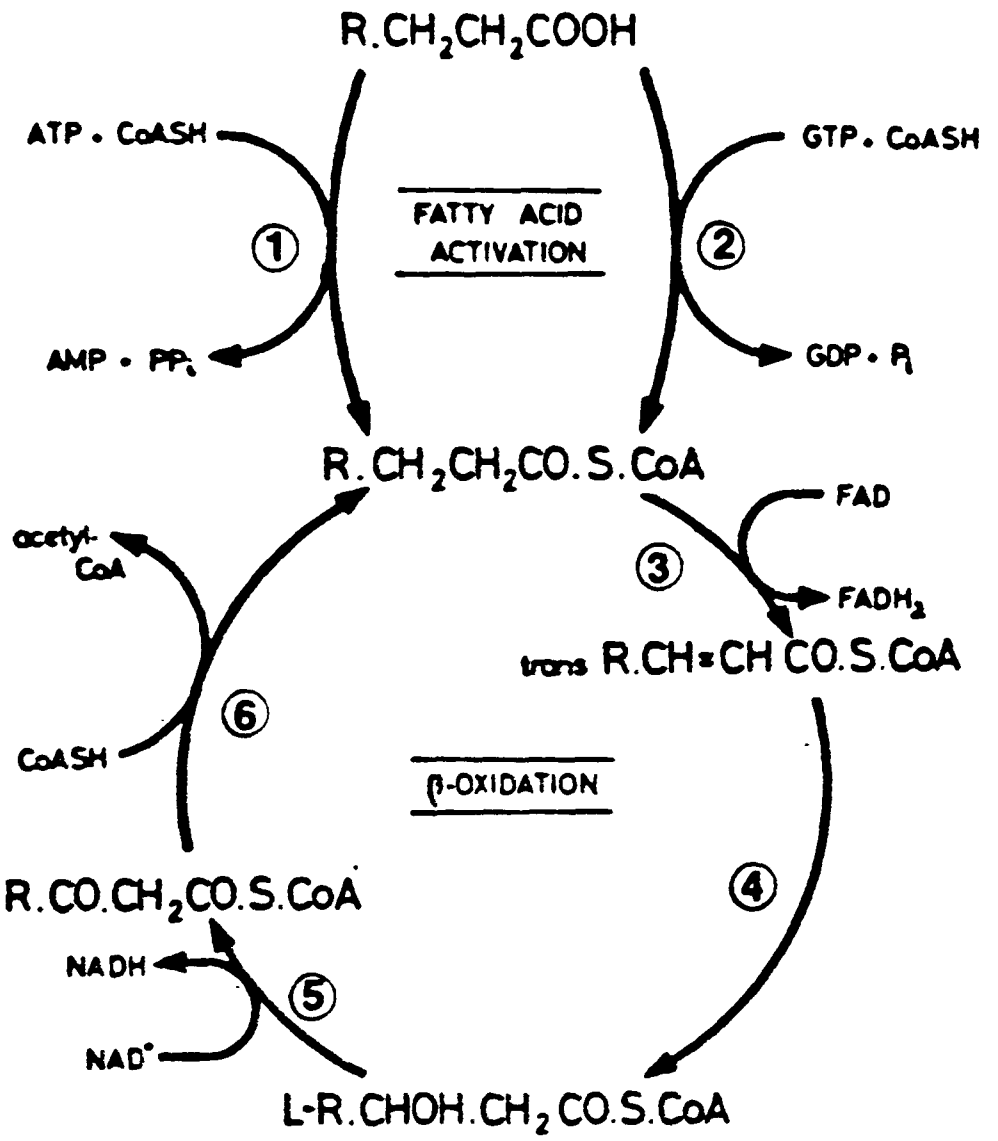


Fig. 1.5 The β-oxidation pathway for fatty acid oxidation

1. ATP-dependent fatty-acyl-CoA synthetase;
2. GTP-dependent fatty-acyl-CoA synthetase;
3. flavoprotein-linked acyl-CoA dehydrogenase;
4. enoyl hydratase;
5. 3-hydroxyacyl-CoA dehydrogenase;
6. β-ketothiolase.

TABLE 1.5 Yield of ATP from catabolism of different metabolic fuels

Fuel	Conditions	ATP yield (moles/mole of fuel utilised)
Glucose	Complete oxidation	38
Glucose	Lactate production	2
Glycogen	Complete oxidation	39
Glycogen	Lactate production	3
Palmitate	Complete oxidation	129
Oleate	Complete oxidation	147
Acetoacetate	Complete oxidation	24

1.4.1. Carbohydrate fuel

The importance of carbohydrates as fuels has been recognised for many years. The form of carbohydrate available to tissues from the blood is usually glucose (exceptions are found for example in insects, many of which contain trehalose in the blood). The endogenous store of carbohydrate is glycogen. Early work on muscle contraction demonstrated that lactate is produced and that it originated from glycogen with the muscle (Fletcher and Hopkins, 1907; Parnas and Wagner, 1914). Later glucose was found to be also degraded by muscle (Best et al., 1926a,b). This established the importance of both glucose and glycogen in muscle contraction and made it possible to understand that the degradation of glucose and glycogen occur through the same pathway. The work of Embden, Meyerhof and Parnas in the 1920's and 1930's (see Needham, 1971) elucidated the reactions that constitute this pathway of glycolysis. Subsequent work has confirmed the importance of glucose and glycogen as fuels for musclular activity in various types of muscle of many species (see Sacktor, 1965; Randle et al., 1966; Crabtree and Newsholme, 1975).

Carbohydrate (i.e. glycogen or glucose) is the only fuel which can be utilised by certain tissues (see Krebs, 1972) to generate ATP (phosphocreatine can also be considered in this category, but the store in cells is very limited). Glycolysis can proceed anaerobically to form lactate, but only a small fraction of the energy available from the complete oxidation of carbohydrates is made available during anaerobic glycolysis. Hence the latter is an inefficient means of ATP formation (see Table 1.5). However,

glycogen is an important fuel for fast twitch, type IIB (white muscle fibres) and is metabolised mainly to lactate. These fibres have a low oxidative and a high glycolytic capacity (as indicated by the maximal activities of glycogen phosphorylase and 6-phospho-fructokinase (see Section 1.6 and discussion section of Chapter 2). Glucose is an important fuel for the slow twitch type I (red) and the fast twitch type IIA muscle fibres. Type I fibres have a high oxidative capacity, and type IIA fibres have a high oxidative and high glycolytic capacity (as indicated by the maximal activities of glycogen phosphorylase, 6-phosphofructokianse, enzymes of the TCA cycle, and enzymes of the fatty acid oxidation pathway, see Newsholme et al., 1978; Newsholme and Leech, 1983).

1.4.2. Fat fuels

Adipose tissue triacylglycerol is the major fuel reserve in man and in most terrestrial animals. In vertebrates, this is made available to tissues as free fatty acids which are released into the blood by adipose tissue following the intracellular hydrolysis of triacylglycerol (lipolysis). Since fatty acids are almost insoluble in water, they are transported in the blood bound to albumin (see Spector et al., 1969). Fats are also transported as triglycerides in the form of lipoprotein particles which originate either in the intestine from dietary fat (chylomicrons) or from endogenous fat synthesis (VLDL) in the liver. The use of this fuel requires an extracellular lipase, known as lipoprotein lipase, in the tissue (Robinson, 1970). The first indication of direct oxidation of fats in animal tissues became apparent from the results of observations on the value of the respiratory exchange ratio

(respiratory quotient) of the whole animal (Evans, 1914; Richardson et al., 1930; Cruickshank and Kosterlitz, 1941). Evidence that free fatty acids may serve as fuels for muscle have been reviewed by Fredrickson and Gordon (1958); George and Berger (1966); Drummond (1967) and Ekelund (1969).

Ketone bodies are acetoacetate and 3-hydroxybutyrate. The synthesis of ketone bodies involves the cooperation of two tissues, adipose tissue and the liver. Fatty acids are first mobilised from adipose tissue into the blood and then are taken up by the liver where they are converted into ketone bodies via the β-oxidation pathway and the HMG-CoA cycle - the whole process is known as the pathway of ketogenesis (see Newsholme and Leech, 1983). The ability of tissues to utilise ketone bodies in vitro has been demonstrated, e.g. in the perfused rat heart and incubated diaphragm (Williamson and Krebs, 1961; Randle et al., 1966) and skeletal muscle (e.g. Beatty et al., 1964; Maizels et al., 1977). The role of ketone bodies may be to provide a fat- fuel that is much more soluble than fatty acids so that they can be transported in the blood at a free concentration similar to that of glucose so that they can compete for utilisation with glucose by tissues such as brain, muscle and intestine (see Newsholme and Leech, 1983). Ketone bodies become particularly important in long term starvation especially in man (see Williamson and Hems, 1970; Krebs et al., 1971; Robinson and Williamson, 1980; Newsholme and Leech, 1983).

1.4.3. Amino acids

Until recently, amino acids were not thought to be very significant as fuels. Oxidation of amino acids to yield energy for

contraction is thought to be insignificant in most muscles (Hicks and Kerly, 1960; Williamson and Krebs, 1961; Newsholme and Randle, 1964; Randle et al., 1966). Oxidation of amino acids is known to occur in the liver but it is considered that this process is one which is used to remove amino acids in excess of those required for protein synthesis. The physiological significance of amino acid metabolism in skeletal muscle of mammals is now considered to be not so much complete oxidation but the ability to convert some of these amino acids to alanine and glutamine (Felig, 1975; Snell, 1980). This is further discussed in the section below concerning glutamine metabolism.

1.4.4. Other fuels

Lactate and acetate are oxidised by certain muscles (Randle et al., 1966; 1968). Glycerol, a product of complete hydrolysis of triacylglycerol, may be oxidised by muscles (Newsholme and Taylor, 1969). However, except perhaps for lactate, and its use by the heart or other red muscle, these fuels are not considered to be quantitatively important.

From the above account, it can be concluded that carbohydrates, fats and ketone bodies are the three major fuels used by muscle. Although carbohydrates can be metabolised under either aerobic or anaerobic conditions, the other two fuels can only be metabolised under aerobic conditions. However, it should be emphasised that the type of fuel used by one muscle varies according to the conditions and the extent and type of muscular acitivity (for review, see Newsholme & Leech, 1983).

1.5. An overview of glutamine metabolism

Since part of the work described in this thesis is concerned with glutamine metabolism in macrophages, a description of the current knowledge of the metabolism of this amino acid is necessary.

The existence of glutamine was first considered by Hlasiwetz and Habermann in 1873 (cited by Meister, 1980) who suggested that the ammonia found in hydrolysates of protein was a result of the hydrolysis of glutamine and asparagine in the protein. The isolation of glutamine from a protein hydrolysate was reported in 1932 by Damadaran et al., after enzymatic hydrolysis of gliadin. The presence in many animal tissues of the enzymes that catalyse the synthesis of glutamine from glutamate plus ammonia and the hydrolysis of glutamine to glutamate and ammonia were first reported by Krebs (1935).

Glutamine is present in many proteins. A finding which suggests it might be an important amino acid is that it is the most abundant (free) amino acid constituent of mammalian plasma and tissues. In man, it accounts for about 20% of the total plasma (free) amino acids with total amount in the body similar to that of glucose (Meister, 1956). It is not a dietary essential amino acid since it can be synthesised in the body. It is considered to be a storage and transport form of both glutamate and ammonia. Thus in the tissue and body fluid, levels of ammonia are maintained at relatively low levels, since elevated levels of ammonia are toxic to many cells. In general, glutamine is transported more effectively across membranes than glutamate; for instance, glutamine readily

crosses the blood-brain barrier, whereas glutamate does not (Bessman et al., 1948; Schwerin et al., 1950).

Glutamine plays a central role in nitrogen metabolism (Meister, 1965). The amide nitrogen of this amino acid is used as a source of purine and pyrimidine ring nitrogens. The amino nitrogens of pyrimidine bases, nicotinamide adenine dinucleotide, glucosamine phosphate, carbamyl phosphate and several other nitrogenous compounds can all be derived from glutamine. The α-amino nitrogen may be transaminated to several ketoacids to form other amino acids such as alanine and serine.

In addition to this nitrogen-donor function, glutamine has several other important metabolic roles in mammals: in the kidney glutamine provides ammonia for acid/base balance (Pitts, 1973); in the brain it is a precursor for the synthesis of the neurotransmitters glutamate and 4-aminobutyrate (Quastel, 1979); in the small intestine it functions as a source of energy and its nitrogen is incorporated into alanine and citrulline. The latter may be used by other tissues, particularly the kidney, for arginine biosynthesis (see Harper, 1986). Alanine is taken up by the liver, and the nitrogen is used for urea synthesis (for review see Windmueller, 1982), and glutamine utilisation and oxidation is considered to be important if not essential for rapidly-dividing cells (for reviews see Krebs, 1980; Kovacevic and McGivan, 1983).

An outline of the metabolism of glutamine in mammalian tissues is presented in Figure 1.6

What is the source(s) of this glutamine for these various tissues and organs? Apart from the glutamine that is produced from

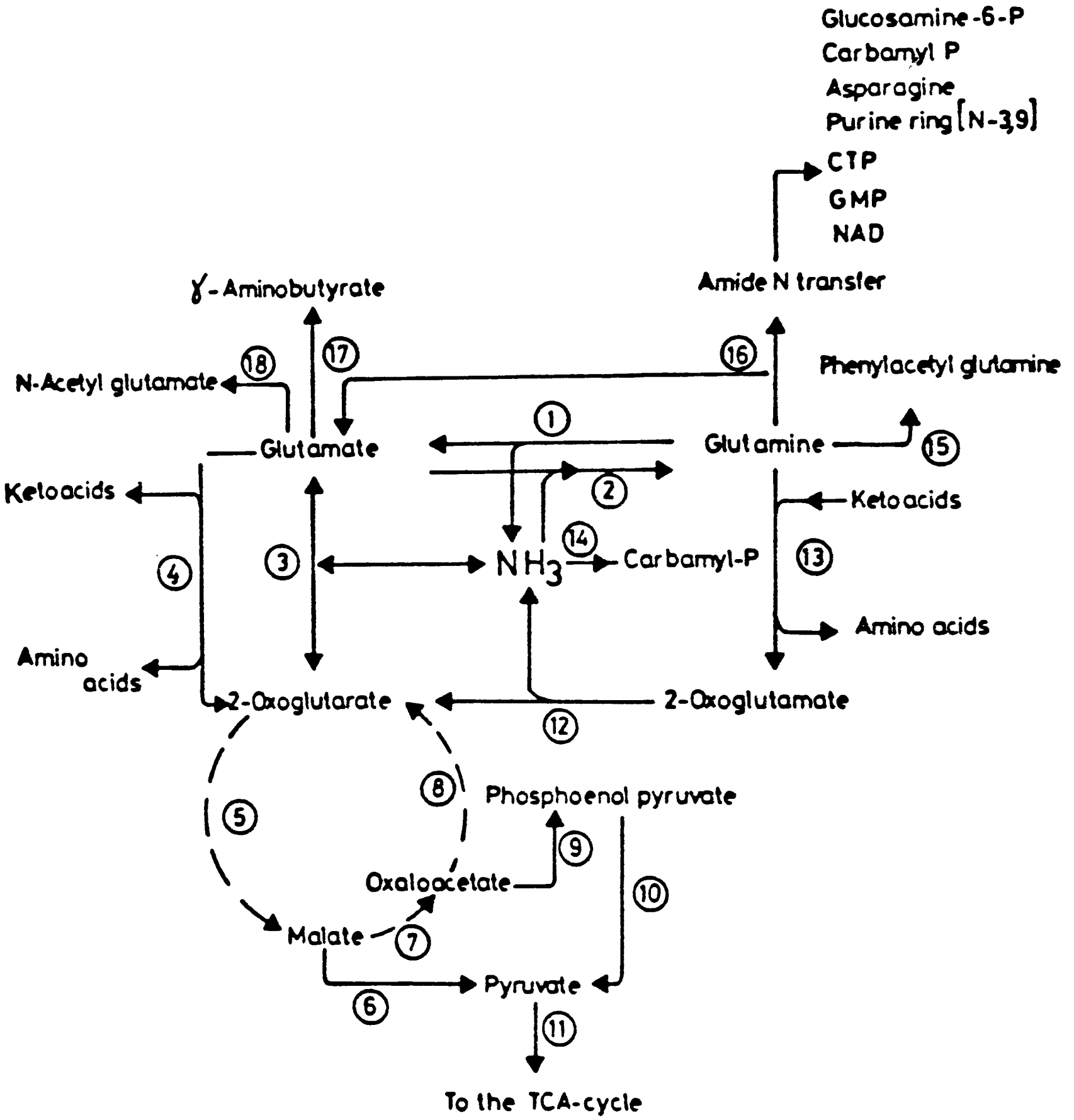

Glucosamine-6-P
Carbamyl P
Asparagine
Purine ring [N-3,9]
CTP
GMP
NAD
Amide N transfer
γ-Aminobutyrate
N-Acetyl glutamate
Phenylacetyl glutamine
Glutamate
Glutamine
Ketoacids
Ketoacids
NH3
Carbamyl-P
Amino acids
Amino acids
2-Oxoglutarate
2-Oxoglutamate
Phosphoenol pyruvate
Oxaloacetate
Malate
Pyruvate
To the TCA-cycle
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18

Fig. 1:6 An outline of glutamine metabolism in mammalian tissues

1. Phosphate-dependent glutaminase; 2. glutamine synthetase 3. glutamate dehydrogenase; 4. transamination; 5. reactions of the TCA-cycle converting 2-oxoglutarate to malate; 6. malic enzyme ($NAD(P^+)$-dependent); 7. malate dehydrogenase (NAD^+-dependent); 8. reactions of the TCA-cycle converting oxaloacetate to 2-oxoglutarate; 9. phosphoenolpyruvate carboxykinase; 10. pyruvate kinase; 11. pyruvate dehydrogenase; 12. α-keto acid ω-amidase; 13. glutamine transaminase; 14. carbamyl phosphate synthetase; 15. phenylacetyl glutamine synthetase; 16. glutamine amidotransferases known to occur in mammalian tissues; 17. glutamate decarboxylase; 18. glutamate N-acylase (adapted from Meister, 1979).

the digestion of dietary protein in the lumen of the small intestine, all of which is metabolised by cells of the small intestine, liver and skeletal muscle are probably the most important tissues in providing glutamine in the blood with skeletal muscle being quantitatively the most important in most conditions.. Measurements of arteriovenous differences of amino acids across forearm or leg muscles in man (see Felig, 1975) and measurements of the pattern of amino acid release from the perfused rat hind-limb of the rat (Ruderman and Berger, 1974) or isolated rat muscle preparations (Odessey et al., 1974; Garber et al., 1976) have shown that the relative proportions of the amino acids released by muscle do not correspond to the fractional amino acid composition of muscle proteins. More than 50% of the total amino acids released by skeletal muscle can be accounted for as glutamine and alanine. Since glutamine is released from skeletal muscle in amounts greater than its occurrence in the muscle protein, it must arise as a result of synthesis within the muscle. Glutamine is synthesised from glutamate and ammonia by the action of the enzyme glutamine synthetase in the following reaction:

$$^{-}OOC\text{-}(CH_2)_2\text{-}CHNH_3^{+}\text{-}COO^{-} + ATP^{4-} + NH_3 \longrightarrow H_2NOC\text{-}(CH_2)_2\text{-}CHNH_3^{+}\text{-}COO^{-} + ADP^{3-} + P_i^{2-}$$

Glutamate and ammonia are not taken up by muscle in appreciable quantities, thus they must be derived from muscle amino acid metabolism. Glutamate is undoubtedly formed from 2-oxoglutarate via transamination. But what is the source of 2-oxo-glutarate and the nitrogen for glutamine synthesis? It has been shown that muscle takes up branched-chain amino acids (leucine,

isoleucine and valine) indeed muscle may be the most important tissue for the metabolism of these amino acids (Miller, 1962). Addition of branched-chain amino acids to the incubation medium of an isolated rat muscle increases the rate of formation and release of glutamine and alanine (Goldberg et al., 1978). These observations led to the suggestion that the nitrogen and some of the carbon of branched-chain amino acids could be used to synthesise glutamine and alanine in muscle (Goldstein and Newsholme, 1976; Snell 1980) and that this was the source of these two amino acids. However, it has been shown, at least in the rat, that branched-chain amino acids are transaminated but not further metabolised since the key enzyme controlling their rate of oxidation (2-oxoisovalerate dehydrogenase) is almost totally in the inactive form in skeletal muscle (Wagenmakers et al., 1984a,b). Hence, it appears that rat muscle takes up branched-chain amino acids primarily to use their nitrogen for the formation of glutamine and alanine. The oxoacids of the branched-chain amino acids are released by muscle (Livesey and Lund, 1980) and metabolised by other tissues (e.g. liver which possesses an active dehydrogenase - see Wagenmakers et al., 1984a,b). For a recent review of this area of amino acid metabolism see Harper (1986).

What is the source of carbon for the synthesis of the penultimate precursor of glutamine, 2-oxoglutarate? Glucose is the most likely candidate. It can provide both oxaloacetate (via pyruvate carboxylase) and acetyl-CoA (via pyruvate dehydrogenase) from pyruvate, and the condensation of these two products will form citrate. Citrate can be metabolised by the reactions of the TCA

cycle to 2-oxoglutarate.

During non-acidotic conditions, most of the glutamine released by skeletal muscle is taken up from the blood by the small intestine. The latter has been shown to use glutamine as an important respiratory substrate (see Windmueller, 1982). One major end-product of glutamine metabolism in the small intestine is alanine (Hanson and Parsons, 1977; Windmueller and Spaeth, 1978; Watford et al., 1979), and the latter is metabolised by the liver; in starvation, most of the carbon of alanine would be converted to glucose.

During acidosis, some of the glutamine released by skeletal muscle will be taken up from the blood by the kidney. In this tissue, glutamine is converted into glutamate in a reaction catalysed by phosphate-dependent glutaminase. The glutamate is then oxidatively deaminated to 2-oxoglutarate via glutamate dehydrogenase. In both these reactions ammonia is produced which enables the kidney to buffer the protons excreted into the tubules. This enables a large quantity of protons to be excreted without substantial loss of other buffers such as phosphate (P itts, 1973). The 2-oxoglutarate formed from glutamine degradation in the kidney is converted into phosphoenolpyruvate which can either be oxidised or converted to glucose via the pathway of gluconeogenesis (for reviews see Goldstein, 1976; Kovacevic and McGivan, 1983).

For further detailed information on glutamine metabolism in mammalian tissues see Meister (1979); Lund (1980); Windmueller (1982); Kovacevic and McGivan (1983).

1.6 Theoretical basis of maximal enzyme activity measurements in vitro.

As part of the study of energy metabolism of the macrophage, the maximal catalytic activities of enzymes likely to be part of energy yielding pathways were measured in vitro. Assay of the activities of enzymes specific to different pathways enables a comparison of the importance of such pathways to be made. However, quantitative information can be obtained only from the activities of certain key enzymes: these enzymes must catalyse reactions far removed from equilibrium in vivo and it must also be demonstrated that the given enzyme acitivity is equivalent to the maximum flux in some tissues (see below). For the theory underlying the identification of such enzymes see Newsholme and Crabtree (1976); Crabtree and Taylor (1978); Crabtree et al., 1979; Newsholme et al. (1980). This method has proved to be particularly useful for comparative studies. The enzyme assays are carried out under optimal conditions of pH, ionic strength, cofactor and substrate concentrations and in the presence of activators required for maximal activity.

There are at least two conditions that must be satisfied before an enzyme activity can be used to provide quantitative information. First, it is necessary to establish which enzymes in the pathway catalyse non-equilibrium reactions (see Newsholme and Start, 1973). Secondly, it is necessary to demonstrate experimentally that the maximum activities of such enzymes in vitro can be used to indicate quantitatively the maximum flux through a reaction. This is done by comparison of the in vitro enzyme

activity with measured or calculated maximum flux through the pathway. A theoretical consideration underlying the use of maximal enzyme activities as indices of maximal fluxes through metabolic pathways is given in Appendix A.

Since the in vitro enzyme activity measurements are carried out under conditions of substrate and cofactor near-saturation of the enzyme, an assumption is implicit in the maximal enzyme activity approach. This is that non-equilibrium enzymes are near-saturated with substrate in vivo when the flux is maximal or close to maximal. From studies of the concentrations of intermediates in several tissues it is known that enzymes catalysing non-equilibrium reactions that are not flux generating in a pathway are not necessarily substrate saturated. In fact the flux-generating step of a particular pathway may be located at a site detached from the tissue under investigation e.g. for the pathway of fatty acid oxidation in muscle, the flux-generating step is triglyceride lipase within adipose tissue (see Newsholme and Leech, 1983). It should also be noted that some reactions which do not approach saturation with substrate in normal conditions of the cell may do so when the flux increases close to the maximal (e.g. hexokinase in the use of glucose in muscle; phosphofructokinase in the use of glycogen in muscle).

In muscle, a number of enzymes have been used to indicate flux through certain major energy-producing pathways (see Table 1.6).

In part of the present work the approach of measuring the maximal catalytic activities of certain key enzyme in vitro to

TABLE 1.6 Enzymes whose maximum in vitro activities may be used as indices of maximum flux through fuel utilisation pathways in muscle

Enzyme	Pathway	Reference
Hexokinase	Glucose uptake and oxidation	Crabtree & Newsholme (1972a) Crabtree & Newsholme (1975)
Phosphorylase 6-Phosphofructokinase	Anaerobic glycolysis	Crabtree & Newsholme (1972a) Crabtree & Newsholme (1975) Zammit & Newsholme (1976)
Pyruvate dehydrogenase	Pyruvate oxidation	Newsholme *et al.* (1980)
Oxoglutarate dehydrogenase	Tricarboxylic acid cycle	Newsholme *et al.* (1980)
Carnitine-palmitoyl transferase	Fatty acid oxidation	Crabtree & Newsholme (1972b) Zammit & Newsholme (1979)
Triacylglycerol lipase	Oxidation of endogenous triglyceride	Crabtree & Newsholme (1975) Zammit & Newsholme (1979)

indicate the maximal flux through some metabolic pathways is applied to macrophages. This is achieved by comparison of the maximal catalytic activities of these enzymes and the similarity to the maximum flux through the given pathways with measured or calculated rates of fuel utilisation by macrophage preparations.

The leucocytes have generally been assumed to utilise glucose to satisfy the major part of their energy requirement (see for example Krebs, 1972). However, a recent systematic study of the fuels utilised by lymphocytes (see Ardawi and Newsholme, 1985a) has indicated that glucose, glutamine, fatty acids and ketone bodies can all be utilised by these cells and all these fuels may contribute significantly to ATP formation. The initial approach to the investigation of fuel metabolism in lymphocytes involved the systematic measurement of both non-equilibrium enzymes and near-equilibrium enzymes in the pathways of major fuel metabolism. The results obtained indicated the possible maximal flux through these pathways, as well as the enzymic components of these proposed pathways. This approach has also been recently applied in investigations of metabolic pathways in neoplastic tissues (Knox, 1976; Weber, 1977a,b; Snell, 1984) liver (Snell, 1984) and foetal tissues (Knox, 1976; Snell, 1984). Thus for the investigation of macrophage fuel metabolism it was considered necessary to measure maximum catalytic activities of the enzymes likely to catalyse key non-equilibrium reactions in the fuel utilisation pathways; this has then been extended by measurement of the rates of fuel utilisation and the fates of these fuels.

The results of enzyme activity measurements and the possible physiological implications of these activities, in the macrophage, are presented in Chapter 2.

CHAPTER 2:

Maximal Enzyme Activites of Energy Metabolism in Macrophages

2.1 INTRODUCTION

Knowledge of the maximal capacity of a metabolic pathway can provide information on the quantitative importance and hence the role of that pathway in a given tissue. In chapter 1 it was explained that an indication of the maximum capacity of a given metabolic pathway may be obtained from a simple biochemical measurement, that is, the maximum *in vitro* catalytic activity of a key enzyme in the given pathway. This approach has been used particularly in muscle: thus it has been shown that the activity of hexokinase provides an indication of the maximal capacity for glycolysis-from-glucose and either phosphorylase or 6-phosphofructokinase provides an indication of glycolysis-from-glycogen (Crabtree & Newsholme, 1972a; Newsholme *et al.*, 1980). Also, the maximum activity of oxoglutarate dehydrogenase is the only enzyme that provides a quantitative index of the maximum capacity of the tricarboxylic acid cycle and hence aerobic capacity in muscle (Read *et al.*, 1977; Cooney *et al.*, 1981; Newsholme & Paul, 1983). A list of such key enzymes for indication of the maximum capacity of various pathways is given in table 1.6.

Despite the fact that some metabolic studies had been carried out on macrophages (see for example Karnovsky *et al.*, 1970, 1975; Simon *et al.*, 1977) none of these studies could claim to be systematic or comprehensive. The question arises how is it possible to obtain, relatively quickly, the information on the metabolic properties of cells or tissues that are metabolically uncharacterised. One means of obtaining this information is from the *in vitro* catalytic activities

of a variety of enzymes including those that are key enzymes as indicated above. A comparison of catalytic activities within the tissue and between different tissues provides information on which some preliminary characteristics of the metabolism can be proposed. This information can then form the basis of further different studies.

This approach has been used in the presence work to provide information on metabolism in the murine macrophage. Since this work followed an earlier study on lymphocytes (see Ardawi & Newsholme, 1985A) it was considered important to extend this earlier work with the lymphocyte for comparison with macrophages. The results are presented and discussed in this chapter.

The following key enzyme activities were measured: hexokinase for the pathway of glycolysis-from-glucose, glycogen phosphorylase for the pathway of anaerobic glycolysis-form-glycogen; oxoglutarate dehydrogenase for the tricarboxylic acid cycle; carnitine-palmitoyl transferase for the pathway of fatty acid oxidation; pyruvate dehydrogenase for the pathway of pyruvate oxidation and glucose-6-phosphate dehydrogenase for the pentose phosphate pathway.

In addition, maximal activities of a number of other enzymes were measured for comparison with other activities reported in this work, and with enzyme activities from other cells and tissues. These activities were as follows:

(i) The glycolytic enzymes; 6-phosphofructokinase, pyruvate kinase and

lactate dehydrogenase.

(ii) The tricarboxylic acid cycle enzymes; citrate synthase, NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenase and NAD^+-linked malate dehydrogenase.

(iii) The activities of some enzymes involved in amino acid and/or pyruvate metabolism; glutamate dehydrogenase, $NADP^+$-linked malate dehydrogenase, phosphoenolpyruvate carboxykianse, pyruvate carboxylase, aspartate aminotransferase and alanine aminotransferase. In particular, the activity of phosphate-depenent glutaminase (hereafter in this chapter termed glutaminase) was measured since it is specific to the pathway of gluamine metabolism, which may be important in the macrophage. Furthermore, it was important to know if glutaminase activity could provide a quantitative indication of the maximum flux through this pathway.

(iv) The activities of enzymes involved in ketone-body metabolism; 3-oxoacid CoA-transferase, acetoacetyl-CoA thiolase and D-3-hydroxybutyrate dehydrogenase.

(v) The pentose phosphate pathway enzyme 6-phosphogluconate dehydrogenase.

(vi) An enzyme likely to be involved in lipid biosynthesis, ATP-citrate lyase.

Although the enzyme activities listed in points (i) to (vi) provide only qualitative information concerning a metabolic pathway, this information nonetheless may be of use in providing more information about a particular pathway.

In this chapter, references are made to enzyme activities measured in tissues of the rat (for which there is much information in

the literature) in comparison to those measured in the murine macrophage. This is justified since absolute activity comparisons are not made, but comparisons of the pattern of enzyme activities within a tissue to those in another tissue or cell type are made. Much useful information may be obtained from such comparisons (see discussion).

EXPERIMENTAL

2.2.1 Preparation of macrophages

Elicited peritoneal macrophages were obtained from 12-16 week-old female mice of the C57 BL/6 strain. Macrophages were elicited by intraperitoneal injection of 1.5 ml of 4% Brewers thioglycollate medium 4 days prior to the harvesting procedure. Mice were killed with diethylether, and peritoneal macrophages immediately harvested as follows; 5 ml of phosphate buffered saline (sterile, pH 7.2-7.4) was injected into the peritoneal cavity, which was then gently massaged using a sterile Pasteur pipette for one minute. Using a sterile, plugged Pasteur pipette the buffer plus suspended cells were removed and placed in a sterile 50 ml plastic centrifuge tube, cooled on ice. Peritoneal cells were pooled from several mice in the plastic centrifugal tube, and were kept at $0^{o}C$.

Each mouse yielded approximately 2×10^{7} cells from the peritoneal cavity. Macrophages were purified by adherence to plastic Petri dishes (see Cohn, 1974) as follows; the cells suspended in buffer were centrifuged at 500 g for 7 min. The cells were then resuspended in Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) foetal calf serum (FCS), 2 mM glutamine and 20 ug gentamicin/ml, and were added to 100 mm tissue culture petri dishes at a density of $2.0 - 2.5 \times 10^{7}$ cells per dish. After incubation for 4 h at $37^{o}C$ in 5% CO_2:95% air, adherent macrophages were washed vigorously with phosphate buffered saline (PBS) three times and then were incubated at 37oC in 5%CO2:95% air in fresh culture medium (described

above) for a period of 24-48 h. This population of cultured cells was at least 94% macrophages (as determined by differential staining, see Johnson et al., 1978). The macrophages were removed from culture, after washing three times with PBS, by gentle scraping into 2-3 mls of PBS using a rubber policeman. The biological viability of these cells was determined to be >85% by exclusion of trypan blue. The cells were centrifuged at 500g for 5 min and resuspended in the relevant extraction buffer for the enzyme to be assayed. The protein content of the culture dishes at the time of assay was 2-4 mg/dish.

2.2.2 Preparation of lymphocytes

Male Wistar albino rats (160-180g) were killed by cervical dislocation and the mesenteric lymph nodes were carefully removed and placed in extraction medium cooled to $0^{o}C$. Quantitatively no significant differences existed between enzyme activities measured in homogenates of lymphocytes extracted from lymph nodes, or homogenates of lymph nodes (Ardawi & Newsholme, 1982). Hence lymph node homogenates were used in all enzyme activity assays, except for pyruvate dehydrogenase.

2.2.3 Preparation of homogenates

Murine macrophages or rat mesenteric lymph nodes were homogenised in a small glass ground homogenizer (1 ml capacity) with 5-10 vol of extraction medium at $0^{o}C$. For all enzymes studied, preliminary experiments established that extraction and assay procedures were such as to produce maximum enzyme activities. The

principles and advice provided by Crabtree et al. (1979) were closely followed in this work.

The whole homogenate was used for enzyme assays without further treatment, except for the following: for the assay of glycogen phosphorylase (E C 2.4.1.1) homogenates were centrifuged at 8000g (in an Eppendorf Micro-centrifuge) for 2 min and the resultant supernatant was used for assay; for the ketone-body-utilising enzymes, 3-oxoacid CoA-trnsferase, acetoacetyl-CoA thiolase and 3-hydroxybutyrate dehydrogenase (EC 2.8.3.5 ,EC 2.3.1.9, and EC 1.1.1.30 respectively). Triton-X-100 was added (to give a concentration of 0.1%) to the homogenates. The homogenates were sonicated (for two separate periods of 2s with a micro-probe of an MSE 100W ultrasonic disintegrator operating at an amplitude of 9 um; the homogenates were maintained at 0-4^{o}C during sonication), after which they were centrifuged for 2 min at 8000g and the resultant supernatants used; for the assay of oxoglutarate dehydrogenase (EC 1.2.4.2) and carnitine palmitoyl-transferase (EC 2.3.1.2.1) it was important to use a mitochondrial preparation of both lymphocytes and macrophages [lymphocytes were homogenized for 2 x 10s at 0^{o}C, using a Polytron homogenizer (PCU-2 at Position 3); macrophages were homogenized in a small ground-glass homogenizer], the homogenate was centrifuged at 300 g for 5 min followed by centrifugation of the resultant supernatant at 8000 g for 2 min and the pellet was resuspended in extraction medium; for pyruvate dehydrogenase (EC 1.2.4.1), macrophages or lymphocytes (prepared as described by Ardawi & Newsholme, 1982) were initially frozen in liquid N_2, then were mixed well in the relevant extraction buffer plus Triton-X-100 (to give a concentration of 0.1%). The

pyruvate dehydrogenase was then fully extracted by freeze/thawing the mixture three times. The resulting homogenate was centrifuged for 30s in a Eppindorf Microfuge at 8000 g. The supernatant was used for the assay of enzyme activity.

The extraction medium for hexokinase consisted of 50 mM-triethanolamine/HCl, 1 mM-EDTA, 2 mM-$MgCl_2$ and 30 mM-mercaptoethanol at pH 7.0 (Crabtree & Newsholme, 1972a). The same extraction medium was used for pyruvate kinase and lactate dehydrogenase, except that the pH was 7.6 and 7.5 respectively. For 6-phosphofructokinase the extraction medium consisted of 50 mM-Tris, 1mM-EDTA and 5mM-$MgSO_4$ at pH 8.2 (Opie & Newsholme, 1967). The extraction medium for phosphorylase consisted of 35mM-glycerol-2-phosphate, 20mM-NaF, 1mM-EDTA and 30mM-mercaptoethanol at pH 6.2 (Cornblath et al. 1963). For the assay of citrate synthase, the extraction medium consisted of 25mM-Tris and 1mM-EDTA at pH 7.4 (Sugden & Newsholme, 1975). For assay of oxoglutarate dehydrogenase, the extraction medium consisted of 250 mM-Mannitol, 5 mM-Tes (potassium salt) and 1 mM-EGTA at pH 7.4 (Cooney et al., 1981). For $NADP^+$-linked isocitrate dehydrogenase, glutamate dehydrogenase, aspartate aminotransferase and alanine aminotransferase, the extraction medium consisted of 50mM-triethanolamine/HCl, 1mM-EDTA, 5mM-$MgCl_2$ and 30 mM-mercaptoethanol adjusted to pH 7.5 with KOH (Sugden & Newsholme, 1975). For the assay of NAD^+-linked isocitrate dehydrogenase, the extraction medium was the same as that used for $NADP^+$-linked enzyme, and this consisted of 20mM-Tris, 10 mM-dithiothreitol, 1 mM-$MgCl_2$, 100 mM-KCl and 250 mM-sucrose at pH 7.6 (Newsholme & Williams, 1978). For both pyruvate carboxylase and

phosphoenolpyruvate carboxykinase, the extraction medium consisted of 300mM-sucrose, 50mM-triethanolamine/HCl and 1mM-EDTA at pH 7.4 and 7.1 respectively (Crabtree et al., 1972). For the assay of 3-oxoacid CoA-transferase and acetoacetyl-CoA thiolase, the extraction medium consisted of 10 mM-Tris, 3 mM-$MgCl_2$, 1 mM-EDTA, 1 mM-mercaptoethanol and 300 mM-sucrose at pH 7.4 (Williamson et al., 1971; Beis et al., 1980). For D-3-hydroxybutyrate dehydrogenase, the extraction medium consisted of 50 mM-triethanolamine/HCl, 1 mM-EDTA, 2 mM-$MgCl_2$ and 30 mM-mercaptoethanol at pH 7.5 (Beis et al., 1980). For the assay of glutaminase, preliminary studies showed that maximal enzyme activities were obtained by using an extraction medium which consisted of 150mM-potassium phosphate buffer (equimolar mixture of K_2HPO_4 and KH_2PO_4), 1 mM-EDTA and 50 mM-Tris at pH 8.6. For the assay of carnitine-palmitoyl transferase, the extraction medium consisted of 100 mM-Tris and 1 mM-EDTA at pH 7.4 (Zammit & Newsholme, 1979). For the assay of pyruvate dehydrogenase, the extraction medium consisted of 50 mM-potassium phosphate (equimolar mixture of K_2HPO_4 and KH_2PO_4), 10 mM-EGTA, 2 mM-dithiothreitol, 1mM- benzamidine, 1mM-phenylmethylsulphonylfluoride and 0.3mM-tosyl-lysylchloromethane at pH 7.0 (Denyer et al. 1986). For the assay of ATP-citrate lyase the extraction medium consisted of 100 mM-Tris, 5 mM-$MgCl_2$ and 1 mM-EDTA at pH 8.0 (Rider, 1983).

For the measurement of enzyme activities reported in this chapter, enzyme assays were performed using similar methods to those reported in the literature (see appendix B). The activities of hexokinase, 6-phosphofructokinase, pyruvate kinase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, citrate synthase and

glutaminase were measured after culture of macrophages for 24 h, 48 h, 72 h, and 96 h. No significant change in activity was observed over this period for any of these enzymes.

2.2.4 Expression of results

All activities are expressed as nmol of substrate utilised/min per mg protein or as umol substrate utilised/min per g dry weight. Protein was determined as described by Bradford (1976). Dry weight was determined by drying a quantity of cells of known protein content at 50°C for 18h. It was found tht 1 mg of macrophage protein was equivalent to 1 mg dry weight. This high value for the protein content may reflect the fact that macrophage proteins probably have little similarity to γ-globulin (the protein used as a standard in the protein assay). However, the protein concentration for a known number of cells is very similar to the value determined by Johnston et al., (1978) using the method of Lowry et al. (1951) and egg lysozyme as a standard.

RESULTS

2.3.1 Glycolytic enzymes

The maximal activities of hexokinase, 6-phosphofructokinase, phosphorylase, pyruvate kinase and lactate dehydrogenase in murine macrophages and some cells of tissues of the rat are presented in Tables 2.1 and 2.2. The activities of all the glycolytic enzymes measured in the macrophage are high, except for phosphorylase; indeed, the activity of hexokinase in macrophages is as high as that in any other major tissue of the body (compare with activities presented in Table 1 of Cooney and Newsholme 1982). The activity reported here for the macrophage is considerably higher than that reported by Kiyotaki et al. (1984); in the present work an ATP-regenerating system is included in the assay to lower the concentration of ADP, which is a potent inhibitor of hexokinase. The activities of macrophage glycolytic enzymes are similar to those reported for heart, intestine and kidney (Table 2.2), tissues which are known to be able to utilise glucose at high rates. Thus the macrophage may also have a high capacity for glucose utilisation.

2.3.2 Activity of pyruvate dehydrogenase and enzymes of tricarboxylic acid cycle

The maximal activities of citrate synthase, NAD^+-linked and $NADP^+$-linked isocitrate dehydrogenase, oxoglutarate dehydrogenase and pyruvate dehydrogenase in murine macrophages and other cells or tissues of the rat are presented in Tables 2.1 and 2.3.

TABLE 2.1

Enzyme Activity Data

Lymphocytes and macrophages were prepared and enzymes extracted and assayed as described in the experimental section. Activities were measured at 25°C, except for glutaminase and phosphoenolpyruvate carboxykinase (37°C).

* Data from Ardawi and Newsholme (1982)

† Data from Curi et al. (1987)

data presented as means ± S.E.M.

Enzyme	Enzyme activity (nmol/min per mg of protein)	
	Lymphocytes	Macrophages
Hexokinase	17.3 ± 0.31	76 ± 1.4
Phosphorylase	3.8 ± 0.04	3.0 ± 0.11
6-Phosphofructokinase	25.7 ± 0.69	22.8 ± 1.0
Pyruvate kinase	403 ± 9.9	447 ±39.3
Lactate dehydrogenase	823 ±64	764 ±13.4
Glucose-6-phosphate dehydrogenase	17.5 ± 0.48	34.5 ± 2.6
6-Phosphogluconate dehydrogenase	21.1 ± 0.29	23.8 ± 0.74
Pyruvate dehydrogenase	3.3 †	3.2 ± 0.26
Citrate synthase	63.7 ± 1.6	108 ± 3.3
NAD^+-linked isocitrate dehydrogenase	6.3 †	5.8 ± 0.08
$NADP^+$-linked isocitrate dehydrogenase	26.9 †	27.4 ± 0.27
ATP-citrate lyase	1.1 †	1.6 ± 0.03
Oxoglutarate dehydrogenase	5.1 ± 0.08	10.1 ± 0.69
3-Oxoacid CoA-transferase	19.9 *	36.6 ± 0.63
Acetoacetyl-CoA thiolase	27.4 *	15.5 ± 0.68
3-Hydroxybutyrate dehydrogenase	4.8 *	0.17 ± 0.02
Carnitine palmitoyltransferase	0.47 ± 0.05	0.75 ± 0.12
Phosphate-dependent glutaminase	39.4 ± 1.3	152 ± 5.5
Glutamate dehydrogenase	34.0 ± 1.6	98.6 ± 3.0
$NADP^+$-linked malate dehydrogenase	3.5 ± 0.26	<0.001
Phosphoenolpyruvate carboxykinase	4.9 ± 0.35	7.6 ± 0.51
Pyruvate carboxylase	2.5 ± 0.19	4.7 ± 0.34
Aspartate aminotransferase	67.4 ± 4.8	118 ± 9.7
Alanine aminotransferase	10.3 ± 2.5	4.4 ± 0.16
NAD^+-linked malate dehydrogenase	683 ± 16.6	452 ±10.6

Table 2.2

The Maximal activities of some glycolytic enzymes in cells of tissues of the mouse or rat

Data are taken from:
a present work
b Ardawi, 1983
c Crabtree and Newsholme, 1972a
d Budohoski et al, 1982
e Cooney and Newsholme, 1982 b

For the presentation of data from c, d and e it was assumed that the wet wt: dry wt ratio = 4.0

Enzyme activities (μmol/min/g dry wt at 25°C)

Animal and cell or tissue	Hexokinase	6-PhosPhofructokinase	Phosphorylase	Pyruvate Kinase	Lactate Dehydrogenase
mouse macrophage [a]	76.0	22.8	3.0	447	764
rat mesenteric lymph node [b]	7.3	14.4	1.8	90.6	397
rat spleen [b]	4.6	8.2	-	170	463
rat heart [c]	24.4	40.0	48.0	-	-
rat quadriceps Fermoris (white muscle) [c]	7.6	188	200	-	-
rat intestine [d]	21.2	39.2	-	-	-
rat kidney [e]	8.0	12.4	-	-	-

Table 2.3

The Maximal activities of some tricarboxylic acid cycle enzymes, plus pyruvate dehydrogenase, in cells or tissues of the mouse or rat

Data are taken from:
a present work
b Ardawi, 1983
c Alp et al, 1976
d Budohoski et al, 1982
e Sugden and Newsholme, 1975
f Read et al, 1977
g Kerby et al (1976); measured at 30°C.

For the presentation of data from c, d, e and f it was assumed that the wet wt: dry wt ratio = 4.0

Enzyme activities (μmol/min per g dry wt at 25°C)

Animal and cell or tissue	Citrate synthase	Isocitrate dehydrogenase		oxoglutarate dehydrogenase	pyruvate dehydrogenase
		NAD linked	$NADP^+$-linked		
murine macrophage [a]	108	5.8	27.4	10.1	3.2
rat mesenteric lymph node [b]	41.0	3.9	2.4	2.1	4.1
rat spleen [b]	26.5	2.4	1.2	1.1	-
rat heart [c]	384	20.0	288	26.8 [f]	23.6 [g]
rat intestine [d]	48.8	-	-	6.0	-
rat brain [e]	54.4	3.2	4.4	-	-

The activity of citrate synthase is higher than the activities of the other enzymes of the tricarboxylic acid cycle in the macrophage, a pattern that is found for all tissues presented in Table 2.3. Macrophage $NADP^+$-linked isocitrate dehydrogenase has an activity several-fold greater than for NAD^+-linked isocitrate dehydrogenase, which is similar to the pattern in rat heart but not to the other tissues presented in table 2.3. Macrophage oxoglutarate dehydrogenase activity is 3-fold greater than that of pyruvate dehydrogenase; in heart, an aerobic tissue that oxidises most of the glucose utilised, the activities of these two enzymes are very similar (see table 2.3 and Paul, 1979). This may indicate either that the rate of oxidation of pyruvate is less than that of other fuels that give rise to acetyl-CoA (e.g. fatty acids) or that oxoglutarate dehydrogenase is involved in another pathway (see below.)

2.3.3 Maximal activities of enzymes involved in glutamine metabolism

The pathway for glutamine metabolism in rat lymphocytes, kidney and intestine has been proposed to include the following enzymes, the activities of which have been measured previously (see legend to Figures 2.1, 2.2 and 2.3 for references): glutaminase, glutamate dehydrogenase, aspartate aminotransferase, alanine aminotransferase, oxoglutarate dehydrogenase, $NADP^+$-linked malate dehydrogenase, and phosphoenolpyruvate carboxykinase (see Figures 2.1, 2.2 and 2.3). The activities of these enzymes plus NAD^+-linked malate dehydrogenase and pyruvate carboxylase, which may be involved in the pathway of amino acid metabolism, were measured in the murine macrophage and rat

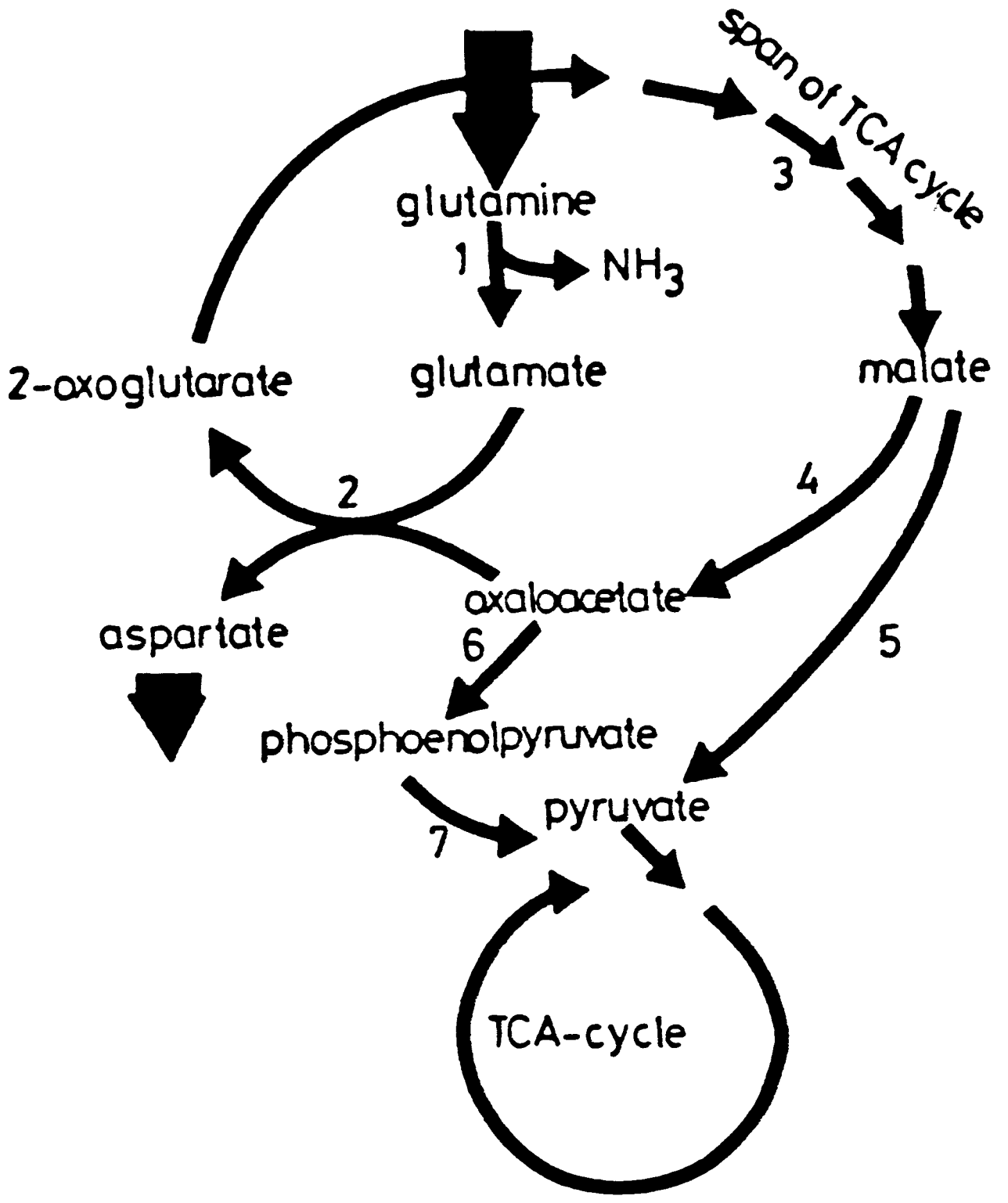


Fig. 2.1 Proposed pathway for glutamine metabolism in lymphocytes (schematic outline)

1. Glutaminase; 2. aspartate aminotransferase; 3. enzymes of the TCA-cycle converting 2-oxoglutarate into malate; 4. malate dehydrogenase (NAD^+-dependent reaction); 5. malic enzyme ($NAD(P^+)$)-dependent reaction; 6. phosphoenolpyruvate carboxykinase; 7. pyruvate kinase. (Ardawi, 1983)

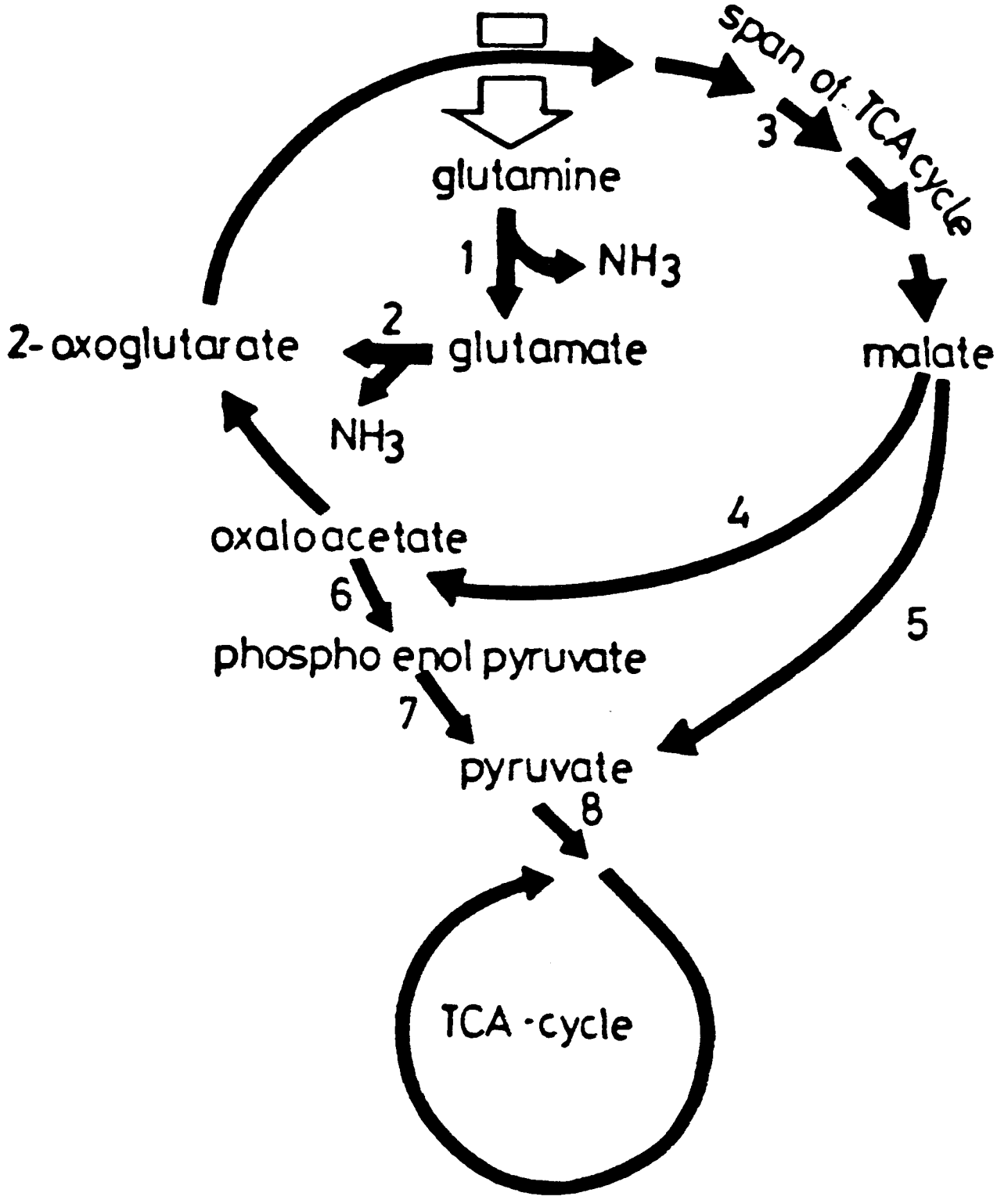


Fig. 2.2 The pathway of glutamine metabolism in kidney (schematic outline)

Glutamine is taken up from both the blood and the glomerular filtrate by the proximal and distal tubules of the nephron. Within the cell, mitochondrial glutaminase hydrolyses glutamine into glutamate with the production of ammonia. The glutamate produced is converted to 2-oxoglutarate by glutamate dehydrogenase (2) accounting for the second molecule of ammonia from glutamine. 2-oxoglutarate is converted to malate by reactions that are considered part of the TCA-cycle (3). The malate is transported out of the mitochondria and then either converted to oxaloacetate by malate dehydrogenase (NAD^+-dependent) reaction (4) or to pyruvate via the 'malic' enzyme ($NAD(P)^+$)-dependent (5); in the former case, oxaloacetate is converted to phosphoenolpyruvate via PEPCK (6) and phosphoenolpyruvate is then converted to pyruvate via pyruvate kinase (7). The pyruvate after entering the mitochondria is converted to acetyl CoA by pyruvate dehydrogenase (8) for complete oxidation in the TCA-cycle.

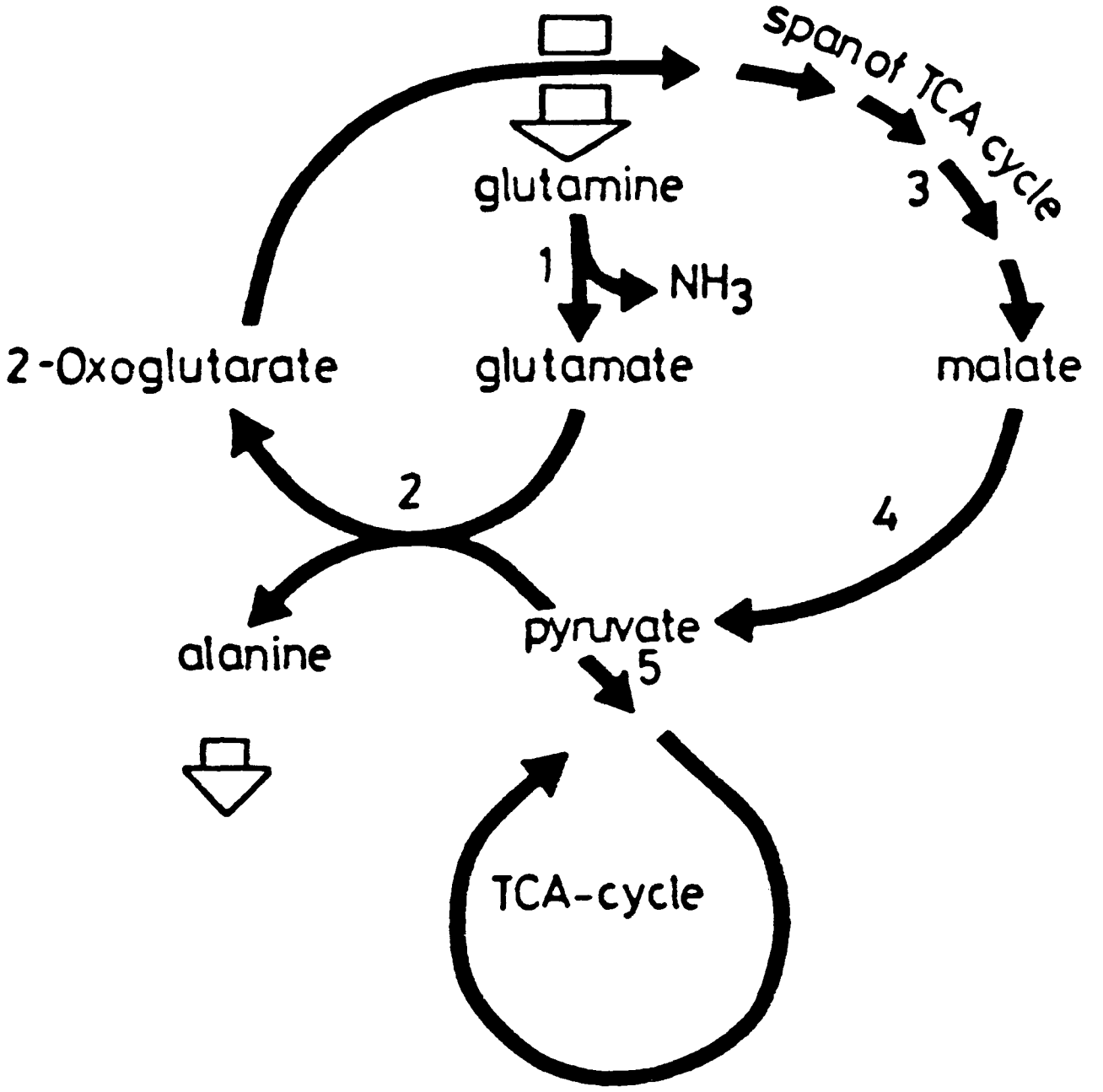


Fig. 2.3 The pathway of glutamine metabolism in small intestine (schematic outline)

Glutamine is metabolised by the epithelial cells of the small intestine and the pathway of metabolism is, in general, similar to that described for the kidney (Fig. 2.2) but there are two specific differences (Hanson & Parsons, 1980); glutamate is converted to 2-oxoglutarate by transamination rather than by the glutamate dehydrogenase reaction; and pyruvate is formed from malate via a mitochondrial reaction catalysed by 'malic' enzyme. The pyruvate produced can either be oxidised via TCA-cycle or converted to alanine via aminotransferase reaction.

1. Glutaminase; 2. alanine aminotransferase; 3. enzymes of the TCA-cycle converting 2-oxoglutarate into malate; 4. 'malic' enzyme ($NAD(P)^+$-dependent reaction); 5. pyruvate dehydrogenase complex.

lymphocyte and are presented in Tables 2.1 and 2.4. The activity of glutaminase in the macrophage is about 4-fold greater than that in the lymphocyte and between 3 and 17-fold greater than those in other tissues presented in Table 2.4. The activity of glutamate dehydrogenase in the macrophage is similar to that of glutaminase (calculated at 25°C), which is in contrast to the kidney, in which the dehydrogenase activity is 4 fold higher than that of glutaminase (see Newsholme et al., 1982). This suggests that an aminotransferase(s) rather than glutamate dehydrogenase, is important in the conversion of glutamate into oxoglutarate in macrophages. Similarly to the lymphocyte, the activity of aspartate aminotransferase is high, whereas that of alanine aminotransferase is low, suggesting that formation of aspartate is more important than the formation of alanine in macrophages. In contrast to the lymphocyte, the activity of $NADP^+$-linked malate dehydrogenase is very low (<0.01 nMol/min per mg protein), suggesting that phosphoenolpyruvate carboxykinase catalyses the major reaction by which intermediates of the tricarboxylic acid cycle are converted into pyruvate for conversion into lactate or complete oxidation (see Goldstein & Newsholme, 1976). Hence it is possible, via this pathway, that glutamine provides acetyl-CoA for complete oxidation by the tricarboxylic acid cycle in macrophages. On the basis of subsequent work, it is suggested that a mitochondrial oxaloacetate decarboxylase could also be involved in the conversion of oxaloacetate to pyruvate. This activity was not, however, measured in the present work.

2.3.4 Ketone body and fatty acid metabolism

Table 2.4

The Maximal activities of some enzymes involved in glutamine metabolism in cells or tissues of the mouse or rat

Data taken from:
a present work; measured at 37°C (except glutamate dehydrogenase)
b Ardawi, 1983; measured at 37°C (except glutamate dehydrogenase)
c Newsholme et al, 1982
d Budohoski et al, 1982

For the presentation of data from c and d it was assumed that the wet wt: dry wt ratio = 4.0

Animal and cell or tissue	Enzyme activities (μmol/min per g dry wt at 25°C) Glutaminase	Glutamate dehydrogenase	Phosphoenolpyruvate-Carboxykinase
murine macrophage [a]	152	98.6	7.6
rat mesenteric lymph node [b]	34.8	13.2	2.7
rat spleen [b]	24.4	-	-
rat kidney [c]	48.4	180.8	11.2
rat intestine [d]	26.8	-	-
rat liver [c]	8.8	40.4	8.8

In most tissues, the enzymes involved in ketone body utilisation are considered to catalyse near-equilibrium reactions, so they cannot be used to provide a quantitative indication of the rate of ketone body utilisation. However, qualitative information on the ability of a tissue to use ketone bodies can be obtained from the activities of D-3-hydroxybutyrate dehydrogenase, acetoacetyl-CoA thiolase and 3-oxoacid CoA-transferase. For macrophages and lymphocytes, these activities are presented in Tables 2.1 and 2.5. The activities of 3-oxoacid CoA-transferase and acetoacetyl CoA-thiolase in the macrophage are comparable to those found in brain and muscle which can utilise ketone bodies at high rates, but the activity of D-3-hydroxybutyrate dehydrogenase is low. However, the D-3-hydroxybutyrate dehydrogenase activity is lower than that of the transferase for all tissues presented in Table 2.5. The presence of ketone body-utilising enzymes in the macrophage may indicate these fuels can be utilised by the macrophage.

The activity of carnitine palmitoyl-transferasee in the macrophage was at least 10-fold loweer than tht in rat heart muscle (Table 2.5) which can oxidise fatty acids at a high rate. However, the carnitine palmitoyl-transferase activity in the macrophage, if expressed as the rate of C_2-unit transport into the mitochondria for oxidation, represents an activity similar to that reported for pyruvate dehydrogenase. Thus fatty acids may provide an important fuel for the macrophage in vivo.

An important enzyme in providing a source of acetyl-CoA in the cytosol for lipid or cholesterol biosynthesis is ATP-citrate lyase.The

Table 2.5

Maximal activities of ketone-body utilising enzymes and Carnitine-Palmitoyl transferase in cells or tissues of the mouse or rat

Data taken from:

a present work
b Ardawi, 1983
c Williamson et al, 1971
d Beis et al, 1980
e Crabtree and Newsholme, 1972a
f Hanson and Carrington, 1981

For the presentation data from c, d, e and f it was assumed that the wet wt: dry wt ratio = 4.0

Enzyme activities (μmol/min per g dry wt at 25°C)

Animal and cell or tissue	D-3-hydroxybutyrate dehydrogenase	3-oxoacid CoA transferate	Acetoacetyl CoA-thiolase	Carnitine-Palmitoyl transferase
murine macrophage [a]	0.2	36.6	15.5	0.75
rat mesenteric lymph node[b]	0.4	1.0	1.4	0.36
rat spleen [b]	0.9	3.3	4.1	0.41
rat brain [c]	2.3	8.4	8.4	-
rat heart [d]	5.6	193	48.8	8.0
rat diaphram [d]	1.2	68.4	18.8	-
rat skeletal muscle (gastrocnemius) [d]	0.5	32.0	18.4	-
(quadriceps) [e]	-	-	-	7.6
rat intestine [f]	2.2	36.0	-	1.28

activity of this enzyme in the macrophage (Table 2.1) is greater than carnitine palmitoyl-transferase. However cholesterol biosynthesis in the macrophage is likely to be low, as the activity of the key enzyme in cholosterol biosynthesis, 3-hydroxy-3-methylglutaryl-CoA synthase, is less than 10% of that of palmitoyltransferase when expressed as C_4-units (see Knight et al., 1983). Hence the lyase may be important in the formation of cytosolic acetyl-CoA that can be used to produce malonyl-CoA either for de novo fatty acid synthesis or for chain elongation of fatty acids. Unfortunately acetyl-CoA carboxylase activity was not assayed in the present work.

2.4.1 DISCUSSION

The overall aim of the work described in this chapter was to establish a metabolic profile for the murine peritoneal macrophage. This was achieved by measurement of the maximal activities of some key and other enzymes involved in glucose, pyruvate, amino acid, ketone body and fatty acid metabolism.

2.4.2 Glycolysis

Glucose and glycogen are considered to be the most important carbohydrates for provision of energy in a variety of tissues. Several tissues including brain, kidney medulla, retina and erythrocytes have an absolute or a major requirement for glucose (Krebs, 1972). In general, vertebrate skeletal muscle that contains a high proportion of type II fibres (known also as white muscle) probably depends on glycogen within the muscle for most of its energy requirement during physical activity and to this end the capacity for glycogenolysis exceeds markedly that of glucose phosphorylation (Crabtree & Newsholme, 1972a). However, for other muscles, and for tissues in general, the dependence on either glucose or glycogen for energy formation is variable. To illustrate such variability a comparison between red and white muscles is useful.

Red muscle (e.g. rat soleus, rabbit semitendinosus) has a good blood supply so that glucose and oxygen can be made available to the muscle and hence it possesses a high capacity for aerobic metabolism.

This type of muscle possesses a high hexokinase activity (see rat heart muscle, Table 2.2) and glucose is considered to be the main carbohydrate fuel for the supply of energy during sustained activity (Crabtree & Newsholme, 1972 a,b). On the other hand, white skeletal muscle (e.g. pectoral muscle of game birds, rabbit adductor longus), which is adapted for powerful contractions for short periods of time,has a poor blood supply and hence the supply of glucose and oxygen is minimal; consequently glycogen rather than glucose is considered to be the main carbohydrate fuel of this muscle. Since anaerobic glycolysis produces only 3 moles of ATP per mole of glucose residue (compared to 38 for complete oxidation) a high capacity of glycolysis is required to produce sufficient ATP for muscular activity. Thus the activities of the key enzymes in the pathway glycolysis-from-glycogen, phosphorylase and 6-phosphofructokinase are very high, relative to hexokinase (Crabtree et al., 1979; and Table 2.2).

On the basis of these considerations and the activities of the glycolytic Enzymes observed in the present work, it is suggested that glucose rather than glycogen is the important carbohydrate fuel for the macrophage. Thus the activity of hexokinase is considerably higher than that of phosphorylase (Tables 2.1 and 2.2). In macrophages, the maximum activity of hexokinase is considerably greater than tht of 6-phosphofructokinase, which is unusual (Crabtree & Newsholme, 1972a; Cooney & Newsholme, 1982). This probably reflects the quantitative importance of the pentose phosphate pathway, a suggestion that is supported by the high activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Table 2.1). The activities of

the last two enzymes are approximately similar to those reported by Kiyotaki et al. (1984) for the macrophage. It should be noted tht the activfty of glucose-6-phosphate dehydrogenase in the murine macrophage is almost 8-fold greaer than that in murine liver (compare data in Table 2.1 with that presented in Newsholme et al., 1979). The pentose phosphate pathway is important in the macrophage not only for the production of ribose phosphate and NADPH for biosynthetic purposes, but also for NADPH for the NADPH oxidase reaction which is responsible for the production of the superoxide radical (see chapter 1 and Rossi et al., 1985). The requirement for NADPH for the oxidase reaction is important when the macrophage is stimulated by exposure to specific agents such as opsonized particles, phorbol esters (e.g., phorbol myristate acetate) or inflammatory mediators (e.g. components of the complement pathway, C5a and C567) or formylated chemotactic peptides (see Rossi et al., 1985).

2.4.3 Glutamine and amino acid metabolism

The glycolytic enzyme activities indicate that the macrophage has the capacity to utilise glucose at a high rate, and previous work has indicated that these cells do utilise this fuel. However such work does not indicate whether glucose is the major fuel for the cell. Since such cells are known to use glucose, it appears to have become accepted - almost by default - that this is the major if not the only fuel. Nonetheless other cells are known to use other fuels in addition to glucose. Thus Zielke et al. (1984) have indicated that glutamine may provide an important source of energy for mammalian cells in culture; however, these cells were proliferating whereas elicited

peritoneal macrophages are not, they are terminally differentiated end-cells.

The present work has provided evidence, for the first time, that glutamine is utilised at a high rate by the macrophage. Preliminary studies on the nature of the pathway by which glutamine may be metabolised in the macrophage has been investigated by measuring the maximum activities of enzymes that are considered to be components of this pathway in other cells or tissues (e.g. lymphocyte, kidney and small intestine of the rat, see Figures 2.1, 2.2. and 2.3 respectively).

If glutamine utilisation leads to oxidation by the macrophage, a pathway must exist for the conversion of glutamine into acetyl-CoA and it is likely that this would be achieved via pyruvate and pyruvate dehydrogenase (see Goldstein & Newsholme, 1976; Snell & Duff, 1977). The conversion of malate to pyruvate can be achieved via the reactions that are catalysed by malate dehydrogenase, phosphoenolpyruvate carboxykinase, $NADP^+$-linked "malic" enzyme or oxaloacetate decarboxylase as suggested for the epithelial cells of the small intestine (Hanson & Parsons, 1980).

The presence of phosphoenolpyruvate carboxykinase but not $NADP^+$-dependent "malic" enzyme activity in the macrophage (Table 2.1) suggests the participation of phosphoenolpyruvate carboxykinase in converting intermediates of the TCA-cycle into pyruvate in these cells, but does not exclude the importance of NAD^+-linked malate dehydrogenase (decarboxylating) in converting malate into pyruvate in

a similar manner to that suggested for the epithelial cells of the small intestine (Watford et al., 1979; Hanson & Parsons, 1980). No attempts were made to measure oxaloacetate decarboxylase or NAD^+-dependent malate dehydrogenase (decarboxylating) in the macrophage due to the lack of satisfactory assays.

2.4.4 Comparison of metabolic fluxes with maximal enzyme activities in macrophages

It has been established, for muscle, that a quantitative indication of the maximum capacity of some metbolic pathways can be obtained from the maximum *in vitro* catalytic activity of some key enzymes in those pathways (Chapter 1). Can the same approach be applied to macrophages? To test this, it is necessary to measure the maximum enzyme activities and compare them with maximal fluxes.

Glycolysis

Comparison of the maximal catalytic activities of hexokinase and 6-phosphofructokinase with that of the glycolytic flux (glycolysis-from-glucose) can be made from the data presented in Table 2.6. Glycolytic flux (see legend to Table 2.6) represents approximately 32% of 6-phosphofructokinase activity and 10% of hexokinase activity. As the glycolytic flux did not increase during stimulation of metabolic activity (e.g. during phagocytosis, Table 4.11) then 6-phosphofructokinase or hexokinase maximum activities are not good indicators of maximum glycolytic flux from glucose in the macrophage. However, in comparison to the other types of cell or

Table 2.6

Maximum flux through glycolysis from glucose, and maximum activities of hexokinase, 6-phosphofructokinase and phosphoylase

Enzyme activities were measured at 25°C but are presented at 37°C to aid comparison with the calculated flux of glycolysis from glucose. Enzyme activities at 37°C are calculated from the Arrhenius equation (assuming that a rise of 10°C increases the rate by a factor of 2.0 (see Crabtree and Newsholme, 1975). For Murine Macrophages the rate of glycolysis was calculated from the maximum observed rate of glucose utilisation (Table 4.11). For rat lymphocytes, colonocytes and sleen slices the glycolytic rate was obtained from the data provided by Ardawi (1983), Ardawi and Newsholme (1985b) and Suter and Weideman (1975). Enzyme activities were calculated from the values reported for Murine Macrophages (the present work), rat lymphocytes and spleen (Ardawi, 1983) and rat Colonocytes (Ardawi and Newsholme, 1985 b)

preparation	conditions	Rate of glycolytic flux (μmol/min per g dry wt)	Enzyme activities (μmol/min per g dry wt)		
			Hexokinase	6-phosphofructokinase	phosphorylase
murine macrophages	5mM-glucose 95% air:5% CO_2	17.5	182	54.7	7.2
rat lymphocytes	5mM-glucose 100% O_2	0.62	17.5	34.6	4.3
rat colonocytes	10mM-glucose 100% O_2	6.9	56.6	43.9	-
rat spleen slices	3mM-glucose 95% O_2:5% CO_2	0.63	11.0	19.7	-

tissue preparation presented in Table 2.6, the macrophage has a much smaller ratio of 6-phosphofructokinase activity/glycolytic flux.

If it is assumed that lactate produced in the absence of exogenous fuel by the macrophage (Table 4.11) is derived from glycogen, then the maximal activity of phosphorylase (Table 2.6) is only slightly in excess of the calculated flux of the pathway glycolysis-from-glycogen. Thus, similarly to white muscle, the maximum activity of phosphorylase may indicate the maximum capacity of glycolysis-from-glycogen in the macrophage. As glycolysis-from-glucose has a much higher flux than glycolysis-from-glycogen in the macrophage, then glucose will be quantitatively the most important substrate for glycolysis.

Tricarboxylic acid cycle

The TCA cycle together with the electron-transport chain is considered to be the most important metabolic pathway for energy production under aerobic conditions. Nonetheless, a detailed investigation into the maximal activities of the enzymes that catalyse key reactions in the cycle in macrophages has not been previously undertaken. The maximal catalytic activities of some key enzymes of the cycle have been measured. Of all the enzymes investigated oxoglutarate dehydrogenase had the lowest maximal activity (Tables 2.1 and 2.3; NAD^+ and $NADP^+$-linked isocitrate dehydrogenase activity are summed for the purpose of this discussion.) This is similar to muscle where this activity has been used as an index of the maximal flux through the TCA cycle (Read et al., 1977; Cooney et al., 1981).

Comparisons of the maximal activity of oxoglutarate dehydrogenase with calculated flux of the TCA cycle (calculated from oxygen uptake data, see legend to Table 2.7) in the macrophage and other cell and tissue preparations can be made from the data presented in Table 2.7. The activity of the dehydrogenase in the macrophage is considerably greater than the flux through the cycle. The flux through the cycle is calculated assuming that oxygen is consumed only for mitochondrial respiration. It is known that some of the oxygen consumed in the macrophage will be utilised for O_2^- production. However, this rate may be low: it has been estimated to be approximately 5% for resting and approximately 20% of total O_2 comsumption for phagocytosing elicited peritoneal macrophages from the guinea pig (Karnovsky et al., 1970).

Hence the oxoglutarate dehydrogenase activity does not appear to provide a satisfactory quantitative assessment of the cycle for the macrophage. The high activity of this enzyme in the macrophage, relative to the calculated rate of the TCA cycle, may indicate that this enzyme has a role in other pathways. This may well be the pathway of glutamine and glutamate catabolism (see below and also Chapter 5 for a discussion of the pathway of glutamine metabolism in the macrophage).

Glutamine utilisation

For the epithelial cells of the small intestine, Pinkus and Windmueller (1977) have suggested that the maximal glutaminase

Table 2.7

Maximum activities of oxoglutarate dehydrogenase and maximum calculated rate of the tricarboxylic acid cycle

Oxogluterate dehydrogenase (OGDH) activity was measured at 25°C but is presented at 37°C to aid comparison with the calculated flux through the TCA cycle. Enzyme activities at 37°C are calculated as described in table 2.6. The flux through the TCA cycle was calculated from the rate of O_2 uptake. From the stoichiometry of the pathway of oxidation of C_2/C_3 units of major fuels, the flux through the cycle is equal to 1/3 of the O_2 uptake. For Murine Macrophages, the rate of O_2 uptake was obtained from the data presented by Karnovsky et al, (1975) for caseinate elicited peritoneal Macrophages. It is assumed caseinate elicited cells have the same oxygen requirement as thioglycollate elicited cells and 1 mg protein = 1 mg dry wt for these cells. It is also assumed that the oxygen requirement of resting and phagocytosing cells is solely for mitochondrial respiration. For rat lymphocytes the rate of the TCA cycle was calculated from the data presented by Ardawi (1983). For rat enterocytes, the rate of the cycle was calculated from the data provided by Watford et al (1979) and OGDH activity was calculated from the data provided by Budohoski et al (1982) and assuming a wet wt/dry wt ratio of 4.0. For isolated working heart the data are taken from Cooney et al, (1981).

preparation	conditions	Rate of TCA cycle (μmol/min per g dry wt)	OGDH activity (μmol/min per g dry wt)	OGDH activity/rate of TCA cycle
murine macrophages (resting)	5mM glucose plus 2mM glutamine;	2.86	24.2	8.5
murine macrophages (phagocytosing)	and 95% air: 5% CO_2	5.86	24.2	4.1
rat lymphocytes	5mM glucose 100% O_2	0.53	5.25	9.9
rat enterocytes	10mM glucose 95% O_2:5% CO_2	6.50	15.0	2.3
rat isolated working heart	purfusion with 5mM-glucose 95% O_2:5% CO_2	5.75	12.1	2.1

activity is sufficient to account for the highest observed rate of glutamine utilisation. Can the flux through the glutamine utilisation pathway in macrophages be indicated by the maximal catalytic activity of gltaminase? A comparison of the maximal activity of glutaminase with the rate of glutamine utilisation in macrophages can be made by reference to Table 2.8. This shows that glutaminase activity is much higher than the rate of glutamine utilisation by macrophages; this is also the case for lymphocytes, colonocytes and kidney tubules. Therefore, glutaminase activity cannot be used to provide a quantitative assessment of the pathway in any of these preparations. However it may be possible that under some conditions the rate of this pathway may be similar to the maximal activity of glutaminase. If this is not the case, it suggests that the transport of glutamine across the cell or mitochondrial membrane may limit the flux.

Fatty acid utilisation

In muscle, the maximal enzyme activity of carnitine palmitoyl transferase can be used as a quantitative index of the maximal rate of fatty acid oxidation (Crabtree & Newsholme, 1972b). Can the flux through the fatty acid oxidation pathway in macrophages be indicated by the maximal catalytic activity of carnitinepalmitoyl transferase?

The maximal activity of this enzyme was found to be substantially greater than the calculated rate of oxidation of oleate by cultured macrophages (Table 2.9). Therefore, the transferase activity cannot be used as a quantitative index of the maximum rate of fatty acid oxidation in macrophages. This is similar to the finding

Table 2.8

Maximal activities of glutaminase and rate of glutamine utilisation

Glutaminase activity was measured at 37°C in all cases. The rate of glutamine utilisation was obtained from data presented for macrophages (the highest observed rate of glutamine utilisation, table 4.11) rat lymphocytes (Ardawi, 1983), rat colonocytes (Ardawi and Newsholme, 1985b) and rat kidney tubules (Vinay et al, 1978). Glutaminase activities are from the same sources.

preparation	conditions	Rate of glutamine utilisation (μmol/min per g dry wt)	glutaminase activity (μmol/min per g dry wt)
murine macrophages	2mM glutamine 95% air:5% CO_2	3.3	152
rat lymphocytes	2mM glutamine 100% O_2	2.7	34.8
rat colonocytes	5mM-glutamine 100% O_2	5.5	27.5
rat kidney tubules	5mM-glutamine 95% O_2:5% CO_2	10.6	97.0

Table 2.9

Maximal activities of carnitine - palmitoyl transferase and rate of oleate oxidation

Carnatine- palmitoyl transferase (CPT) activity was measured at 25°C but is presented at 37°C to aid comparison with the calculated rate of oleate oxidation. Enzyme activities at 37°C are calculated as described in table 2.6. For murine macrophages the rate of oleate oxidation was calculated from the maximum observed rate (Table 4.15). The data for rat lyphocytes was obtained from Ardawi (1983). For rat intestine the rate of oleate oxidation has been reported by Windmueller and Spaeth (1978), and CPT activity was calculated from the value reported by Hanson and Carrington (1981), assuming wet wt/dry wt = 4.0. CPT activity has been divided by 2 because of the dual involvement of the enzyme in fatty acid transport into the mitochondria (McGarry et al, 1978).

preparation	conditions	Rate of oleate oxidation (μmol/ min per g dry wt)	CPT activity (μmol/min per g dry wt)
murine macrophages	0.3 mM-oleate 95% air:5% CO_2	0.17	0.90
rat lymphocytes	0.5 mM-oleate 100% O_2	0.05	0.45
rat intestine	1mM-oleate infused into artery in vivo	0.03	1.32

for rat lymphocytes and intestine (Table 2.9).

The present work suggests that maximum fluxes through some metabolic pathways in murine peritoneal macrophages cannot be assessed by the catalytic activities of certain key enzymes in these pathways. The reason why macrophages are different from muscle in this respect may be explained by the following considerations. Regulation of a flux through an individual enzyme, provided it is not flux-generating can be achieved by changes in the concentration of its pathway-substrate (internal regulation - see Newsholme & Crabtree, 1979). A greater sensitivity will be achieved if the substrate concentration is well below the K_m value of the enzyme. Hence, when the maximum activity is measured *in vitro* at concentrations of pathway substrate that approach saturation, the activity is well in excess of the flux. If this view is correct, it would suggest that transport processes, e.g. glucose transport across the cell membrane, and glutamine transport across the cell or mitochondrial membranes provide major limiting and therefore, regulating processes in glycolysis and glutamine oxidation respectively. Also the concentration of fatty acyl CoA, the substrate for carnitine-palmitoyl transferase, may be limiting *in vivo* due to the requirement for activated fatty acids in triacylglycerol and phospholipid synthesis (see Chapter 4). Only direct studies on the regulatory mechanisms of these pathways will provide definitive evidence for this hypothesis.

Although these present studies have not led to the development of a simple tool to investigate precise rates of fuel utilisation in macrophages, they have provided useful information on the activities

of enzymes in the important energy-producing pathways in macrophages and have provided information on which to construct a preliminary metabolic profile. Using this information, and that presented in chapters 3 and 4 concerning fuel utilisation and its fate, a more comprehensive profile of energy-producing processes in this cell is created.

The enzyme activities reported in this chapter suggest that the macrophage may utilise glucose, glutamine, fatty acids and ketone bodies. The rates of utilisation of these fuels and their metabolic fates are presented in chapters 3 (for 1h, short term incubations) and 4 (for 2-4 day longer term incubations).

CHAPTER 3:

Rates of utilisation and fates of glucose, glutamine, pyruvate, fatty acids and ketone bodies by incubated macrophages

3.1 INTRODUCTION

The fundamental importance of macrophages to the immune response is well-established. Through their abundance, distribution, motility, responsivity and versatility, macrophages are involved in almost every aspect of the immune and inflammatory responses, from acute to delayed hypersensitivity, from the first breach of epithelium to its eventual repair (see chapter 1). Despite the large volume of work concerning secretory and endocytic properties and recent work concerning the surface properties of macrophages (Van Furth, 1985), there has been no detailed study on the fuels that can be used by these cells. Although the use of glucose by the macrophage has been studied this has been largely directed to an understanding of its role in the "respiratory burst" and in the process of phagocytosis (Kiyotaki et al., 1984; Karnovsky et al.,1970), its precise quantitative role in the provision of energy for the macrophage does not appear to have been considered. Furthermore, other fuels are known to be used by other tissues including both short and long chain fatty acids, amino acids, ketone bodies and possibly other sugars. However, there have been no systematic studies on the importance of such fuels for providing energy for the important activities of the macrophages in the immune system.

The aim of the work, which is described in this chapter, was to assess the rates of utilisation and metabolic fate of fuels or potential fuels such as glucose, glutamine, fatty acids, and ketone bodies and any interaction between glucose and the other substrates by

incubated murine peritoneal macrophages. The incubation system was maintained as simple as possible to aid interpretation of results. Thus the incubation was carried out in 10 ml Erlenmeyer flasks containing cells, incubation medium and other additions. The availability of isolated macrophages offers a biological preparation well-suited for metabolic studies; thus the preparation of cells and their incubation are not difficult, rates of substrate utilisation and the fate of the substrates can be studied either in isolation or in the presence of other substrates which enables metabolic interactions to be discovered. The simplicity means that there is no interference from other substances (e.g. growth factors) that are present in an *in situ* or cell culture system. Other advantages of the incubated cell system are as follows:

(i) The cells retain membrane structure, internal organisation and remain biochemically viable.

(ii) The problem of diffusion of substrates and other metabolites to and from cells is minimal.

(iii) If whole organs or tissue preparations are used, several cell types may be present, which interferes with the interpretation of the results. With the cell incubation system, a homogenous cell preparation can be prepared prior to the start of the incubation.

Thus for the short term study (1-2h) of macrophage metabolism , fuel utilisation, fate and interaction, the cell incubation system appeared to be very useful and hence was used in this study.

EXPERIMENTAL

3.2.1 Preparation of Macrophages

Macrophages were obtained from the peritoneal cavity of 12-16 week-old female mice of the C57 BL/6 strain. A volume (1.5 ml) of thioglycollate broth was injected into the peritoneal cavity and peritoneal cells were harvested 4 days later. Macrophages were purified by adherence to plastic petri dishes (see the experimental section of chapter 2 and Cohn, 1974). After removal from culture, the pooled suspension of cells was tested for biological viability by trypan blue exclusion. This was always >85%. The cell suspension was kept at 0-4^{o}C until addition to the incubation (always less than 60 min.).

3.2.2 Incubation procedure

Incubations were performed at 37^{o}C in 10 ml Erlenmeyer flasks that had been silicone-treated. Freshly-prepared macrophages (at a denisty of 3-6 mg protein or approximately 2-4 x 10^{7} cells per ml of incubation medium) were incubated in a total volume of 1.0 ml of standard incubation medium. The latter consisted of phosphate buffered saline (A plus B, see appendix C) supplemented with 1.5% defatted bovine serum albumin. In some experiments albumin was omitted. Albumin had been treated with charcoal to remove fatty acids (Chen, 1967) and dialysed against 0.9% NaCl overnight, prior to use. The pH of the incubation medium was 7.2-7.4. The incubation medium was chilled on ice and oxygenated by bubbling 100% O_2 through the medium for 20-30 min prior to the start of the experiment. Macrophages were

preincubated at 37°C in the above medium in the absence of substrate for 15-20 min, after which the substrate was added, the flasks were gassed with 100% O_2 for 20 s and were shaken continuously for the period of incubation (90-100 oscillations per min). Incubations were carried out at 37°C in a Gallenkamp shaking water bath.

Incubations were terminated by the addition of 200 ul $HClO_4$ (25% w/v) to the incubation flask and the mixture was immediately cooled on ice. Precipitated protein was removed by centrifugation at 8500 g (in an Eppendorf microcentrifuge) for 3 min. The supernatant was neutralised with KOH and KOH/triethanolamine (appendix C), and after cooling to 0°C the $KClO_4$ removed by centrifugation at 8500 g for 3 min. The supernatant was stored at -20°C until required for metabolite analysis.

In experiments in which ^{14}C-labelled substrates were used and the rate of $^{14}CO_2$ produced was to be determined, flasks with plastic centre wells suspended above the incubation medium were employed. These centre wells contained 0.2ml of a 1:1 mixture of phenylethylamine : methanol, which was adsorbed onto Whatman No.1 filter paper (see Leighton et al.,1985). The incubation was terminated by addition of 0.2 ml of 25% w/v $HClO_4$ (injected through the subaseal) and the $^{14}CO_2$ produced was collected in the centre well. The phenylethylamine/methanol mixture was added before the termination of the incubation by injection through the subaseal using a 1 ml capacity syringe. After addition of $HClO_4$ the flasks were returned to the water bath for a further 1 h incubation to facilitate the release and collection of $^{14}CO_2$. After this period, the centre well was removed

from the flask, placed in a scintillation vial containing 1 ml of H_2O, and 10 ml of scintillant was added and mixed. The radioactivity was then determined in a Beckman scintillation counter (Model L 7500).

3.2.3 Specific radioactivities of substrates

The specific radioactivities of the substrates at the final concentration in the incubation medium were as follows:

[U-^{14}C]-glucose : 0.4 uCi/mMol

[1-^{14}C]/[3-^{14}C] pyruvate: 1.0 uci/mMol

[U-^{14}C]-glutamine: 1.0 uCi/mMol

[1-^{14}C]-butyrate: 2.0 uCi/mMol

[1-^{14}C]-oleate: 1.0 uCi/mMol

3.2.4 Analytical Methods

Metabolites in neutralised extracts of cells plus medium were determined spectrophotometrically (using a Gilford Stasar III Spectrophotometer) using enzymic methods by following the changes in absorption at 340 nm of or reduced pyridine nucleotides, in a 1.0 cm light path cell containing the reaction mixture. The following metabolites were measured by the usual spectrophotometric methods: glucose by the coupled hexokinase and glucose-6-phosphate dehydrogenase method (Bergmeyer et al., 1974); glutamine and ammonia (by the method of Windmueller and Spaeth, 1974, except that asparaginase was dialysed for 48h against 4 changes of 80 mM potassium phosphate buffer, pH 6.6 prior to use); glutamate (Bernt and Bergmeyer, 1974); aspartate (Bergmeyer et al., 1974); lactate (Eagle

and Jones, 1978); pyruvate (Czok and Lamprecht, 1974); acetoacetate (Mellanby & Williamson, 1974); 3-hydroxybutyrate (Williamson & Mellanby, 1974); alanine (Williamson, 1974); and ADP and AMP (Jaworek et al., 1974). The ATP content of the macrophage was assayed by a luciferin-luciferase method (Stanley and Williams, 1969). In some experiments, analysis of the concentration of amino acids was carried out using an automated amino acid analyser (Model LKB 4400) using a buffer system described in Appendix C.

3.2.5 Measurement of rate of oxygen consumption

Oxygen consumption was measured polarographically by means of a Clark-type oxygen electrode. The basal rate was determined for every measurement before the addition of substrate, inhibitor or uncoupler. Macrophages, at a density of 4-5 mg protein per incubation, were incubated in 1.2 ml of PBS (A + B, see appendix C) pH 7.2-7.4, at 37°C in a glass jacketed vial which was rapidly stirred for 15-20 min, during which time oxygen consumption was linear with time.

Macrophages were cultured for 48 hours in serum-free medium (in the presence of defatted B.S.A.) in either 95% air/5% CO_2 or 95% O_2/5% CO_2, prior to being removed from culture and treated as described above.

3.2.6 Protein determination

A small volume of cells was removed from the suspension to be

used for an incubation experiment, and solubilised with triethanolamine/KOH (Appendix C). The protein concentration was then determined by the method of Bradford (1976), using γ-globulin as a standard.

3.2.7 Dry weight determination

A volume of cells (containing ~ 1 x 10^8 cells) of known protein concentration was pipetted into a pre-weighed glass tube; after centrifugation at 400g for 5 min, the supernatant was carefully removed, firstly by aspiration and finally by absorption with filter paper. The remaining pellet was then dried at 50°C to constant weight. It was found that 1 mg of macrophage protein (using a γ-globulin standard) was equivalent to 1 mg dry weight, within 10% error.

3.2.8 Biochemical Viability of Macrophages

One means of assessing the metabolic viability of cells during in vitro incubation is to measure the concentration of ATP, ADP and AMP. Maintenance of the normal concentrations of these nucleotides and specially their concentration ratios is considered to be an indication of biochemical viability (Newsholme & Start, 1973; Newsholme et al., 1986). Therefore, the concentrations of these nucleotides were measured in macrophages as soon as possible after removal from culture (30-60 min) and then after 30, 60 and 90 min of incubation in the standard incubation medium plus or minus 2 mM glutamine (table 3.1). Preincubation was not used in these experiments. The concentrations of ATP and the ATP/AMP concentration ratios were slightly low at zero

TABLE 3.1 - Concentrations of adenine nucleotides in incubated peritoneal murine macrophages.

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

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

time (this probably reflects the effect of the period of 30-60 min in which the cells are kept on ice, that is, the period between removal of the cells from culture and the start of the incubation) but were increased at 30 and especially by 60 min of incubation. The ATP/AMP concentration ratios, which is considered to be the best indicator of biochemical viability, and the increase in the total adenine nucleotide concentration over the period of incubation are similar to the results reported for rat lymphocytes by Ardawi & Newsholme (1983); they are in contrast with the situation found in rat enterocytes (Watford et al., 1979) and rat colonocytes (Ardawi & Newsholme, 1985), in which the total adenine nucleotide concentration and the [ATP]/[AMP] ratio decreased during incubation. The increase in the total adenine nucleotide concentration in the macrophage probably reflects the synthesis of purine nucleotides, possibly from adenosine produced during the period on ice.

In addition, it was found that rates of glucose and pyruvate utilisation and lactate production were linear over 60 min of incubation (see Results section) and the rate of O_2 consumption was linear over 20 min (results not shown). These results indicate that isolated peritoneal macrophages provide a satisfactory system for short-term metabolic studies.

RESULTS

3.3.1 Rates of utilisation of glucose, glutamine, pyruvate, acetoacetate and 3-hydroxybutyrate

At an initial concentration of 5 mM, glucose is used at a rate of more than 300 nmol/h per mg protein by incubated macrophages and almost all of this glucose utilisation can be accounted for as lactate (table 3.2). In identical incubation conditions, it was observed that the rate of glucose utilisation by incubated lymphocytes was 40 nmol/h per mg protein, that is, the rate of utilisation by the macrophage is approximately 8-9 fold greater than that by rat lymphocytes (see table 3.7). The rates of glucose utilisation and lactate production are approximately linear with time (Fig. 3.1).

Glutamine, at an initial concentration of 2 mM, is utilised at a rate of approximately 100 nmol/h per mg protein by incubated macrophages (table 3.2). The rates of production of glutamate, aspartate and lactate are high; surprisingly, the total rate of formation of these three end-products is considerably higher than the rate of utilisation of glutamine. This problem is addressed in section 3.4.2. The production rates of a number of amino acids, when macrophages are incubated in the presence of glutamine, are shown in table 3.3. The observed rate of glutamine utilisation by the macrophage is similar to that of the rat lymphocyte (see table 3.8).

Pyruvate was utilised at a rate of 73 nmol/hr per mg protein by the incubated macrophage (Figure 3.2), and it was linear over 60 min. This rate is similar to that of the rat lymphocyte incubated under

TABLE 3.2- Rate of utilisation of glucose and glutamine and production of lactate, glutamate and aspartate by isolated incubated murine peritoneal macrophages.

Macrophages were incubated as described in experimental Section for 60 minutes at a density of 3-6 mg protein/ml. Rates are given as means ± S.E.M.; the number of separate experiments was at least four. A negative sign indicates utilisation; differences in rates between incubations with glucose and glucose plus glutamine for glucose and glutamine utilisation and aspartate production that are statistically significant (Student's t test) are indicated by *($P < 0.05$) or +($P < 0.01$).

Rates of lactate, glutamate or aspartate production in the presence of glucose, glutamine or both substrates have been corrected for endogenous (i.e., blank) rate of production.

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

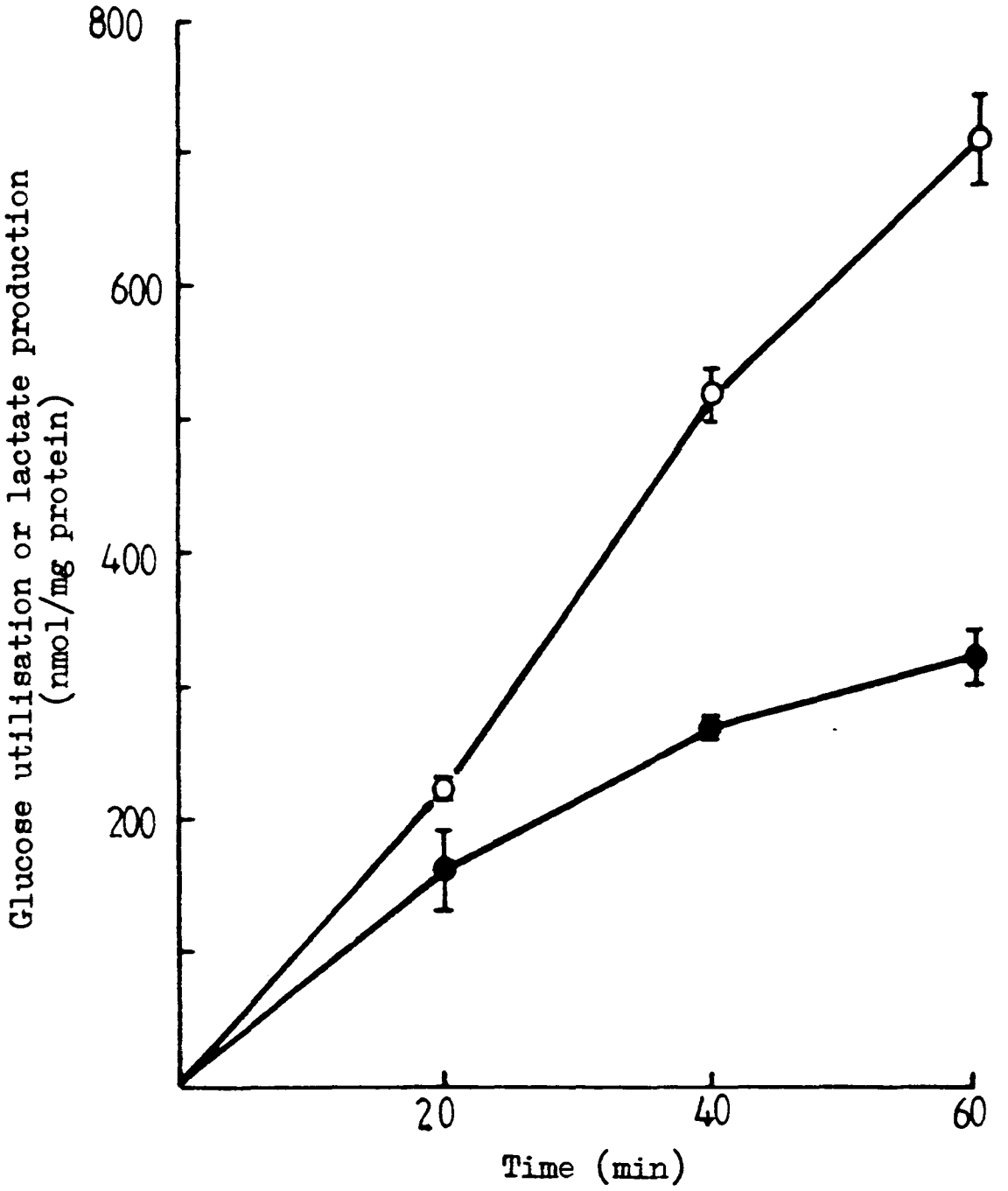


Figure 3.1 Time course of glucose utilisation (●) and lactate production (O) by incubated macrophages.

Incubations were terminated at the times indicated and glucose and lactate concentrations measured as described in the experimental section. The initial concentration of glucose was 5 mM. Results are presented as means of four separate experiments. Bars represent ± S.E.M. Cells were incubated at a density of 3-6 mg protein/ml.

TABLE 3.3 Production rates of amino acids by incubated macrophages.

Macrophages were incubated as described in the experimental section for 60 min at a density of 3-6 mg protein/ml. Incubations were terminated as described in the experimental section, and a sample of neutralised medium, containing intracellular and extracellular metabolites, was analysed for amino acid content using an amino acid analyser.

Amino acid	Production rate (nmol/h per mg protein
Glutamate	111.3
Aspartate	26.3
Alanine	8.2
Valine	2.9
Methionine	6.1
Leucine	3.1
Tyrosine	3.8
Phenylalanine	2.7
Lysine	1.5

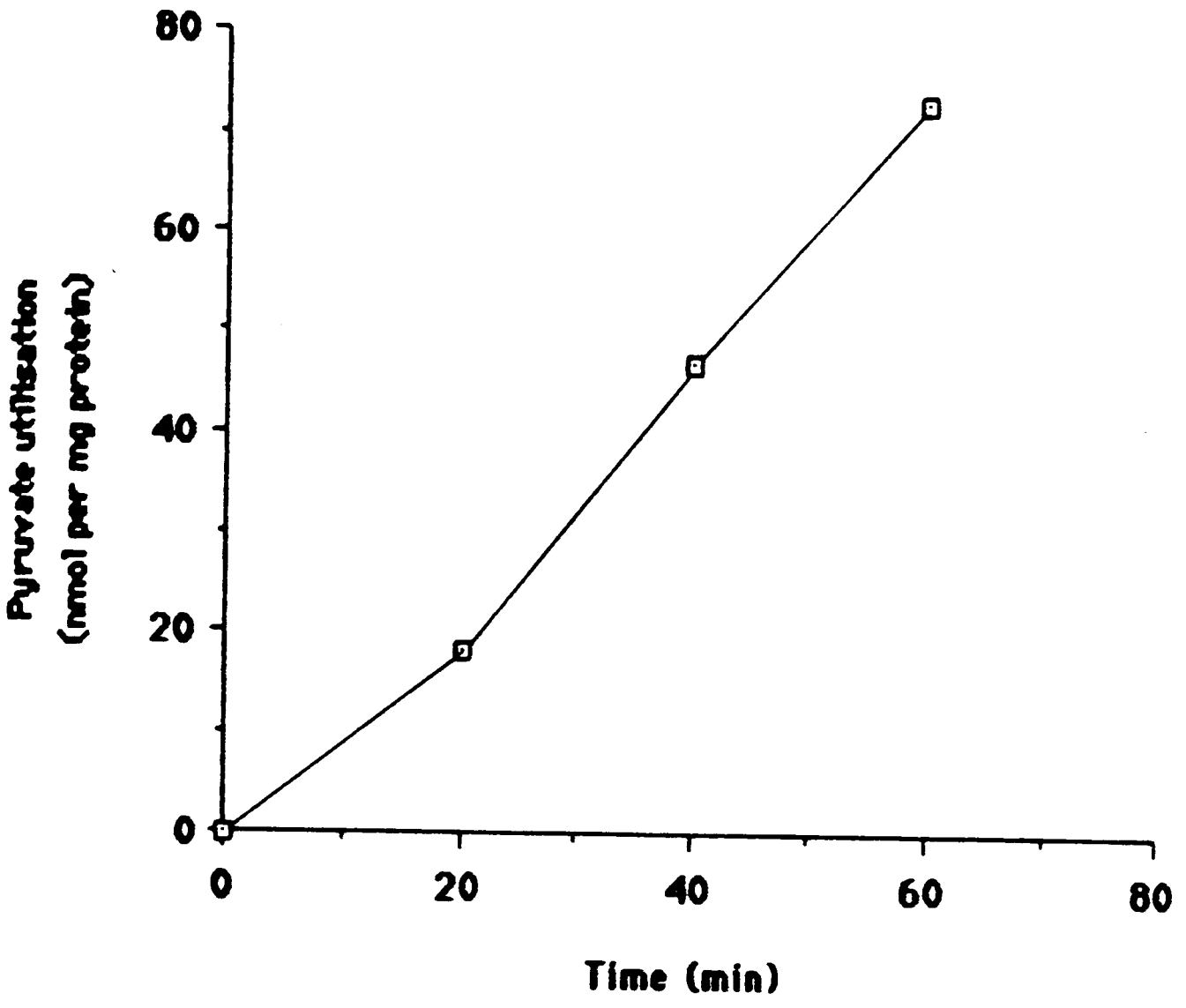


Figure 3.2 - Rate of pyruvate utilisation by incubated macrophages

Macrophages were incubated as described in the experimental section. Incubations were terminated at the times indicated, and pyruvate utilisation was measured as described in the experimental section. Results are presented as means of three separate experiments (each consists of 2-3 separate incubations). S.E.M. values are not given but they are less than 10% of means.

similar conditions (see Curi et al., 1987).

Rates of utilisation of either acetoacetate or 3-hydroxybutyrate by the incubated macrophage were not detectable (data not shown). This result was unexpected, especially for acetoacetate, since the macrophage contains the ketone-body utilising enzymes 3-oxoacid CoA-transferase and acetoacetyl-CoA thiolase (see table 2.1). These enzymes are also present in the rat lymphocyte (Ardawi & Newsholme, 1982) which utilise ketone bodies at a rate similar to that of glucose (Ardawi & Newsholme, 1984). A possible explanation for this difference will be given in the discussion section of this chapter.

3.3.2 Interaction between glucose and glutamine metabolism

When 2 mM-glutamine was included in the incubation medium containing 5 mM-glucose, the rate of glucose utilisation by macrophages was decreased by 16%, and the rate of glutamine utilisation was decreased by 39% (table 3.2). In the presence of glucose, the rate of aspartate production was decreased by 47%. However, addition of the alternative substrates to medium containing one of them caused no statistically significant change in the rate of glutamate or lactate production; these findings are different to those observed with lymphocytes (Ardawi & Newsholme, 1983, 1984).

3.3.3 The effect of ketone bodies and fatty acids on rates of glucose metabolism by incubated macrophages

The rates of glucose utilisation or lactate production by

incubated macrophages were not significantly changed by 3 mM-acetoacetate or 3 mM 3-hydroxybutyrate; nor were they changed by 0.5 mM butyrate or 0.3 mM oleate (results not shown).

3.3.4 Rates of oxidation of glucose, glutamine, pyruvate, short and long chain fatty acids by incubated macrophages

The rates of oxidation of glucose, glutamine, pyruvate, butyrate and oleate have been investigated by following the production of $^{14}CO_2$ from [^{14}C]-labelled substrates. These rates are presented in table 3.4, together with actual rates of substrate utilisation.

A time course for the oxidation of pyruvate is shown in Figure 3.3. [1-^{14}C]-Pyruvate can lose $^{14}CO_2$ in the pyruvate dehydrogenase reaction or, if it is metabolised via pyruvate carboxylase and the label randomized, in the TCA cycle via the decarboxylation reactions of this cycle, or by the action of PEPCK. However, [3-^{14}C]-pyruvate will lose $^{14}CO_2$ primarily from the decarboxylation reactions of the TCA cycle or by the action of PEPCK. The difference observed in the rates of oxidation of [1-^{14}C]- and [3-^{14}C]-pyruvate (Figure 3.3) suggests that most of the pyruvate is metabolised via pyruvate dehydrogenase and that only a small proportion is converted to oxaloacetate and oxidised by reactions of the TCA cycle.

The effect of the concentration of oleate on its rate of oxidation is shown in Figure 3.4. The oleate was added to the incubation as a complex with albumin (see Appendix C). The albumin concentration was varied between 1.5 and 2.5% (w/v) in these

TABLE 3.4 - Rates of utilisation and oxidation of glucose, glutamine, pyruvate, butyrate and oleate by isolated incubated murine peritoneal macrophages.

Macrophages were incubated for 60 min at a density of 3-6 mg/ml and rates of utilisation and oxidation were measured as described in the experimental section. Results are presented as means ± S.E.M. for at least four separate experimenta.

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

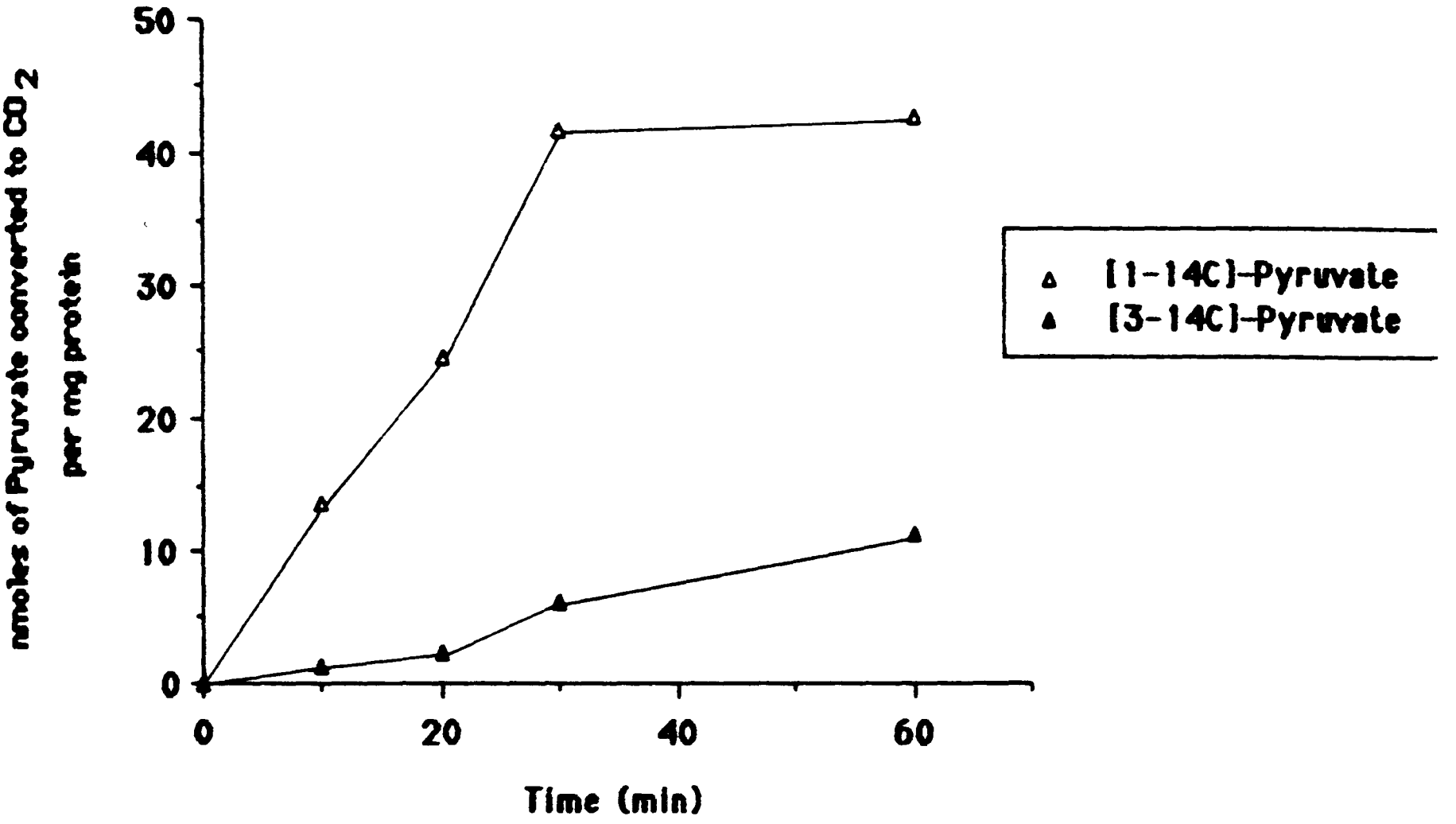


Figure 3.3 - Rates of pyruvate converted to CO_2 by incubated macrophages

Macrophages were incubated as described in the experimental section. Incubations were terminated at the times indicated, and $^{14}CO_2$ collected and measured as described in the experimental section. Results are presented as means of three separate experiments (each consists of 2-3 separate incubations). S.E.M. values are not given but they are less than 10% of means.

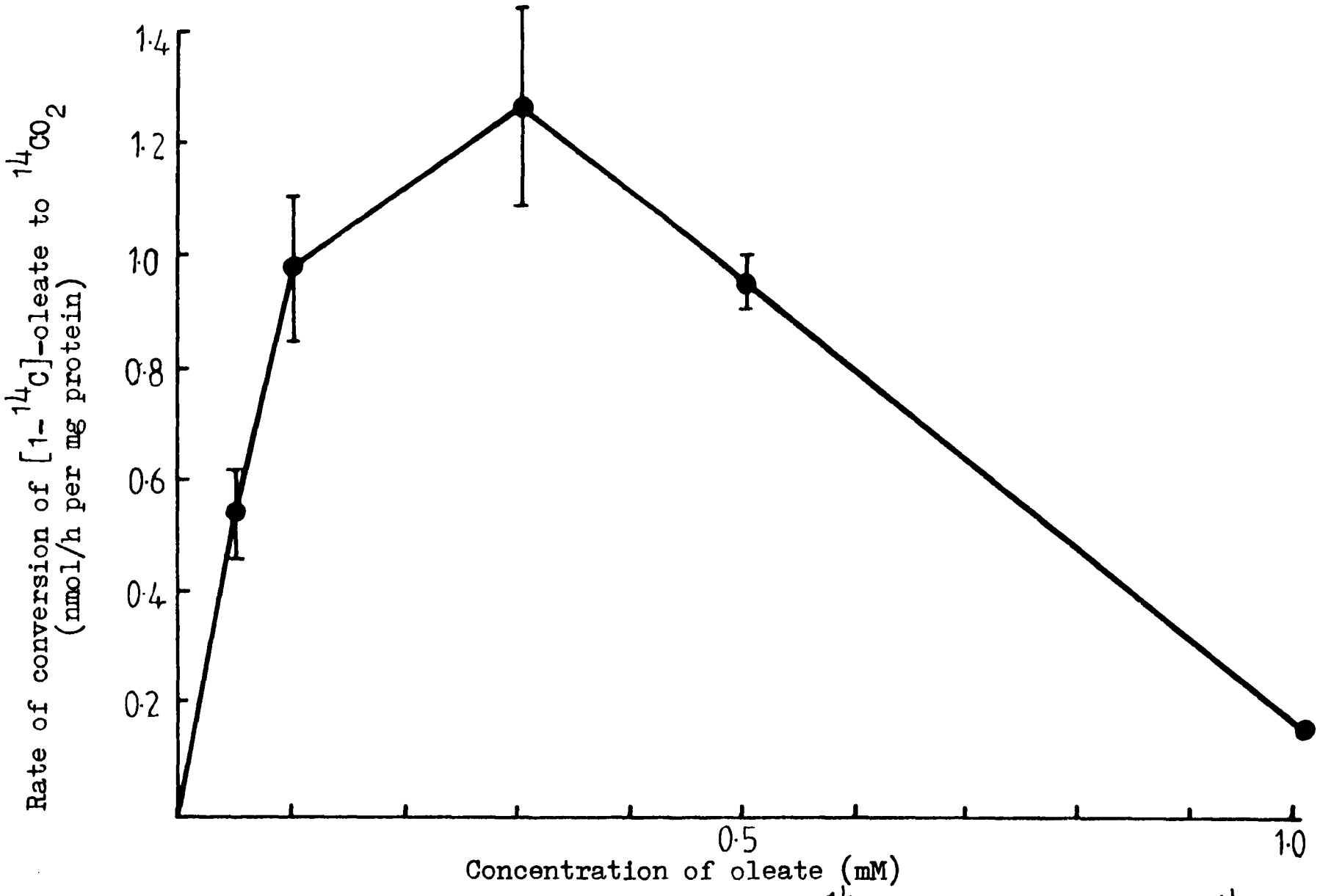


Figure 3.4 The effect of concentration of oleate on the rate of $^{14}CO_2$ production from [1-^{14}C]-oleate by incubated macrophages.

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

experiments. At a concentration of albumin of 2.5%, the optimal concentration of oleate for $^{14}CO_2$ production was found to be 0.3 mM and, in contrast to lymphocytes and other tissues (see Ardawi & Newsholme, 1985a), the rate of oxidation decreased dramatically as the concentration of oleate was increased to 1mM. The Rate of uptake of oleate by the macrophage has been shown to be saturated at 0.2 mM by Lokesh and Wrann, 1984. This is in complete contrast to other tissues, for example, the rat heart, which exhibits a threshold effect: below about 0.35 mM very little or no fatty acid uptake occurs (Opie, 1968). The significance of this difference will be discussed in chapter 6.

3.3.5 Rates of Oxygen Consumption by the Incubated Macrophage

Macrophages that had been cultured in 95% air/5%CO_2 for 48h, utilised oxygen at a rate of almost 50nmol/h per mg protein in the absence of any added substrate and this was decreased by about 75% in the presence of KCN (table 3.5); this indicates that most of the oxygen consumed required the participation of cytochrome oxidase and was not involved in production of toxic oxygen products. This is consistent with the finding that macrophages, which are cultured at a high density (as in this experiment) lose some of their capacity to secrete toxic oxygen products (Johnston et al., 1978). The addition of an uncoupling agent (CCCP) doubled the rate of oxygen consumption (table 3.5). However, even this latter rate is only 4% that which would be expected if oxoglutarate dehydrogenase provided a quantitative index of the maximum flux through the TCA cycle, as it does for muscle tissue (Newsholme and Paul, 1983). This suggests that the role of oxoglutarate dehydrogenase may not be so much as part of

TABLE 3.5- Oxygen consumption by isolated peritoneal murine macrophages cultured in air and oxygen.

Macrophages were cultured and prepared as described in the experimental section. Culture in air was 95% air plus 5% CO_2 and culture in oxygen was 95% O_2 plus 5% CO_2. Oxygen consumption was measured polargraphically by means of a Clark-type oxygen electrode. The basal rate was determined for every measurement before the addition of substrate, inhibitor or uncoupler. Macrophages at a density of 4–5 mg protein/1.2 ml buffer(pH 7.2–7.4) were incubated at 37°C in a glass jacketted vial which was rapidly stirred for 15–20 min during which time oxygen consumption was linear. Values of O_2 consumption are presented as means ± S.E.M. with the number of separate determinations given in parenthesis. Significant difference (Student's *t* test) from basal oxygen consumption is indicated by * ($P < 0.05$), $^{+}(P < 0.01)$, $^{++}(P < 0.001)$.

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

the 'classical' TCA cycle but as part of the pathway for utilisation of gluamine (see Figures 5.1 and 5.6). Of the various substrates that were added to the incubated medium [glucose (5 mM), pyruvate (1mM), alanine (2mM), acetoacetate (3mM), 3-hydroxybutyrate (3mM), oleate (0.5mM) and butyrate (0.5mM] only succinate or pyruvate significantly increased the rate of oxygen uptake and addition of succinate plus glutamate or succinate plus glutamine did not increase the rate above that in the presence of succinate alone (table 3.5). This suggests that metabolism of endogenous substrates can provide much if not all of the energy required by these cells, at least for a short period of incubation (15 min). Similar findings were obtained for macrophages that had been cultured in 95% oxygen/5% CO_2 except that the rate of oxygen consumption in the absence of added substrate was lower (20.5 nmol/h per mg protein) but was increased to a higher rate (157 nmol/h per mg protein) by the addition of the uncoupling agent CCCP, plus succinate (table 3.5).

3.3.6 Effect of albumin on glucose and glutamine metabolism.

It is well-established that pinocytosis and phagocytosis occur at high rates in inflammatory macrophages (see Cohn, 1978). Both these processes could result in the uptake of protein (i.e., albumin) from the incubation medium. It is likely that this protein will be digested by the lysosomes followed by release and subsequent utilisation of the constituent amino acids. Consequently, it was considered important to investigate the rates of utilisation of glucose and glutamine in the presence and absence of albumin in incubation medium. Omission of albumin from the incubation medium had no effect on the rates of

glucose utilisation or lactate formation, but glutamine metabolism was considerably modified. In the absence of albumin, the rate of glutamine utilisation was almost 100% higher (table 3.6) and there was a 40% increase in the rate of glutamate production, but there were small decreases in the rates of aspartate and lactate production. Furthermore, in the absence of albumin, the rate of glutamine utilisation was almost linear with time for 60 min whereas, in the presence of albumin, the rate almost reached a plateau after 40 min (Figure 3.5). These effects could be explained by use of endogenously produced amino acids: thus, it is likely that, after about 40 min of incubation, amino acid production from lysosomal degradation of albumin would be making a quantitatively significant contribution to metabolism. Interestingly, the overall rate of glutamate production is higher in the absence of albumin but there is little difference in the rate of aspartate production (Figure 3.5). The reason for this is unclear.

3.3.7 Rates of glutamine utilisation by incubated macrophages at different Glutamine concentrations in the absence of albumin

The rate of glutamine utilisation by incubated macrophages does not approach saturation until the concentration of extracellular glutamine exceeds 20mM (Figure 3.6). A double-reciprocal plot of the rate of utilisation versus glutamine concentration (Figure 3.7) indicates that the concentration of glutamine which produces 50% of the maximal rate of utilisation is about 9.6mM. It is of interest that this value is similar to the intracellular concentration of glutamine in the macrophage after a short exposure to medium containing 2mM

TABLE 3.6 - Rates of utilisation of glucose and glutamine and production of lactate glutamate and aspartate by murine macrophages incubated with or without albumin.

Macrophages were incubated as described in experimental section for 60 min at a density of 3-6 mg/m Rates are given as means ± S.E.M.; the number of experiments is given in parentheses. A negative sign indicates utilisation; differences in rates between incubations with and without albumin (1.5% that are statistically significant (Student's t test) are indicated by *(P<0.05) or +(P<0.01).

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

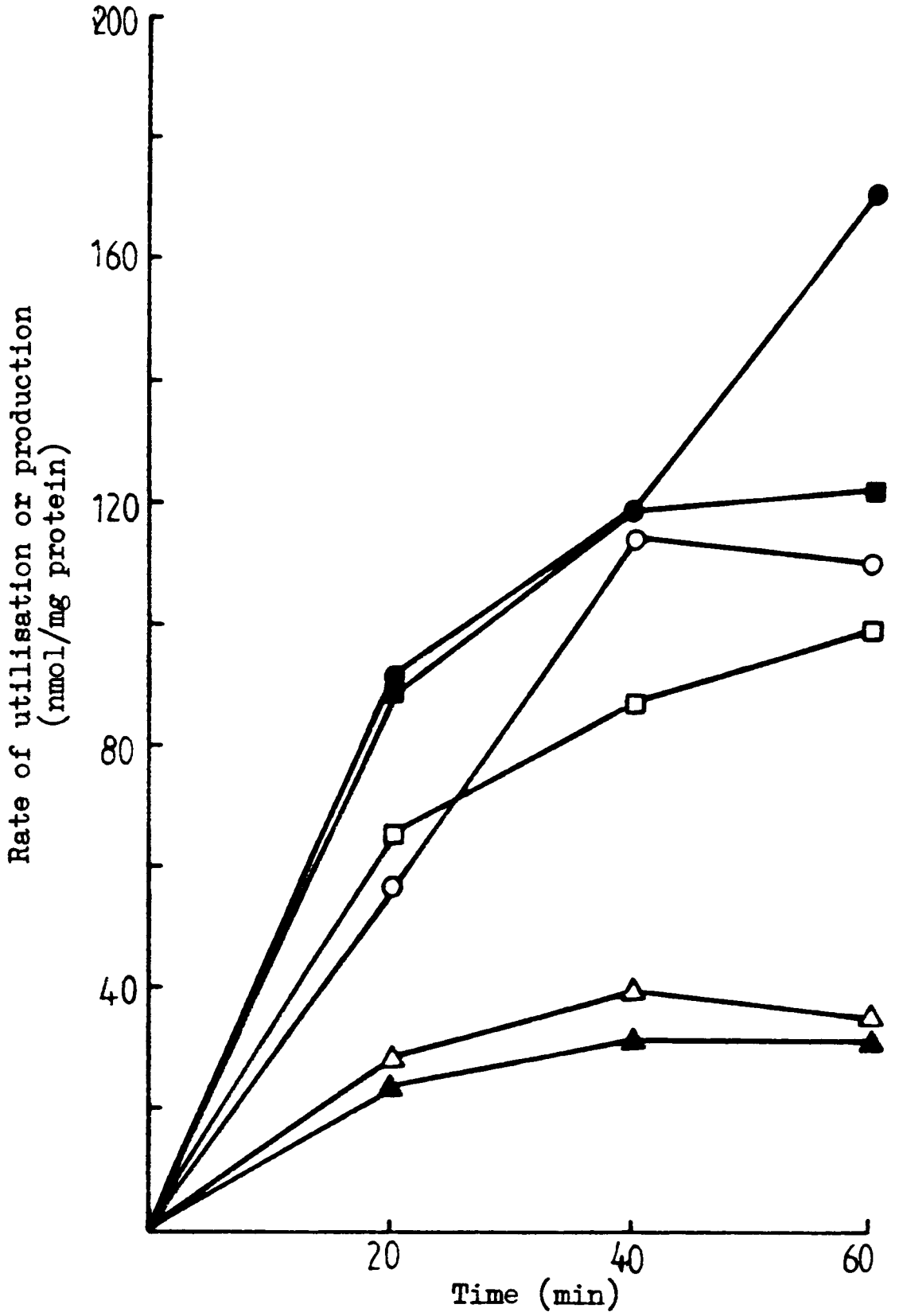


Figure 3.5 Rates of glutamine utilisation (○,●) Glutamate (□,■) and aspartate (Δ,▲) production by incubated macrophages in the presence or absence of albumin.

Incubations of macrophages were terminated at the times indicated and glutamine, glutamate and aspartate concentrations measured as described in the experimental section. The initial concentration of glutamine was 2 mM. Open symbols represent incubations in the presence of 1.5% (w/v) albumin in the incubation medium, filled symbols represent incubations in the absence of albumin. Cells were incubated at a density of 3-6 mg protein/ml. Results are presented as the means of four separate experiments. S.E.M. values are not given but they are less than 10% of means.

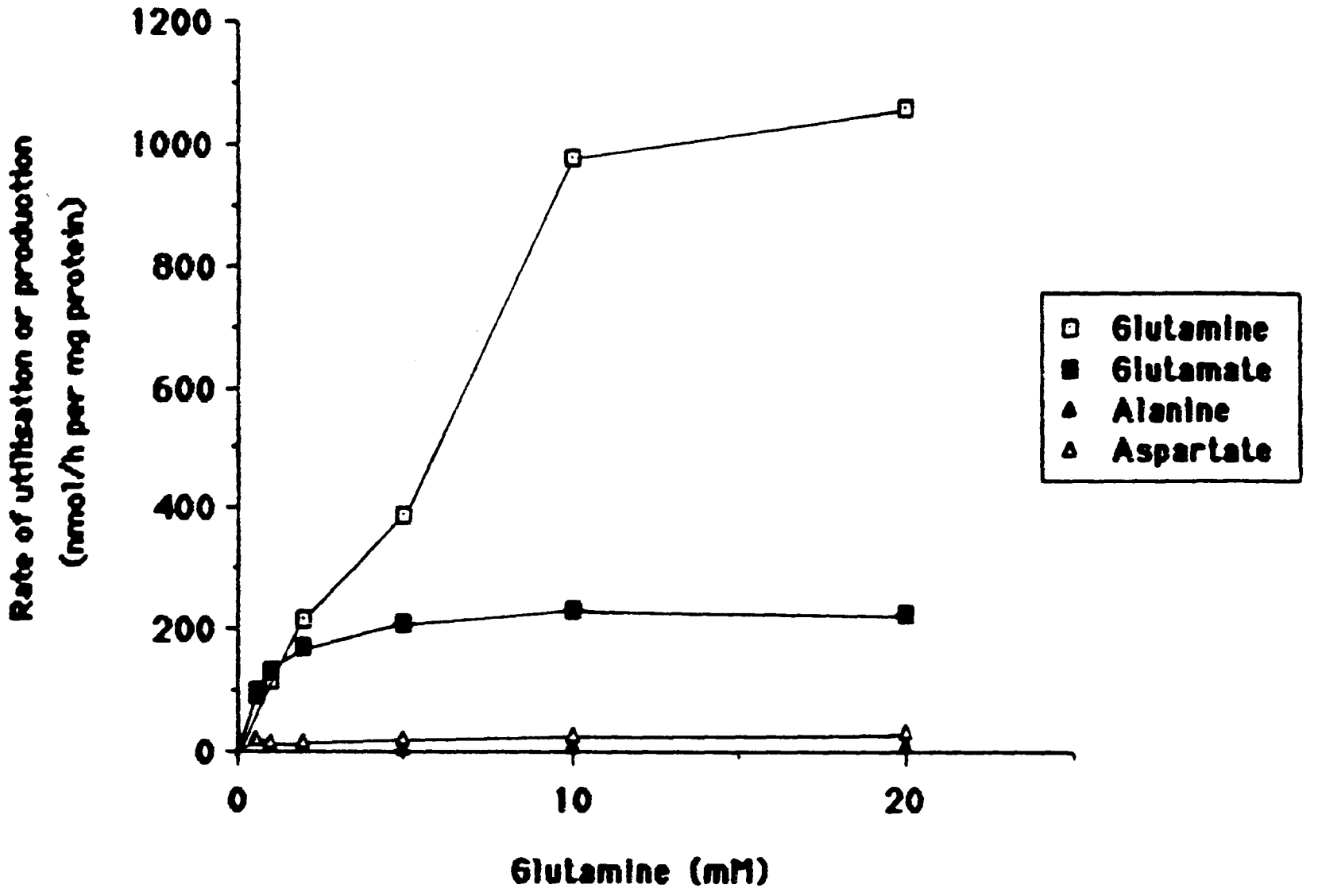


Figure 3.6 - The rates of glutamine utilisation; glutamate, aspartate and alanine production versus glutamine concentration.

Macrophages were incubated as described in the experimental section. Incubations were terminated after 1 h and glutamine utilisation or glutamate, aspartate, alanine production were measured as described in the experimental section. Results are presented as means of three separate experiments (each consists of 2-3 separate incubations). S.E.M. values are not given but they are less than 10% of means.

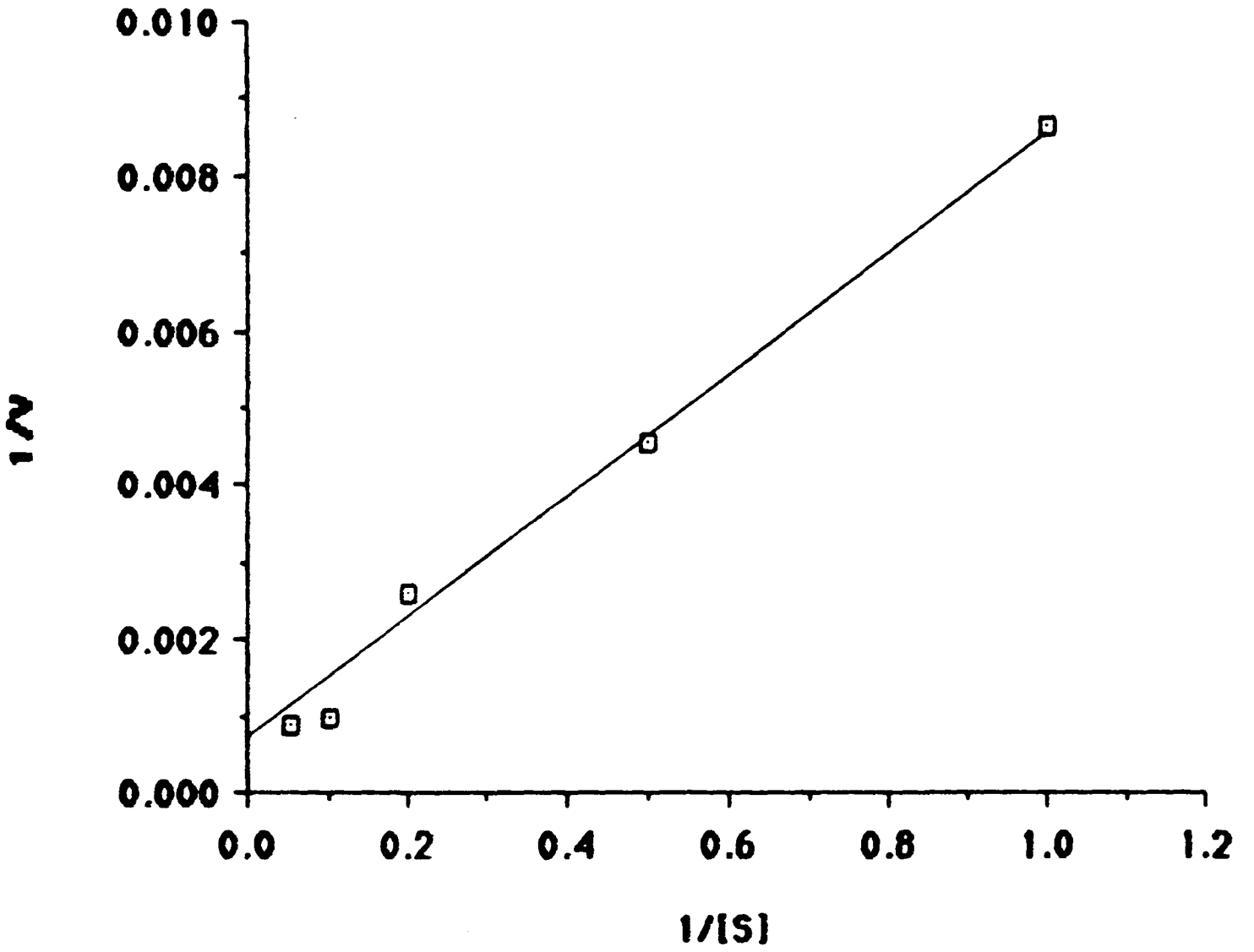


Figure 3.7 - Double-reciprocal plot of the rate of glutamine utilisation by incubated macrophages versus glutamine concentration.

Macrophages were incubated as described in the experimental section. Incubations were terminated after 1 h and glutamine utilisation was measured as described in the experimental section. Results are presented as means of three separate experiments (each consists of 2-3 separate incubations). The units used in this figure are, [S]: mM; V: nmol/h per mg protein.

glutamine (see chapter 5). The rates of glutamate, aspartate and alanine production approach saturation at approximately 10mM glutamine (Figure 3.6). The rate of alanine production in these short-term incubations (1h) was never more than 8% of the rate of glutamine utilsation. The fate of the glutamine that is not recovered as glutamate, aspartate, alanine or lactate (the rate of production of lactate at higher concentrations of glutamine did not exceed that found at 2mM glutamine-data not shown in Figure 3.6) is uncertain; it is possible that at the higher concentrations of glutamine, its rate of oxidation may increase disproportionately. This possibility was not investigated, but it should be noted here that macrophages in culture appear to oxidise a much greater proportion of the glutamine than in the short-term incubations (see chapter 4).

DISCUSSION

3.4.1 Glucose Metabolism

The work presented in this chapter demonstrates, for the first time, that the rate of glucose utilisation by the incubated macrophage is extremely high. It is considered that this is not an abnormal response to incubation since the macrophage utilises glucose at even higher rates when in culture (Chapter 4). The rate of glucose utilisation reported in this chapter for elicited macrophages (Table 3.2) is high in comparison with almost all other tissues; the rate in isolated soleus muscle of the rat is approximately 100 nmol/h per mg of protein (Challiss *et al.*, 1986) and that for rat brain *in situ* is approximately 300 nmol/h per mg of protein (Hawkins *et al.*, 1971). The rate of glucose utilisation by the macrophage is approximately 10 fold higher than cells isolated from lymphoid tissues and is comparable to the rates reported for cells such as enterocytes and colonocytes (table 3.7) which are known to utilise glucose at high rates.

Of particular interest is the fact that almost all of the glucose utilised by the macrophage can be accounted for as lactate (Table 3.2 and Fig. 3.1) and this is also the case for macrophages in culture (Chapter 4). This occurs despite the fact that the cells have oxygen available and hence it can be described as aerobic glycolysis. The actual proportion of the glucose utilised that is oxidised to CO_2 (as indicated by conversion of U-^{14}C-glucose to $^{14}CO_2$ by the incubated macrophage is very small - probably about 3% (Table 3.4). Since some glucose could have been converted to lactate via the pentose phosphate pathway and hence lost CO_2 in the third reaction of that pathway

TABLE 3.7 Rates of utilisation of glucose by various *in vitro* cell incubations.

Type of cell	Conditions of incubation	Rate of utilisation (μmol/h per g dry wt.)
Murine macrophages[a]	5 mM glucose 100% O_2	339
Rat lymphocytes[b]	5 mM glucose 100% O_2	37.2
Rat thymocytes[c]	5 mM glucose 100% O_2	22.2
Rat enterocytes[d]	10 mM glucose 95% O_2: 5% CO_2	720
Rat colonocytes[e]	10 mM glucose 95% O_2: 5% CO_2	413

a: present work

b: Ardawi and Newsholme (1983

c: Hume *et al.* (1978)

d: Watford *et al.* (1979)

e: Ardawi and Newsholme (1985b)

perhaps none of the carbon of the utilised glucose is oxidised by the TCA cycle. Consistent with this view, Stubbs et al. (1973) reported that, of the CO_2 produced from glucose by the macrophage, very little was derived from the TCA cycle: it was considered that CO_2 was produced via glucose utilisation by the pentose phosphate pathway. This characteristic of the macrophage, to convert glucose almost stoichiometrically into lactate at a high rate, is similar to that reported, many years ago, for proliferating cells (see Warburg, 1956 a,b): it is also characteristic for cells that have the potential for rapid cell division [e.g. lymphocytes - see Roos & Loos, 1973; Hume et al., 1978; Ardawi & Newsholme, 1983) and also for enterocytes (Watford et al.,1979) and colonocytes (Roediger, 1982)]. However, some of the enterocytes and colonocytes will not be dividing (see Hansen & Parsons, 1987). They are perhaps similar to macrophages, that is they are terminally differentiated and have little ability to divide. The possible reason for this metabolic similarity bewtween rapidly-dividing cells, those with the potential for rapid cell division and terminally-differentiated cells such as macrophages, colonocytes and enterocytes will be discussed in Chapter 6.

The high rate of glycolysis in the macrophage will provide a considerable amount of ATP which will satisfy at least part of the energy requirement of these cells (see Chapter 4, section 4.4.7). Other possible fuels for the macrophage that were investigated in this work were glutamine, long and short-chain fatty acids and ketone bodies. Of these potential fuels, only glutamine was utilised at a high rate. However, when glucose and glutamine were present in the incubation medium together, glutamine had little effect on the rate of

glycolysis: it was slightly inhibited (table 3.2). Thus either the energy requirement of the macrophage is increased when both glucose and glutamine are being utilised or an endogenous fuel(s) is being consumed when glutamine is omitted from the incubation medium, and that the rate of endogenous fuel utilisation is inhibited by glutamine.

3.4.2 Glutamine Metabolism

The possible importance of glutamine metabolism in macrophages was indicated by the observations that these cells also possess a high activity of glutaminase, together with high activities of enzymes capable of metabolising glutamate to malate or pyruvate (see chapter 2). The work presented in this chapter has established, for the first time, that glutamine is utilised by incubated macrophages at a very high rate (see tables 3.2 and 3.6). Indeed the rate of glutamine utilisation by the incubated macrophage is as high as that reported for any other type of cell (table 3.8). The major end-products of glutamine metabolism appear to be glutamate, aspartate, lactate and alanine (see tables 3.2, 3.3. and 3.6). Of the glutamine metabolised beyond glutamate, a greater proportion gives rise to aspartate rather than alanine (Table 3.3). This finding is expected on the basis of the maximum activities of aspartate aminotransferase and alanine aminotransferase (see Table 2.1).

Somewhat surprisingly it was observed that the total rate of production of glutamate, lactate plus aspartate by these cells is considerably higher than the rate of utilisation of glutamine. This

TABLE 3.8 Rates of utilisation of glutamine by various *in vitro* cell incubations

Type of cell	Conditions of incubation	Rate of utilisation (μmol/h per g dry wt.)
Murine macrophages[a]	2 mM glutamine (plus 1.5% BSA) 100% O_2	102
Murine macrophages[a]	2 mM glutamine (BSA omitted) 100% O_2	186
Murine macrophages[a]	5 mM glutamine (BSA omitted) 100% O_2	391
Rat lymphocytes[b]	2 mM glutamine 100% O_2	160
Rat enterocytes[c]	5 mM glutamine 95% O_2: 5% CO_2	660
Rat colonocytes[d]	5 mM glutamine 100% O_2	331

a: present work

b: Ardawi and Newsholme (1983)

c: Watford *et al*. (1979)

d: Ardawi and Newsholme (1985b)

could have been due to uptake and digestion of albumin from the medium by these cells. When experiments were carried out in the absence of added albumin to the incubation medium, the rate of end-product formation was almost identical to that of glutamine utilised. This supports the view that the rate of formation of amino acids from the albumin, which enters the cell via endocytosis and is subsequently digested by lysosomes, was sufficient to interfere in the metabolic studies. The rate of endocytosis and association of the endocytotic vacuole with the lysosome does appear to be sufficiently rapid in macrophages to explain these findings - that is significant digestion of albumin within one hour of incubation. Thus Kielian & Cohn (1980) have shown that a large proportion of phagocytosed zymosan particles are associated with lysosomes within 1h of exposure of murine macrophages to these particles. Further, many of the amino acids liberated by albumin digestion could be transaminated with oxoglutarate so producing glutamate, thus contributing to the very high rate of production of glutamate when albumin is present in the incubation medium (table 3.6).

The data presented in table 3.4 indicate that little of the glutamine utilised by the macrophage is oxidised. There are several possible pathways by which glutamine could be either completely or partially oxidised by macrophages: (i) For complete oxidation of glutamine, 2-oxoglutarate derived from glutamine is metabolised via a pathway which involves five decarboxylation reactions, three within the TCA cycle [oxoglutarate dehyddrogenase(twice) plus isocitrate dehydrogenase] and phosphoenolpyruvate carboxykinase plus pyruvate dehydrogenase (Figure 3.8).

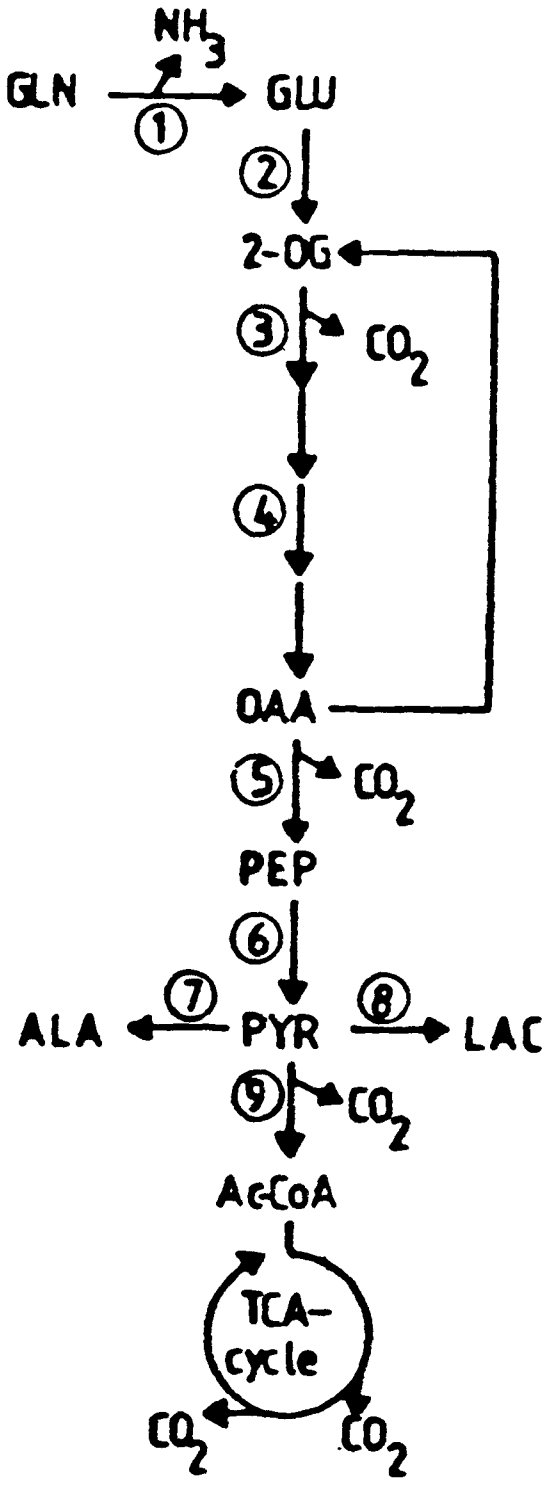


Fig. 3: 8 Possible pathway(s) for the oxidation of glutamine in macrophages

1. Glutaminase; 2. transamination; 3. oxoglutarate dehydrogenase; 4. reactions that are considered part of the TCA-cycle; 5. phosphoenolpyruvate carboxykinase; 6. pyruvate kinase; 7. transamination; 8. lactate dehydrogenase; 9. pyruvate dehydrogenase.

(ii) For partial oxidation of glutamine, 2-oxoglutarate derived from glutamine is metabolised until the stage of oxaloacetate formation with one decarboxylation step at the level of oxoglutarate dehydrogenase (top part of Figure 3.8); hence only 20% of the carbon of glutamine would be converted to CO_2.

(iii) 2-Oxoglutarate derived from glutamine is metabolised to pyruvate via phosphoenolpyruvate carboxykinase; the pyruvate can either be oxidised in the TCA cycle resulting in complete oxidation of glutamine or be converted to alanine or lactate with only 40% of the carbon of glutamine converted to CO_2 (Figure 3.8).

Calculations, based on the amount of $^{14}CO_2$ produced from [U-^{14}C]-glutamine, over 1 h of incubation, indicate that the percentage of utilised glutamine that is metabolised by the pathways described in (i), (ii) and (iii) would be 9%, 47% and 23% respectively. Since it can be estimated that up to 30% of utilised glutamine can be metabolised beyond glutamate, to aspartate and lactate in these experiments (Table 3.6), it is suggested that the pathway of glutamine utilisation in the macrophage involves only partial oxidation. The glutamine that is metabolised beyond glutamate will give rise mainly to oxaloacetate which will be transaminated to aspartate, with a smaller amount giving rise to pyruvate and hence lactate or alanine (see Figure 3.8).

The ability of the macrophage to utilise glutamine at a high rate and metabolise it via a pathway of partial oxidation is similar to that reported for tumour cells (see McKeehan, 1982; Kovacevic & McGivan, 1983) and normal cells with proliferative potential (e.g.

lymphocytes, thymocytes) (see Ardawi & Newsholme, 1985a). Thus there are two common metabolic characteristics of all of these types of cells: both glutamine and glucose are utilised at high rates, but both substrates are only partially oxidised (see chapter 6 for further discussion).

3.4.3 Glucose and Glutamine as fuels for the macrophage

Despite the fact that glucose and glutamine are only partially oxidised, the high fluxes through these pathways will provide substantial rates of ATP generation. The pathway of glucose utilisation can provide ATP via substrate level phosphorylation, whereas the pathway of glutamine utilisation will provide ATP primarily by mitochondrial oxidative phosphorylation. Calculations based on the highest observed rate of glucose utilisation (and assuming glucose is metabolised solely to lactate) indicate that approximately 700 nmol ATP/h per mg protein will be generated from this fuel. Calculations based on the highest observed rate of glutamine utilisation [assuming approximately 30% of glutamine is metabolised beyond glutamate (Table 3.6) and as far as aspartate (Figure 3.8)] indicate that approximately 500 nmol ATP/h per mg protein will be generated from this fuel. When both fuels are present in the incubation medium the rate of ATP generation is calculated as 930 nmol/h per mg protein, and in these conditions (making the assumptions concerning the pathways of metabolism as described above and using the rates of utilisation reported in table 3.6) glucose will contribute 62% and glutamine will contribute 38% to the energy requirement of the cell. It should be noted that any endogenous fuel

contribution to energy generation has not been taken into account in these calculations.

Since the macrophage possess the enzymic potential for oxidation of acetyl-CoA via the TCA cycle, but little acetyl-CoA appears to be produced from either glucose or glutamine, the question arises as to which substrates can be oxidised by the this cycle in these cells. To answer this question, rates of utilisation and/or oxidation of fatty acids and ketone bodies were measured in these cells.

3.4.4 Rates of fatty acid and ketone body utilisation

The short-chain fatty acid butyrate was oxidised by the macrophage at a very low rate (table 3.4). The long-chain fatty acid oleate was oxidised at a maximal rate of 1.3 nmol/h per mg protein (Figure 3.4). This is considerably lower than expected on the basis of carnitine palmitoyl transferase activity. The low oxidation rate for oleate observed over 1 h of incubation may be due to the dilution of radioactivity at the level of fatty acyl CoA because of turnover of phospholipid or triglyceride (see the discussion section of Chapter 4). Higher rates of utilisation are observed for cultured macrophages (see table 4.15; Lokesh & Wrann, 1984). The rate of oleate utilisation by macrophages in culture is found to be 20-30 nmol/hr per mg protein, and the process approaches saturation at about 0.2 mM oleate. Much of the oleate utilised by the cells in culture is incorporated into cellular lipids. Macrophages are known to have a marked plasma membrane activity - even at a normal unstimulated pinocytic rate they can interiorise an area of plasma membrane equivalent to their total

surface area every 35 min (Steinman et al., 1976). Since pinocytosis probably involves phospholipid turnover, it is likely that exogenous fatty acids will become incorporated into phospholipid very Readily (Lokesh & Wrann, 1984). In addition, exogenous fatty acid can also be taken up and incorporated into triacylglcyerol, which may provide an important energy store for these cells (see Chapter 4). These findings with the cultured cell support the view that considerable turnover of fatty acids in either phospholipids or triacylglycerol may occur in these cells.

The rate of utilisation of either acetoacetate or 3-hydroxybutyrate were so low as to be undetectable. This is surprising since these cells possess the enzymic capacity for utilising at least acetoacetate (see Chapter 2). The pathway by which acetoacetate is catabolised involves the enzymes acetoacetyl CoA thiolase and 3-oxoacid CoA transferase, which are considered to catalyse near-equilibrium reactions. Consequently, if the acetyl CoA concentration in the mitochondria is high this would prevent high rates of acetoacetate utilisation, due to product inhibition of the pathway. A high rate of fatty acid oxidation would be expected to elevate the acetyl CoA level and hence inhibit ketone body oxidation. In these experiments these fatty acids are probably derived from endogenous triglyceride (Chapter 4). Hence it is possible to argue that ketone bodies may provide an important fuel for these cells 'in vivo' when the endogenous triglyceride store has been depleted. This hypothesis has not been tested.

3.4.5 Pyruvate Metabolism

When pyruvate was added to the incubation medium at a concentration of 1 mM, the rate of pyruvate oxidation was high in comparison to the rate of oxidation of pyruvate derived from glucose (tables 3.4 and 6.3). Why are the rates different? The overall rate of pyruvate oxidation by the mitochondria is likely to be regulated by the availability of mitochondrial pyruvate (i.e. intramitochondrial concentration) and the activity of pyruvate dehydrogenase. There will be competition for glycolytically-produced pyruvate between the mitochondrial pyruvate transporter (for reaction with pyruvate dehydrogenase) and cytosolic lactate dehydrogenase. The lactate dehydrogenase has a very high activity (764 nmol/min per mg protein) in comparison to pyruvate dehydrogenase (3.2 nmol/min per mg protein) and hence is likely to compete very effectively for this pyruvate. Furthermore the high rate of glycolysis will lead to a high rate of conversion of NAD^+ to NADH. A low capacity of the mitochondrial shuttles for oxidation of cytosolic NADH would maintain a high cytosolic NADH/NAD^+ concentration ratio which would also favour the lactate dehydrogenase reaction. When pyruvate is supplied extracellularly, especially in the absence of glucose, the NAD^+/NADH concentration ratio should be high and the cytosolic pyruvate concentration should remain high - hence favouring transport into the mitochondria and oxidation via the pyruvate dehydrogenase reaction. Hence in the absence of extracellular glucose when the rate of cytosolic NADH production is low, the fate of pyruvate should be almost exclusively oxidation via pyruvate dehydrogenase. This is indeed the case. Under these conditions, the rate of [1-^{14}C]-pyruvate conversion to $^{14}CO_2$ (i.e. pyruvate dehydrogenase activity) is almost

identical to the rate of pyruvate utilisation (Figures 3.2. and 3.3). Interestingly, the results suggest that after 30 min incubation the rate of [1^{14}C]-pyruvate oxidation is markedly decreased (Figure 3.3). This could be due to a marked increase in the acetyl-CoA concentration ratio which could lead to an inhibition of pyruvate dehydrogenase activity. It would be of considerable interest to measure the concentration of acetyl-CoA in the mitochondria of the macrophage at several time points over a 60 min period of incubation, to study the possible mechanism of inhibition of pyruvate dehydrogenase. This has not been done. The inhibition of the enzyme could be achieved either either through an interconversion cycle (see Randle et al., 1978, for review) or by an end-product inhibition which causes an accumulation of the dihydrolipoamide component of the enzyme complex, in the acetylated form (see Randle et al., 1978). This would inhibit the decarboxylation step in the pyruvate dehydrogenase reaction. Some preliminary results were obtained to suggest that an interconversion cycle does not exist in these cells. Thus, incubation of macrophages in the presence of dichloroacetate, which normally activates pyruvate dehydrogenase via the interconversion cycle had no effect on the activity of macrophage pyruvate dehydrogenase isolated from these cells (results not shown). This finding has subsequently been extended to the lymphocytes from which mitochondria can be more easily prepared. The evidence in lymphocyte mitochondria suggests the cycle does not exist in these cells (see Curi et al. 1987).

Over a period of 60 min the rate of [3-^{14}C]-pyruvate oxidation is approximately linear and shows no dramatic decrease as that for [1-^{14}C]-pyruvate (Figure 3.3.). This suggests that this oxidation rate

reflects pyruvate metabolism via pyruvate carboxylase, randomisation of the [^{14}C] label, and the loss of $^{14}CO_2$ in the reactions of the TCA cycle or the phosphoenolpyruvate carboxykinase reaction. Since pyruvate carboxylase is activated by acetyl-CoA, the maintenance of the linear rate between 30-60 min of incubation suggests either that the acetyl-CoA concentration is not increased (see above) or that the enzyme approaches maximum activation by acetyl-CoA long before any increase in the concentration of this activator.

On the basis of the change in slope of the pyruvate oxidation curve (Figure 3.3), it is tempting to speculate that extracellular pyruvate is metabolised by pyruvate dehydrogenase to produce acetyl-CoA which is first used to provide energy for refilling ATP and phosphagen stores depleted in preparation of the cells. After this the oxidation rate slows and the citrate formed may be transported out of the mitochondria rather than suffering metabolism in the cycle. This cytosolic citrate could either be transported out of the cell or be metabolised via ATP-citrate lyase to give rise to oxaloacetate and acetyl-CoA. Cytosolic acetyl-CoA may be utilised for the synthesis of lipid or cholesterol. Thus the macrophage may be able to utilise pyruvate for the formation of acetyl-CoA either for energy generation or for the formation of storage triacylglycerol or cholesteryl ester. The former could be utilised for energy formation under conditions of fuel starvation or for other purposes which are discussed in chapter 4. Alternatively, the acetyl-CoA (actually malonyl CoA) could be important for chain elongation in fatty acid metabolism to provide the correct chain length fatty acid for phospholipid synthesis for incorporation into the cell membranes: the fluidity of the membrane is

dependent on the fatty acid composition of the membrane (see Martin et al., 1976)

3.4.6 Oxygen Consumption

Macrophages which had been cultured in 95% air/5% CO_2, utilised oxygen at a rate of almost 50nmol/h per mg protein. This rate, however, is approximately 10-fold lower than the rate of oxygen consumption reported for elicited peritoneal macrophages, measured in cell culture conditions (Karnovsky et al., 1970; Karnovsky et al., 1975). This cannot be attributed to a greater proportion of O_2 being utilised for O_2^- production for cultured cells, compared to the cells removed from culture in this study, since Johnson et al. (1978) have measured O_2^- release from unstimulated thioglycollate elicited murine peritoneal macrophages and their results suggest that only a small fraction of the oxygen consumed may be involved with superoxide formation. The macrophages used for the oxygen consumption experiments described in this chapter were cultured for 48h in the absence of serum (but in the presence of albumin), hence removing any extracellular supply of lipid-fuel for these cells. Endogenous lipid may, however, be utilised by the macrophages: this appears to be the case during a period of culture (see chapter 4). The basal rate of oxygen consumption by macrophages removed from culture after 48 h was approximately 50 nmol/h per mg protein. Since 1nMol of oleate requires 26nmol of O_2 for complete oxidation - this suggests that fatty acids could account for the basal rate of oxygen consumption if oxidised at a rate of approximately 2nmol/h per mg protein, and therefore long-chain fatty acids might represent a major fuel for macrophages

in vivo.

Glucose or glutamine would not be expected to alter significantly rates of oxygen consumption in such short term incubations, since it has been shown (see above) that little of the carbon of glutamine or glucose is oxidised by these cells. In contrast, pyruvate or succinate stimulate oxygen consumption by the macrophage. Pyruvate may achieve this by activating pyruvate dehdyrogenase (which will increase the level of acetyl-CoA); it will also, via pyruvate carboxylase, raise the oxaloacetate level so providing both substrate and cosubstrate for the TCA cycle. Succinate when transported into the mitochondria will bypass the regulatory steps in the TCA cycle, and give rise to reducing equivalents for oxidation by the electron transport chain.

Macrophages cultured in 95% O_2/5% CO_2 do not appear to have any difference in their oxidative capacity (Table 3.5) compared to macrophages cultured in 95% air/ 5% CO_2. This suggests that the macrophages normally cultured in the latter gaseous environment are not subject to oxygen starvation, and that fuel utilisation via non-oxidative pathways is not due to local hypoxic conditions.

CHAPTER 4

Metabolism of glucose, glutamine and long chain fatty acids by cultured macrophages

4.1 INTRODUCTION

Mammalian cells are cultured in medium that contains carbohydrate, amino acids, salts, vitamins and growth factors. Almost thirty years ago, Eagle (1959) identified the basic composition of tissue culture medium, except for the growth factors which were provided by added serum. Eagle's work has led to the development of culture media which contain the essential amino acids required by man (Rose, 1938) plus glutamine, tyrosine and cysteine. Eagle (1956, 1959) observed that the omission of any one of these amino acids resulted in cell death, and that glutamine concentration requirement of cells was 10-100 fold greater than for any other amino acid. The amount of glutamine used for amido transfer reactions, that is donation of nitrogen for, for example, purine synthesis and for protein synthesis is very small and it has been proposed that glutamine is required to provide a significant portion of the energy for the cultured mammalian cells (Zielke et al., 1976, 1978; Kovacevic and Morris, 1972; Donnelly and Scheffler, 1976; Reitzer et al., 1979). The most abundant carbon compound (fuel) available to cultured cells is glucose (5-20 mM), and this is closely followed by glutamine (2-6 mM). Glucose is metabolised to lactate at a high rate with only a small proportion of the glucose carbon being oxidised to CO_2 via the tricarboxylic acid cycle (Morell & Froesch, 1973). It has been shown, with different types of cultured cell, that the rate of glucose metabolism is not directly related to cell growth (Bryant et al., 1958; Graff et al., 1965; Bissell et al., 1972; Morell and Froesch, 1973; Rheinwald and Green, 1974). Hence it has been suggested that glucose is required not for energy generation but for the provision of precursors for macromolecular synthesis i.e.

an anabolic role (incorporation into nucleoside triphosphates and nucleic acids). In support of this it has been found that when glutamine is present, glucose need only be present at a very low concentration (70 nmol daily supplementation) (Zielke et al.,1976, 1978).

In general, very little work has been done on the metabolic profile of cells in culture. Thus there have been few studies describing the metabolism of cultured macrophages. To the author's knowledge, previous work has been largely concerned with metabolism that occurs during phagocytosis, emphasis being directed towards glucose metabolism, oxygen consumption and superoxide production (Hard, 1970; Karnovsky et al., 1970; Stubbs et al., 1973; Drath and Karnovsky, 1975; Rossi et al., 1975; Michl et al., 1976).

The results presented in Chapter 3 indicate that the macrophage can metabolise and utilise glucose and glutamine at high rates and that they have the ability to oxidise long-chain fatty acids. The question arises whether this is due to 'abnormality' of short-term incubations. The culture experiments described in this chapter have provided the conditions for observing metabolism of these substrates over several days which is important in determining the end-products of both glutamine and fatty acid metabolism under what might be considered to be more physiological conditions.

In addition, short-term incubtions in culture conditions (1 hour) were also performed to assess the contribution of a number of fuels to the process of phagocytosis of zymosan particles.

Recent reports have described uptake and incorporation of different classes of lipid into the macrophage. Cholesterol is accumulated due to the uptake of LDL (Fogelman et al., 1985) and is stored mainly as cholesterol-ester. Long-chain fatty acids (oleate or palmitate) are incorporated into macrophage phospholipids or stores of triglyceride (Lokesch and Wrann, 1984; Von Hodenberg et al., 1984). Experiments on quantitative balance described in this chapter attempt to assess the proportion of fatty acid which is incorporated into lipids and that which is oxidised. Hence the overall aim of the work presented in this chapter was to assess quantitatively and systematically the fuel metabolism in the cultured murine peritoneal macrophage.

EXPERIMENTAL

4.2.1 Cell preparation and culture

Elicited peritoneal macrophages were obtained from 12-16 week-old female mice of the C57 BL/6 strain, bred in the Sir William Dunn School of Pathology, Oxford. Cells were harvested as described in chapter 2, the mice were killed with diethylether and the cells removed after intraperitoneal injection of 5 ml of phosphate buffered saline pH 7.2-7.4. Macrophages were elicited by intraperitoneal injection of 1.5 ml of 4% Brewers thioglycollate medium 4 days prior to the harvesting procedure. Harvested cells were pooled in sterile 50 ml plastic centrifuge tubes, and were centrifuged at 500g for 7 min. The cells were resuspended in Eagle's minimum essential medium (MEM) supplemented with 10% (v/v) FCS, 2mM glutamine and 20 ug gentamicin/ml. These cells were plated at a density of 2.0-2.5 x 10^7 cells per 100 mm tissue culture Petri dish, or 8 - 10 x 10^5 cells per well in a 24 well tissue culture plate. After incubation for 4 h at 37^{o}C in 95% air 5% CO_2 adherent macrophages were washed vigorously with PBS three times and then were incubated at 37^{o}C in 5% CO_2:95% air in the relevant culture medium (described below) for a period not exceeding 96 h. The volume of culture medium used was 25 ml for a 100 mm tissue culture dish or 2 ml for a well in a 24 well plate. The biological viability of these cells was determined to be >95% by exclusion of trypan blue. Johnston et al. (1978) have shown that the percentage of cells not identifiable as macrophages after 24 h in culture was <6%. The methods employed by this group for the preparation and culture of cells were identical to those used in the present work.

4.2.2 Culture media and supplements

Two variants of MEM were used for these experiments. MEM with Earle's salts (without L-glutamine) was obtained from Gibco-Biocult Ltd., while an identical medium but without D-glucose was supplied by staff of the Tissue Culture Laboratory, Sir William Dunn School of Pathology, Oxford. The exact composition of the Gibco-Biocult MEM is given in Appendix C.

Foetal calf serum (FCS) was routinely heat inactivated (56^{o}C for 30 min) before use. It was used at a concentration of 5% (v/v) in all cultures which were supplemented with FCS.

Stock solutions of sterile glutamine (200 mM) were obtained from the Tissue Culture Laboratory of the Sir William Dunn School of Pathology, Oxford. Gentamicin was added to all culture media at a concentration of 20 ug/ml.

Bovine serum albumin (BSA) was de-fatted before use by the method of Chen (1967). It was used at a concentration of 0.1% in the media which contained this supplement.

All media plus supplements, either separatrely or when mixed, were filtered through millipore filters (pore size of 0.22 umetre) before use.

4.2.3 Media used for measurement of glucose and glutamine.

The medium used for initial 4-hour incubation consisted of MEM plus glutamine, gentamicin and either 5% foetal calf serum (henceforth referred to as serum) or 0.1% defatted bovine serum albumin (henceforth referred to as albumin). Serum at a concentration of 5% was chosen since this is a routinely used cell culture concentration; the final concentration of glucose, glutamine or fatty acid in the medium but derived from the serum would be less than 4% of the final concentration of these substrates that were routinely present. Albumin was used at a concentration of 0.1% as this was the lowest concentration of protein that would sustain cell number over several days in culture, in the presence of both glucose and glutamine (table 4.1a). This was important since now the effect of serum on macrophage rates of utilisation of glucose or glutamine and their fates could be determined.

Variations in the type of culture system and the media used, in the experiments described in this chapter, are given in Table 4.1b. It should be noted that cell lysis occurred when culture media was deficient in both glucose and serum. The reason for this has not been investigated but it may be related to an extremely low extracellular pyruvate concentration under these conditions: it has recently been reported (O'Donnell-Tomey et al., 1987) that pyruvate reacts rapidly, stoichiometrically and nonenzymatically with H_2O_2 and in so doing protects cells from the cytolytic effect of H_2O_2. The latter is an immediate product of O_2^- metabolism, a radical known to be released by macrophages (Chapter 1).

Table 4.1 Amount of protein on culture plates containing macrophages after 96 hour culture

The concentration of glutamine, when present, was initially 2 mM and glucose 5 mM.

Conditions of culture	Amount of protein (mg/100 mm tissue-culture plate)
MEM (plus glucose) glutamine and albumin (0.1%)	2.75*†
MEM (plus glucose) glutamine and foetal calf serum (5%)	3.44*†
MEM (plus glucose) albumin (0.1%)	1.93
MEM (plus glucose) foetal calf serum	2.67
MEM glutamine and foetal calf serum (5%)	2.52
MEM foetal calf serum (5%)	2.01

* Values used to calculate rates of utilization of substrate and production of metabolites.

† No difference in amount of protein compared with zero time.

TABLE 4.1.b The concentrations of glucose, glutamine and oleate in the media were 5, 2 and 0.3 mM respectively. Serum was used at a concentration of 5% (v/v) while albumin was used at a concentration of 0.1% (w/v).

Type of culture system	Approx. no. of cells per incubation	Volume of medium per incubation	Substrates present in medium	Serum or albumin supplement
100 mM tissue culture dish	2×10^7	25 ml	1. glucose plus glutamine	serum
			2. glucose plus glutamine	albumin
95% air: 5% CO_2			3. glucose	serum
			4. glucose	albumin
37°C			5. glutamine	serum
			6. glutamine	albumin
			7. none	serum
			8. none	albumin
24-well plate	1×10^6	2 ml	1. glucose plus glutamine	serum
95% air: 5% CO_2 37°C			2. glucose plus glutamine plus oleate	serum
			3. glucose plus oleate	serum
			4. glutamine	serum
			5. glutamine plus oleate	serum
			6. oleate	serum

The rates of glucose and glutamine utilisation and the production of metabolites after 23 h of culture are presented in table 4.2 in units of nmol/h per mg protein, for ease of comparison with rates given in chapter 3. The data for the cultures in which cell lysis occurred are also included (Note that,of the large quantity of glutamine that is utilised in this condition, most is converted to glutamate and ammonia).

For the culture conditions in which cells werre incubated in 24-well plates, with glucose as the only substrate, the protein concentration decreased markedly. The reason for this is unclear, but the results from these incubations are omitted.

4.2.4 Radioactive Substrates

[2-^{14}C]-Glucose This was used at a specific radioactivity of 0.5 uCi/ml of culture medium. The culture medium glucose concentration was always 5mM. [2-^{14}C]-Glucose was chosen since this carbon is lost primarily in the TCA cycle. There is thus minimal loss of this carbon in the pentose phosphate pathway. Further intermediates of the pentose phosphate pathway or glycolysis, that are incorporated into cellular macromolecules will be detected in the total cellular radioactivity, as described below.

[U-^{14}C]-Glutamine This was used at specific radioactivity of 0.36 uCi/ml of culture medium. The glutamine concentration in the culture medium was always 2 mM. [U-14C]-Glutamine was chosen so that the formation of CO_2

Table 4.2 Rates of substrate utilization and metabolite production by macrophages during 23 hour culture.

Macrophages were cultured using 100 mM tissue culture dishes as described in the experimental section. Rate of utilisation or production of metabolites were measured as described in the experimental section. Glucose, when present, was used at an initial concentration of 5 mM, glutamine 2 mM, serum 5% (v/v) and albumin 0.1% (w/v).

Additions to culture medium	Rates of utilization or production (nmol/h per mg protein)					
	Glucose	Lactate	Glutamine	Ammonia	Glutamate	Aspartate
Glucose, glutamine & albumin	570±22.5	888±23.6	61±6.0	59±2.3	7.2±0.38	6.6±0.57
Glucose, glutamine & serum	561±22.8	920±21.0	55±8.0	56±3.7	11±2.1	1.5±0.18
Glucose & albumin	487±11.4	872±35	-	35±1.9	<0.1	<0.1
Glucose & serum	462±10.0	874±11.3	-	-	3.2±0.70	<0.1
Glutamine & albumin	-	<0.1	214±2.3	253±16.6	153±7.7	13.4±2.4
Glutamine & serum	-	64±6.9	89±5.8	109±3.4	18±1.9	3.1±0.36
Albumin	-	<0.1	-	-	5.3±0.60	<0.1
Serum	-	67±5.4	-	-	<0.1	<0.1

from this substrate would result in a loss of radioactivity from the medium plus cells at the end of the culture (see below).

[3-^{14}C]-Pyruvate This was used at a specific radioactivity of 1.0 uCi/ml of culture medium. This substrate was only used in the experiments to determine incorporation into lipid.

Total pyruvate concentration in the culture medium was 1 mM. [3-^{14}C]-Pyruvate was used to avoid loss of $^{14}CO_2$ in the pyruvate dehydrogenase reaction.

[1-^{14}C]-oleic acid This was used at a specific radioactivity of 0.25 uCi/ml of culture medium. The culture medium concentration of oleate was always 0.3 mM. This is a concentration of fatty acid that is present in the plasma of fed animals and it has been shown that the rate of oleate incorporation into macrophages is saturated at 0.2 mM (Lokesh and Wrann, 1984). The [^{14}C] from the 1-position will be lost during oxidation of the oleate, whereas that incorporated into lipid will be detected in the total cell radioactivity (see below). Oleate was always added to culture medium as a complex with albumin. This complex was prepared as described in Appendix C.

4.2.5 Sampling Methods

Samples of medium (1 ml) were removed from 100 mm tissue culture dishes at the time points shown in the results section. The 1ml aliquots were kept at -20^{o}C until analyses were to be carried out.

For the 24 well plates, at the time points shown in the results section, 1 ml of medium was removed from each well to be sampled, and was stored at -20°C until analyses were to be carried out.

All experiments involving radiolabelled subtrates were carried out in 24 well plates. At the time points shown in the results section, 1 ml of medium was removed from each well to be sampled and was stored at -20°C until analysis of radioactivity. The remaining medium was removed by aspiration, and the cells washed three times with PBS. A volume (200 ul) of nonidet P40 detergent was added to the cells and mixed until callular integrity was destroyed. This cell lysate was then kept at -20°C until analysis of radioactivity. Four wells were treated identically to the rest for each time point, except that the cells were not disrupted with detergent but were resuspended in PBS for protein determination.

For experiments in which 100 mm tissue culture dishes were used, 4 dishes were used for every experimental condition shown, and the experiment was repeated at least twice. For experiments in which 24 well plates were used eight wells were used for every experimental condition shown, and the experiment was repeated at least twice.

4.2.6 Analytical methods

Metabolite concentrations were determined spectrophotometrically (using a Gilford Stasar III spectrophotometer) using enzymatic methods and changes in absorbance at 340 nM of pyridine nucleotides. Details of the methods are given in Chapter 3.

For the determination of radioactivity, 100 ul of sample (medium or cell lysate) was added to 900 ul of water and to this mixture 10 ml of scintillation fluid was added. The radioactivity was then measured using a Beckman liquid-scintillation Counter (Model LS 7500).

4.2.7 Lipid extraction and separation

Cells which had been cultured for between 60 and 80 h in the presence of a radioactive substrate were washed with PBS and gently scraped up into a small volume of PBS. They were then transferred into a glass test tube and were pelleted by centrifugation. The PBS was removed by aspiration and 10 ml of chloroform : methanol (2:1) was added. The cells were resuspended in the organic solvent and were then sonicated for two separate periods of 10 seconds with a micro-probe of an MSE 100W ultrasonic disintegrator operating at an amplitude of 12 um. They were then left at room temperature for 30 minutes. A volume (2.5 ml) of water was added, and the contents of the tube were thoroughly mixed. After low speed centrifugation the upper phase was removed by aspiration, and the lower phase was dried down under nitrogen: this was then resuspended in a very small volume of chloroform and applied to a silica gel thin layer chromatography (TLC) plate. This was developed in Hexane : diethylether : acetic acid (60:30:1, vol:vol:vol). The standards used were triolein, cholesterol and oleic acid, and the position of the standards on the TLC plates was determined by a charring method. Phospholipids were identified by reaction with phosphomolybdic acid (see Hofman, 1962). The radioactivities associated with each class of lipid were deteremined

by scraping the silica gel containing the lipid off the TLC plate, and incubating it overnight in tissue solubilizer at room temperature. A volume (16 ml) of scintillant was then added and the radioactivity was measured in a Beckman liquid Scintillation Counter (Model LS 7500).

4.2.8 Phagocytosis

Phagocytosis experiments were always performed using 24-well plates. Each well contained approximately 8 x 10^5 macrophages. Before the addition of zymosan particles (boiled yeast cell walls), culture medium was aspirated from each well, and the cells were washed 3 times with PBS. A volume (300 ul) of new medium for the phagocytosis experiment was then added, followed by 10 ul of a suspension of zymosan particles (25 mg/ml). The culture plates were well shaken, to ensure dispersion of zymosan particles in each well. The 24 well plates were then centrifuged at 1000 rpm for 5 min so that all the zymosa n particles made contact with the macrophages.

The culture plates were then incubated at 37°C in 5% CO_2:95% air for 60 min. Preliminary studies indicated that a steady-rate of uptake of zymosan was achieved after 30 min; this is in agreement with the findings of Ezekowitz et al. (1983). At the end of 60 min incubation, the 300 ul of incubation medium was removed and stored at -20°C until analyses were to be carried out. The cells were then washed 3 times with PBS. The rate of Zymosan particle ingestion was assessed as follows: Zymosan particles that were attached to the cells rather than ingested were removed by treatment with 2.5% (w/v) trypsin for 15 min at 37oC. The cells were then washed 3 times with PBS. Cells were

disrupted by the addition of 200 ul of nonidet P40 detergent, and the number of zymosan particles released were counted using a haemocytometer. The extent of phagocytosis could then be expressed as the mean number of particles ingested per cell.

The media for phagocytosis consisted of phosphate-buffered salines A and B (see appendix C) 5 mM glucose, or 2 mM glutamine or both, 5% (v/v) serum and 20 mM Hepes. The effect of phagocytosis on the rates of utilisation of glucose and glutamine and the rate of formation of lactate were studied. The above medium was chosen so that the effect of phagocytosis on rates of glucose and glutamine utilisation could be determined in the absence of other compounds that could be utilised (e.g., other amino acids). Preliminary studies established that the rate of phagocytosis was the same in this artificial medium in comparison to that in normal culture medium.

4.2.9 Spontaneous decomposition of glutamine

This was determined as 5% per day in a culture medium incubation at $37^{o}C$ in 5% CO_2 and 95% air in the absence of cells. The initial glutamine concentration was 2 mM. The degree of glutamine decomposition at all experimental time points was taken into account in any calculations, as was the effect on the rates of ammonia and glutamate formation.

4.2.10 Measurement of the protein concentration

For 100mm tissue culture dishes the following procedure was

used; after the initial 4 h incubation (of freshly harvested cells), macrophage cultures to be used for meaurement of protein concentration i.e., the zero-time cultures, (MEM + glutamine + 5% serum or 0.1% albumin) were washed 3 times with PBS, and then the cells were scraped up, using a rubber policeman, into a small volume of PBS. A sample of this cell suspension was removed and solubilised with KOH/triethanolamine (Appendix C). Protein was determined by the method of Bradford (1976). The same procedure was followed at 96 hours for all tissue culture plates, and the results are shown in table 4.1. Note that the protein concentration did not change for the cultures containing both glucose and glutamine, over the 96 hour period. All calculations were based on protein content of the zero time cultures.

For 24-well plates the following procedure was used; 4 wells were treated identically to 8 other wells that were to be used for experimental analysis. For each time point the protein content was determined from these 4 wells (by scraping up the cells and solubilising in KOH/triethanolamine), thus an average protein concentration value was obtained for a well at each time point, and for every experimental condition.

The protein values varied according to the type of medium used to culture the cells; it varied between 103 - 190 ug protein per well for between 8×10^5 - 1×10^6 cells plated, findings similar to those reported by Johnson et al. (1978): this work indicated that the protein content for 10^6 thioglycollate-elicited cells is 143 ug.

4.2.11 Amino acid analysis of medium

The analysis of the concentration of the amino acids in the culture or incubation media was sometimes carried out using an LKB 4400 amino acid analyser. This is further described in Appendix C. However the concentrations of glutamine, serine and threonine could not be determined using this technique, since they co-elute.

RESULTS

4.3.1 Glucose Metabolism

The time courses of glucose utilisation and lactate production over 3-4 days by macrophages incubated in 100 mm dishes or 24-well plates are given in Figures 4.1 and 4.2. The rates of glucose utilisation and lactate production were linear over this period in the 24-well plates provided the glucose concentration in the culture medium was always above 1 mM (Fig. 4.2). Where the cells are plated at a higher density in the 100 mm tissue culture dishes, the glucose concentration decreased more rapidly, which probably explains the deviation from linearity observed in figure 4.1.

The rate of glucose utilisation is at least 5-fold greater than that of glutamine when they are present in the same culture medium. Lactate production accounts for most of the glucose utilised. At all time points studied, the rate of glucose utilisation was greater when glutamine was also present in the culture medium (Figure 4.1)

The presence of oleate in the culture medium had no effect either on the rate of glucose utilisation or that of lactate production (Figure 4.2). Although lactate is produced even in the absence of added glucose (Figure 4.3), the rate of production was never more than 15% of that produced in the presence of added glucose.

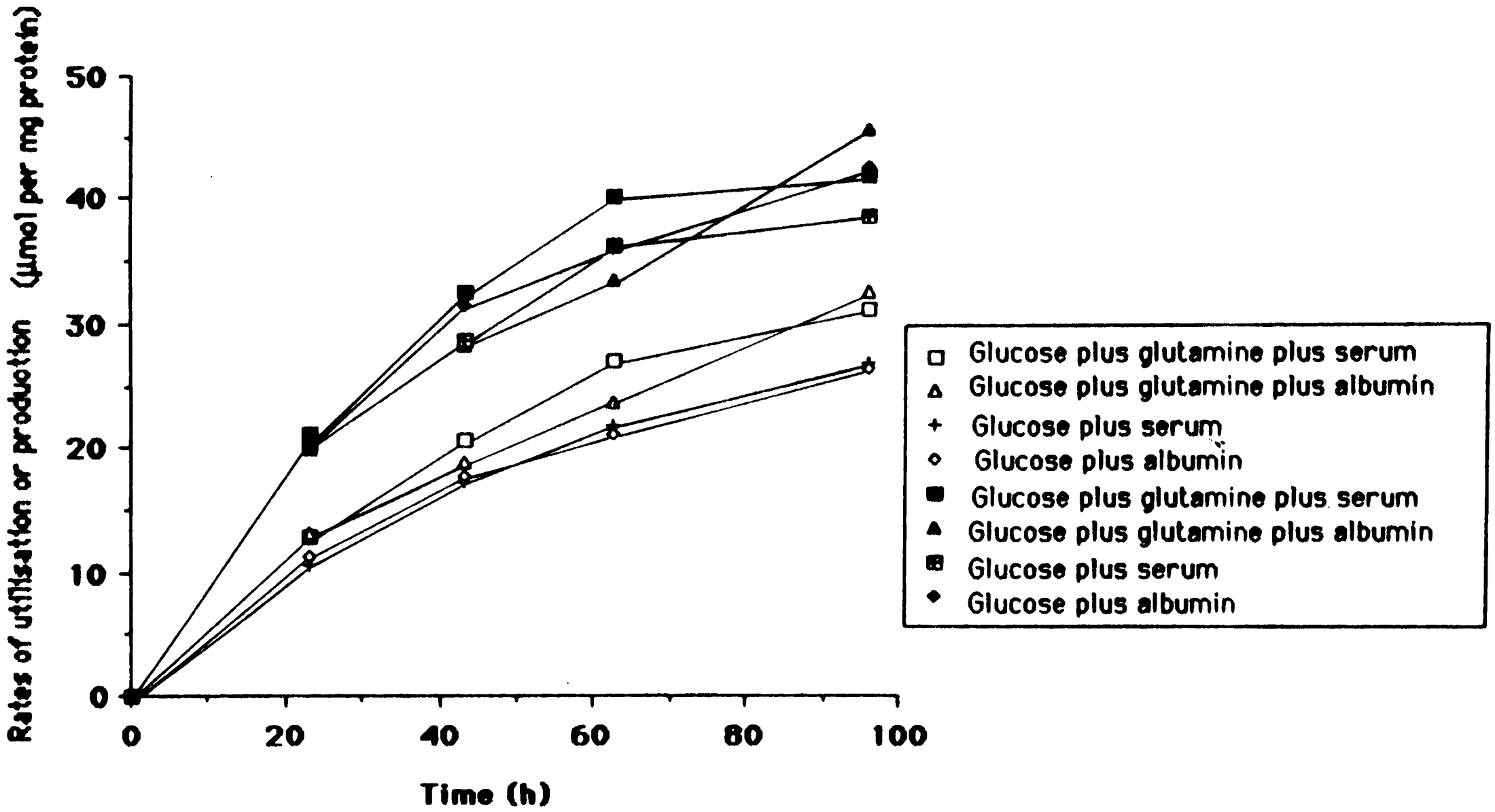


Figure 4.1 - Rates of glucose utilisation and lactate production by macrophages in different conditions of culture.

Macrophages were cultured using 100 mm tissue culture dishes as described in the experimental section. The variations in conditions of culture are given above, the open symbols represent glutcose utilisation, the closed symbols represent lactate production. Glucose was present at an initial concentration of 5 mM, glutamine 2 mM, serum 5% (v/v) and albumin 0.1% (w/v). S.E.M. values are not given but they are less than 10% of means.

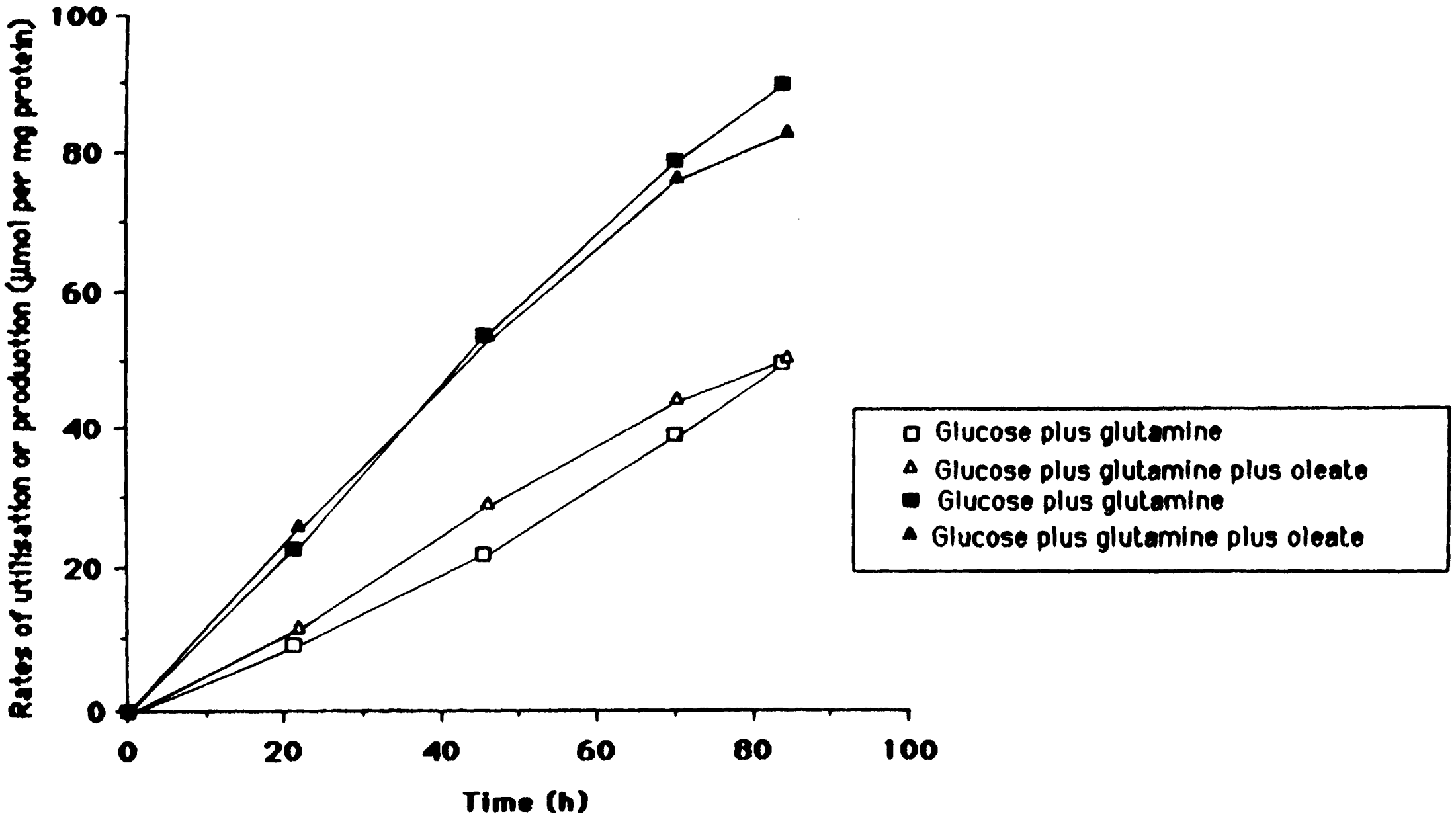


Figure 4.2 - Effect of oleate on rates of glucose utilisation and lactate production by macrophages in culture.
Macrophages were cultured using 24-well plates as described in the experimental section. The variations in conditions of culture are given above, the open symbols represent glucose utilisation, the closed symbols represent lactate production. Glucose was present at an initial concentration of 5 mM, glutamine 2 mM and oleate 0.3 mM. All culture media contained 5% (v/v) serum. S.E.M. values are not given but they are less than 10% of means.

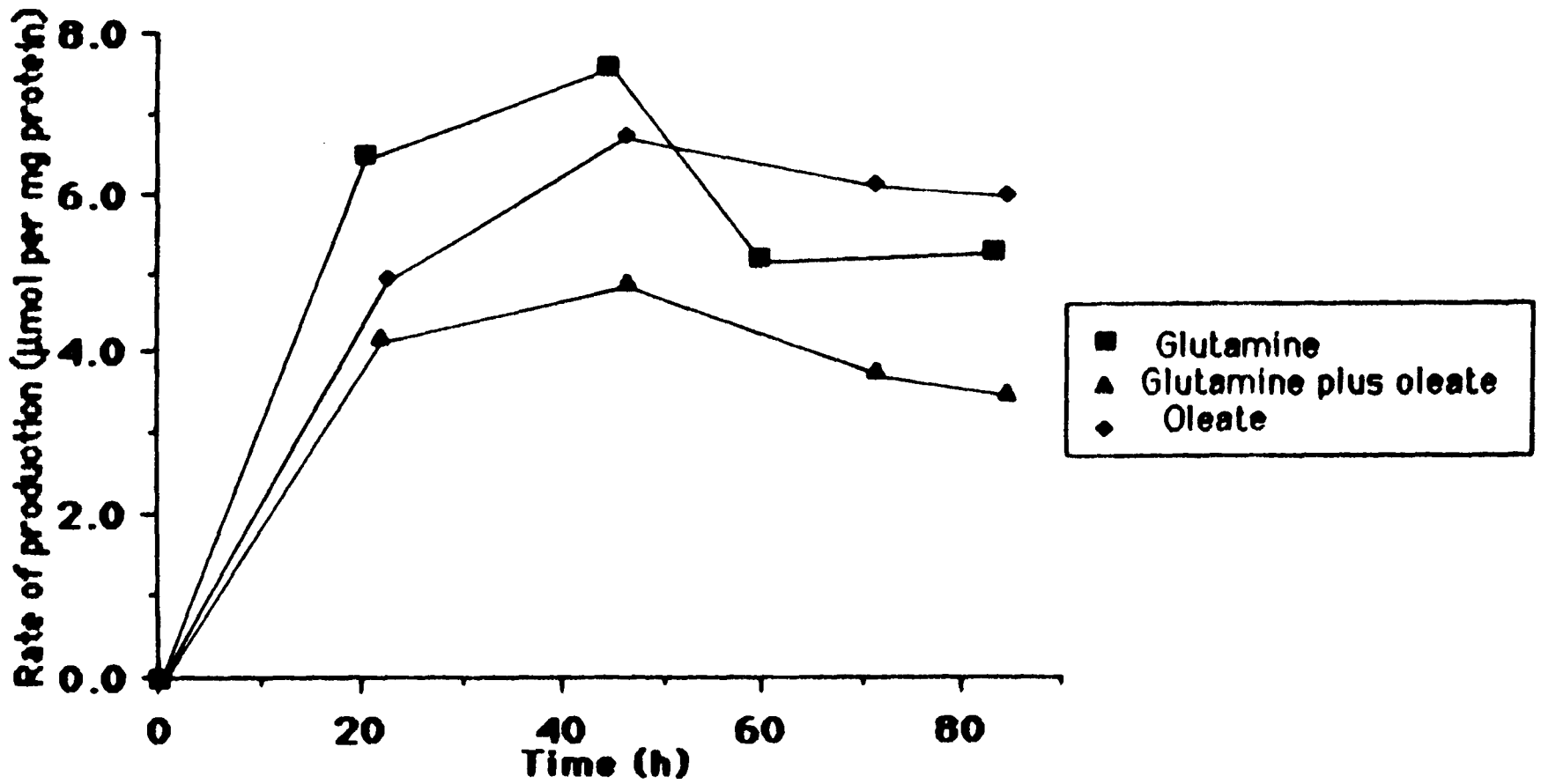


Figure 4.3 - Lactate production, in the absence of added glucose, by macrophages in culture.

Macrophages were cultured using 24-well plates as described in the experimental section. The variations in conditions of culture are given above. Glutamine was present at an initial concentration of 2 mM, and oleate 0.3 mM. All culture media contained 5% (v/v) serum. S.E.M. values are not given but they are less than 10% of means.

4.3.2 Glutamine metabolism

The time courses of glutamine utilisation and ammonia production over 4 days of culture in a 100 mm dish are given in Figure 4.4. The amount of glutamine utilised and the degree of linearity of this utilisation is slightly greater when the cell density (number of cells per ml of medium) is less, as in the 24-well plate cultures (Figure 4.5). The reason for this is unclear.

The presence of glucose decreased the rate of utilisation of glutamine by approximately 40%, over the whole time period of culture (Figures 4.4 and 4.5). When glucose was present in the culture medium, the rate of production of ammonia was identical to the rate of glutamine utilisation (Figure 4.4). However when glucose was omitted, not only was the rate of glutamine utilisation markedly increased, but the rate of ammonia production was increased over and above that predicted from the increment in the rate of glutamine utilisation.

Oleate had no effect on the rate of glutamine utilisation (Figure 4.5).

The time courses for glutamine utilisation and glutamate production for the cultures that contained glutamine are given in Figure 4.6. It is interesting to note that the rate of glutamate production is much lower than the rate of glutamine utilisation for each condition. When glucose is omitted from the culture medium, the rates of both glutamine utilisation and glutamate production are increased. The time course of aspartate production is not given since

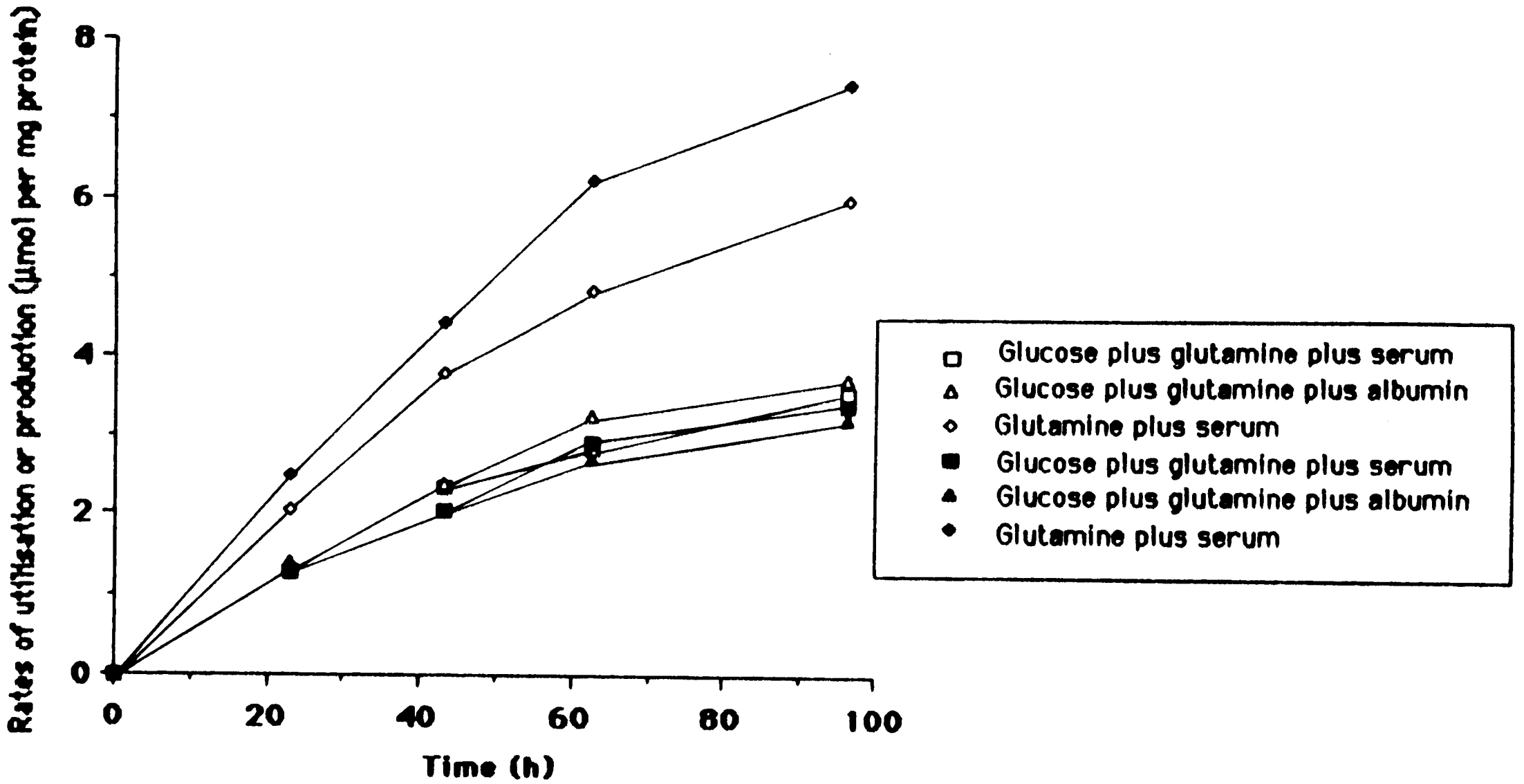


Figure 4.4 - Rates of glutamine utilisation and ammonia production by macrophages in different conditions of culture.

Macrophages were cultured using 100 mm tissue culture dishes as described in the experimental section. The variations in conditions of culture are given above, the open symbols represent glutamine utilisation, the closed symbols represent ammonia production. Glucose was present at an initial concentration of 5 mM, glutamine 2 mM, serum 5% (v/v) and albumin 0.1% (w/v). S.E.M. values are not given but they are less than 10% of means.

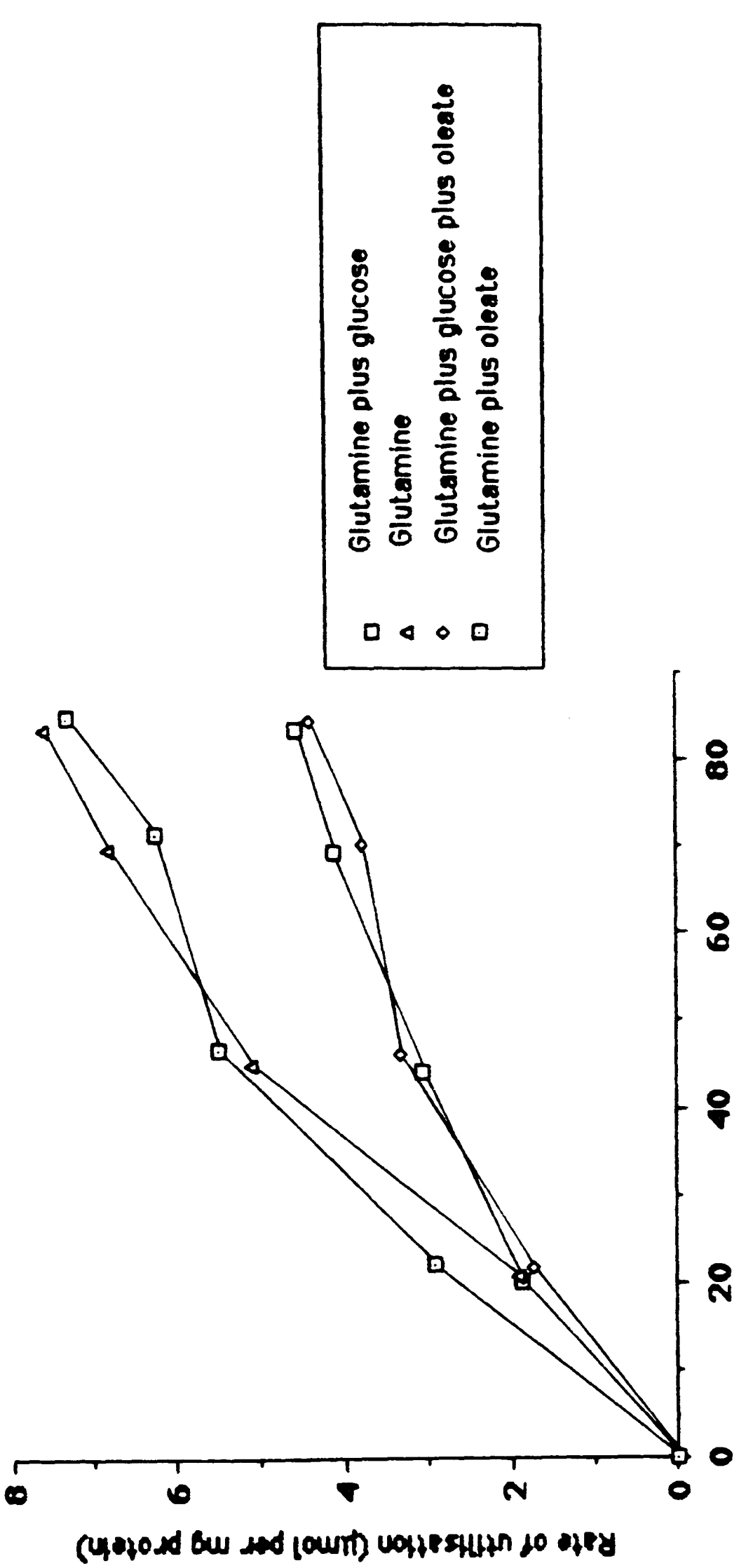


Figure 4.5 - Effect of oleate on the rates of glutamine utilisation by macrophages in culture.

Macrophages were cultured using 24-well plates as described in the experimental section. The variations in conditions of culture are given above. Glucose was present at an initial concentration of 5 mM, glutamine 2 mM and oleate 0.3 mM. All culture media contained 5% (v/v) serum. S.E.M. values are not given but they are less than 10% of means.

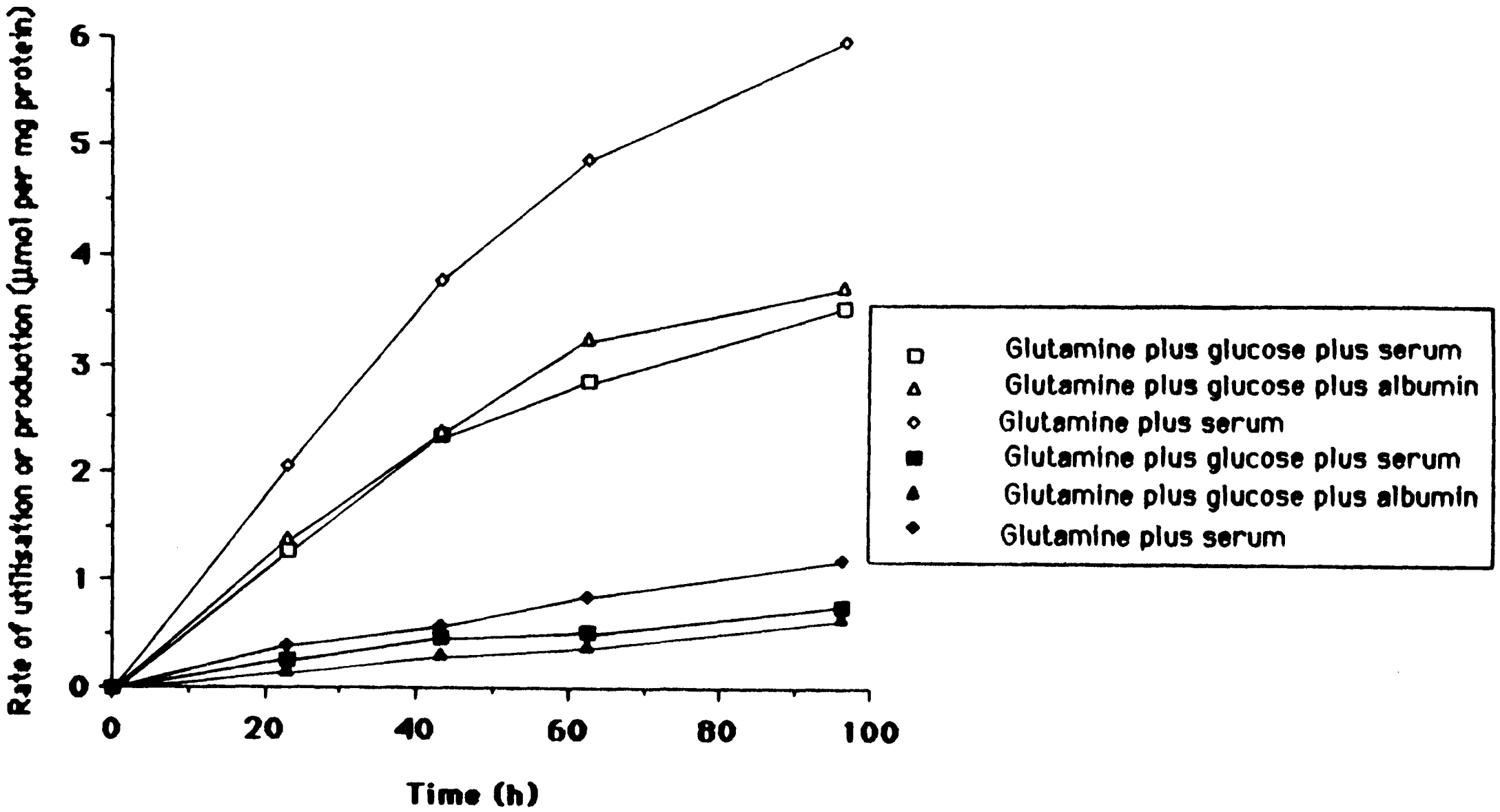


Figure 4.6 - Rates of glutamine utilisation and glutamate production by macrophages in different conditions of culture.

Macrophages were cultured using 100 mm tissue culture dishes as described in the experimental section. The variations in conditions of culture are given above, the open symbols represent glutamine utilisation, the closed symbols represent glutamate production. Glucose was present at an initial concentration of 5 mM, glutamine 2 mM, serum 5% (v/v) and albumin 0.1% (w/v). S.E.M. values are not given but they are less than 10% of means.

the amount of aspartate produced was always less than 5% of the amount of glutamine utilised.

The time courses of alanine and glutamate production in the cultures containing glutamine are given in Figure 4.7. (Note that these experiments were performed with 5% serum supplement and not with albumin.) The rate of alanine production is highest when both glucose and glutamine are present in the culture medium. The decrease in the rate of alanine production when glucose is omitted is almost identical to the increased rate of ammonia production (table 4.13b). The percentage of glutamine metabolised to glutamate is almost identical whether glucose is present or not (table 4.13b).

4.3.3 Analysis of the amino acid composition of culture medium after 96 hours incubation

Changes in the concentration of 11 different amino acids are expressed as rates of utilisation or production by macrophages over a 96 hour incubation (table 4.3). The fresh culture medium contains 13 amino acids (including glutamine, if added, see appendix C) and, of these 13, the concentrations of 12 were measured by the amino acid analyser (all but tryptophan) and in addition, those of alanine, aspartate and glutamate were also measured. However, the concentrations of threonine and glutamine could not be measured with accuracy by the analyser. Rates of lysine and histidine metabolism are not given since their concentrations did not change over the 96 h, in any of the culture conditions. [The concentrations of glutamate, aspartate and alanine were measured using both the amino acid analyser

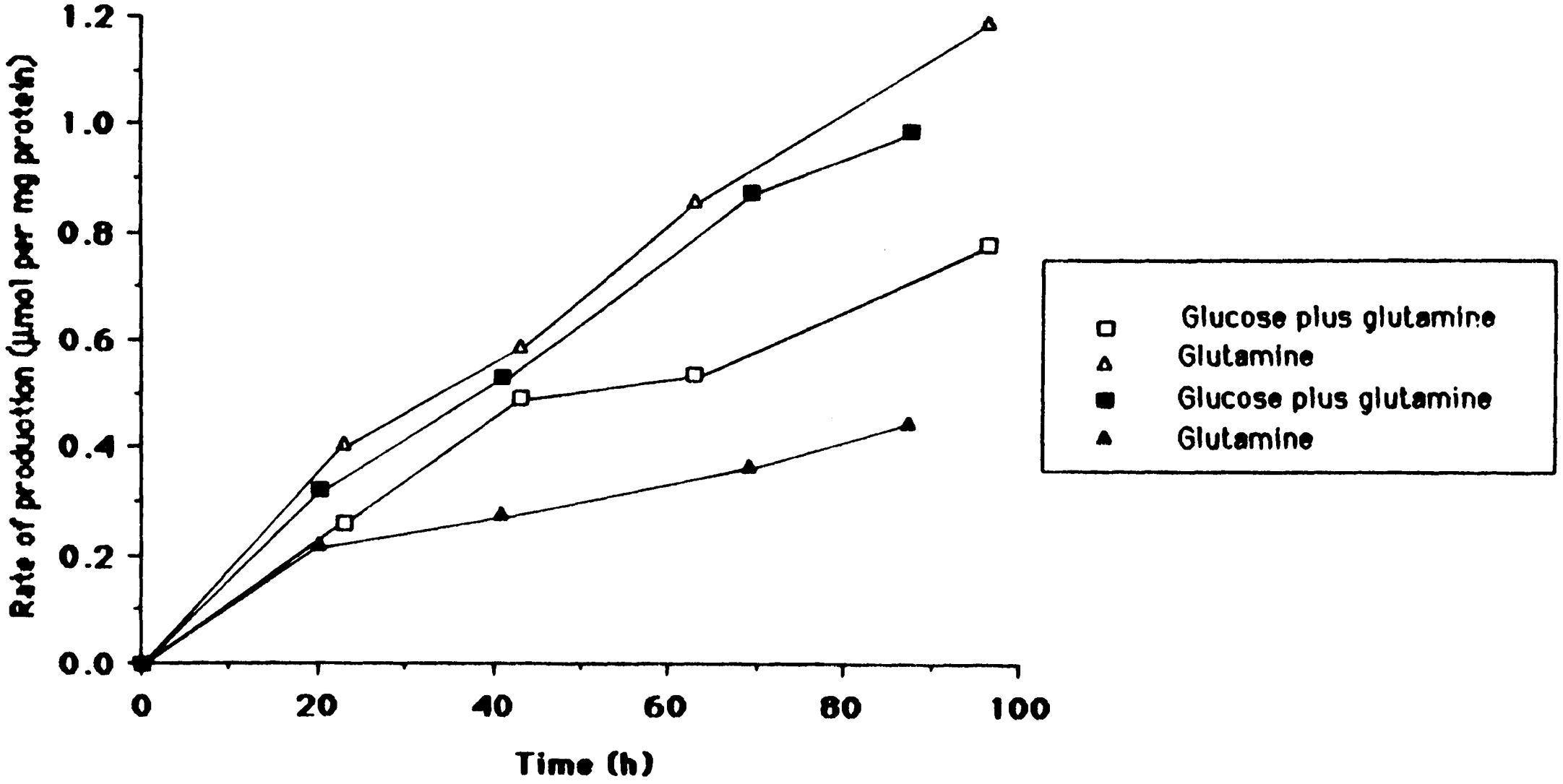


Figure 4.7 - Rates of glutamate and alanine production by macrophages in different conditions of culture.

Macrophages were cultured using 100 mm tissue culture dishes as described in the experimental section. The variations in conditions of culture are given above, the open symbols represent glutamate production, the closed symbols represent alanine production. Glucose was present at an initial concentration of 5 mM and glutamine 2 mM. All culture media contained 5% (v/v) serum. S.E.M. values are not given but they are less than 10% of means.

Table 4.3 Rates of amino acid utilization or production by macrophages in culture with various additions to culture medium

The concentration of glutamine, when present, was initially 2 mM. All media contained glucose (initially 5 mM) except that denoted by *. The final concentration of serum in the culture media was 5% (v/v) and albumin 0.1% (w/v). The rates of amino acid utilisation or production were measured as described in the experimental section.

Amino acid	Rates of utilization or production (μmol/mg protein per 96 h)					
Additions ...	glutamine and albumin	glutamine and serum	albumin	serum	glutamine and serum	serum
Aspartate	0.046	0.037	0	0	0.115	0
Glutamate	0.840	0.878	0.255	0.204	1.32	0.282
Glycine	0.344	0.165	0.262	0.224	0.404	0.513
Alanine	1.86	1.69	0.214	0.144	0.686	0.045
Valine	0.153	0.085	0	-0.138	-0.096	-0.314
Methonine	0.137	0.030	0.110	0.011	0.071	0.077
Isoleucine	0.053	0.030	-0.345	-0.508	-0.141	-0.673
Leucine	0.046	-0.105	-0.255	-0.414	-0.096	-0.609
Tyrosine	0.137	0.067	0.166	0.088	-0.038	-0.019
Phenylalanine	0.122	0.098	0.152	0.094	0	0
Arginine	-3.17	-2.97	-3.19	-2.74	-3.14	-3.186

and enzymatically (table 4.4)].

4.3.4 Rates of incorporation of [2-^{14}c]-GLUCOSE, [u-^{14}C]-glutamine and [1-^{14}C]-oleate into cell contents

The time courses of incoporation of [2-^{14}C]-glucose and [U-^{14}C]-glutamine into the cell contents of cultured peritoneal macrophages over 84 h are given in Figure 4.8. The rates of incorporation of both glucose and glutamine were approximately linear over this period. The incorporation was measured in optimal culture conditions (that is glucose, glutamine and serum were all present). The rate of incorporation of glucose into cell contents was slightly higher than that of glutamine.

The time courses of incorporation of [1-^{14}C]-oleate into the cell contents of cultured peritoneal macrophages over a time course of 84h in several conditions of culture are given in Figure 4.9. When both glutamine and glucose were present in the culture medium, the rate of incorporation of oleate into cell contents was considerably higher than if either or both of these components were omitted from the culture medium.

4.3.5 Rates of oxidation of oleate, glutamine and glucose

The apparent rates of oxidation of [1-^{14}C]-oleate, [U-^{14}C]-glutamine and [2-^{14}C]-glucose are calculated from the difference between the amount of radioactivity present at zero time and that in the medium plus that incorporated into cellular material,

TABLE 4.4 Rates of glutamate, aspartate and alanine production by macrophages in culture, measured by enzymic methodology or amino acid autoanalysis.

All media contained glutamine and serum. Glutamine was present at an initial concentration of 2 mM, and serum 5% (v/v).

	Rate of production (μmol/mg protein per 96 h)			
	enzymic assay		amino acid analyser	
	Glucose present (5 mM)	Glucose omitted	Glucose present (5 mM)	Glucose omitted
Glutamate	0.784	1.193	0.674	1.039
Aspartate	0.165	0.183	0.037	0.115
Alanine	1.089	0.492	1.475	0.641

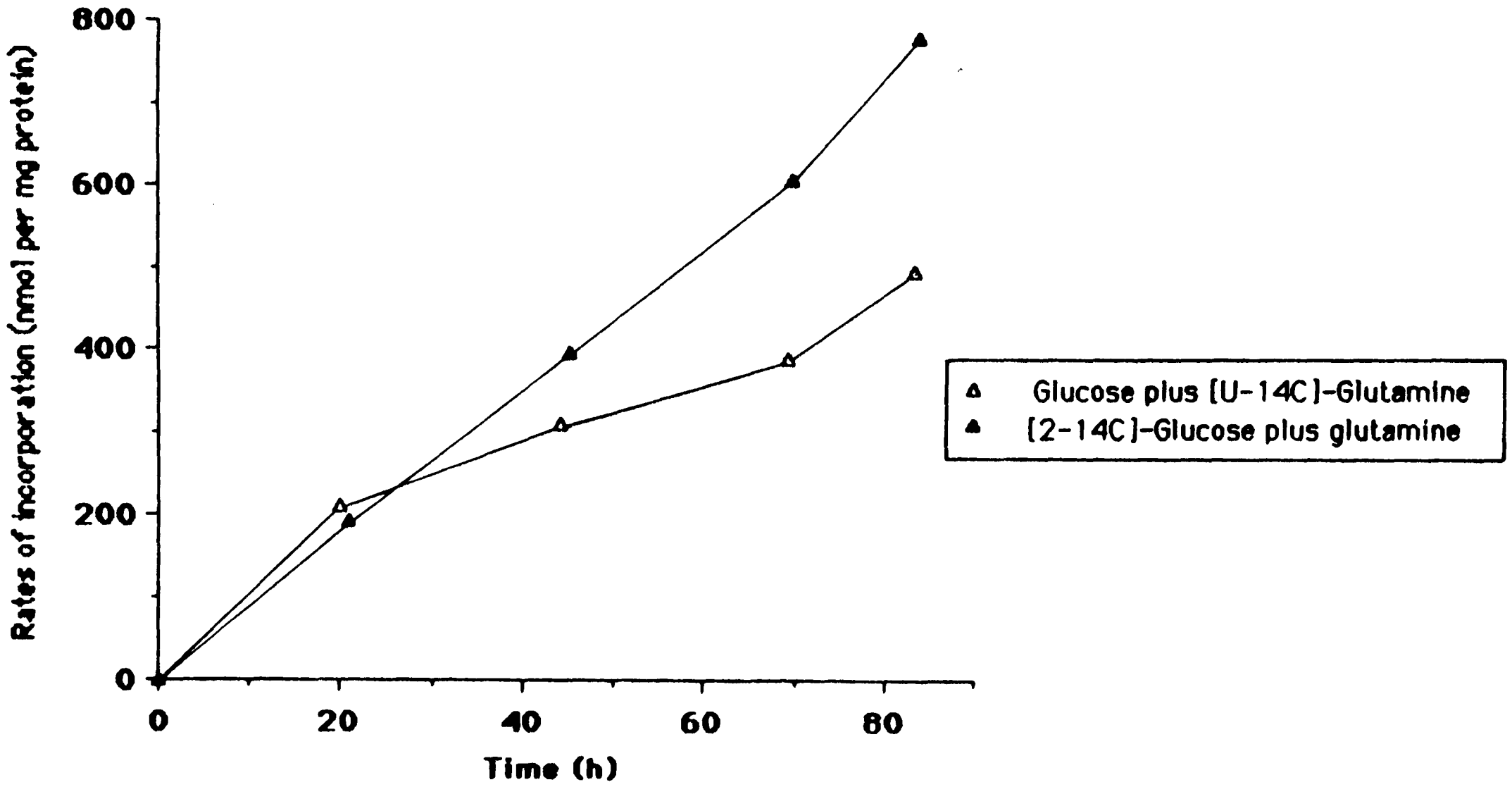


Figure 4.8 - Rates of radiolabelled glucose or glutamine incorporation into cell material.

Macrophages were cultured in 24-well plates as described in the experimental section. Incubations were terminated at the times indicated and the incorporation of radiolabelled substrate into cellular material was measured as described in the experimental section. Glucose was present at an initial concentration of 5 mM and glutamine 2 mM. All culture media contained 5% (v/v) serum. S.E.M. values are not given but they are less than 10% of means.

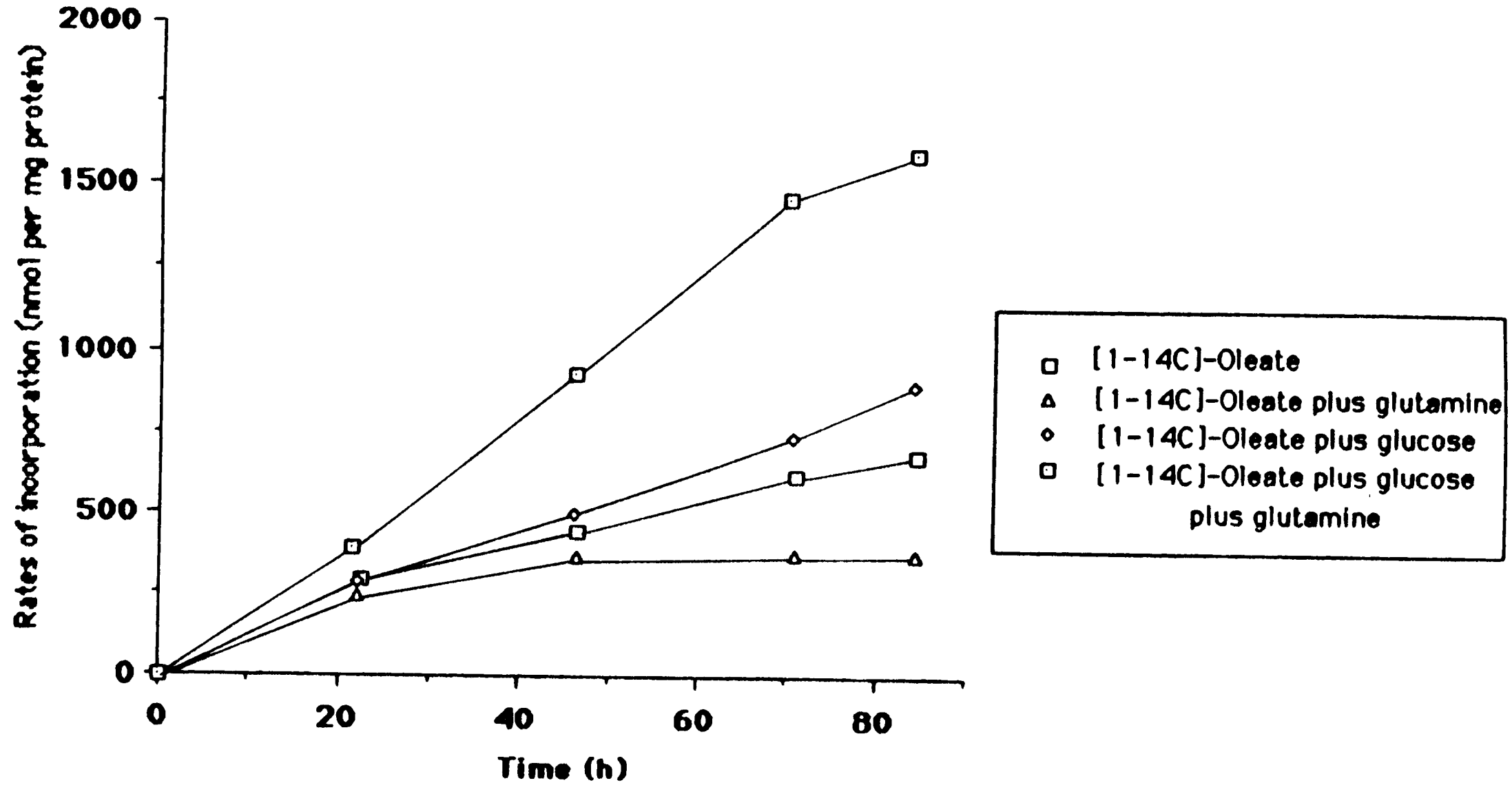


Figure 4.9 - Rates of radiolabelled oleate incorporation into cell material in different conditions of culture.

Macrophages were cultured in 24-well plates as described in the experimental section. Incubations were terminated at the times indicated and the incorporation of radiolabelled oleate into cellular material was measured as described in the experimental section. The variations in conditions of culture are given above. Glucose was present at an initial concentration of 5 mM, glutamine 2 mM and oleate 0.3 mM. All culture media contained 5% (v/v) serum. S.E.M. values are not given but they are less than 10% of means.

at any particular time point. It is considered that the radioactivity not recovered is lost as $^{14}CO_2$. Since this was only calculated as a difference and was not measured in these experiments, the rates are described as apparent oxidation rates: they are expressed as nmol substrate oxidised to CO_2 per mg protein.

The time courses of oleate oxidation in different conditions are shown in Figure 4.10. As might be expected, the highest rate was observed when glucose and glutamine were omitted from the culture medium, while the lowest rate was observed when both glucose and glutamine were present.

The results of a series of experiments to determine the rate of incorporation into cell contents and the apparent oxidation rates of [2-^{14}C]-glucose and [U-^{14}C]-glutamine in different culture conditions, after 82h of incubation are given in table 4.5. The rate of oxidation of glucose was highest when oleate was included in the culture medium; it was lowest when glutamine was included in the medium. The rate of incorporation of ^{14}C from glucose into cell contents was highest when both glutamine and oleate were included in the medium; it was slightly less when glutamine was present, but was lowest when oleate was present. The rate of oxidation of glutamine was highest when oleate was present in the culture medium; it was lowest when glucose and oleate were present. The rate of incorporation of [^{14}C] from [^{14}C]-glutamine into cell contents was similar for all conditions, but the highest rate was observed when glucose was the only other addition to the medium. To identify all the cell constituents into which the radioactivity was incorporated would be a large task but, since it was

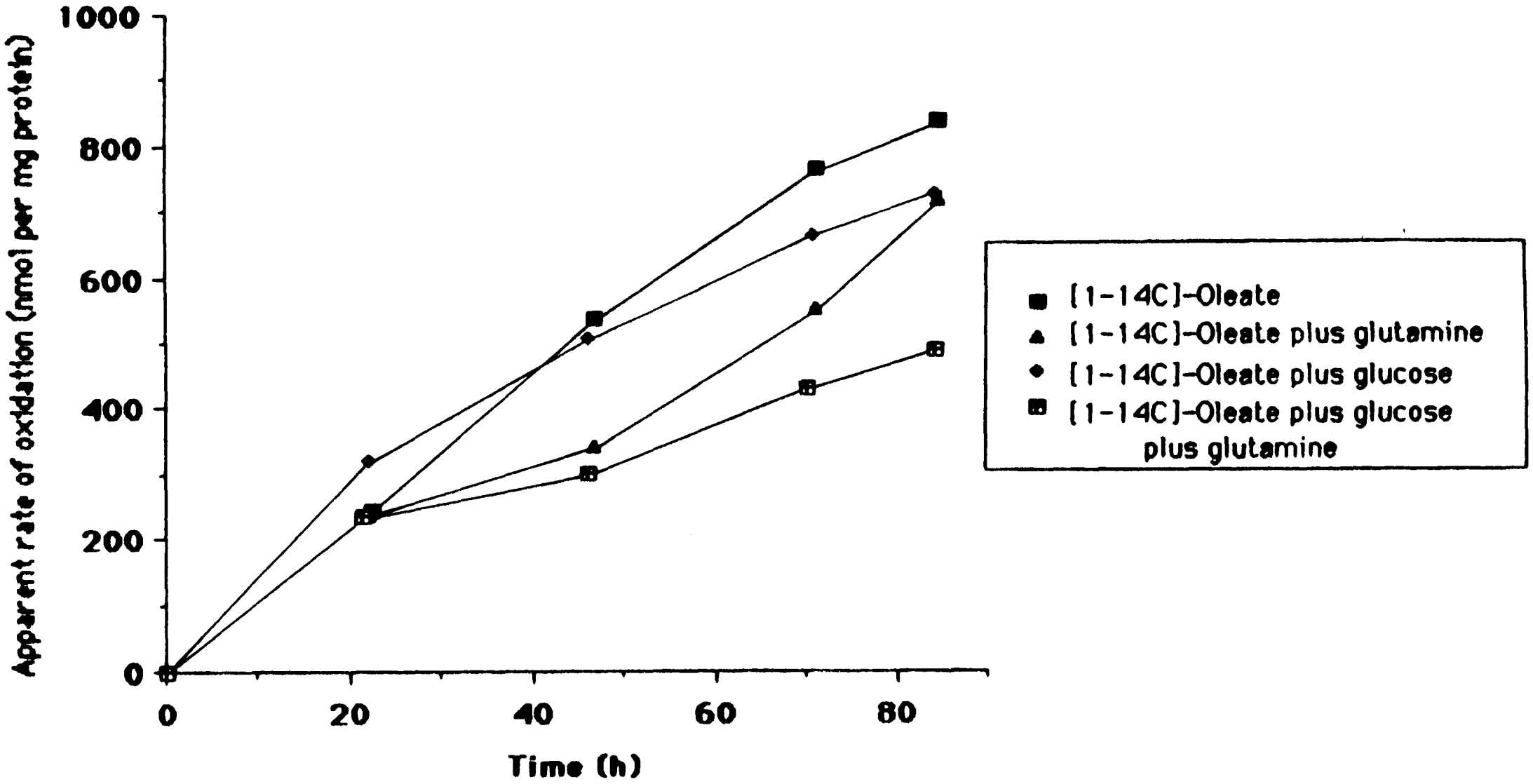


Figure 4.10 - Apparent rates of oxidation of radiolabelled oleate by macrophages in different conditions of culture.

Macrophages were cultured in 24-well plates as described in the experimental section. Incubations were terminated at the times indicated and the apparent rate of oxidation (apparent rate of conversion to CO_2) was measured as described in the experimental section. The variations in conditions of culture are given above. Glucose was present at an initial concentration of 5 mM, glutamine 2 mM and oleate 0.3 mM. All culture media contained 5% (v/v) serum. S.E.M. values are not given but they are less than 10% of means.

Table 4.5 Effects of glucose, glutamine or oleate on the rates of radiolabelled glucose or glutamine incorporation into cellular material or oxidation by macrophages during 82 h in culture

Incorporation of radiolabelled substrate into cellular material or CO_2 was measured as described in the experimental section. Glucose was present at an initial concentration of 5mM, glutamine at 2mM and oleate 0.3mM. The average protein concentration present at the end of the incubation for each condition was as follows: glucose plus glutamine (± oleate), 0.122 mg/well; glucose plus oleate, o.116 mg/well; glutamine (± oleate), 0.103 mg/well.

Incubation conditions	Rates (μmol/82 h per mg protein)	
	incorporation into cell material	apparent oxidation
[2-^{14}C]-glucose plus glutamine	0.78 ± 0.05	2.1 ± 0.13
[2-^{14}C]-glucose plus glutamine plus oleate	1.01 ± 0.08	2.4 ± 0.19
[2-^{14}C]-glucose plus oleate	0.37 ± 0.05	3.2 ± 0.43
[U-^{14}C]-glutamine	0.43 ± 0.03	4.5 ± 0.31
[U-^{14}C]-glutamine plus glucose	0.51 ± 0.01	3.2 ± 0.03
[U-^{14}C]-glutamine plus glucose plus oleate	0.35 ± 0.01	3.2 ± 0.06
[U-^{14}C]-glutamine plus oleate	0.35 ± 0.04	5.3 ± 0.54

considered that lipid was likely to be the major constituent, this was analysed further.

4.3.6 Incorporation of radioactivity from [1-^{14}C]-oleate, [2-^{14}C]-glucose, [U-^{14}C]-glutamine and [3-^{14}C]-pyruvate into cellular lipids

Macrophages were incubated for 68h in the relevant culture medium prior to lipid extraction and analysis. Oleate, glutamine and glucose (of which only one was radiolabelled) were all present in the culture medium used. Pyruvate, however, was added to medium deficient in any other substrate. The incorporation of label into four classes of lipid was investigated: triacylglycerol, fatty acid, cholesterol and phospholipid. They were separated by thin layer chromatography (T.L.C.) using a solvent system of hexane: diethylether: acetic acid, 60:30:1 (vol:vol:vol). The R_f values were as follows: triolein 0.875, oleate 0.530, cholesterol 0.394 and phospholipid 0.031 (average value). The proportion of radioactivity in the lower and upper phases when sonicated cells are extracted in chloroform:methanol are given in table 4.6. When cells were incubated with [1-^{14}C]-oleate, radioactivity was associated almost totally with the organic phase, whereas when cells were incubated with [U-^{14}C]-glutamine the radioactivity was associated almost totally with the aqueous phase. This suggests that oleate is incorporated primarily into lipid, while glutamine is incoporated primarily into non-lipid material. The latter was not further investigated. Most (72%) of the radioactivity from [3-^{14}C]-pyruvate was associated with the organic phase, whereas only 28% of radioactivity from glucose was associated with this phase.

Table 4.6 The percentage of radioactivity incorporated into cellular material that separates into the aquaeous upper phase and the chloroform lower phase after macrophages were cultured in the presence of radiolabelled glucose, glutamine, pyruvate or oleate for 68 hours

Cells from 36 wells were combined and the lipid and non-lipid cellular material was separated as follows: (i) disruption of cellular integrity by sonication; (ii) extraction of the dispersed cellular contents in 2:1 chloroform: method, as described in the experimental section. Oleate, glutamine and glucose (of which only one was labelled) were all present in the culture medium used. Pyruvate, however, was added to medium deficient in any other substrate. Glucose was present at an initial concentration of 5mM, glutamine at 2mM, pyruvate 1mM and oleate o.3mM.

Radiolabelled substrate	Upper phase		Lower phase	
	dpm/	percentage	dpm/	percentage
[2-^{14}C]-glucose	12,989	72.3	4,983	27.7
[U-^{14}C]-glutamine	15,988	98.1	311	1.9
[3-^{14}C]-pyruvate	4,914	28.5	12,349	71.5
[1-^{14}C]-oleate	12,788	0.5	2,678,096	99.5

Approximately 80% of the radioactivity in the oleate incorporated into cell lipid is associated with triacylglycerol, about 18% is associated with phospholipid while only 2% is recovered as free fatty acid (table 4.7). Thus most of the oleate is incorporated into triacylglycerol. The latter may represent a fuel store for the cell. Pyruvate is incorporated approximately equally into all four classes of lipid, whereas glucose is incorporated mainly into phospholipid and triacylglycerol. From the data presented in table 4.8, it is clear that in the conditions of cell culture used in this work, the rates of synthesis of lipid from precursors such as pyruvate or glucose were low, whereas the rate of incorporation of fatty acid into cellular lipid was high.

4.3.7 Release of free fatty acids from triacylglycerol enriched macrophages

Macrophages were incubated in the presence of [1-^{14}C]-oleate, glucose and glutamine for 64h. The triacylglycerol content of the cells is considerably increased under these conditions (Figure 4.9). After 64 h of incubation, the medium was replaced with a fatty acid free culture medium containing 1.5% defatted albumin, glucose and glutamine. After a further 25 h of incubation, approximately 17% of the radioactivity originally associated with the cells was released into the medium, and this value was increased to 20% after 42 h of incubation (table 4.9). The increase in the concentration of fatty acids in the medium after 19 h incubation (in fatty acid free medium) was 0.054 mM. This is equivalent to a rate of release of free fatty acid from the macrophage of approximately 16 nmol/h per mg protein.

Table 4.7 The percentage distribution of radioactivity into various lipid components of macrophages after 68 hours of culture with radio-labelled glucose, glutamine, pyruvate or oleate

Cells from 36 wells were combined and the lipids extracted as described in the experimental section. Oleate, glutamine and glucose (of which only one was labelled) were all present in the culture medium used. Pyruvate, however, was added to medium deficient in any other substrate. Glucose was present at an initial concentration of 5mM, pyruvate at 1mM and oleate 0.3mM.

Radiolabelled substrate	Fatty acid	Triglyceride	Phospholipid	Cholesterol	Unidentified lipids
[2-^{14}C]-glucose	<0.1	33.3	62.5	4.2	<0.1
[3-^{14}C]-pyruvate	10	19.7	29.0	38.3	2.9
[1-^{14}C]-oleate	1.8	78.8	17.5	2.9	0.6

Table 4.8 Relative rates of incorporation of radioactivity into lipid fractions of macrophages after 68 hours of culture in the presence of radiolabelled pyruvate, glucose or oleate

Rates of incorporation are calculated from the total radioactivity in the four lipid classes extracted and analysed. See legends for Tables 4.6 and 4.7 for experimental details. Pyruvate was present at an initial concentration of 1mM, glucose at 5mM and oleate 0.3mM.

	Relative rates of incorporation			
Substrate	fatty acid	triglyceride	phospholipid	cholesterol
[3-^{14}C]-pyruvate	1.0	1.0	1.0	1.0
[2-^{14}C]-glucose	<0.1	6.5	8.2	0.42
[1-^{14}C]-oleate	-	1057	152	-

Table 4.9 Depletion of triacylglycerol from triacylglycerol rich macrophages, when incubated in media in which fatty acids were ommited

Type of macrophage	Release of radioactivity into medium (as percentage of radioactivity in triacylglycerol rich macrophages at start of experiment)			Triacylglycerol concentration in macrophage (as percentage of concentration in macrophages at start of experiment)	
Time from start of experiment	20h	25h	30h	20h	30h
Elicited peritoneal[a]		17%			
Resident peritoneal[b]			50%		56%
J 774[b]	50%			50%	

a: Present work, culture media not replaced.

b: Von Hodenberg et al. (1984), culture media replaced hourly.

4.3.8 Phagocytosis of zymosan particles - the effect on rates of utilisation of glucose and glutamine

The degree of phagocytosis (i.e. the average number of particles ingested per cell) was measured in cells incubated in either culture medium or buffered incubation medium in the absence of added substrate or in the presence of a variety of substrates for 1 h (table 4.10). No differences in the degree of phagocytosis were observed. The value of 6-7 particles ingested per cell is similar to the value reported by Michl et al. (1976). Furthermore, the degree of phagocytosis was almost identical if the experiment was performed after 4 or 48 h of culture.

Of considerable interest is the observation that phagocytosis had no effect on the rates of utilisation of either glucose or glutamine (table 4.11). Nor was there any effect of phagocytosis on the rate of formation of lactate in these experiments, which is in agreement with the results of Karnovsky et al. (1970). The media from these experiments were analysed for the concentrations of amino acids released by the cells. There was only a small increse in the concentrations of alanine and glycine which occurred for all conditions of incubation. This is surprising as a large amount of glutamate would be expected to be released due to the high rate of utilisation of glutamine over the 1 h of incubation in these experiments (cf. Table 3.3). Macrophage glutamate metabolism is further discussed in section 4.4.2.

Table 4.10 Effect of substrates in the incubation medium on degree of phagocytosis by macrophages in culture

Macrophages were cultured in standard conditions (see experimental section) for either 4 h or 48 h. A phagocytic load of zymosan particles was then given in fresh incubation medium, and the experiment was terminated after 1 h. The number of particles ingested per cell was determined as described in the experimental section.

Substrate added to incubation medium	Number of particles ingested per cell	
	4 hours culture	48 hours culture
None	6.2 ± 0.3	6.2 ± 0.2
Glucose (5mM)	7.4 ± 1.1	6.5 ± 0.1
Glutamine (2mM)	6.2 ± 0.9	6.5 ± 1.2
Glucose (5mM/plus glutamine (2mM)	6.6 ± 1.0	6.5 ± 0.2
MEM + glutamine (2mM)	7.2 ± 1.1	-

Table 4.11 Effect of phagocytosis of zymosan particles on the rates of utilisation of glucose and/or glutamine and formation of lactate by macrophages in culture

Macrophages were cultured in standard conditions (see experimental section) for 48 h. 300µl of fresh inculation medium was added to washed cells, and a phagocytic load of zymosan particles was given. Cells were allowed to phagocytose for 1 h, and the incubation medium was removed and analysed as described in the experimental section.

Substrate present	Rates of (µmol/h per mg proteins)					
	glucose utilisation		glutamine utilisation		lactate production	
	control	zymosan	control	zymosan	control	zymosan
None	-	-	-	-	639 ± 46	647 ± 18
Glucose	945 ± 107	1149 ± 114	-	-	2062 ± 116	2153 ± 87
Glutamine	-	-	182 ± 49	216 ± 18		
Glucose plus glutamine	907 ± 118	1127 ± 177	145 ± 36	172 ± 49	2048 ± 125	2278 ± 72

DISCUSSION

4.4.1 Glucose Metabolism

The rate of glucose utilisation by the cultured macrophage is very high, and remains high over at least 3-4 days of culture (tables 4.2 and 4.11, Figures 4.1 and 4.2). The high rates of glucose utilisation by the macrophage are comparable to the rates of utilisation observed for enterocytes and colonocytes during incubation: these latter cells are considered to utilise glucose at high rates (table 3.7). Almost all of the glucose utilised by the macrophage can be accounted for as lactate (table 4.12), which is similar to cultured human diploid fibroblasts (Zielke et al., 1978), cultured HeLa cells (Reitzer et al., 1979) and cultured chick cells (Bissell et al., 1972).

The rate of glucose utilisation is 5-10 fold greater than that of glutamine utilisation when they are both present in the culture medium. The rate of glucose utilisation increased between 16-25% in the presence of glutamine (Figure 4.1). This could be brought about by the rapid increase in intracellular ammonia concentration, due to glutamine metabolism (table 5.3) since ammonia is known to be an activator of 6-phosphofructokinase (Muntz and Hurwitz, 1951; Muntz, 1953; Hichman and Weidemann, 1975). However, it is likely that the transport of glucose limits the rate of glycolysis in macrophages, as it does in lymphocytes (see Ardawi, 1983) so that an activation of this enzyme would not necessarily lead to an

increase in the rate of glycolysis.

The fate of metabolised glucose after 82h of incubation is given in table 4.12. The fate of glucose appears to be similar, whether glutamine or oleate or both were present in the culture medium: 80-90% of the glucose is accounted for as lactate, <2% is accounted for as glucose incorporated into cellular macromolecules, and 4-8% isaccounted for as CO_2. The low rate of oxidation of glucose is similar to the findings for cultured fibroblasts (Morell and Froesch, 1973) and cultured HeLa cells (Reitzer et al., 1979). The proportion of glucose that was estimated to enter the TCA cycle for oxidation in cultured HeLa cells was 4% (Reitzer et al. 1979) which is identical to the results obtained for macrophages in the present work. An even smaller proportion of glucose is oxidised by guinea pig elicted peritoneal macrophages (Karnovsky et al., 1970; the amount of [6-^{14}C]-glucose converted to $^{14}CO_2$ by the cells is less than 0.5%). The results obtained by Karnovsky et al. (1970) are similar to those reported here for murine peritoneal macrophages, concerning the fate of metabolised glucose (i.e., approximately 90% of glucose is metabolised to lactate). The contribution of glucose to the energy requirement of the cell will be discussed in section 4.4.7. The significance of high rates of glycolysis in rapidly-dividing cells in culture and in macrophages will be discussed in chapter 6. A similar explanation is proposed.

TABLE 4.12

Fate of metabolised glucose.

Substrate present	Glucose utilization (µmol/ mg protein per 82h)	% of metabolised Glucose		
		Cellular Material	CO_2 (from TCA cycle)	Lactate
5 mM Glucose 2 mM Glutamine 0.3 mM Oleate	50.0	2.0	4.8	82.0
5 mM Glucose 2mM Glutamine	49.0	1.6	4.2	89.8
5 mM Glucose 0.3 mM Oleate	41.5	0.9	7.8	91.3

4.4.2 Glutamine Metabolism

The rate of gluamine utilisation by the cultured macrophage is very high, and the present results show that this is the case for the cells during 3-4 days of cultures (tables 4.2 and 4.11; Figures 4.4 and 4.5). The high rates of gluatmine utilisation by the macrophage in culture are similar to those reported for the incubated macrophage in chapter 3. Since cells in culture represent a much more physiological environment than the incubation presented in chapter 3, these latter findings strongly support the view that this high rate of utilisation of gluamine occurs *in vivo*. These rates are as high as those reported for other types of cell in which rates of glutamine utilisation are considered to be high (tables 3.8 and 5.2). However, of considerable interest is the observation that the fate of glutamine when measured after 3-4 days of culture is very different to that found after 1h of incubation (chapter 3). The major end-products of glutamine metabolism appear to be CO_2, glutamate, ammonia and alanine (table 4.13), which is similar to the fate of glutamine described for cultured human diploid fibroblasts and HeLa cells (table 4.14). It is calculated for the macrophage, that approximately 80% of utilised glutamine was metabolised beyond glutamate. Of this, most is oxidised to CO_2 (table 4.13). Thus the cultured macrophage, in contrast to the macrophage during short-term incubation, appears to oxidise much of the glutamine that is utilised. This suggests that glutamine is a respiratory fuel for these cells - a role that has

TABLE 4.13a

Fate of glutamine carbon.

Substrate present	Glutamine utilization (µmol/ mg protein per 82h)	% Glutamine carbon			
		Cellular material	CO_2	Glutamate	Aspartate
2 mM Glutamine	7.55	5.6	58.9	18.4	2.1
2 mM Glutamine 0.3 mM Oleate	7.15	4.9	74.0	___	___
5 mM Glucose 2 mM Glutamine	4.55	11.1	71.2	18.7	2.9
5 mM Glucose 2 mM Glutamine 0.3 mM Oleate	4.30	8.0	73.7	___	___

TABLE 4.13b

Fate of glutamine nitrogen.

Substrate present	Glutamine utilization (µmol/ mg protein per 82h)	% Glutamine nitrogen			
		Ammonia	Alanine	Glutamate	Aspartate
2 mM Glutamine	7.55	63.2	4.7	9.2	2.1
5 mM Glucose 2 mM Glutamine	4.55	48.5	18.2	9.4	2.9

Table 4.14 Products of glutamine metabolism by cultured mammalian cells

Product	Percent of metabolised glutamine	Type of cell
CO_2	35-56	HeLa[a]; L-M mouse cells[b]
Glutamate	17	Human diploid fibroblasts[c]
Lactate	13	HeLa[a]; human diploid fibroblasts[c]
Pyruvate	2	HeLa[a]
Citrate	3	HeLa[a]
Malate	1	HeLa[a]
Aspartate	<1	Hela[a]
Cellular Material	2	HeLa[a]; human diploid fibroblasts[c]

a: Reitzer et al. (1979)

b: Stoner and Merchant (1972)

c: Zielke et al. (1980)

Adapted from Zielke et al. (1984)

been suggested for other cell types although the proportion of glutamine metabolised to CO_2 may not be as high as found in the present work: enterocytes (Neptune, 1965 Winmueller and Spaeth, 1974; Watford et al., 1979), reticulocytes (Rapoport et a., 1971), stimulated lymphocytes (Ardawi and Newsholme, 1982), fibroblasts in culture (Donnelly and Scheffer, 1976; Zielke et al., 1984) and malignant cells (Kovacevic and Morris, 1972; Reitzer et al., 1979). [The contribution of glutamine to the energy requirement of the cells will be discussed in section 4.4.7] Studies reported in chapter 3 demonstrated that after a one hour incubation period, glutamate (measured in both cells plus medium) is the major end-product of glutamine metabolism, by the incubated macrophage (tables 3.2, 3.3 and 3.6). Of interest is the fact that very litle of this glutamate was recovered in the medum of cultured cells (table 4.2 and Figure 4.6). Similarly, little glutamate is found in the medium of cultured cells after 1h in culture, over which time the rate of utilisation glutamine was high (table 4.11); less than 10% of the utilised glutamine was found as glutamate in the medium. These findings suggest that the intracellular concentration of glutamate is very high after a short (a few hours) exposure to medium containing glutamine. Indeed, a high glutamate concentration has been found after a short period of culture (table 5.3). Furthermore, the intracellular glutamate concentration decreases quite markedly over several days of culture (A.H. Gordon, personal communication), even though a high rate of gluatmine utilisation is maintained over this period.

The following hypothesis can be formulated on the basis of these findings The glutamine that is utilised initially at a high rate by the macrophage is 'trapped' within the cell as glutamate. Glutamate (and other dicarboxylic acids) traverses plasma membranes relatively poorly (Eagle et al. 1956; Hems et al., 1968). [In support of this, glutamate is taken up and oxidised much more slowly than glutamine when applied to various cells that utilise glutamine at a high rate (Rapoport et al., 1971 Traybum and Van Heyningen, 1973; Lavietes et al., 1974; Windmueller and Spaeth, 1975; Lazo, 1981] The intracellular glutamate is then metabolised to CO_2 more slowly. This ability to take up glutamine at a high rate and 'trap' it as glutamate may represent a physiologically important process in the macrophage. If glutamine/glutamate metabolism is initially important in these cells either for energy production or other reasons (see chapter 6), a marked decrease in the extracellular concentration of glutamine could seriously impair the functioning of thse cells. An intracellular 'store' of glutamine/glutamate could be of considerable importance in the maintenance of the normal functioning of the macrophage until more extracellular glutamine becomes available.

Possible pathways of glutamine metabolism have been discussed in chapter 3 (see Figure 3.8) and a suggestion for the macrphage glutamine utilisation pathways will be presented in chapter 5 (see Figure 5.6).

The rate of glutamine utilisation was decreased

approximately 40% by the presence of glucose in the culture medium (Figures 4.4 and 4.5). A similar decrease was observed with incubated macrophages (chapter 3). The inhibition of glutamine utilisation by glucose has been reported for cultured HeLa cells (Reitzer et al. 1979), human diploid fibroblasts (Zielkeet al., 1978) and proliferating thymocytes (Brand, 1985). The mechanism(s) by which glucose could influence glutamine utilisation in the macrophage is not known, but reactions that could have regulatory roles in the kidney are shown in figure 5.1.

The proportion of glutamine nitrogen recovered in alanine increased by an identical amount to the decrease in the proportion found in ammonia (table 4.13). The increased cytosolic concentration of pyruvate, which should occur when glucose is metabolised by the macrophage, may well be utilised as substrate for transamination catalysed by alanine aminotransferase. Glutamate may well attain high cytosolic concentrations in the macrophage due to the action of a non-mitochondrial phosphate dependent glutaminase which is present in the macrophage (chapter 5). Part of this cytosolic glutamate may be transaminated to form alanine provided the pyruvate is available (i.e., the concentration is sufficiently high).

4.4.3 Metabolism of some other amino acids by the macrophage

The rates of utilisation or production by macrophages of 11 amino acids over 96 hours in culture are presented in table 4.3. In the six culture conditions in which cellular integrity was

preserved throughout the period of culture, some general trends were observed. Glutamate, glycine and alanine were produced in the absence of added glutamine, at a rate approximately 2-fold greater than that of other amino acids. For glutamate and alanine, these rates were increased approximately 9- and 18-fold in the presence of added glutamine respectively. Four other amino acids appeared to be utilised by the macrophage. These were valine, leucine, and isoleucine (the branched-chain amino acids) and arginine. The presence of glutamine in the culture media markedly inhibited the rate of utilisation of the branched-chain amino acids. However, the maximal observed rate of branched-chain amino acid utilisation was <5% of the rate of glutamine utilisation under the same conditions. Muscle is known to be a major tissue for the uptake of branched-chain amino acids (Miller, 1962) but other tissues that can utilise these amino acids include intestine (Harper, 1986), and tumour cells (Lazo, 1981). These results demonstrate that branched-chain amino acids can also be used by the macrophages. Arginine is utilised at a very high rate in all conditions of culture. The utilisation rate of arginine was approximately half that found for glutamine. However it is known that macrophages can release the enzyme arginase in culture conditions. The release of this enzyme has been reported to be responsible for the killing of tumour cells (Currie, 1978) and Schistosomula of Schistosoma Mansoni, a helminth (Olds *et al.*, 1980), by depleting these cells of a source of arginine required for cell proliferation.

4.4.4 Oleate Metabolism

Oleate was selected to study the metabolism of long chain fatty acids since it has been used in previous studies with macrophages (Lokesh and Wrann, 1984; Von Hodenberg et al. 1984). There are two major fates of oleate, incorporation into cellular lipids (e.g., triacylglycerol, phospholipid) and oxidation to CO_2. Both these fates have been studied for the first time in macrophages and are reported in the present work (table 4.15 and Figures 4.9 and 4.10). Oleate metabolism did not have a significant effect on either glucose or glutamine metabolism (Figures 4.2 and 4.5, tables 4.12 and 4.14), except to increase the proportion of glutamine oxidised to CO_2 when glucose was omitted from the culture medium. However, the presence of glucose and/or glutamine had marked effects on rates of oleate utilisation and its fate (table 4.15). The highest rate of utilisation and the highest proportion incorporated into cellular material (76%) was observed in the presence of both glutamine and gluose. If glutamine and glucose are able to satisfy the major energy requirement of the cell under these conditions, then little oleate would be required for oxidation. The utilisation rate of oleate is depressed by 20-50% if either glucose or glutamine or both are omitted from the culture medium, but the proportion of oleate oxidised under these conditions increases to between 45 and 66% of the oleate used. This suggests that not only do these alternative substrates decrease the rate of oxidation of oleate, but that they stimulate its utilisation for phospholipid and triacylglycerol synthesis. The higher oxidation

TABLE 4.15

Fate of metabolised oleate.

Substrate present	Oleate utilization (µmol/ mg protein per 82h)	% of metabolised oleate	
		Cellular material	CO_2
5 mM Glucose 2 mM Glutamine 0.3 mM Oleate	2.09	76.5	23.5
5 mM Glucose 0.3 mM Oleate	1.62	55.2	44.8
0.3 mM Oleate	1.53	44.9	55.1
2 mM Glutamine 0.3 mM Oleate	1.09	34.3	65.7

rate probably reflects the greater role of oleate in satisfying the energy requirement of the cell in conditions where glucose and/or glutamine are not available. Sumbilla et al. (1981) have calculated that the rate of pamitate oxidation over a 3h incubation by cultured human diploid fibroblasts was 0.33 nmol/h per mg protein (the palmitate concentration was 0.2 mM). For macrophages it can be calculated that the rate of oxidation of oleate would be approximately 10 nmol/h per mg protein when no other substrate is present. Thus, in comparison the rate of fatty acid oxidation by the macrophage is very high. This suggests ether that the energy requirement might be very different for these different types of cell, or that marked dilution of label occurred in the experiments with diploid fibroblasts.

The contribution of oleate to the energy requirement of the macrophage will be discussed in section 4.4.7).

4.4.5 Incorporation of [^{14}C] from [1-^{14}C]-oleate, [2-^{14}C]-glucose, [U-^{14}C]-glutamine or [3-^{14}C]-pyruvate into lipid, and release of fatty acid from triacylglycerol enriched macrophages

The results presented in tables 4.6 and 4.7 indicate that of the oleate incorporated into cellular material, 96% is preferentially incorporated into triacylglycerol and phospholipid. The proportion of oleate associated with triacylglycerol is approximately 80%. From the results presented by Lokesh and Wrann (1984) and Von Hodenberg et al. (1984) using

shorter periods of incubation with oleate, it is clear that oleateis preferentially incorporated into phospholipid over the first 24h of incubation. However, the stimulation of triacylglycerol synthesis in the macrophage, and the increase in intracellular triacylglycerol concentration (Van Hodenberg et al., 1984) ensures that in longer periods of incubation in the presence of oleate (i.e., 68h in the present work) the proportion of incorporated fatty acid associated with triacylglycerol increases steadily over the period of incubation. Triacylglycerol is not the only type of lipid that can be accumulated and stored in the macrophage. Cholesteryl ester accumulation is important in forming the 'foam cells' of atherosclerotic lesions, these cells being derived from monocyte-macrophages (Mahley, 1979; Schaffner et al., 1980; Fowler et al., 1979; Gerrity, 1981). Cholesteryl ester accumulation probably occurs due to low density lipoprotein uptake by the macrophage (Fogelman et al., 1985). However, the present work raises the question as to whether some of the accumulated lipid in foam cells is triacylglycerol. The ability of macrophages to take up oleate, even at concentration normally present in the plasma of the fed animal, leads to this interesting question. One idea in the progressive development of atherosclerotic plaques is that the death of the macrophages (and possibly smooth muscle cells) is caused by an overloading of lipid and this exacerbates the whole poblem of inflammation and macrophage invasion. It would be of considerable interest if plasma fatty acids as well as LDL contributed to lipid overload.

The rate of synthesis of lipid from precursors such as glucose or pyruvate is low (table 4.8). If expressed as the percentage incorporation into lipid from the original extracellular concentration of substrate, then approximately 35% of oleate and <1% of pyruvate or glucose or glutamine are incorportated into chloroform soluble lipid, after 68h of incubation. Also Werb and Cohn (1971) have reported a very low rate of cholesterol synthesis (<1% of the cell cholesterol concentration in 6h) in murine peritoneal macrophages from [1-^{14}C]-acetate. Therefore, the source of accumulated lipid (fatty acid in triacylglycerol, cholesterol in cholesteryl ester) appears to be primarily from exogenous sources.

Fatty acids are released into medium by triacylglycerol enriched cultured murine macrophages (present work and Van Hodenberg et al., 1984). To the author's knowledge, adipocytes are the only other cell type known to release free fatty acids (see Newsholme and Start, 1973) although the rate of fatty acid release is much greater for adipocytes than for the macrophage. However, since only one sampling point, at 19h after the start of the incubation in fatty acid free medium, was used, this value for fatty acid release from the macrophages may be an underestimate. Von Hodenberg et al. (1984) have reported that considerable re-uptake of released fatty acids occurs if the incubation medium is not replaced regularly (i.e, every hour, see table 4.9). The enzyme responsible for the formation of fatty acid within the macrophage is presumably a triacylglycerol lipase but no studies have been done on this enzyme. There is no

evidence to support the view that triacylglycerol is secreted by the macrophage and hydrolysed extracelllulary by lipoprotein lipase (Von Hodenberg et al., 1984), which is known to be secreted by the macrophage (see Mahoney et al., 1985). The role of this latter lipase would, therefore, be to utilise exo genous substrate (i.e., VLDL) in the vicinity of the macrophage. The significance of the release of both fatty acids and lipo protein lipase by the macrophage is unclear. It is tempting to speculate that the release of fatty acids might be involved in the local killing of bacteria, since there is some evidence that fatty acids can decrease bacterial growth (Freese et al., 1973; Fay and Farias, 1975).

Long chain fatty acids can enter the cell by diffusion so that their rate of uptake is proportional to the difference in concentrations across the plasma membrane. Once inside the cells, fatty acids are 'activated' by conversion to fatty-acyl CoA. The fate of the latter can be either esterification or oxidation. In cells other than adipocytes, triacylglycerol acts as a endogenous fuel reserve, so that their ultimate fate will be oxidation. The macrophage, however, has been shown to be capable of fatty acid release. Thus the role of triacylglycerol in the macrophage may be 3-fold:

(i) To provide a fuel reserve for the cell;

(ii) As a source of free fatty acids to released in vivo during a response to bacterial infection. Fatty acids have been shown to be effective in inhibiting growth of bacteria in vitro (Freese et al., 1973; Fay and Farias, 1975);

(iii) To buffer the intracellular concentration of fatty acyl CoA, which at high concetrations can be damaging to the cell (see Newsholme and Crabtree, 1976; Newholme and Leech, 1983).

Since macrophages appear to possess both the enzymic capacity for synthesis of triacylglycerol and its hydrolysis, the potential for a triacylglycerol-fatty acid substrate cycle exists in the macrophage (see Newsholme and Crabtree, 1976, for a full description of this cycle). The role of this putative cycle in these cells in unclear, except it is possible that it might act as a buffer system to maintain a fairly constant concentration of the fatty acyl CoA level in the macrophage to prevent damage to the cell, to enable phospholipid recycling or synthesis to occur when required or to allow fatty acid oxidation for energy formation.

The present results suggest that measurements of the rate of this cycle might be worthy of investigation in these cells (see Brooks et al. (1983) for details of the method). One important point about this cycle is that it uses a considerable amount of energy (see Baldwin, 1973). If the capacity of this cycle is high, then stimulation of the rate of cycling could increase the use of ATP by these cells to such an extent, that if other fuels were not available or if oxygen tension was low, the cells might die. This is an interesting speculation in view of the known death of some macrophages during lipid accumulation in atherosclerotic plaques.

4.4.6 Glucose and glutamine utilisation during zymosan particle phagocytosis

Macrophages are known to have a large endocytotic capacity. At their usual unstimulated pinocytotic rate, murine macrophages internalise an area of plasma membrane equivalent to their total surface area every 35 min (Steinman et al., 1976). Their phagocytotic activity is greater; over a 10 min period they can engulf sufficient red cells to cause the internalisation of half or more of their total surface area (Mahoney et al., 1977). The possible mechanisms of phagocytosis and energy requirements have been reviewed by Silverstein and Loike (1980). Karnovsky et al., (1970, 1975) have shown phagocytosis is accompanied by increases in rates of oxygen consumption and [1-^{14}C] and [6-^{14}C]-glucose oxidation. The increase in oxygen consumption was greater than that required for increased superoxide generation under these conditions. Thus an increased ene rgy demand may be satis fied by a increase in mitochondrial respiration. However, possible fuels, with the exception of glucose, for phagocytosis have not been previously investigated.

The work presented in section 4.3.8 suggests that endogenous fuel utilisation may provide much, if not all the energy requirements for phagocytosis. Candidates for stored endogenous fuel would be long-chain fatty acids (derived from triacylglycerol), glycogen and protein. Lactate produced in the absence of added substrate is probably produced from glycogen utilisation. However, there is no increase in this rate during

zymosan particle ingestion. Can the increased energy demand during phagocytosis be satisfied by fatty acid oxidation? Karnovsky et al. (1970) have shown that the oxygen consumption of guinea-pig elicted peritonal macrophages increases approximately 50% during phagocytosis. About half the increase is due to mitochondrial respiration, as shown by the addition of KCN. The rate of oxygen consumption for non-phagocytosing cells reported by Karnovsky et al. (1970) is in good agreement with the rate that can be calculated for murine macrophages cultured in the presence of glucose and glutamine. As virtually all glucose is converted to lactate even when the cells are phagocytosing, then little could be oxidised by the TCA cycle. Thus an increase in oxygen consumption of the magnitude observed cannot be explained by a greater rate of glucose oxidation via the TCA cycle. This increase in oxygen consumption, however, is equivalent to the utilisation of 3.7 nmol of oleate/h per mg protien. As the calculated maximal rate of oleate oxidation is approximately 10 nmol/h per mg protein, then fatty acids could satisfy the increase in energy demand during phagocytosis. This would represent a novel role for fatty acid oxidation.

Endogenous triacylglycerol stores would, however, be utilised very quickly under these conditions. Loike et al., (1979) have reported that in glucose-free medium, after 6h of incubation, the number of particles ingested per macrophage was markedly reduced, while after only 1h of incubation little difference was observed, which supports the view than an endogenous fuel is depleted during this time. An alternative

explanation is that other energy utilising processes could be decreased over this period to permit the use of energy for phagocytosis. These discussions indicate that much further work is needed to identify the contribution of endogenous and exogenous fuels to the process of phagocytosis.

4.4.7 The contribution of glucose, glutamine and oleate to the energy requirement of cultured murine peritoneal macrophages

The results presented in this chapter suggests that glucose, glutamine or oleate can contribute to ATP generation for macrophages in culture, whether present singly or together in the cultue medium. On the basis of the estimated contribution of each fuel to CO_2 and lactate production during culture, it is calculated that the contribution to ATP formation is 54, 24 and 22% for glucose, glutamine and oleate respectively, when all three are present in the culture medium (table 4.16). Even though 80-90% of glucose is converted to lactate, the small proportion that is oxidised contributes considerably to ATP formation (table 4.17).

When glucose is omitted from the culture medium, the contribution from both glutamine ad oleate to ATP formation increases (table 4.16). Under these conditions the utilisation of glutamine markedly increases, while that of oleate decreases. However, the decrease in oleate utilisation is coupled to a large increase in the proportion of oleate oxidised to CO_2 (table 4.15) and thus with an increase in ATP formation. Oleate utilisation

TABLE 4.16

Contribution of glucose, glutamine and oleate to the energy requirement of cultured peritoneal macrophages.

It was assumed that the following number of mols of ATP can be generated from the metabolism of 1 mol of substrate as shown below;

Glucose 38 mol ATP (CO_2 as end-product)
Glucose 2 mol ATP (Lactate as end-product)
Glutamine 24 mol ATP (CO_2 as end - product)
Oleate 146 mol ATP (CO_2 as end-product)

It is assumed that ATP generation is maximal in the presence of glucose, glutamine and oleate
ATP generation is calculated from the rate of production of CO_2 or lactate from the above fuels. This data is given in Tables 4.12, 4.13 and 4.15.

Substrates present	ATP generated (µmol/ mg protein)	% contribution of substrate to total ATP generation	% of maximum ATP generation
5 mM Glucose	173	54	100
2 mM Glutamine	76	24	
0.3 mM Oleate	71	22	
5 mM Glucose	166	68	76
2 mM Glutamine	78	32	
5 mM Glucose	199	65	96
0.3 mM Oleate	105	35	
2 mM Glutamine	127	55	72
0.3 mM Oleate	104	45	
2 mM Glutamine	107	100	33
0.3 mM Oleate	122	100	38

TABLE 4.17

Contribution of anaerobic and aerobic metabolism of glucose to ATP generation from this substrate.

Substrate present	ATP generated (μmoles/ mg protein)		% of ATP generated from glucose	
	ANAEROBIC	AEROBIC	ANAEROBIC	AEROBIC
5 mM Glucose 2 mM Glutamine 0.3 mM Oleate	82	91	47	53
5 mM Glucose 2 mM Glutamine	88	78	53	47
5 mM Glucose 0.3 mM Oleate	76	123	38	62

is also decreased when glutamine is omitted from the cultue medium, but under these conditions the proportion of oleate oxidised to CO_2 is increased, and therefore, the contribution to ATP formation is increased. In fact, when glutamine is omitted from the medium, glucose and oleate can increase their contribution to the formation of ATP markedly (tables 4.12 and 4.15), so that the rate of formation of ATP remains almost identical to that observed when all three fuels are present in the culture medium.

Glutamine has been estimated to provide 40%, 30% and >50% of the energy requirement of Chinese hamster fibroblasts (Donnelly and Scheffler, 1976), human diploid fibroblasts (Zielke *et al.*, 1978) and cultured HeLa cells (Reitzer *et al.*, 1979) respectively. Thus the results for the contribution of glutamine to ATP formation in the murine macrophage (table 4.16) are in agreement with the findings from other types of cultured mammalian cells.

Thus it is likely *in vivo* that all these fuels (namely glucose, glutamine and long-chain fatty acids) contribute significantly to energy formation in the macrophage. Work previous to that presented in this chapter suggested only that glucose was important. The intriging question is how the rates of utilisation and oxidation of these three important fuels are regulated in the macrophage - and how the integration of regulation is achieved. A preliminary investigation into the control of glutamine metabolism is presented in chapter 5.

CHAPTER 5

The subcellular localisation of some enzymes of glutamine metabolism and some properties of phosphate-dependent glutaminase of the macrophage

5.1 INTRODUCTION

Phosphate-dependent glutaminase is widely distributed among tissues of the mammal (table 5.1). Two isoenzymes have been identified, a kidney-type and a liver-type (see review by Lund, 1980). Tissues in which it has a relatively high activity, in all species investigated, include brain, kidney, small intestine and lymphoid tissues (Krebs, 1935; Horowitz and Knox, 1968; Ardawi & Newsholme, 1984). The physiological role of the pathway in which this enzyme is involved appears to be different in each tissue. In the kidney the pathway is involved in acid-base balance: the ammonia produced from glutamine buffers the protons that are excreted by the kidney during acidosis ensuring that a large quantity of protons can be excreted (see Pitts, 1973). The brain enzyme appears to be immunologically (Curthoys et al., 1976) and kinetically (Svenneby, 1972) identical with the kidney enzyme, but its function is probably quite different. It may act as the source of two neurotransmitters, glutamate and 4-aminobutyrate (Quastel, 1979). The small intestine of the rat derives much of its energy from the oxidation of glutamine via a pathway that includes phosphate-dependent glutamase (for review see Windmueller, 1982). This is also the case for neoplastic cells (see Kovacek & McGivan, 1983; Moreadith & Lehninger, 1984) and human fibroblasts (Sumbilla et al., 1981). [Whether the major role of the pathway for utilistion of glutamine in these cells is energy production will be discussed in Chapter 6].

The importance of the glutamine metabolising pathway in the macrophage is suggested by the high activity of glutaminase in these

Table 5.1. The maximal activities of glutaminase in various tissues of the rat

The results are presented as means ±S.E.M. for tissues from four separate animals.

Tissue	Glutaminase activity (nmol/min per mg of protein)
Brain	180±0.1
Kidney	85±5.3
Liver	13±1.5
Soleus muscle	12.6±0.3
Gastrocnemius muscle	<0.1
White adipose tissue	24.9±0.8
Brown adipose tissue	39.0±3.2
Lymph node(mesenteric)	33±0.5

cells (chapter 2 and table 2.1), together with the high rates of glutamine utilisation observed in both short term incubations (Chapter 3) and longer term culture (Chapter 4 and table 5.2). Some enzyme activities that might constitute a glutamine utilisation pathway have been measured and are reported in Chapter 2. In this chapter, the intracellular distribution of some of these enzymes is reported: this was studied by differential and sucrose gradient centrifugation. This work provides prima facie evidence of a unique characteristic of glutamine metabolism in the macrophage.

The first step in understanding the nature of a metabolic pathway and how it may be controlled is to identify equilibrium nature of the individual reactions of the pathway. To do this, the mass action ratio (T) for a particular reaction in a pathway is compared with the apparent equilibrium constant (Keq) of the reaction (Newsholme & Start, 1973): in addition these two quantities can be used to calculate ΔG for the reaction ($\Delta G = -RT\ln K/T$ - see Newsholme & Leech, 1983).

It was indicated in Chapter 1 that if the ratio of Keq/T is > 5 for a given enzyme-catalysed reaction, or if the value of ΔG is >4kJ, it is probable that the enzyme catalyses a non-equilibrium reaction. Calculations based on the data given in table 5.3 indicate that glutaminase catalyses a non-equilibrium reaction in the macrophage. Other reactions or processes which may also be non-equilibrium in the pathway of glutamine metabolism include the transport of glutamine across the cellular or the mitochondrial membranes and the reactions catalysed by oxoglutarate dehydrogenase and phosphoenolpyruvate

Table 5.2. Rates of glutamine utilization by several types of cell during incubation or culture

The data are obtained from the following sources: (a) present work (see Table 4.2 & Fig. 3.6); (b) Ardawi (1983); (c) Watford et al. (1979); (d) Vinay et al. (1978); (e) Ardawi & Newsholme (1985); (f) Reitzer et al. (1979).

Type of cell	Conditions of culture or incubation	Rate of glutamine utilization (μmol/h per g dry wt.)
Macrophages	incubated cells, 2 mM-glutamine, 100% O_2	186[a]
	incubated cells, 5 mM-glutamine, 100% O_2	391[a]
Lymphocytes	incubated cells, 2 mM-glutamine, 100% O_2	162[b]
Enterocytes	incubated cells, 5 mM-glutamine, 95% O_2; 5% CO_2	66[c]
Kidney tubules	incubated cells, 5 mM-glutamine, 95% O_2; 5% CO_2	636[d]
Colonocytes	incubated cells, 5 mM-glutamine, 100% O_2	331[e]
Macrophages	cultured cells, 2 mM-glutamine, 95% air; 5% CO_2	89.4[a*]
Hela tumour cell	cultured cells, 2 mM-glutamine, 95% air; 5% CO_2	220[f†]

* Rate expressed as nmol/h per mg protein (after 23 h of culture).

† This rate (expressed as nmol/h per mg protein) was calculated using the average protein concentration over the culture period (50 h).

Table 5.3. Calculation of the free energy change for the reaction catalysed by glutaminase in the murine macrophage

The intracellular concentrations of glutamine, glutamate and ammonia were obtained from Dr. A. H. Gordon, M.R.C. London (Personal Communication) and were measured after a 2 hour incubation of the cells in fresh culture medium. The mass action ratio for the glutaminase reaction was calculated from the equation [Glutamate] [Ammonia] / [Glutamine] [H_2O]. The standard free energy change for this reaction is -14.2 kJ/mole (Lehninger, 1975).

Metabolite	Intracellular concentration (mM)
Glutamine	7.6
Glutamate	19.5
Ammonia	14.0

The concentration of water is assumed, by convention, to be unity.

Mass action ratio is 35.9

Apparent equilibrium constant = 308

$\Delta G = -RT \ln K/\uparrow$

$= -RT \ln 308/35.9$

$= -5.5$

and Table 5.4

carboxykinase (PEPCK) (Fig. 5.1). This is based on the work described for the kidney (Newsholme et al., 1982). Since concentrations of the substrates and products of these reactions have not been measured in the macrophage, another step in the analysis of the structure of a metabolic pathway is the identification of the flux-generating step. So far in the work reported in this thesis, it has been shown that increasing the medium concentration of glutamine above the normal physiological level increases the rate of glutamine utilisation: this means that a flux-generating step for glutamine utilisation is not present in the macrophage and must be present in some other tissue (see Newsholme and Leech, 1983, for discussion of flux-generating steps). However, it can be shown that the rate of glutamine utilization by the macrophage can be changed independently of the extracellular glutamine concentration, which indicates that there is external control (i.e., it is regulated by factors other than the substrate concentration - see Newsholme & Crabtree, 1979) of the glutamine pathway in the macrophage: for the incubated macrophage, the rate of glutamine utilization is increased if protein or glucose is omitted from the incubation medium (Chapter 3). However it is unclear which reaction(s) is influenced by external regulation. To identify the step(s) would require measurement of the concentrations of the intermediates in the pathway including cytosolic and mitochondrial glutamine concentrations under two different flux conditions. However, glutaminase from other tissues has properties which may be important in external control (Ardawi, 1983, for rat lymphocytes; Goldstein, 1976, and Curthoys et al., 1984, for rat kidney). Therefore it seemed important to study glutaminase from the macrophage and the results of the study of the properties are presented in this Chapter.

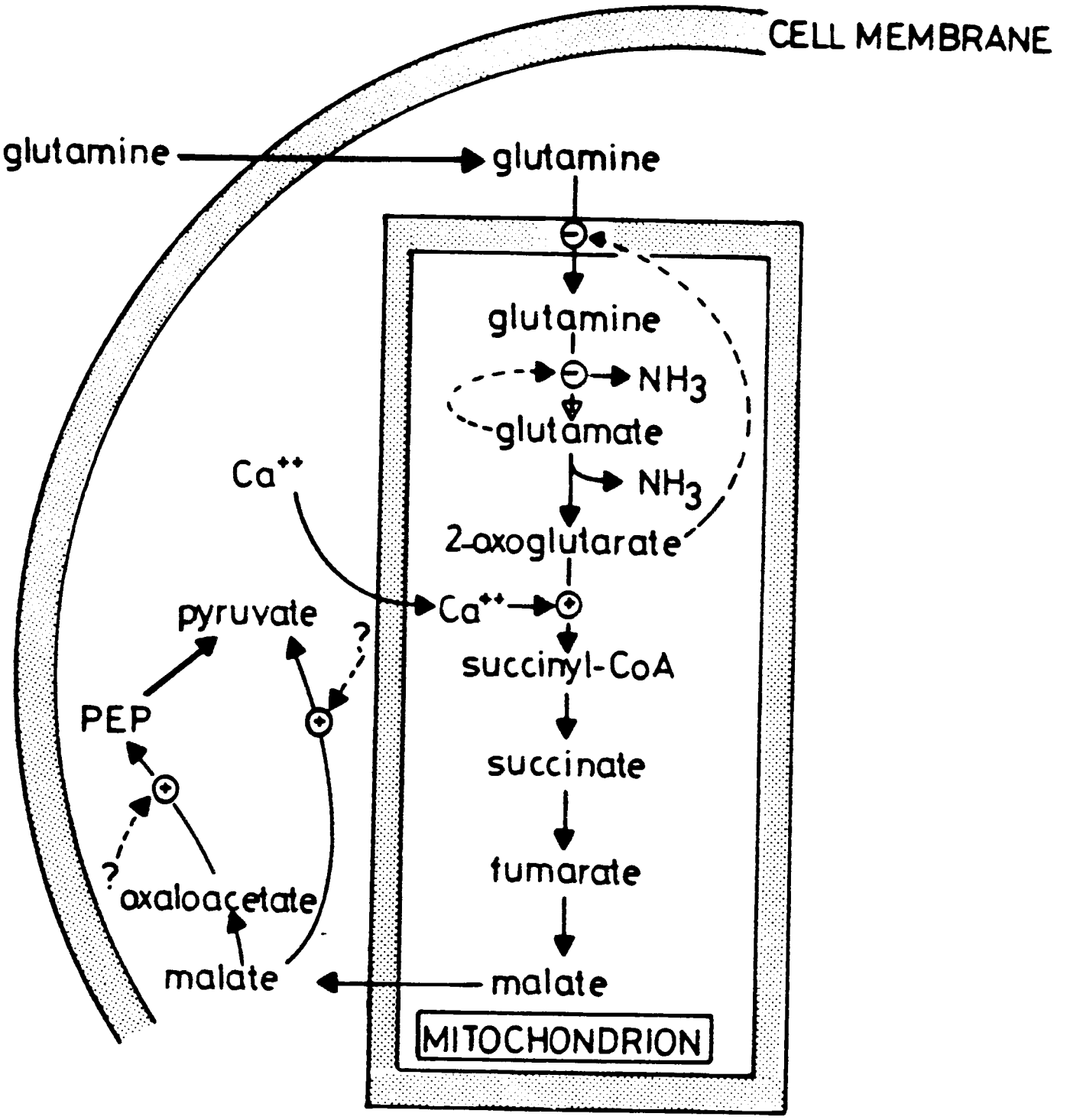


Figure 5.1 Control of glutamine metabolism in the kidney

Adapted from Newsholme and Leech (1983).

Table 5.4. Properties of some 'non-equilibrium' enzymes and transport systems in the renal glutamine pathway

Non-equilibrium enzymes or transport systems	Properties of enzymes			
	K_m for pathway substrate	Physiological concentration of pathway-substrate	Activators	Inhibitors
Glutamine transport across cell membrane		1 mM	N.K.	N.K.
Glutamine transport across the mitochondrial membrane	2.1 mM (glutamine)	2 mM*	N.K.	2-oxoglutarate
Glutaminase	5 mM (glutamine)	2 mM*	phosphate	(glutamate, 2-oxoglutarate
Oxoglutarate dehydrogenase	0.3 mM (2-oxoglutarate)	2.6 mM	H^+, Ca^{2+}	N.K.
Malate transport across mitochondrial membrane	N.K. (malate)	0.6 mM*	N.K.	N.K.
Phosphoenolpyruvate carboxykinase	N.K. (oxaloacetate)	1.0 μM†	N.K.	N.K.

Data taken from Newsholme & Leech (1983).

N.K. = not known.

* Assuming an equal distribution between cytosolic and mitochondrial compartments.

† Assuming an equal distribution of malate between cytosolic and mitochondrial compartments and calculation of concentration of oxaloacetate from cytosolic $NAD^+/NADH$ ratio.

As an important initial step in determining the pathway of glutamine metabolism in the macrophage, it is necessary to know the intracellular localisation of the enzymes proposed to be involved in this pathway. The first part of the results section of this chapter concerns the localisation of glutaminase and other glutamine pathway enzymes. Some properties of glutaminase in a crude homogenate of macrophages are presented in the latter part of the results section.

EXPERIMENTAL

5.2.1 Preparation of homogenates for subcellular distribution studies

Elicited peritoneal macrophages were obtained from 12-16 week-old female mice of the C57 BL/6 strain, bred in the Sir William Dunn School of Pathology, Oxford. Macrophages were obtained from the peritoneal cavity of mice 4 days after injection of 1.5 ml of Brewers thioglycollate broth intraperitoneally, and purified by adherence to plastic petri dishes (see Cohn, 1974; and the experimental section of Chapter 2). The purified cells after being gently scraped up into a small volume of phosphate-buffered saline pH 7.2-7.4 (Dulbecco & Vogt, 1954) were centrifuged at 500g for 5 min. These cells were then resuspended in 2 ml of extraction medium which consisted of 250mM-sucrose, 1mM-EDTA, 5mM-HEPES [4-(2-hydroxyethyl)-1-pipemazine-ethane-sulphonic acid] and 1% (w/v) bovine serum albumin, pH 7.4. The cell suspension was then homogenised for 2 x 10s bursts at $0^{o}C$ in a polytron homogenizer (PCU-2 at Position 3).

5.2.2 Subcellular fractionation

The homogenate was centrifuged at 600g for 5 min, and the supernatant was then centrifuged at 8500g for 15 min using a Sorvall centrifuge. The pellet obtained was resuspended in the washing medium (extraction medium minus EGTA) and was washed once by centrifugation at 8500g for 15 min. The supernatant from the first 8500g centrifugation was kept on ice and is termed the cytosolic fraction. The final pellet was resuspended in washing medium and was termed the mitochondrial fraction. No further centrifugation steps were performed

to separate microsomes from the cytosol. The crude nuclear fraction (pellet from the initial 600g spin) was not used further; preliminary experiments established that enzyme activities of interest in the present work were almost non-detectable in the nuclear fraction. Mitochondrial integrity was assessed by measuring the activity of citrate synthase in the mitochondrial fraction plus or minus 0.05% triton-x-100. Preparations were only used if there was at least a 10-fold increase in activity on addition of triton.

The distribution of the cytoplasmic (lactate dehydrogenase)(L) and mitochondrial (citrate synthase)(C) marker enzymes were determined in each subcellular fractionation experiment. The percentage activities of any enzyme measured in the particulate pellet was assumed to be that in the mitochondrial fraction (X), whereas the percentage activity measured in the supernatant was assumed to be cytoplasmic (Y). By solving simultaneous linear equations, as shown below, an estimation of the subcellular location of macrophage enzymes was made:

$$Lm \times C + Cm \times M = X$$

$$Lc \times C + Cc \times M = Y$$

where M and C represent the corrected percentage distribution of any macrophage enzyme in the mitochondrial and cytoplasmic fractions respectively. Lm, Lc, Cm and Cc are the percentage recoveries of the marker enzymes in the two subcellular fractions.

5.2.3 Enzyme activity assays

Enzyme activities reported in this chapter were measured

according to the procedures described in Chapter 2, and appendix B.

5.2.4 Sucrose Gradients

Sucrose solutions were prepared in 1% (w/v) bovine serum albumin and 1 mM-EDTA at pH 7.0. For the intracellular distribution of glutaminase and marker enzymes, a continuous linear sucrose gradient (30-50%) was used. Murine elicited peritoneal macrophages were homogenised in an isolation medium consisting of 150 mM-potassium phosphate (equimolar mixture of K_2HPO_4 and KH_2PO_4), 1 mM-EDTA, 50 mM-Tris and 250 mM-sucrose, using a polytron homogeniser (PCU-2 at position 3, 2 x 10 s at 0^oC). Homogenates were centrifuged at 650 g for 5 min, so removing cell debris. The resulting supernatant was layered onto the prepared sucrose gradient (which had been left to equilibrate for at least one hour at 0^oC) and this was centrifuged in a SW40 rotor using a Beckman L2-65B ultracentrifuge at 80,000 g for 3 hours. After centrifugation, the contents of the tube were collected carefully by aspiration using a Pasteur pipette. Aliquots of the gradient were removed and separated according to the position of the distinct bands observed at irregular intervals down the tube. All aliquots were kept at $0-4^oC$ prior to assay of enzyme activities, which occurred as soon as possible after centrifugation. Results are presented in both tables and figures for the percentage distribution of activity and in tabular form for absolute enzyme activities. The percentage distribution and in addition the percentage volume of each fraction in which the enzyme activity was measured are given in Table 5.7 and Figure 5.2. The latter point is important since the distribution of the enzyme activity must be seen as a function of density of the material with which the enzyme is associated, and not

as a function of volume of the fraction in which it is measured. The absolute enzyme activities in each of the fractions (expressed as umol/min/ml fraction) are given in Table 5.8. Activities of all enzymes shown should be distributed as a function of the density of material the enzyme is associated with; hence there is a large difference in the distribution of activity of the cytosolic marker, lactate dehydrogenase, and the mitochondrial markers citrate synthase and glutamate dehydrogenase. The distribution of glutaminase plus the marker enzymes will be further discussed in the results section.

5.2.5 Preparation of homogenates for the study of some properties of glutaminase

Cells were prepared by the standard procedure (chapter 2). The purified cells, after being gently scraped up into a small volume of phosphate-buffered saline pH 7.2-7.4 (Dulbecco & Vogt, 1954) were centrifuged at 500g for 5 min. These cells were then resuspended in 1 ml of extraction medium and were homogenised using a 1ml capacity small ground-glass homogeniser at $0^{\circ}C$. The extraction medium consisted of either 150 mM phosphate (equimolar mixture of K_2HPO_4 and KH_2PO_4), 50 mM Tris, 1 mM EDTA, pH 8.6 for studies on enzyme inhibition and determination of the K_m for glutamine, or 100 mM Tris, 1 mM EDTA, pH 8.6 for studies on phosphate activation.

After homogenisation, the crude homogenate was diluted up to 5 times with the relevant extraction buffer, before addition to the assay medium. Glutaminase was assayed by the sampling method of Curthoys and Lowry, 1973, as soon as possible after homogenisation.

5.2.6 Assay medium for glutaminase

For all studies on glutaminase activities except for phosphate activation and determination of the K_m for glutamine, the assay medium consisted of 50 mM phosphate buffer (equimolar mixture of K_2HPO_4 and KH_2PO_4), 0.2mM-EDTA, 50 mM-Tris, 20 mM glutamine and 0.05% (v/v) triton-x-100, pH 8.6. For the determination of the K_m for glutamine, the above assay medium was used but the concentration of glutamine was varied over the range 0-50 mM. The K_m was determined using a Hanes plot (Figure 5.4).

For study of phosphate activation, the following assay medium was used; 100 mM-Tris, 0.2 mM-EDTA, 20 mM-glutamine and 0.05% (v/v) triton-x-100, pH 8.6. Phosphate was added over the range 0-200 mM. The concentration of phosphate required for half-maximal activation of glutaminase was calculated directly from the curve shown in Figure 5.5.

Triton-x-100 is included in all assays of glutaminase at a final concentration of 0.05% (v/v). However, 74% of the maximal glutaminase activity in a crude macrophage homogenate is found in the absence of triton-x-100. This is not the case for other tissue or cell preparations where detergents are required to release more than 50% of glutaminase activity.

In all assays, it was demonstrated that the activity was linear with enzyme concentration and was linear with time for up to 20 min.

RESULTS

5.3.1 Intracellular distribution of glutaminase and other enzymes involved in glutamine metabolism

Differential centrifugation

The percent activity of each enzyme recovered after differential centrifugation is given in Table 5.5. At least 95% of enzyme activity in the whole homogenate was recovered in the two subcellular fractions obtained in this technique. The enzyme activities in the mitochondrial and cytosolic fractions are given in Table 5.6: the activities of glutamate dehydrogenase and pyruvate carboxylase were present exclusively in the mitochondrial fraction; the activities of PEPCK and pyruvate kinase were present exclusively in the cytosolic fraction; the activities of malate dehydrogenase, aspartate aminotransferase, phosphate-dependent glutaminase and phosphate-independent glutaminase were present in both compartments. This distribution of activities is similar to that previously reported for rat lymphocytes (Curi et al., 1986) with the exception of phosphate-dependent glutminase. To the author's knowledge this is the first report of a significant activity of phosphate-dependent glutaminase in a non-mitochondrial compartment: for previous reports of the localisation of phosphate-dependent glutaminase see discussion .

5.3.2 Sucrose gradient centrifugation

The subcellular distribution of phosphate dependent glutaminase was further characterised by examining the distribution of this enzyme through a 30-50% sucrose gradient together with several marker

Table 5.5. Percent activity in cytosol and mitochondria of enzymes involved in glutamine metabolism in peritoneal murine macrophages

Results are presented as means ±S.E.M. Maximal activities are given in Table 5.6. Percent activity is corrected for 100% for each enzyme. At least 95% activity was recovered in the two fractions.

Enzyme	Percent activities recovered after centrifugation	
	Cytosol	Mitochondria
Lactate dehydrogenase	91.8±2.0	8.2±2.0
Citrate synthase	22.3±0.5	77.7±4.6
Pyruvate kinase	97.4±1.9	2.6±0.1
Pyruvate carboxylase	19.4±6.6	80.6±10.2
Phosphoenolpyruvate carboxylase	99.3±4.5	0.7±0.1
Malate dehydrogenase	71.5±0.8	28.5±0.1
Aspartate aminotransferase	58.4±5.1	41.6±2.2
Phosphate-independent glutaminase	81.1±5.4	18.9±1.4
Phosphate-dependent glutaminase	62.3±4.6	37.7±1.2
Glutamate dehydrogenase	9.0±1.7	91.0±5.5

Table 5.6. Maximal activities and corrected distribution of activities between mitochondria and cytosol of enzymes involved in glutamine metabolism in peritoneal murine macrophages

Macrophages were homogenised, mitochondrial and cytosolic fractions prepared and enzyme activities measured as described in Methods section. Results are presented as means ±S.E.M. for four separate experiments.

Enzyme	Maximal Activity (nmol/min per mg protein)	Percent distribution	
		Cytosol	Mitochondria
Lactate dehydrogenase	764±13.4	100	0
Citrate synthase	108±3.3	0	100
Pyruvate kinase	447±39.3	100	0
Pyruvate carboxylase	4.7±0.34	0	100
Phosphoenolpyruvate carboxylase	7.6±0.51	100	0
Malate dehydrogenase	452±10.6	78	22
Aspartate aminotransferase	118±9.7	60.5	39.5
Phosphate-independent glutaminase	16.7±0.5	85	15
Phosphate-dependent glutaminase	135.3±4.9	55.5	44.5
Glutamate dehydrogenase	98.6±3.0	0	100

enzymes: lactate dehydrogenase as a cytosolic marker, glutamate dehydrogenase and citrate synthase as mitochondrial markers, and 5′-nucleotidase as a plasma membrane marker. The latter enzyme has been shown to e associated with the plasma membrane in macrophages (Werb & Cohn, 1972). The results from the sucrose gradient centrifugation are given in Tables 5.7, 5.8 and Fig. 5.2. The distribution of phosphate-dependent glutaminase through the gradient is similar to that of 5′-nucleotidase, citrate synthase and glutamate dehydrogenase, but is completely different to lactate dehydrogenase. These results suggest that phosphate-dependent glutaminase activity is associated only with particulate material from the cell.

5.3.3 A preliminary study of some properties of macrophage glutaminase

The data presented above suggest that phosphate-dependent glutaminase is present in both mitochondrial and non-mitochondrial compartments of the cell. The experiments to determine some properties of glutaminase have been carried out with a crude homogenate of macrophages so that no distinction has been made between the differently-localised enzymes.

A plot of activity of glutaminase versus concentration of glutamine is given in Figure 5.3: the plot is hyperbolic as indicated by the fact that a plot of activity/substrate concentration versus substrate concentration is linear (Fig. 5.4); the K_m for glutamine obtained from this plot is 4.8 mM. This value is similar to values reported for the enzyme from rat mesenteric lymph nodes, kidney, small intestine, bovine spleen and Ehrlich ascites tumour cells (see Table

Table 5.7. Percent distribution of enzyme activities from mouse macrophage on a 30-50% sucrose gradient

Details are given in text

Fraction number	Vol. of fraction (ml)	Percent volume of fraction	Percent distribution				
			Glutaminase	Lactate dehydrogenase	5'-Nucleotidase	Citrate synthase	Glutamate dehydrogenase
I	0.6	5.5	0.3	28.7	0	5.5	2.1
II	1.9	17.3	9.7	35.4	10.5	16.4	15.8
III	3.2	29.1	71.1	26.2	72.7	45.4	57.2
IV	3.1	28.2	14.9	5.2	8.4	24.5	19.2
V	2.0	18.2	2.2	3.5	5.2	5.2	3.4
VI	0.2	1.8	1.8	1.1	3.2	3.0	2.2

Table 5.8. The distribution of some enzyme activities in homogenate of murine macrophages on a 30-50% sucrose gradient

Activities are presented as means ±S.E.M. for four separate experiments.

Fraction number	Vol. of fraction (ml)	Enzyme activities (µmol/min per ml gradient)				
		Glutaminase	Lactate dehydrogenase	5'-Nucleotidase*	Citrate synthase	Glutamate dehydrogenase
I	0.6	0.010±0.004	5.32±0.071	<0.1	0.163±0.009	0.110±0.005
II	1.9	0.109±0.002	2.07±0.095	4.90±0.20	0.155±0.005	0.259±0.037
III	3.2	0.472±0.006	0.911±0.054	20.1±2.00	0.255±0.017	0.557±0.012
IV	3.1	0.102±0.002	0.185±0.021	2.40±0.51	0.142±0.002	0.193±0.003
V	2.0	0.023±0.002	0.197±0.009	2.30±0.10	0.047±0.002	0.053±0.005
VI	0.2	0.195±0.009	0.603±0.023	14±1.0	0.218±0.004	0.343±0.021

* nmol/min per ml gradient

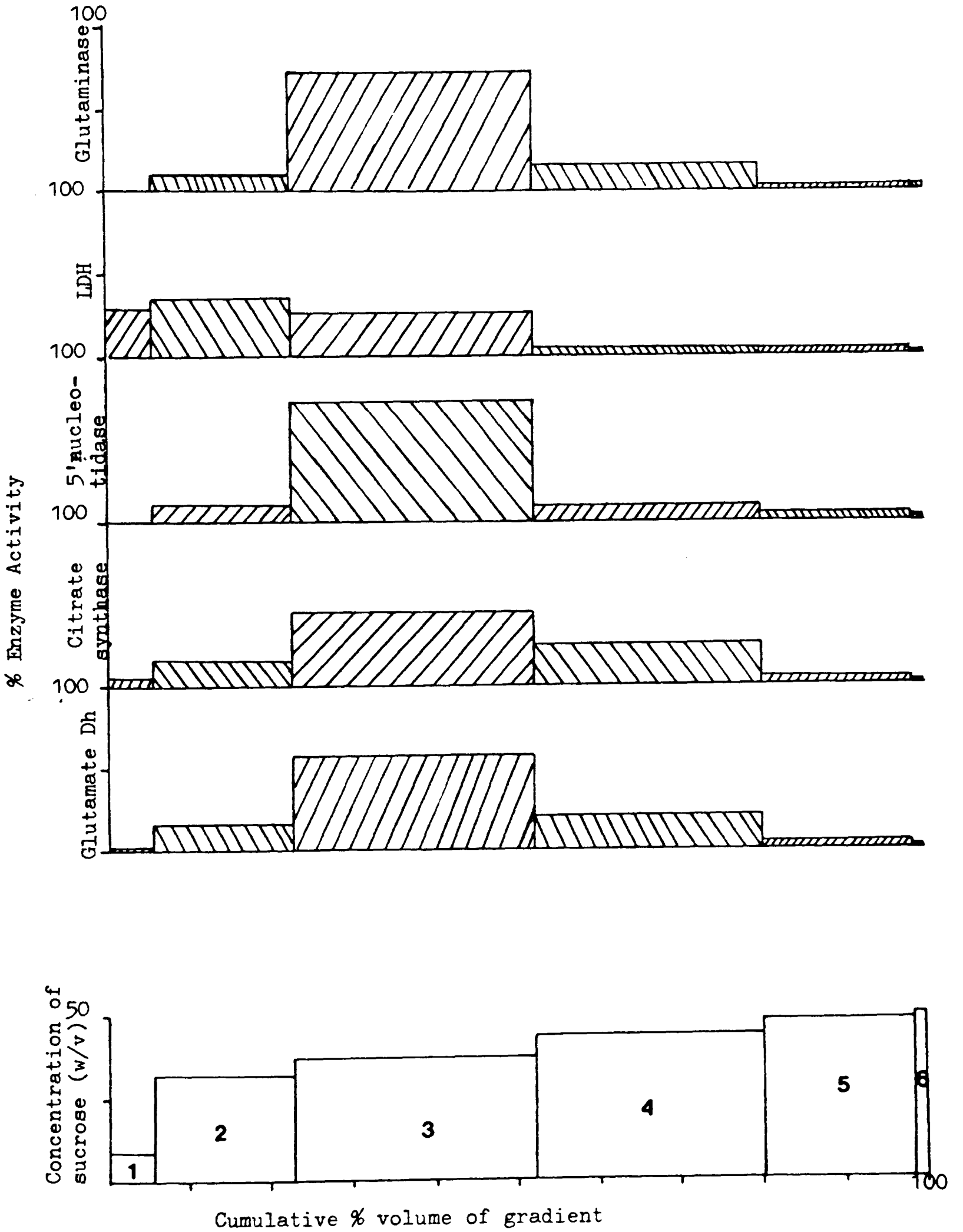


Figure 5.2

% Enzyme Activity through a 30-50% sucrose gradient.

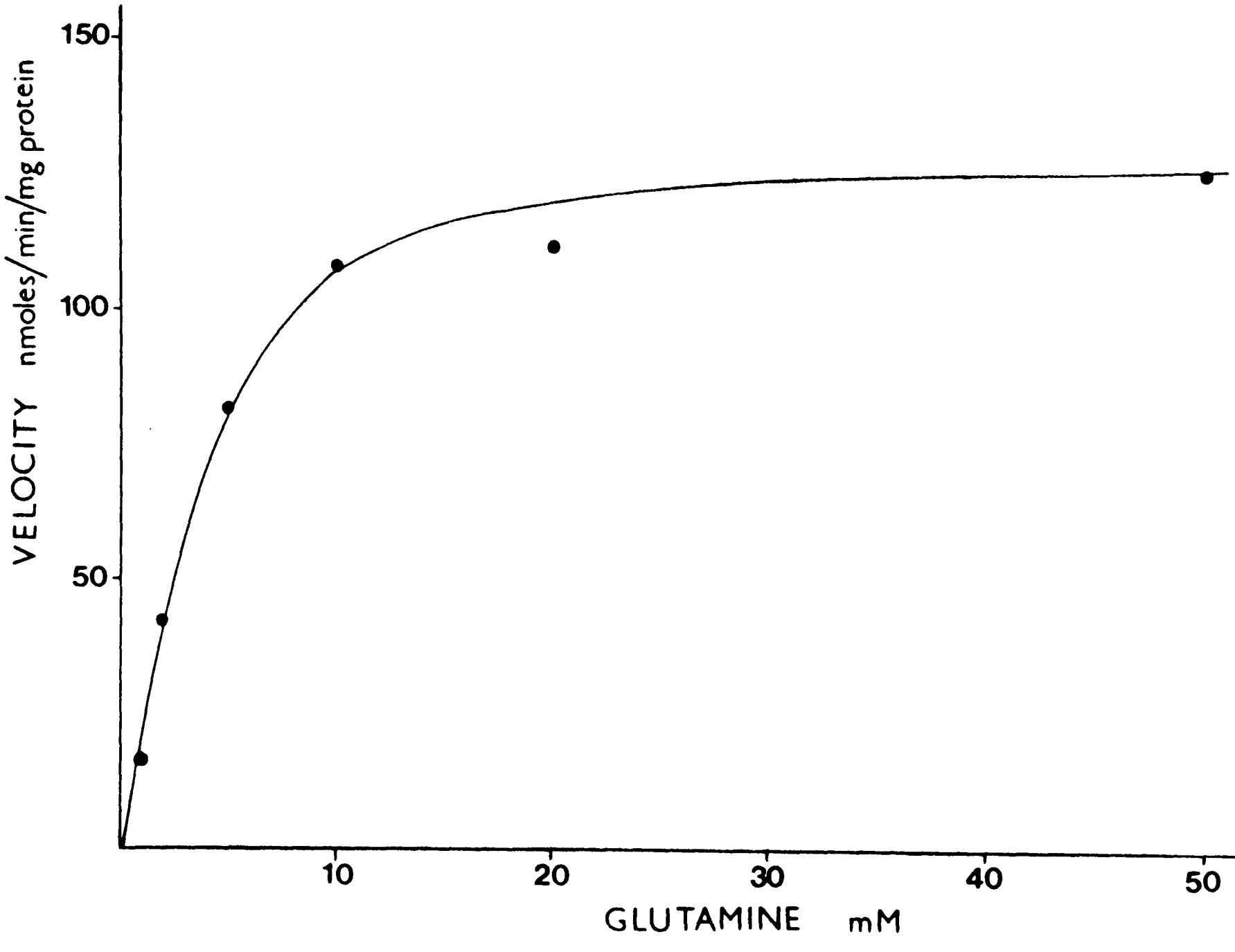


Figure 5.3 Velocity Vs. glutamine concentration.

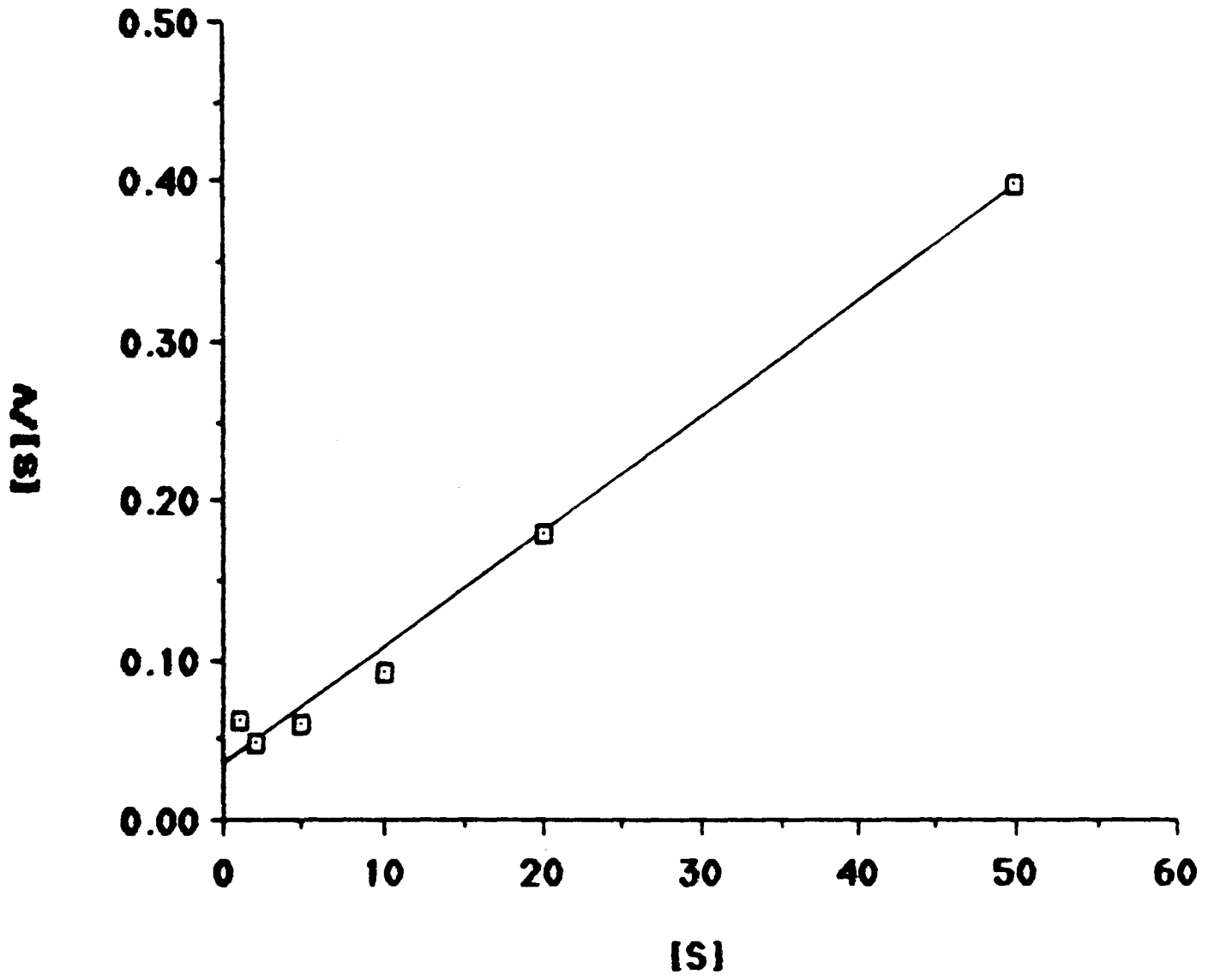


Figure 5.4 - Macrophage glutaminase activity: A Hanes plot

Glutamine was assayed by the sampling method described in Appendix B, at various concentrations of glutamine as indicated. Each point is the mean of two separate determinations measured in triplicate. The units used in this figure are, [S]: mM; V: nmol/min per mg protein.

Table 5.9. Km values of glutaminase from different sources for glutamine

Data are taken from (a) Ardawi & Newsholme (1984); (b) Pinkus & Windmueller (1977); (c) Shapiro et al. (1982); (d) Ardawi (1983); (e) Kovacevic (1974); (f) present work; (g) Huang & Knox (1976); (h) Chui & Boeker (1979).

Tissue or cell	Km (mM)
Rat mesenteric lymph nodes	2.0[a]
Rat small intestine	2.2[b]
Rat kidney	4.0[c]
Bovine spleen	4.0[d]
Ehrlich ascites tumour cells	4.5[e]
Mouse peritoneal macrophage	4.8[f]
Rat liver	28.0[g]
Bovine brain	30.0[h]

5.9).

Phosphate is known to be an activator of glutaminase (Sayre & Roberts, 1958; Svenneby, 1972; Curthoys et al., 1976; Pinkus & Windmueller, 1977). For glutaminase in crude homogenates of murine macrophages, a plot of activity against phosphate concentration is sigmoid (Figure 5.5); 50% activtion across at about 17 mM. This is similar to that reported for the enzyme from bovine spleen (Ardawi, 1983) but is higher than that for the enzyme from rat mesenteric lymph node (Ardawi and Newsholme, 1984b) and liver (Lund, 1980). However this value is much lower than the reported values for rat kidney (Horrowitz & Knox, 1968) and bovine brain (Chiu & Boeker, 1979). Macrophage glutaminase only shows maximal activity when extracted in the presence of phosphate. Thus less than half the maximal activity is observed when enzyme is activated by high concentrations of phosphate after extraction in the absence of phosphate (Fig. 5.5).

The effects of glutamate, ammonia, 2-oxoglutarate and citrate on the activity of macrophage phosphate-dependent glutaminase are given in Table 5.10. All these compounds are likely intermediates in the pathway for the metabolism of glutamine by the macrophage (see Chapters 3 and 4. Only glutamate and 2-oxoglutarate have any significant effect on the enzyme activity: both compounds inhibit the activity but high concentrations were required (Table 5.10).

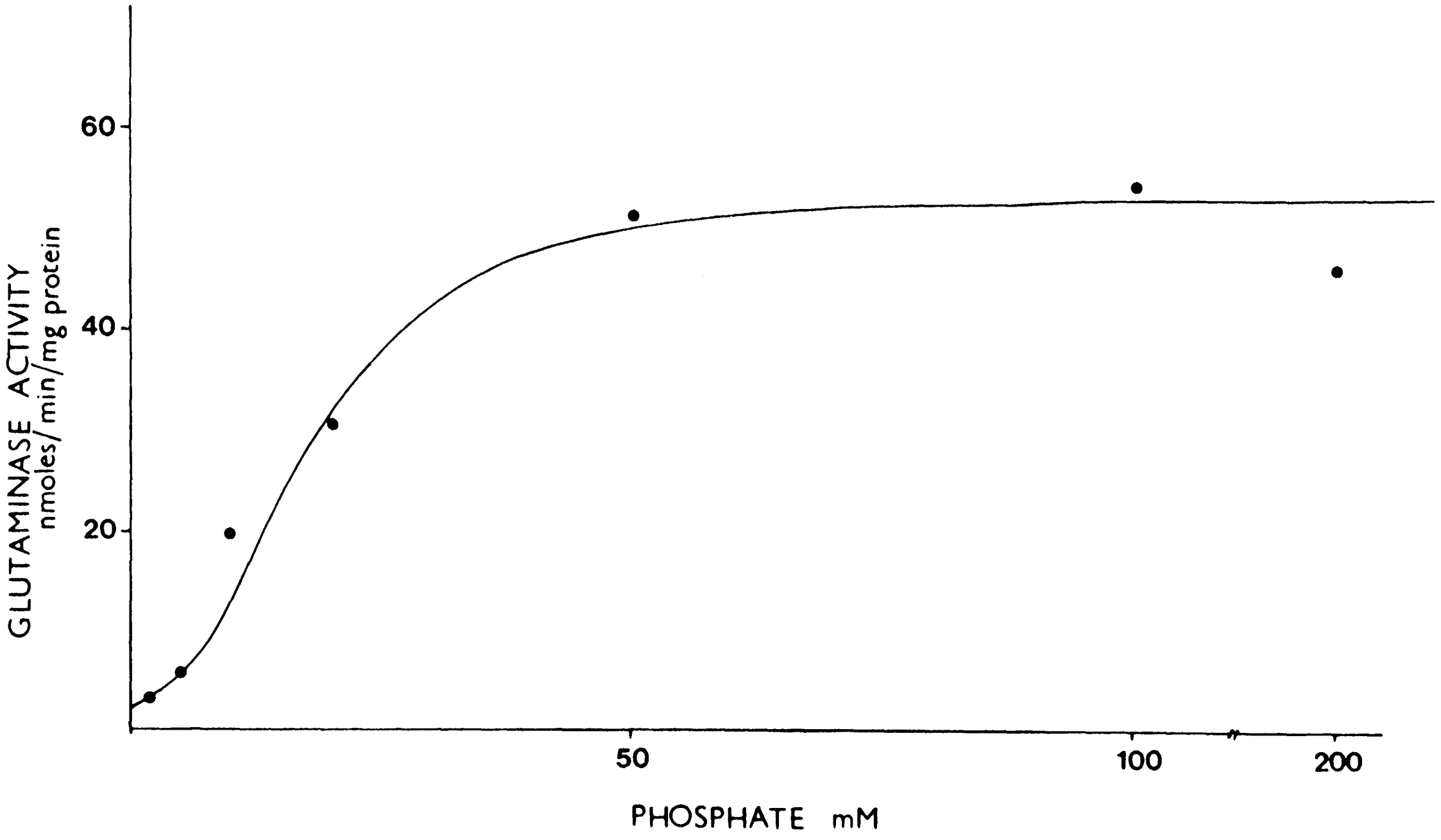


Figure 5.5

Activation of glutaminase by phosphate

Table 5.10. Inhibition of murine macrophage glutaminase

Control activity was 114.0 nmol/min per mg protein. The concentration of the glutamine was 20 mM.

Addition to assay medium	Percent inhibition
Glutamate (2 mM)	0
Ammonium chloride (2 mM)	0
α-Ketoglutarate (2 mM)	6
Citrate (2 mM)	11
Glutamate (5 mM)	0
Glutamate (10 mM)	6
Citrate (10 mM)	24
Glutamate (20 mM)	48
Ammonium chloride (20 mM)	10
α-Ketoglutarate (20 mM)	73
Citrate (20 mM)	24

5.4.1 DISCUSSION

Glutaminase is known to be present in many tissues of the mammal (table 5.1). It is present in different lymphoid tissues of the rat, and in mesenteric lymph nodes of six different mammals that have been investigated (Ardawi, 1983; table 5.12). However, on a protein basis, the activity of glutaminase in the macrophage is higher than any of those in lymphoid tissues (e.g. it is 2-3fold that in rat mesenteric lymph nodes).

The rates of glutamine utilisation by incubated and cultured macrophages are given in chapters 3 and 4. From a knowledge of the values of V_{max} and K_m of glutaminase presented in this Chapter, the rate of reaction in vivo can be calculated, assuming that activators are fully effective but that glutamate has no inhibitory effect; it is also assumed that the intracellular steady-state concentration of glutamine is 3.7 mM (the mean of five determinations over 12 days in culture, A.H. Gordon, personal communication). The calculated activity of the enzyme is 66 nmol/min per mg protein. The highest observed rate of glutamine utilisation by the macrophage is 18 nmol/min per mg protein (Figure 36). This suggests that glutaminase may not be fully activated by phosphate or that the intramitochondrial concentration of glutamine is considerably less than 3.7 mM. Alternatively, other inhibitors of glutaminase so far not discovered may exist in the macrophage or possibly that enzyme activity is regulated by some process as yet not investigated (e.g. covalent modification).

Table 5.12. Activities of glutaminase in lymphoid tissues of different animals

Lymphoid tissues were isolated, extracted and glutaminase was assayed as described in Methods section. Activities were measured at 37 °C and are presented as means ±S.E.M. with the numbers of separate animals given in parentheses.

Data from Ardawi (1983).

Animal	Tissue	Glutaminase activity (μmol/min per g fresh wt.)
Gerbil	Mesenteric lymph node	5.6±1.20 (3)
Guinea pig	Mesenteric lymph node	6.0±0.45 (5)
Hamster	Mesenteric lymph node	6.6±0.70 (4)
Mouse	Mesenteric lymph node	9.2±0.46 (6)
Rabbit	Mesenteric lymph node	4.6±0.78 (3)
Rat	Mesenteric lymph node	8.7±0.43 (15)
	Thymus	6.0±0.96 (6)
	Spleen	6.1±0.91 (5)
	Peyer's patches	10.6±1.00 (5)
	Bone marrow	3.7±0.15 (6)

5.4.2 Phosphate-dependent and independent glutaminases

In all tissues so far studied phosphate-dependent glutaminase is localised within the mitochondria, probably attached to the inner side of the inner mitochondrial membrane, kidney (Katunuma et al., 1967; Crompton et al., 1973; Curthoys & Weiss, 1974; Kalra & Brosnan, 1974), brain (Katunuma et al., 1967), liver (Guha, 1961; Kalra & Brosnan, 1973), small intestine (Pinkus & Windmueller, 1977), Ehrlich ascites tumor cells (Kovacevic, 1974) and lymphocytes (Ardawi and Newsholme, 1984b).

Curthoys and Weiss (1974) demonstrated, using differential centrifugation of rat kidney homogenate, that of the non-nuclear phosphate-dependent glutaminase activity (74% of total) 92% was associated with the mitochondrial fraction. [For the phosphate-independent glutaminase, of the non-nuclear activity (95% of total) 34% of this activity was associated with the mitochondria. In the present work, it was found that only 15% of macrophage phosphate-independent glutaminase was associated with the mitochondria (table 5.6)]. In contrast to the previous finding a considerable proportion of phosphate-dependent glutaminase was shown to be non-mitochondrial (55%). To examine this further the distribution of phosphate-dependent glutaminase through a sucrose gradient was investigated. The results support a mitochondrial localisation of part of the activity but, on the basis of the distribution of 5'nucleotidase, the non-mitochondrial activity may be associated with the plasma membrane. In the macrophage, there is evidence that some enzymes normally associated with the inner mitochondrial membrane, are

also found in association with the plasma membrane. Thus proteins of the electron transport chains are normally confined to the inner mitochondrial membrane and the endoplasmic reticulum of cells. However, recent studies with both neutrophils and macrophages have shown the existence of a NADPH oxidase system in the plasma membrane; this system is capable of reducing molecular oxygen to O_2^- (see Rossi et al., 1985, and Berton & Gordon, 1985, for reviews); the NADPH oxidase is considered to be an electron transport system in which a flavoprotein, a b-type cytochrome and possibly quinones are involved. Whether glutaminase plays any functional role in this activity is not known.

Phosphate-dependent glutaminase is not secreted by macrophages. Samples of media from cultured macrophages taken after 48 and 96h contained no detectable glutaminase activity (results not shown).

So far it has been considered that phosphate-dependent glutaminase is the only enzyme capable of utilisi ng glutamine. In most mammalian tissues that utilise glutamine, its metabolism can be initiated by deamidation (the most common), transamidation or transamination. Transamination (catalysed by aminotransferase) involves the transfer of the 2-amino group to a 2-keto acid to form an amino acid, while transamidation involves the transfer of the nitrogen in the amide group to a completely different class of compound, such as in the synthesis of purines and pyrimidines (catalysed by amidotransferases).

Glutamine that is transaminated gives rise to 2-oxoglutaramate which is deaminated by ω-deaminase to give 2-oxoglutarate (Cooper & Meister, 1972). Glutamine that is utilised for amidotransferase reactions gives rise to glutamate. Katunuma et al. (1967) reported that rat kidney contains two distinct activities that deamidate glutamine; a phosphate-dependent glutaminase and a phosphate-independent and maleate stimulated glutaminase. The former glutaminase is immunologically identical to the glutaminase contained in rat brain and intestine; but distinct from liver glutminase (Curthoys et al., 1976). The phosphate-independent glutaminase activity is a partial reaction of γ-glutamyltranspeptidase (Curthoys & Kehlenschmidt, 1975). Maleate increases the apparent glutaminase activity of te latter, both by inhibiting transpeptidation and stimulating the hydrolytic activity. For the macrophage, the maximum activity of phosphate-independent glutaminase is 16.7 nmol/min per mg protein, which represents approximately 12% of the phosphate-dependent activity. The phosphate-independent activity is measured in the presence of maleate, so that it could represent -glutamyltranspeptidase activity. The macrophage is unlikely to contain any appreciable activity of the glutamine transaminase or transamidase enzymes, since the rate of formation of glutamate or ammonia in the absence of either phosphate or maleate is very low. No activity was measured when glutamine was replaced by asparagine in both phosphate dependent and independent glutaminase assay media (results not shown); this indicates that the glutaminase activities measured were not part of a general deamidation activity.

5.4.3 Some properties of macrophage glutaminase

The K_m for macrophage glutaminase was found to be 4.8 mM, which is similar to the K_m values for the enzyme from other cell types and tissues (Table 5.9).

The concentration of phosphate required for half-maximal activity of glutaminase was found to be 17 mM. Rat lymphocyte glutaminase can be activated by compounds other than phosphate including phosphoenolpyruvate, leucine, sulphate, ATP, CTP, GTP and ITP (Ardawi and Newsholme, 1984b). It is likely that some or all of the compounds will activate macrophage glutaminase, but they were not tested in the present work. The mechanism for phosphate activation has been described for the enzyme from a number of tissues. Phosphate is essential for polymerisation of kidney glutaminase, and the extent of polymerisation is correlated with activation of the enzyme (Godfrey et al., 1977). Chui & Bocker (1979) have shown that phosphate increases the V_{max} for bovine brain glutaminase and decreases its K_m for glutamine. Phosphate also decreases the K_m for the rat kidney enzyme (Shapiro et al., 1982).

Glutamate is known to be a potent inhibitor of glutaminase from brain and kidney (Krebs, 1935; Sayre and Roberts, 1958; Goldstein, 1966; Svenneby, 1970; Crompton et al., 1973; Shapiro et al., 1982) from spleen (Ardawi, 1983) mesenteric lymph nodes (Ardawi and Newsholme, 1984b). The potency of inhibition by glutamate varies considerably according to the tissue (see table 5.11) but for macrophage glutaminase, glutamate is a poor inhibitor (table 5.10). Other inhibitors of glutaminase have been described: Ardawi and

Table 5.11. Inhibition of glutaminase from different sources by glutamate

Data are obtained from (a) Ardawi & Newsholme (1984); (b) Ardawi (1983); (c) Goldstein (1966); (d) Chui & Backer (1979); (e) present work.

Tissue or cell	Concentration of glutamate that causes 50% inhibition (mM)
Rat mesenteric lymph nodes	0.1[a]
Bovine spleen	0.1-0.5[b]
Rat kidney	2.0[c]
Bovine brain	19.0[d]
Murine macrophage	20.0[e]

Newsholme (1984b) have shown that 2-oxoglutarate, glutamate, citrate or ammonium chloride (at 2 mM) inhibit lymphocyte glutaminase by 97, 72, 35 and 32% respectively; Goldstein (1966) and Ardawi (1983) have shown that, of these compounds, only glutamate causes marked inhibition (at 2 mM) of glutaminase from rat kidney or bovine spleen. A similarly poor response was observed for the macrophage enzyme (Table 5.10). Goldstein concludes that the most effective inhibitors of glutaminase are four or five-carbon dicarboxylic acids with an amino, hydroxyl or carbonyl group on the α-carbon e.g. glutamate, 2-oxoglutarate, malate or oxaloacetate. Although this may also be the case for macrophage glutaminase, high concentrations of glutamate or 2-oxoglutarate are required to cause significant inhibition. In this respect macrophage glutaminase shows a similarity to the liver enzyme (see review by Lund, 1980).

5.4.4 Intracellular distribution of some enzymes of the pathway utilising glutamine in the macrophage

On the basis of the enzyme-distribution study reported in tables 5.5 and 5.6, it is suggested that, of the glutamine that is transported into the mitochondria, it will be deamidated to form glutamate which will be transaminated or deaminated to form 2-oxoglutarate which will be metabolised via the enzymes of the TCA cycle to malate. It is likely that further metabolism of this malate will proceed in the cytosol due to mitochondrial transport of malate. In the cytosol, malate is likely to be converted to oxaloacetate, to phosphoenolpyruvate and then to pyruvate. The latter may be converted to lactate or alanine; alternatively it may be transported into the

mitochondria where it will be converted to acetyl CoA and then completely oxidised by the TCA cycle; in addition, pyruvate, within the mitochondria, may be carboxylated to form oxaloacetate catalysed by pyruvate carboxylase. Evidence was provided in chapter 3 to suggest that very little if any of the pyruvate derived from glutamine is oxidised in short-term incubations (i.e. 60 min.). However, this may not be the case for cells in culture (see table 6.3). Since phosphate-dependent glutaminase has a non-mitochondrial and a mitochondrial location in the macrophate, some glutamate may be produced in the cytosol and this could either be transported directly into the mitochondria or may undergo transamination with pyruvate to produce alanine and 2-oxoglutarate, catalysed by alanine aminotransferase in the cytosol. There is some evidence that alanine aminotransferase is a cytosolic enzyme in the macrophage (see chapter 4). The metabolic fates of glutamine in the macrophage are summarised in Fig 5.6.

The intracellular concentration of glutamine in the macrophage after 2 h in culture is 7.6 mM (A.H. Gordon, personal communication) which was 3.8 fold greater than the medium concentration at the time of sampling (Table 5.3) which suggests that glutamine is actively transported into the macrophage. A concentration ratio of 10 has been reported for liver (Windmueller, 1984) and Ardawi & Newsholme (1986) have suggested that cellular transport of glutamine into the lymphocyte is active. The concentrations of glutamine and glutamate decreased in the macrophage after 24 h of culture, that of glutamate falling well below 10 mM (A.H. Gordon, personal communication), a concentration which causes little inhibition of glutaminase (Table

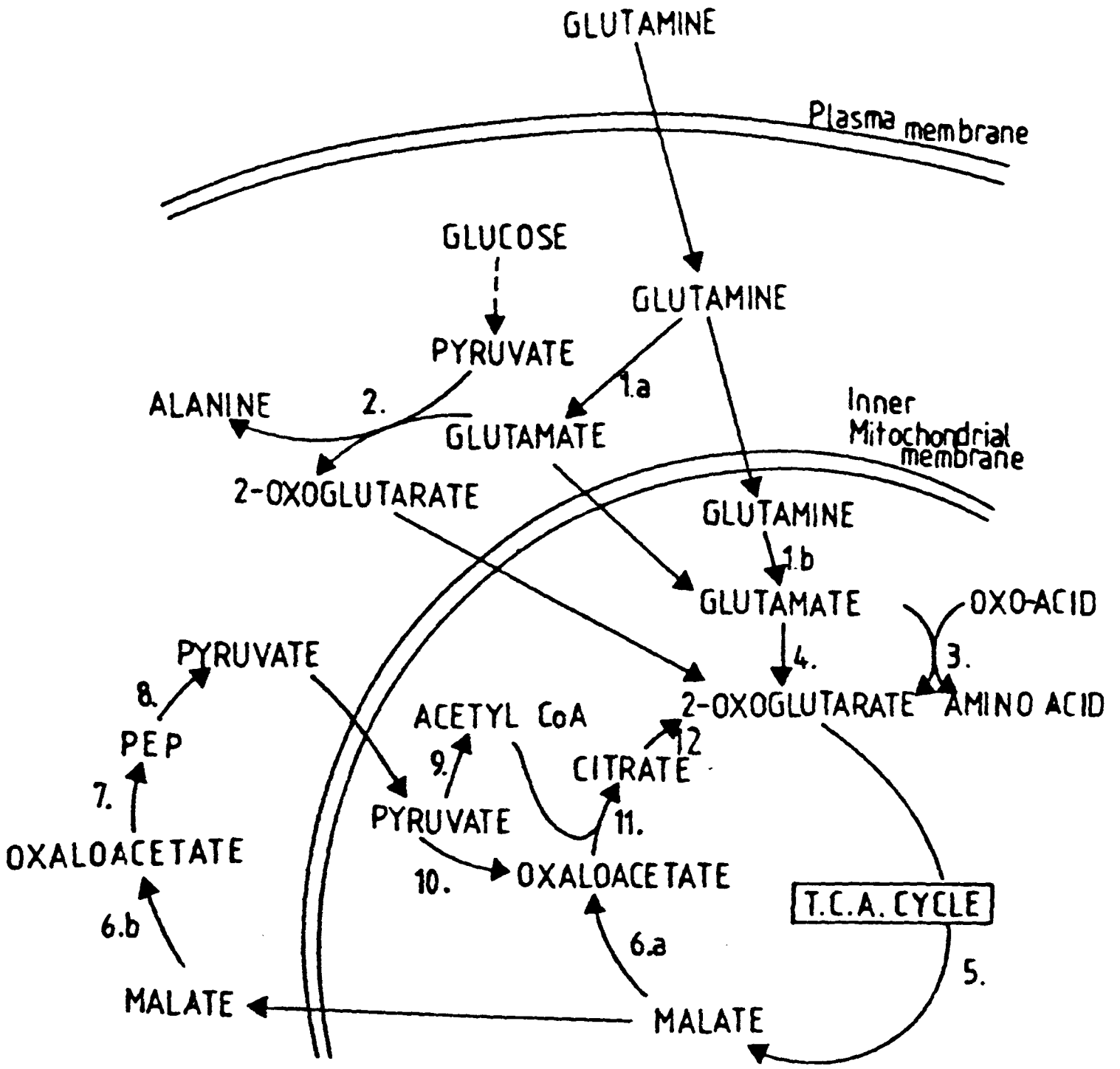


Figure 5.6 The pathway of glutamine metabolism in the murine macrophage.

Non-mitochondrial glutaminase (1.a); mitochondrial glutaminase (1.b); alanine aminotransferase (2); amino acid aminotransferase (e.g. aspartate aminotransferase) (3); glutamate dehydrogenase (4) T.C.A. cycle enzymes: oxoglutarate dehydrogenase, succinyl-CoA synthetase, succinate dehydrogenase, fumarase (5); malate dehydrogenase (NAD^+-linked) mitochondrial (6.a); malate dehydrogenase (NAD^+-linked) cytosolic (6.b); PEPCK (7); pyruvate kinase (8); pyruvate dehydrogenase (9); pyruvate carboxylase (10); citrate synthase (11); T.C.A. cycle enzymes: aconitase, isocitrate dehydrogenase (NAD^+ and $NADP^+$-linked) (12).

5.10). Thus although the intracellular concentration of glutamate in the macrophage appears to be high enough to inhibit glutaminase, by perhaps 50% (Table 5.3), after a short exposure to fresh medium (which contains a high concentration of glutamine), the concentration of glutamate may fall rapidly to levels that would cause little inhibition of the enzyme.

Glutaminase plays an important role in both the utilisation and metabolism of glutamine in the macrophage. Glutamine is probably converted to glutamate in both the cytosolic and mitochondrial compartments of the macrophage, so that transport of this glutamate into the mitochondrion may limit the rate of metabolism of glutamine in the cytosol. However, the precise role of cytosolic glutaminase in the macrophage is unclear. It is possible that it acts as a means of trapping glutamine in the form of glutamate in the macrophage for subsequent use in the provision of energy and/or carbon and nitrogen for the process of purine and pyrimidine synthesis (see chapter 6 for further discussion). This would also be consistent with the poor inhibition of the macrophage glutaminase by glutamate, and this might be particularly the case for the cytosolic enzyme, but this possibility has not been examined in the present work.

CHAPTER 6

General Discussion

CHAPTER 6

General Discussion

This chapter is divided into two major sections. In the first, the types of fuel utilised and the integration of metabolism of these fuels by the macrophage, are discussed. In the second section, the physiological importance of high rates of utilisation of glucose and glutamine for the macrophage is discussed.

6.1.1. Fuel utilisation by tissues of the body

Fuels are required to provide energy for the energy-demanding processes of the cell. According to the conditions, various fuels (e.g. glucose, fatty acids, ketone bodies and amino acids) are available in the blood and are utilised at markedly different rates by different tissues. The fuels that are available arise either from dietary intake or endogenous fuel reserves. The daily dietary intake of an average man consists of about 220 g carbohydrates, 70 g protein and 60 g fat (Cahill and Owen, 1968). This satisfies the energy requirement of all the tissues of the body. In the fed state most tissues use glucose for energy provision. One important exception is the liver in which amino acid and short-chain fatty acid metabolism is important in the fed state, since these compounds are available and liver is the major site of their catabolism. Glucose serves as a fuel for many tissues and is an obligatory fuel for several tissues including the brain (except during prolonged starvation), erythrocytes, renal medulla and testes (see Krebs, 1972). Indeed, in this review by Krebs (1972), he also included the white blood cells. The work in this thesis, while confirming the use of this fuel by macrophages,

has provided evidence that this white cell can utilise fuels other than glucose.

The pattern of fuel utilisation by any tissue may change from one condition to another. For example, the fuels for the brain, skeletal muscle and liver are different in the prolonged fasted state: glucose is the sole fuel for the brain in the fed state but, during prolonged starvation (in man), ketone bodies become the predominant fuel (Owen et al., 1967, 1969). Skeletal muscle can use glucose, fatty acids and ketone bodies as fuels, depending on its metabolic activity and the physiological conditions of the animal; it also contains stored glycogen as a fuel reserve (see Newsholme and Leech, 1983). The liver, in the fed state, takes up both glucose and 3-carbon compounds such as lactate and converts them into either glycogen or fatty acids; the latter are converted into triacylglycerol and are released into the blood in the form of very-low-density-lipoproteins. During starvation the liver mobilizes glycogen for glucose release, synthesizes ketone bodies from fatty acids that have been mobilised from adipose tissue and converts lactate and other compounds into glucose (see Cahill, 1976; Newsholme, 1976). In adipose tissue, triacylglycerol is continually broken down to glycerol and fatty acids by the action of a triglyceride lipase, the glycerol is released and is eventually taken up by the liver for gluconeogenesis; the fatty acids may be re-esterified with glycerol-3-phosphate derived from glucose in the adipocyte, especially in the fed state; but during conditions such as starvation and stress, fatty acids are mobilised and released into the circulation (see Hales et al., 1978).

During prolonged starvation, the concentration of glucose in the circulation decreases by not more than 25%, whereas those of fatty acids and ketone bodies increase up to 5- and perhaps 300-fold respectively (Owen et al., 1969; Aoki et al., 1971). This increase in concentration of fatty acids and ketone bodies in the bloodstream is of considerable physiological significance since this is a signal for an increase in their rate of oxidation by a number of tissues including heart, skeletal muscle, brain and kidney cortex, and this increase in fatty acid oxidation decreases the rate of glucose utilisation (see Cahill, 1976; Newsholme, 1976). This effect is part of the glucose/fatty acid/ketone body cycle (see Newsholme and Leech, 1983) for maintenance of the plasma glucose level and conservation of carbohydrate. This above discussion is necessary to explain the importance of the findings concerning macrophage fuel metabolism.

6.1.2. Fuels utilised by macrophages

In part of the work presented in this thesis, evidence was presented to show that glucose, glutamine and fatty acids all play a role in the supply of energy for macrophages. The rates of utilisation of these fuels and the resulting rates of ATP generation (and utilisation) in the macrophage (Table 4.16) suggest this cell is extremely metabolically active, e.g. the ATP production rate calculated for the macrophage in optimal culture conditions is approximately 25% of that of the working heart (of the rat). The oxygen requirements of the macrophage exposed to a variety of exogenous fuels can be calculated using the information provided in Tables 4.12., 4.13a and 4.15, which also indicate the proportion of

each fuel that is oxidised to CO_2. These calculated oxygen consumption data are given in Table 6.1, and may be compared to the measured oxygen consumption rates reported by Karnovsky et al. (1975) and given in Table 6.2. There is close agreement between these two values. Furthermore, the rate of oxygen consumption by macrophages can be compared to the oxygen requirement reported for a number of different tissues by Krebs and Johnson (1948) (see Table 6.2). In agreement with the conclusion reached above, based on fuel consumption rates, it is found that the rate of oxygen consumption by macrophages is similar to tissues such as heart or kidney, and both these tissues have large energy demands (see Table 6.2). Although some of the oxygen consumed by macrophages is undoubtably due to the production of reactive metabolites of oxygen (e.g. O_2-, H_2O_2), the proportion required for mitochondrial respiration is much greater (approximately 95% in non-phagocytosing, and 80% in phagosytosing elicited macrophages; see Karnovsky et al., 1970). Thus the macrophage (or at least the elicited macrophage) must be classified as a cell with an extremely high demand for energy: this suggests that the original view that such cells rely solely on the conversion of glucose to lactate (or even on phosphocreatine) for ATP formation (Krebs, 1972) cannot any longer be tenable.

How does the macrophage satisfy this demand for energy? In Chapter 4 evidence was provided that fatty acids and glutamine could be oxidised by the macrophage, while glucose was utilised at a very high rate, but very little was oxidised. These points are further discussed below.

TABLE 6.1 Calculated oxygen consumption rates of elicited peritoneal macrophages utilising a combination of different fuels.

Fuels Present in medium	rate of utilisation (nMol/h per mg protein)	rate of oxidation (nMol/h per mg protein)	O_2 required (nMol/h per mg protein)	Total O_2 required (nMol/h per mg protein)
Glucose	610	29.3	176	559
Glutamine	52	38.3	230	
Oleate	25	5.9	153	
Glucose	598	25.1	151	386
Glutamine	55	39.2	235	
Glucose	506	39.5	237	471
Oleate	20	9.0	234	
Glutamine	87	64.4	386	607
Oleate	13	8.5	221	
Glutamine	92	54.2	325	325
Oleate	19	10.5	273	273

Macrophages were cultured for 82 h, after which time fuel utilisation was measured. The proportion of each fuel oxidised to CO_2 is given in Tables 4.12, 4.13a and 4.15. It is assumed that the complete oxidation of 1 mol of glucose will require 6 mols of O_2, 1 mol of glutamine will require 6 mols of O_2 and 1 mol of oleate will require 26 mols of O_2. Glucose was present at an initial concentration of 5 mM, glutamine 2 mM and oleate 0.3 mM.

TABLE 6.2 Oxygen consumption of some mammalian tissue preparations

Species and tissue	medium	substrate(s) present	O_2 consumption n^2Mol/h per mg dry wt
Rat, liver (sliced)	bicarbonate-saline	glucose (11mM)	520
Rat, liver (sliced)	phosphate-saline	none	549
Rat, kidney (sliced, cortex)	bicarbonate-saline	glucose (11mM)	1085
Rat, kidney (sliced, cortex)	phosphate-saline	glucose (4.4mM)	1355
Rat, kidney (sliced, cortex)	bicarbonate-saline	none	938
Rabbit, peritoneal exudate	citrate-bicarbonate-saline	glucose (11mM)	219
Rat, diaphram (intact)	bicarbonate-saline	none	304
Sheep, heart (minced)	phosphate-saline	none	696
Rat, adipose tissue	phosphate-saline	none	48
Rat, abdominal fat	bicarbonate-saline	glucose (11mM)	4
Mouse, caseinate elicited macrophages (resting)*	Eagle's minimal essential medium plus glutamine	glucose (5mM) plus glutamine (2mM)	515
Mouse, Caseinate elicited Macrophages (phagocytosing)*	Eagle's minimal essential medium plus glutamine	glucose (5mM) plus glutamine (2mM)	1054

Data was obtained from Krebs and Johnson (1948) and is converted to the units of nmol/hr/mg dry wt. assuming 1 µmol O_2 occupies a volume of 22.4 µl.

*These values were calculated assuming 1 µmol O_2 occupies a volume of 22.4 µl and 1 mg macrophage protein = 1 mg dry wt. and were obtained from Karnovsky et al. (1975).

6.1.3. The integration of macrophage fuel metabolism

Glucose is utilised at a markedly high rate by the macrophage, and it is almost stoichiometrically converted to lactate (Table 4.12). This is in contrast to 'normal' tissues that are described above (e.g. kidney, muscle, brain). It has been proposed, for tumours, that the large accumulation of lactate observed during glycolysis (in aerobic conditions) may be due to a deficiency of substrates for the mitochondrial NADH-shuttle systems (e.g. malate/aspartate shuttle) in which NADH produced in the glycolytic pathway by the glyceraldehyde-phosphate dehydrogenase reaction is oxidised in the mitochondria (see Kovacevik and McGivan, 1983). However, this is unlikely to be the case for macrophages since glutamine metabolism will provide the necessary substrates for the shuttle, and the proportion of glucose metabolised to lactate is not significantly different in the presence or absence of glutamine (Table 4.12). The possibility that the mitochondrial membrane carriers involved in this shuttle system in the macrophage are deficient in either number or activity cannot be ruled out, and further work in this area is required. Nonetheless it seems that the production of cytosolic NADH in the macrophage is utilised perhaps exclusively in the lactate dehydrogenase reaction, so that pyruvate conversion to lactate is favoured by the 'availability' of NADH. The low rate of oxidation of pyruvate derived from glucose may also be due to effective competition for pyruvate due to the high activity of lactate dehydrogenase; the activity of the latter is more than 200-fold higher than that of pyruvate dehydrogenase (Table 2.1).

The rates of C_2/C_3 unit (derived from the fuels glucose, glutamine and oleate, e.g. C_3 represents pyruvate derived from either glucose or glutamine, C_2 represents acetyl CoA derived from oleate) oxidation by the TCA cycle in the macrophage are given in Table 6.3. It is interesting to note that in culture approximately all the pyruvate derived from glutamine metabolism is oxidised by the TCA cycle, whereas the ratio of pyruvate generated/pyruvate oxidised is approximately 20 for pyruvate derived from glucose. This suggests that pyruvate derived from glutamine is preferentially oxidised by the TCA cycle. In Chapter 5 it was suggested that pyruvate derived from glutamine is generated in the cytosol, in which case the cell would need to be able to differentiate between pyruvate derived from glutamine and pyruvate derived from glucose. This difficult problem would be avoided if pyruvate derived from glutamine was generated in the mitochondrion: this could be achieved, for example, by the presence of an enzyme such as oxaloacetate decarboxylase. In this case, mitochondrially derived pyruvate would not be exposed to lactate dehydrogenase (unless it were transported out of the mitochondria) and metabolism via pyruvate dehydrogenase would be favoured. There are, however, no measurements of the activity of oxaloacetate decarboxylase in macrophage mitochondria.

The data given in Table 6.3 indicate that the rate of pyruvate oxidation (derived from both glucose and glutamine utilisation) is approximately 100 nmol/h per mg protein if the cells are cultured in the presence of both glucose and glutamine. The observed maximal rate of pyruvate oxidation reported in Chapter 3,

TABLE 6.3 Rate of C_2/C_3 unit oxidation by the TCA cycle when combinations of different fuels are being utilised.

The rate of C_2/C_3 unit oxidation by the TCA cycle (i.e. acetyl units derived from the fatty acid oxidation pathway, pyruvate derived from the pathway of oxidation of glucose or glutamine) was calculated using the data concerning the amount of each fuel that is fully oxidised (see Chapter 4) and assuming that glucose and oleate are metabolised according to the pathways described in Chapter 1, and glutamine is metabolised according to the pathway described in Chapter 5.

Glucose was present at an initial concentration of 5 mM, glutamine 2 mM and oleate 0.3 mM.
*This rate was calculated from the data presented in Chapter 3 concerning pyruvate utilisation and oxidation. The oxidation rate presented in this Table, however, is extrapolated (to 60 min) from the maximal rate observed up to 30 min of incubation.

Fuels present in medium	Rate of utilisation (nmol/h per mg protein)	Rate of C_2/C_3 unit oxidation by TCA cycle (nmol/h per mg protein	Total rate of C_2/C_3 unit oxidation
Glucose	610	59	152
Glutamine	52	39	
Oleate	25	54	
Glucose	598	50	90
Glutamine	55	40	
Glucose	506	79	159
Oleate	20	80	
Glutamine	87	65	144
Oleate	13	79	
Glutamine	92	54	54
Oleate	19	93	93
Pyruvate*	73	84	84

if extrapolated to 60 min, would onl y be slightly less than 100 nmol/h per mg protein (Table 6.3). As the rate of oxidation of pyruvate derived from glucose or glutamine is increased in the absence of one of these fuels (Table 6.3) then this suggests that either the pyruvate dehydrogenase capacity for oxidation is saturated at approximately 100 nmol pyruvate oxidised/h per mg protein or (if pyruvate derived from both these fuels is generated in the cytosol) that mitochondrial pyruvate transport is rate-limiting for pyruvate oxidation. The maximal rate of pyruvate dehydrogenase, calculated from the activity given in Table 2.1, at 37°C is approximately 460 nmol/h per mg protein. However, the concentrations of allosteric effectors regulating pyruvate dehydrogenase activity may never reach optimal values *in vivo*, thus the maximal activity given above may never occur *in vivo*.

During starvation the concentration of fatty acids in the blood increase approximately 5-fold (see Newsholme and Leech, 1983). The increase in blood concentration of fatty acids (due to hydrolysis of triacylglycerol within white adipose tissue) is normally a signal for their oxidation by tissues such as kidney, heart and aerobic skeletal muscle. It has been reported by Lokesh and Wrann (1984) that the uptake of the long-chain fatty acid oleate (which represents 43% of all long-chain fatty acids found in the blood of man, - Havel *et al*., 1964) by murine macrophages, is saturated at approximately 0.2 mM. This concentration is below that found in the fed state of most animals (Newsholme and Leech, 1983). Thus long-chain fatty acids may be utilised by macrophages *in vivo*, even in the fed state. In starvation, the concentrations of glucose

and glutamine in the blood remain relatively stable (see Newsholme and Leech, 1983), thus the macrophage should utilise glucose, glutamine and long-chain fatty acids at approximately the same rates as observed in the fed state of the animal, even if the animal is subjected to prolonged starvation. That is, the changes in the blood fuel levels known to occur in the starved state (see above) should make no difference to the fuels used by the macrophage. Most tissues (except those with an absolute requirement for glucose) will oxidise fatty acids or ketone bodies during prolonged starvation, and the oxidation of these compounds leads to the inhibition of glucose utilisation and oxidation via a well established control mechanism, known as the glucose/fatty acid/ketone body cycle (see Newsholme and Leech, 1983). However, the rate of utilisation of glucose, and the rate of utilisation of glutamine by macrophages was unaffected by the addition of oleate to the culture medium (Figures 4.2 and 4.5). This lack of effect of oleate and the lack of a change in fuel utilisation during conditions such as starvation suggest that the high rates of utilisation of glucose and glutamine by macrophages may occur not only for the generation of energy but for other purposes, that is, they cannot be replaced by other fuels. This other function may be related to the role of the macrophage in the immune response. Thus, the maintenance of the high rates of glucose and glutamine utilisation, even during prolonged starvation, would be justified if the metabolism of these compounds was essential to the full expression of the functional activity of the macrophage, for example the activity in response to an immunological challenge. This consideration has led to a search for a specific

role for high rates of glucose and glutamine utilisation in the macrophage: one possible answer is discussed in Section 6.2.

There are two major fates for utilised long-chain fatty acids in the macrophage; these and their importance to the macrophage are discussed below:

(i) Oleate is oxidised by the macrophage. Oleate makes a contribution of approximately 30% to the acetyl-CoA production for oxidation, when glucose, glutamine and oleate are present in the culture medium (Table 6.3). Thus oleate makes a significant contribution to the energy requirement of the macrophage (Table 4.16). However, in the presence of both glucose and glutamine (when the highest utilisation rate of oleate was observed) the major fate of the oleate that is utilised by the macrophage is incorporation into cellular lipids (Table 4.15). (Nonetheless, that which is oxidised can produce a significant rate of ATP formation.) This is further discussed below.

(ii) Utilised oleate can be incorporated into cellular triacylglycerol and phospholipid (Table 4.7). Incorporation into phospholipid may occur due to new plasma membrane synthesis; thus even at their usual unstimulated pinocytic rate, murine macrophages interiorise an area of plasma membrane equivalent to their total surface area every 35 min due to formation of pinocytic vesicles (Steinman et al., 1976). Evidence that new plasma membrane synthesis occurs after endocytosis is discussed in Section 6.2.

The role of endogenous triacylglycerol synthesis in the macrophage may be 3-fold:

1. To act as an energy store to be used when the exogenous fatty

acid concentration is low or if other fuels are unavailable.

2. To buffer intracellular fatty acid concentrations especially when high intracellular concentrations occur, [high concentrations of fatty acids can have detergent effects e.g. they can inhibit enzymes non-specifically and uncouple oxidative phosphorylation (see Newsholme and Leech, 1983].

3. Murine macrophages have been found to release fatty acids from triacylglycerol into the medium (Chapter 4, discussion; Von Hodenburg et al., 1984). If these fatty acids are released in response to bacterial invasion this may be important, long chain fatty acids have been reported to possess antibacterial activity (Freese et al., 1873; Fay and Farias, 1975).

The rates of utilisation of glutamine and oleate are markedly lower than that of glucose (Table 6.3) but, since the rates of glutamine and oleate oxidation are higher than that of glucose, the utilisation of these two fuels satisfy approximately 50% of the energy requirement of the macrophage (Table 4.16), the question arises as to why glucose is not oxidised at a higher rate, which would not only reduce the requirement for glutamine and oleate oxidation, but also reduce the requirement for glucose. This question is the same as that raised above. The importance of the high rates of glucose and glutamine utilisation by the macrophage are discussed in the following Section.

6.2. Physiological importance of high rates of glycolysis and glutaminolysis in macrophages

It has been many years since Warburg's original observation that tumour cells converted glucose to lactate at a very

high rate (Warburg, 1926; also see Warburg, 1956a,b); rapidly growing ascites tumour cells converted an amount of glucose equal to 30% of their dry weight to lactate per hour. This was found to be characteristic of other rapidly-dividing cells, e.g. absorptive cells of the small intestine (Hanson and Parsons, 1977), cells of the chorion during the first few days of embryonic development (see Warburg, 1956b) and in resting and stimulated lymphocytes (Roos and Loos, 1973). Also lymphocytes can oxidise other fuels such as glutamine, fatty acids and ketone bodies (Ardawi and Newsholme, 1983, 1984). The significance of the high rate of glucose utilisation in rapidly-dividing cells has received much attention, and several proposals have been put forward: (i) it provides precursors for a number of macromolecules that are important during cell division, e.g. glucose-6-phosphate is the substrate for the pentose phosphate pathway that provides reducing power and ribose for RNA and DNA synthesis, and glycerol phosphate is essential for the formation of phospholipids for membranes. Hence it has been suggested that a high glycolytic flux is necessary to provide precursors at a sufficient rate for the biosynthetic processes. However, it seems that the rate of glycolysis is well in excess of the rate of use of its intermediates for biosynthesis. Thus, there was no difference in the rate of cell proliferation when human diploid fibroblasts were cultured in the presence of 5 mM glucose, or in the presence of a very low concentration of glucose - but with a small daily addition (70 nmoles per 5.5 ml medium) of glucose (Zielke et al., 1978). Also, some transformed cell lines have been shown to exhibit normal or subnormal rates of glucose uptake (Molten

et al., 1977; (ii) the high rate of glycolysis may be necessary to maintain high concentrations of glycolytic intermediates to satisfy the demand for biosynthesis. However, the concentrations of glycolytic intermediates do not appear to be very different in tumour cells from normal cells (see Williamson et al., 1970) and this is also the case for concanavalin-A stimulated lymphocytes in comparison to non-stimulated lymphocytes (see Ardawi, 1983); (iii) the genetic changes that are responsible for malignancy may also coincidently increase the glycolytic capacity (see Newsholme and Leech, 1983): this will be extremely difficult to test until the precise understanding of the role of genes in tumour development is more advanced.

The work of Ardawi & Newsholme (1985) on lymphocytes and the work presented in this thesis show that this high flux is not unique to glycolysis. High rates of glutaminolysis [a term applied to the utilisation of glutamine via a pathway that does not result in total oxidation (McKeehan, 1982)] characterise many cells that also exhibit high rates of glycolysis; proliferating cells in culture (Zielke et al., 1978; Donnelly and Scheffler, 1976); Stoner and Merchant, 1972) tumour cells (see Kovacevic and McGivan, 1983) and cells with proliferative potential e.g. lymphocytes (Ardawi and Newsholme, 1983) and enterocytes (Watford et al., 1979). As not all glutamine is completely oxidised by these cells (McKeehan, 1982; Zielke et al., 1984) the pathway has some similarity to glycolysis and has been termed glutaminolysis (McKeehan, 1982). Also in a similar manner to glycolysis, glutaminolysis will provide precursors for biosynthetic processes [both glutamine and aspartate (a product

of glutamine metabolism) will provide nitrogen for purine and pyrimidine synthesis, and aspartate will provide carbon for pyrimidine syntheis]. The maximum rate of precursor utilisation for the provision of precursors for RNA and DNA synthesis may be <1% of the rate of glutaminolysis in lymphocytes (Ardawi, 1983). It has been suggested that the role for glutamine utilisation may be energy provision. This has been the explanation for high rates of glutamine utilisation by tumour cells (see Kovacevic and McGivan, 1983) and enterocytes (Watford et al., 1979). However, if the role of glutamine utilisation and oxidation was solely to provide energy, the question may be raised as to why the rate of fatty acid or keton e body oxidation could not be increased to provide more energy and decrease the demand for glutamine or why more of the glucose that is converted to lactate could not be oxidised (see Ardawi and Newsholme, 1985)

The above question may also be applied to the macrophage. This cell also displays high rates of glucose and glutamine utilisation and as described in Section 6.1.3 can oxidise fatty acids, but the maximal capacity of fatty acid oxidation (Table 2.9) is never observed in the macrophage. Thus glutamine utilisation appears to be important for the macrophage, and this is reflected in the high utilisation rates observed for this cell (Table 5.2). The macrophage possesses the characteristics of high rates of glucose and glutamine utilisation as described for proliferating cells or cells with proliferative potential. Two further points should be made concerning these pathways, which suggest that it is a high rate that is important. For glycolysis,

there is no control over the rate of glycolysis by fatty acid oxidation as there is in other cells (e.g. skeletal muscle, heart - see above). Thus despite the cells hav ing a high oxidative capacity - they maintain a high rate of glycolysis and exhibit no inhibitory response to fatty acids. For glutaminolysis, the rate of the utilisation of glutamine by macrophages during incubation is similar to that of macrophages in culture - despite the fact that in culture much more of the carbon of the glutamine is oxidised. Although the precise biochemical reason for this difference is not known, and it may be related to time of experimentation (e.g. 60 min compared to 80-100 hours), the finding that, in two distinct conditions of metabolism, the rates of glutamine utilisation are the same suggests that the rate is functionally important.

One important point for the significance of high rates of glycolysis and glutaminolysis in these cells is that, unlike the above cells, the macrophage is a terminally differentiated end-cell with little ability to proliferate (for review see Gordon, 1986). Why then should the macrophage disp lay high rates of glucose and glutamine utilisation? Any theory must take into account <u>both</u> types of cell. The macrophage is known to have an extremely diverse secretory capacity (Table 1.3; Takemura and Werb, 1984) and the capacity is high: thus the secreted products can reach high local concentrations in the interstitial fluid when the cells are stimulated to produce particular product(s) (Gordon, 1986). This secretory activity is an important part of the function of these cells in the immune response. Furthermore, macrophages do not, in general, store large amounts of preformed secretory products in

granules, unlike other leukocytes, but respond to particular stimuli by new macromolecular synthesis or by generating active species such as prostaglandins from precursor pools in the plasma membrane. Thus proteins must be synthesised de novo prior to secretion, hence the secretory activity must be preceded by mRNA synthesis. Indeed the macrophage is known to possess a very high rate of protein synthesis (Hammer & Rannels, 1981). Rates of RNA and protein synthesis have been shown to be increased (approximately 2-fold) in the macrophage cell lines J774.1 and PU5-1.8 upon stimulation with immunoadjuvants (such as bacillus calmette-Guerin cell wall, lipopolysaccharide from E. Coli. and the synthetic N-acetyl-muramyl-L-alanyl-D-iso-glutamine; Prosser et al., 1984). During macrophage activation the secretory properties of the cell change dramatically (see Table 1.4), so that mRNA synthesis must be increased presumably utilising purine and pyrimidines that are formed from precursors that are derived from the pathway of glutaminolysis. To provide precursors precisely when required for RNA synthesis the rate of glutaminolysis must be high (see below). In addition, phagocytosis and pinocytosis require the interiorisation of a large amount of plasma membrane (see Steinman et al., 1983 for review), and Werb and Cohn (1972) have shown that phagocytosis is followed by the synthesis of new plasma membrane. Although there is evidence that proteins of the plasma membrane can be returned (re-cycled) to the plasma membrane following the fusion of phagosome and lysosome (Muller et al., 1980), the synthesis of new membrane phospholipids will require the precursor glycerol-3-phosphate which is derived from the glycolytic pathway. If the pathway of glycolysis was slow - it may not, at the

particular time required, be able to produce glycerol 3-phosphate at a sufficient rate for the phospholipid requirement.

The glycolytic pathway has another important role in the macrophage; it provides glucose-6-phosphate which is a substrate for glucose-6-phosphate dehydrogenase, the first enzyme in the pentose phosphate pathway. The importance of this pathway for the macrophage is at least four-fold: (i) it provides NADPH for the macrophage NADPH oxidase which is responsible for the production of O_2^- (see Rossi et al., 1985); (ii) it provides NADPH required for macromolecular synthesis; (iii) it provdes ribose for the synthesis of RNA; (iv) it provides NADPH for re-generation of reduced glutathione - necessary for repair of damage caused by oxygen radicles. Thus both the glucose and glutamine utilising pathways in the macrophage give rise to important intermediates which may be used as precursors for macromolecular synthesis, or, in the case of glucose, can be used to generate the NADPH required for O_2^- production. Furthermore, these intermediates may be required in large amounts but only at very precise times. Thus high rates of macromolecular synthesis or O_2^- production are not continually occurring in the macrophage, therefore why should high rates of gliucose and glutamine utilisation occur continouslsy? Since rapidly dividing cells, and those with proliferative potential, also have high rates of glucose and glutamine utilisation, is there a common feature that would explain these characteristics? Application of the recently developed quantitative theory of metabolic control to branched pathways (Crabtree and Newsholme, 1985) can provide an answer.

6.2.1. Theory of control of branched pathways and application to glycolysis and glutaminolysis

It can b e shown that if a metabolic flux, J, divides into two fluxes J_a and J_b, and J_a is regulated by a factor X, the highest sensitivity of flux J_a to changes in the regulator X is achieved when the fluxes J and $J_b \gg J_a$ (see Figure 6.1). In non-mathematical terms high sensitivity is achieved because the rate of the biosynthetic pathways (that is J_a) can be increased markedly without decreasing significantly the concentration of the metabolic intermediate(s) of the main pathway (B) which would "oppose" the stimulation of the biosynthetic pathway. This principle is relevant to the discussion of glycolysis and glutaminolysis in the macrophage, rapidly dividing cells, and cells with proliferative potential. Both pathways exhibit high rates and both pathways provide precursors for biosynthetic pathways, but the fluxes through these biosynthetic pathways are normally very small in comparison to the fluxes through glycolysis and glutaminolysis (Fig. 6.2). However, the flux through the biosynthetic pathways will need to increase considerably during the periods when the rate of synthesis of mRNA is increased, and similarly when the rate of formation of phospholipd and species such as O_2- is increased. Since the timing of the synthesis of these macromolecules and O_2- in relation to macrophage stimulation (e.g. secretory activity and/or endocytosis) will be very important for the function of these cells (and possibly vital for survival of the organism) the sensitivity of the biosynthetic processes to their specific regulators would be expected to be very high.

One further component of the problem should also be

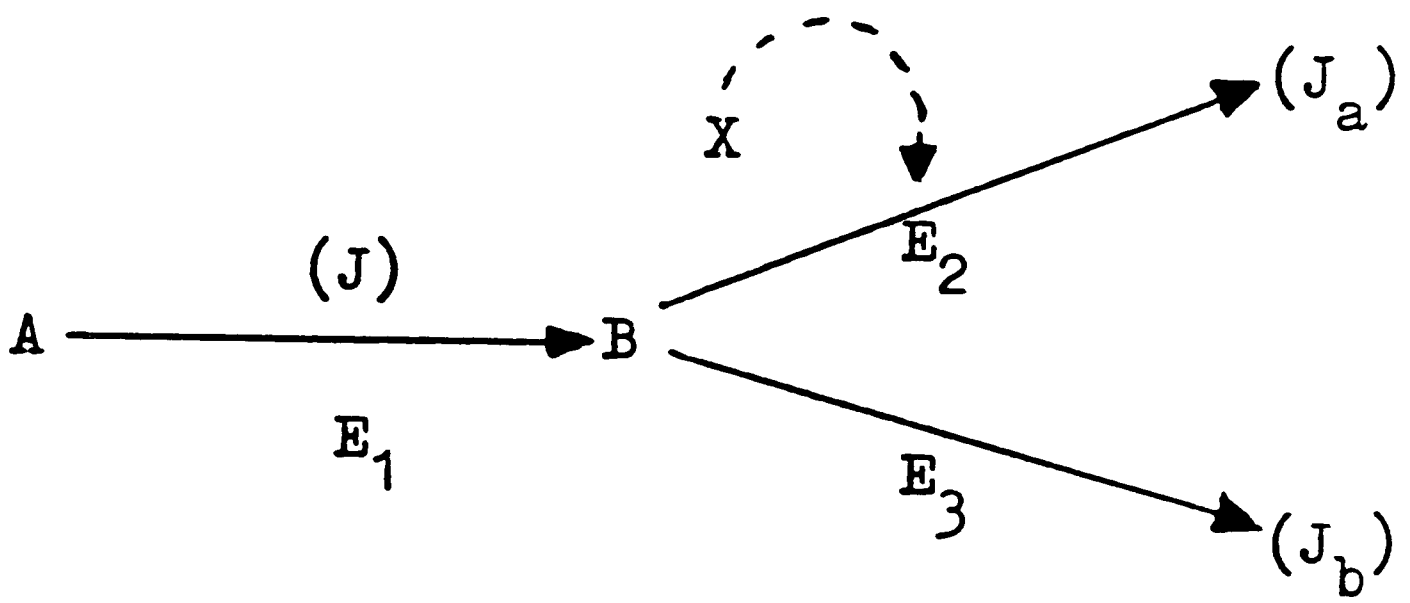


Figure 6.1 Regulatory analysis of branched pathways

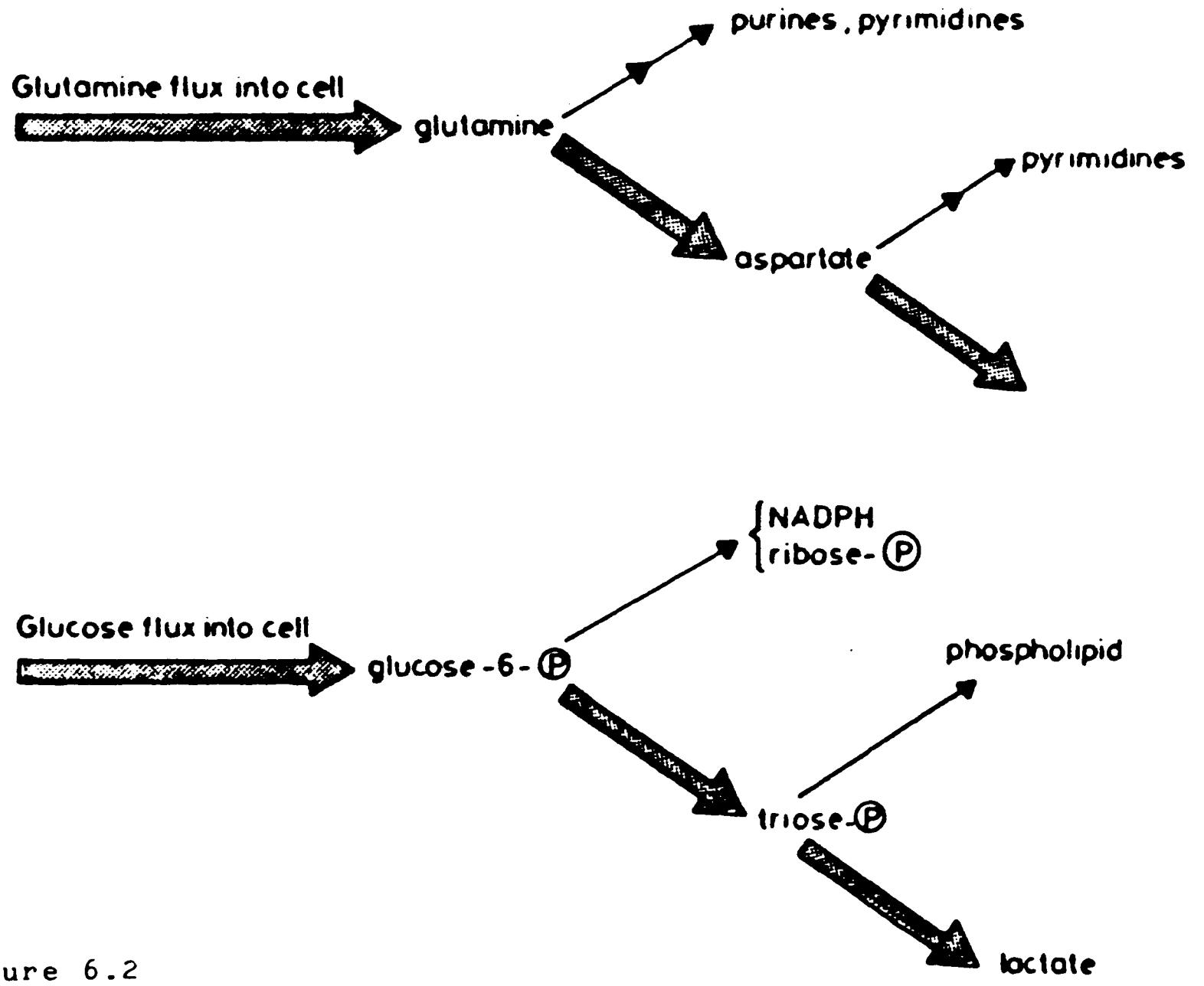


Figure 6.2

The oxidative and biosynthetic branches of the glutaminolytic and glycolytic pathways in macrophages and rapidly dividing cells. For glutaminolysis, glutamine provides nitrogen for both the purine and pyrimidine rings and aspartate provides both nitrogen and carbon for the pyrimidine ring (see Newsholme & Leech). For glycolysis, glucose 6-phosphate is the substrate for the pentose phosphate pathway which provides both ribose and NADPH and triose phosphate provides glycerol 3-phosphate which is a component of phospholipids in membranes.

considered; that is the source of the glutamine for use by macrophages, rapidly dividing cells, and cells with proliferative potential. The major source is skeletal muscle (see Chapter 1) and the source of glucose, especially during injury or sepsis, is the liver. The physiological significance of this is that, if the rate of glycolysis or glutaminolysis was not sufficient in these cells, a feedback information system would need to be established to link the cells of the immune system with the muscle and liver. A sensitive feedback system for macromolecular synthesis would be for the precursor (B, Figure 6.1) to "communicate" directly (e.g. by feedback inhibition) with the flux-generating step of the overall pathway (i.e. E_1, Figure 6.1) so that an increase in demand for macromolecular sysnthesis would be made by increasing total flux (J). However, for both pathways, glycolysis-from-glucose and glutaminolysis in macrophages, rapidly dividing cells and cells with proliferative potential, a flux-generating step will be in other tissues namely muscle and liver (see Newsholme and Leech, 1983 for theoretical considerations of a flux-generating step). Even if a sensitive feedback mechanism were developed to link the cells with the flux-generating step, it could only operate by providing a higher concentration of glutamine or glucose in the bloodstream and this would increase the rate of utilisation of these compounds by other tissues, and so interfere in the supply of these key substrates for the cells urgently requireing them. Furthermore it should be appreciated that, for example, the provision of glycerol 3-phosphate for phospholipid formation for macrophages in a wound would require the glycerol phosphate concentration within the

macrophage to communicate directly with the liver ! But such a complex (and potentially ineffective) direct feedback control mechanism is not required if cells maintain continuously high rates of glycolysis and glutaminolysis. Although at first sight this may appear wasteful, it provides a simple and effective control system that can permit macromolecular synthesis (and in the case of the macrophage, synthesis of O_2-) and hence maintain cell responsiveness to specific stimuli whenever required. For these cells such requirements are necessary for no less a role than survival of the organism.

6.3 Further aspects of macrophage glutamine metabolism

Some recent work has focussed attention on glutamine metabolism in λ-carrageenan wounded skeletal muscle (Albina et al., 1987). Wounded muscle is characterised by necrosis; rapid and extensive infiltration by inflammatory cells which, after an early phase of polymorphonuclear cell predominance, comprise mainly macrophages and fibroblasts (O'Connor et al., 1982); in addition, there is increased net skeletal muscle protein breakdown and alterations in the intracellular pattern of free amino acids (Albina et al., 1986). The most pronounced change in amino acid concentration is that of glutamine, which in wounded muscles shows a 50% decrease in the intracellular concentration five days after wounding (Albina et al., 1986). Glutamine metabolism in wounded muscle is characterised by the following: radiolabelled CO_2 and glutamate production from radiolabelled glutamine were approxim ately 100% greater in wounded compared with non-wounded muscle; radiolabelled CO_2 production from radiolabelled glutamine

was approximately the same in wounded muscle incubations as a reconstituted system of non-wounded muscle plus λ-carrageenan elicited peritoneal macrophages (at approximately the same number as found in a wounded muscle preparation); 6-diazo-5-oxo-L-norleucine (a potent glutaminase inhibitor) inhibited radiolabelled CO_2 production from radiolabelled glutamine approximately 90% in the muscle incubations (Albina et al., 1987). As glutamine synthetase has no appreciable activity in the macrophage (Albina et al., 1987), these results suggest that cells of the inflammatory infiltrate and especially macrophages are using glutamine at the site of the wound, and the source of this glutamine is muscle. These data are consistent, and indeed are predicted, by the findings presented in this thesis.

The macrophage, in isolation, will not make a large demand on the total glutamine supply, within the animal, due to the small number of macrophages compared to lymphocytes, fibroblasts and endothelial cells all of which utilise glutamine. However, during conditions such as sepsis, trauma, burns or surgery, all of these cells will increase in number and activity due to their roles in defence and repair, and hence the demand for glutamine will increase. It has been reported that the rate of release of glutamine by skeletal muscle is increased in conditions of sepsis, trauma, burns or surgery (Askanazi et al., 1980); Aulick and Wilmore, 1979; Lund and Williamson, 1985; Ardawi, 1987). Indeed the muscle wasting observed in severe cases of the above conditions is probably due to protein degradation in muscle, which will provide amino acids as a source of nitrogen for the synthesis of glutamine

(see Chapter 1). This glutamine can then be utilised by cells involved in cell division (lymphocytes, fibroblasts, endothelial cells, macrophage precursors), increased secretory activity (macrophages) and for ammonia formation in the kidney (as acidosis can occur in the conditions listed above). The work reported in this thesis has thus enabled an explanation for the negative nitrogen balance of injury, sepsis, burns and surgery to be put forward. The link between skeletal muscle metabolism and that of the cells involved in defence and repair, as described above, may be considered to be the provision of glutamine for the macrophage and other cells of the immune system and repair. This concept is fully supported by the results reported by Albina et al. (1987) and those described in this thesis.

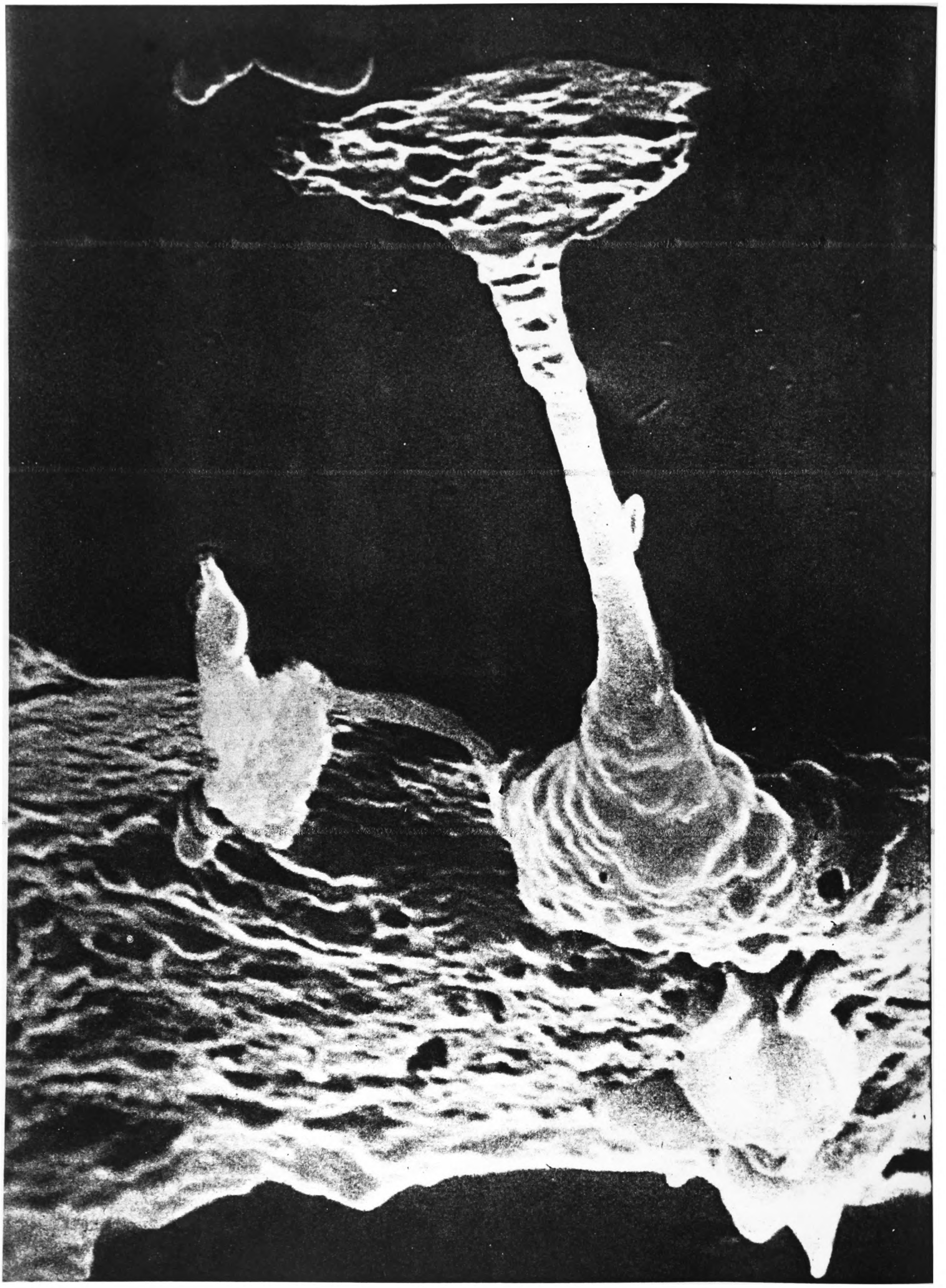
The macrophages of the body are essential for host defence and repair, and the work described in this thesis has shown that glutamine and glucose are utilised by these cells at high rates; it has been suggested by the author that the mcrophage has an absolute requirement for high rates of utilisation of these fuels so that it can display a full functional activity. Activation of macrophages (increase in size, increase in secretory activity etc. see Table 1.4) by the products of specifically stimulated T-lymphocytes e.g. γ-interferon and lymphokines, represents a common, non-specific antigenic response to infection by many important pathogens. The failure of macrophage activation contributes to the immuno-pathology of diseases such as AIDS and leprosy. The work presented in this thesis suggests that the provision of a satisfactory mixture of fuels could be essential to the functioning of the macrophage in

these conditions, and the failure of fuel supply could contribute to ill health and ultimately death during severe sepsis. Further work in this field, both in vitro and in vivo, is essential to provide a greater insight into the role in pathology of the complex metabolism of key cells of the immune system such as the macrophage.

A Macrophage

Cultured on a glass converslip (dark area), showing characteristics of an activated cell e.g. spreading out on the coverslip and ruffling of the membrane. The macrophage is extending a process towards a bacterial spore (bottom right hand corner).

[From National Geographic]



APPENDICES

APPENDIX A

Theoretical consideration underlying the use of maximal enzyme activities as indices of maximal fluxes through metabolic pathways

It has been established, both theoretically and experimentally, that reactions in a metabolic pathway can be divided into two types: those that are near-equilibrium and those that are far removed from equilibrium (non-equilibrium) (see Newsholme and Start, 1973; Crabtree and Taylor, 1978; Newsholme and Leech, 1983). This must not imply that there are some reactions in a pathway at equilibrium: if there is a net flux through a pathway, all the reactions of that pathway will be removed from equilibrium. However, there is a functional difference in that some reactions are very close to equilibrium and some are very far-removed from equilibrium. The usual method for differentiation between these two type of reactions is by comparison of the mass-action ratio (T) with the equilibrium cnstant (K_{eq}) for that reaction. The mass action ratio is the ratio [products]/[substrates] for a reaction, as measured in the living tissue under physiological conditions. If the value of (K_{eq}) is at least five-fold greater than the value of (T) the reaction is assumed to be non-equilibrium; if the difference is less than five-fold the reaction is assumed to be near equilibrium (see Newsholme and Crabtree, 1976).

It is also possible to differentiate between the two types of reactions kinetically. In a non-equilibrium reaction, the rate of the reverse component of the reaction is very much less than the rate of the forward component of the reaction, as the reaction proceeds in the living cell. Therefore, the rate in the forward direction, <u>in vivo</u>, is almost identical to the flux through the reaction in the cell. On the other hand, in a near-equilibrium reaction, the rate of the forward and reverse components of the reaction are very similar, but the rate of both components is very much larger than the overall flux (see Newsholme and Crabtree, 1979). This may be clarified by the following example. Consider two reactions converting substrate A into product B. The rates of the forward and reverse reactions are V_f and V_r respectively. In each case, the net flux is 10 units:

$$A \underset{V_r = 0.01}{\overset{V_f = 10.01}{\rightleftharpoons}} B \qquad (1)$$

$$A \underset{V_r = 90}{\overset{V_f = 100}{\rightleftharpoons}} B \qquad (2)$$

Reaction (2) is much closer to equilibrium than reaction (1) since the ratio of the rates of the forward and reverse reactions is closer to unity (although the flux

through either reaction is 10 units). In the cell under steady-state conditions, the rate of the forward and reverse reactions must conform to the net flux through the pathway. If the activity of an enzyme that catalyses a near-equilibrium reaction is measured uni-directionally in the forward direction *in vitro* at saturating substrate concentration(s) (i.e. the maximal activity of the enzyme is measured), the rate will be considerably higher than the maximum net flux through the pathway and hence through the reaction *in vivo*. However, if the activity of an enzyme that catalyses a non-equilibrium reaction is measured under similar conditions, the rate of the foward reaction may approximate to the maximum net flux through the pathway *in vivo*. Consequently, the maximum catalytic activity of an enzyme that catalyses a non-equilibrium reaction may provide quantitative information about the maximum rate of flux through that pathway in the living cell.

APPENDIX B

Assay of enzyme activities

All the enzymes were assayed by following the rate of change of absorbance at A_{340} except citrate synthase carnitine palmitoyltransferase and pyruvate carboxylase which were assayed by following the rate of change at A_{412}; 3-oxo-acid CoA transferase and acetoacetyl-CoA thiolase were assayed by following the rate of change at A_{303}; pyruvate dehydrogenase was assayed by following the rate of change at 460 nm. All spectrophotometric measurements were performed in a Gilford recording spectrophotometer (Model 240) and 25°C unless otherwise stated.

Hexokinase was assayed as described by Crabtree & Newsholme (1972). The assay medium consisted of 75 mM-Tris/HCl, 7.5 mM $MgCl_2$, 0.8 mM-EDTA, 1.5 mM-KCl, 4 mM-mercaptoethanol, 0.4 mM-$NADP^+$, 2.5 mM-ATP, 1 mM-glucose, 10 mM-creatine phosphate, 1.8 units creatine kinase, 1.4 units glucose 6-phosphate dehydrogenase, 0.05% (v/v) Triton-X-100, to which 10-15 µl of homogenate was added. The total assay volume was 1.0 ml at pH 7.5. The assay was initiated by the addition of glucose. Controls from which glucose was omitted were run concurrently.

6-Phosphofructokinase was assayed as described by Opie & Newsholme (1967). The assay medium consisted of 50 mM-Tris/HCl, 6.7 mM-$MgCl_2$, 200 mM-KCl, 1 mM-mercapto-ethanol, 1 mM-ATP, 2 mM-AMP, 0.2 mM-NAD^+, 5 µg antimycin A,

3 mM-fructose-6-phosphate, 0.16 units glycerol-3-phosphate dehydrogenase, 0.9 units aldolase, 9.6 units triosephosphate isomerase and 0.05% (v/v) Triton-X-100 to which 5-10 µl of homogenate was added. The total assay volume was 1.0 ml at pH 8.2. The assay was initiated by the addition of fructose-6-phosphate which was omitted from concurrently run controls.

Pyruvate kinase was assayed as described by Zammit et al. (1978). The assay medium consisted of 160 mM-triethanolamine/HCl, 10 mM-$MgCl_2$, 80 mM-KCl, 0.17 mM-NADH, 5 mM-ADP, 2 mM-PEP, 9 units lactate dehydrogenase and 0.05% (v/v) Triton-X-100 to which 5 µl of homogenate was added. The total assay volume was 1.0 ml at pH 7.35. The assay was initiated by the addition of PEP which was omitted from concurrently run controls.

Lactate dehydrogenase was assayed as described by Zammit & Newsholme (1976). The assay medium consisted of 60 mM-Tris/HCl, 0.17 mM-NADH 1.0 mM-pyruvate, 5 µg antimycin A and 0.05% (v/v) Triton-X-100 to which 5 µl of homogenate was added. The total assay volume was 1.0 ml at pH 7.5. The assay was initiated by the addition of pyruvate which was omitted from concurrently run controls.

Glucose-6-phosphate dehydrogenase was assayed as described by Bergmeyer et al. 1974). The assay medium consisted of 86 mM-Triethanolamine hydrochloride, 6.9 mM $MgCl_2$, 0.4 mM-$NADP^+$ 1.2 mM-glucose-6-phosphate, 0.05% (v/v) Triton-X-100 and 1.2 units of 6-phosphogluconate

dehydrogenase, to which 10-20 µl of homogenate was added. The total assay volume was 1.0 ml at pH 7.6. The assay was initiated by the addition of glucose-6-phosphate. Controls from which glucose-6-phosphate was omitted were run concurrentluy.

6-Phosphogluconate dehydrogenase was assayed as described for glucose-6-phosphate dehydrogenase except that glucose-6-phosphate and 6-phosphogluconate dehydrogenase were omitted, and 3 mM 6-phosphogluconate was included. The assay was initiated by the addition of 6-phosphogluconate. Controls from which 6-phosphogluconate was omitted were r un concurrently.

Phosphorylase was assayed as described by Bergmeyer et al. 1974). The assay medium consisted of 50 mM-phosphate buffer (equimolar mixture of K_2HPO_4/KH_2PO_4), 1 mM-EDTA, 0.34 mM-$NADP^+$, 8 µM-glucose-1,6-bisphosphate, 15 mM-$MgCl_2$, 1 mM-AMP, 1.4 units glucose-6-phosphate dehydrogenase, 1 unit phosphoglucomutase and 6 mg glycogen to which 30-50 µl supernatant was added. The total assay volume was 1.0 ml at pH 6.8. The assay was initiated by the addition of glycogen which was omitted from concurrently run controls.

Citrate synthase was assayed as described by Alp et al. (1976). The assay medium consisted of 50 mM-Tris/HCl, 1 mM-EDTA, 0.2 mM-DTNB, 0.1 mM-acetyl-CoA, 0.5 mM-oxaloacetate and 0.05% (v/v) Triton-X-100 to which 5 µl of homogenate was added. The total assay volume was 1.0 ml at pH 8.1. The assay was initiated by the addition of

oxaloacetate which was omitted from concurrently run controls.

Oxoglutarate dehydrogenase was assayed as described by Cooney et al. 1981). The assay medium consisted of 250 mM-mannitol, 10 mM-phosphate buffer (equimolar mixture of K_2HPO_4/KH_2PO_4), 10 mM-Tris/HCl, 10 mM-KCl, 5 mM-$MgCl_2$, 0.625 mM-CoA, 2 mM-NAD^+. 10 mM-2-oxoglutarate and 0.05% (v/v) Triton-X-100 to which 20-30 µl of crude mitochondrial preparation was added. The total assay volume was 1.0 ml at pH 7.4. The assay was initiated by the addition of 2-oxoglutarate which was omitted from concurrently run controls. The addition of 2.5 mM-ADP did not make any difference in the assay of the enzyme.

NAD^+- and $NADP^+$-linked isocitrate dehydrogenase were assayed as described by Alp et al. (1976). The assay medium for NAD^+-linked isocitrate dehydrogenase consisted of 70 mM-Tris/HCl, 8 mM-$MgCl_2$, 2 mM-NAD^+, 2 mM-ADP, 1 mM-$MnCl_2$, 3 mM-DL-isocitrate, 20 mM-citrate and 5 µg antimycin A to which 10-20 µl of supernatant was added. The total assay volume was 1.0 ml at pH 7.1. The assay was initiated by the addition of isocitrate plus citrate which were omitted from concurrently run controls. The assay medium for $NADP^+$-linked isocitrate dehydrogenase was the same as that of the NAD^+-linked enzyme, except that: NAD^+ was replaced by 0.5 mM-$NADP^+$ and both ADP and antimycin A were omitted. The final pH was 7.5.

Pyruvate dehydrogenase was assayed by coupling the reaction

with arylamine acetyltransferase and following the change in absorption due to acetylation of P-(P-aminophenylazo)-benzenesulphonic acid as described by Coore et al. 1971). The assay medium consisted of 100 mM-Tris, 0.5 mM EDTA, 1 mM-$MgCl_2$, 5 mM-mercaptoethanol, 1 mM-thiamine pyrophosphate, 0.5 mM-NAD^+, 0.1 mM CoA, 1 mM pyruvate, 10 µg-P-(P-aminophenylazo)-benzenesulphonic acid, 0.1 units-arylamine acetyltransferase and 0.05 units of both dihydrolipoamide acetyltransferase and dihydrolipoamide reductase to which 20 µl of homogenate was added. The total assay volume was 1.0 ml at pH 7.4. The assay was initiated by the addition of pyruvate. Controls from which pyruvate was omitted were run concurrently.

Glutamate dehydrogenase was assayed as described by Williamson et al. (1967), 105 mM-ammonium acetate, 2 mM-ADP, 5 µg antimycin A, 8 mM-2-oxoglutarate and 0.05% (v/v) Triton-X-100 to which 10-20 µl of homogenate was added. The total assay volume was 1.0 ml at pH 7.5. The assay was initiated by the addition of 2-oxoglutarate which was omitted from concurrently run controls.

Aspartate aminotransferase and alanine aminotransferase were assayed as described by Sugden & Newsholme (1975). The assaymedium for aspartate aminotransferase consisted of 75 mM-phosphate buffer (equimolar mixture of K_2HPO_4/KH_2PO_4), 10 mM-2-oxoglutarate, 50 µg pyridoxal phosphate, 0.2 mM-NADH, 5 µg antimycin A, 0.05% (v/v) Triton-X-100, 7.2 units malate dehydrogenase (in glycerol)

and L-aspartate at various concentrations (0.25, 0.50 and 1.0 mM) of aspartate adjusted to pH 7.5 with $KHCO_3$) to which 10-20 µl of homogenate was added. The total assay volume was 1.0 ml at pH 7.5. The assay was initiated by the addition of 2-oxoglutarate which was omitted from concurrently run controls. Maximum activities of the enzyme were obtained by extrapolation to infinite substrate concentration by means of a double reciprocal plot. The assay medium for alanine aminotransferase was the same as for aspartate aminotransferase except that L-aspartate was replaced by DL-alanine (0.25, 0.5, 1.0 mM); malate dehydrogenase (was replaced by Lactate dehydrogenase (in glycerol) and 30-40 µl homogenate was added.

Malic enzyme was assayed as described by Newsholme & Williams (1978). The assay medium consisted of 50 mM-Tris/HCl, 1 mM-$MnCl_2$, 0.2 mM-$NADP^+$, 2 mM-malate (neutralised with KOH), 100 mM-KCl and 0.05% (v/v) Triton-X-100 to which 10-20 µl of homogenate was added. The total assay volume was 1.0 ml at pH 7.4. The assay was initiated by the addition of malate which was omitted from concurrently run controls.

Pyruvate carboxylase was assayed as described by Martin & Denton (1970). The assay medium consisted of 100 mM-triethanolamine/HCl. 5 mM-$MgCl_2$, 0.2 mM-DTNB, 2.5 mM-ATP, 0.75 mM-acetyl-CoA 50 mM-$NaHCO_3$, 5 mM-creatine-phosphate, 10 mM-pyruvate and 0.05% 9v/v) Triton-X-100 (to which 10-15 µl homogenate was added. Immediately before

the assay 5.8 units of creatine kinase and 0.64 units citrate synthase were added. The total assay volume was 1.0 ml at pH 7.5. The assay was initiated by the addition of pyruvate which was omitted from concurrently run controls.

Phosphoenolpyruvate carboxykinase was assayed radiochemically as described by Ballard and Hanson (1967). The assay medium consisted of 100 mM-imidazole, 2 mM $MnCl_2$ 1 . mM-dithiothreitol, 100 mM-$NaHCO_3$ (containing 2 µCi $NaH^{14}CO_3$), 2.5 mM-NADH, 1.25 mM-IDP,m 1.5 mM-PEP, 5 µg antimycin A, 3.0 units (dialysed) malate dehydrogenase and 0.05% (v/v) Triton-X-100 to which 30-50 µl homogenate was added. The total assay volume was 1.0 ml at pH 7.0. The assay was initiated by the addition of homogenate (after 2 min pre-incubation of assay medium). Samples, in duplicate, were incubated at 37°C for 10 min (the time course of the reaction was linear throughout). The reaction was stopped with 0.6 ml 40% (w/v) $HClO_4$. Samples were centrifuged for 2 min in an Eppendorf microcentrifuge; supernatants were bubbled with cold CO_2 for 4 min (to remove $^{14}CO_2$) and 0.3 ml was removed for radioactivity measurement. IDP was omitted from controls.

3-Oxo acid CoA-transferase was assayed in the direction of acetoacetate formation (Williamson et al.,. 1971). The assay medium consisted of 50 mM-Tris/HCl, 10 mM-$MgCl_2$, 5 mM-Iodo-acetamide, 0.05-0.07-mM-acetoacetyl-CoA and 50-mM-succinate to which 10-20 µl supernatant was added.

The total assay volume was 1.0 ml at pH 8.5. Before addition of the substrate, all components of the assay medium were placed in a silica cuvette and the rate of change in A_{303} was followed. This rate served as a control to indicate acetoacetyl-CoA hydrolase activity. After 2-3 min, the enzyme assay was initiated by the addition of succinate which was omitted from concurrently run controls.

Acetoacetyl-CoA thiolase was assayed in the direction of acetyl-CoA formation (Williamson et al., 1971). The assay medium consisted of 50 mM-Tris/HCl, 5 mM-$MgCl_2$, 0.1 mM-CoA, 0.07 mM-acetoacetyl-CoA. The total assay volume was 1.0 ml at pH 7.5. Before the addition of supernatant, the rate of change in A_{303} was followed to account for the spontaneous hydrolysis of acetoacetyl-CoA. 10-20 µl supernatant was then added to initiate the enzyme assay, and the new rate of change of A_{303} was followed in a silica cuvette.

D-3-Hydroxybutyrate dehydrogenase was assayed in the direction of D-3-hydroxybutyrate oxidation (Williamson et al., 1971). The assay medium consisted of 50 mM-Tris/HCl, 50 mM-nicotinamide, 2 mM-dithiothreitol, 2 mM-NAD^+, 5 µg antimycin A and 20 mM-DL-3-hydroxybutyrate to which 40-60 µl supernatant was added. The total assay volume was 1.0 ml at pH 8.5. The assay was initiated by the addition of the supernatant. DL-3-hydroxybutyrate was omitted from concurrently run controls.

Carnitine palmitoyltransferase was assayed as described by

Bieber et al. 1972). The assay medium consisted of 60 mM-Tris, 1.5 mM-EDTA, 1.25 mM-L(-)carnitine, 0.25 mM-5,5'-dithiobis-(2-nitrobenzoic acid) and 35 µM-palmitoyl-CoA and 0.05% Triton-X-100, to which 10-20 µl of mitochondrial suspension was added. The total assay volume was 1.0 ml at pH 8.0. The assay was initiated by the addition of crude mitochondrial suspension. Controls in which D(+)-carnitine replaced L(-)-carnitine were run concurrently.

Phosphate-dependent glutaminase was assayed by the sampling method (glutamate formation) as described by Curthoys and Lowry (1973). The assay medium consisted of 50 mM-phosphate buffer (equimolar mixture of KH_2PO_4 and K_2HPO_4, 0.2 mM-EDTA, 50 mM-Tris/HCl, 20 mM-glutamine and 0.05% (v/v) Triton-X-100 to which 50-100 µl homogenate was added. The total assay volume was 1.0 ml at pH 8.6. Assay media, in triplicate, were incubated at 37°C for 10 min (the time course of the reaction was linear throughout) in which the assay was initiated by the addition of freshly prepared glutamine. Reaction was stopped with 0.2 ml 25% (w/v) $HClO_4$, neutralised and the amount of formed glutamate was determined as described by Bernt and Bergmeyer (1974). Concurrent controls were run in which either glutamine or the homogenate was omitted, thus accounting for non-enzymatic glutamine hydrolysis or endogenous glutamate respectively.

Phosphate-independent glutaminase was assayed as described

for the phosphate-dependent enzyme except that the assay medium consisted of 60 mM-maleate, 0.2 mM-EDTA, 10 mM-glutamine and 0.05% (v/v) Triton-X-100 to which 100 µl homogenate was added. The total assay volume was 1.0 ml at pH 6.6

ATP-citrate lyase was assayed as described by Rider (1983). The assay medium consisted of 50 mM-Tris, 10 mM-$MgCl_2$, 100 mM-KCl, 5 mM-ATP, 0.2 mM-CoA, 20 mM-citrate, 0.15 mM-NADH, 5 mM-creatine phosphate, 1.8 units-creatine kinase, 4 units-malate dehydrogenase, 10 mM-mercaptoethanol, 5 µg-antimycin A and 0.05% (v/v) Triton-X-100 to which 20 µl of homogenate was added. The total assay volume was 1.0 ml at pH 8.0. The assay was initiated by the addition of CoA. Controls from which CoA was omitted were run concurrently.

APPENDIX C

Miscellaneous

(i) MEM culture medium

Eagle's minimum essential medium, with Earle's salts, without L-glutamine was obtained from Gibco Biocult Ltd. It consisted of the following:

Compound	Concentration (mg/L)
$CaCl_2$ (anhyd.)	200.0
KCl	400.0
$MgSO_4.7H_2O$	200.0
NaCl	6800.00
$NaHCo_3$	2200.0
$NaH_2PO_4.H_2O$	140.0
Glucose	1000.0
Phenol red	10.0
L-Arginine.HCl	126.0
L-cystine	24.0
L.Histidine.HCl.H_2O	42.0
L-Isoleucine	52.0
L-Leucine	52.0
L-Lysine.HCl	72.5
L-Methionine	15.0
L-Phenylalanine	32.5
L-Threonine	48.0
L-Tryptophan	10.0
L-Tyrosine	36.0
L-Valine	46.0

Compound	Concentration (mg/L)
D-Ca pantothenate	1.0
Choline chloride	1.0
Folic acid	1.0
i-Inositol	2.0
Nicotinamide	1.0
Pyridoxal.HCl	1.0
Riboflavin	0.1
Thiamine.HCl	1.0

(ii) Phosphate-buffered saline PBS

PBS (type A, and the supplement to produce type A + B) was obtained from Oxoid Ltd. It was used at pH 7.2-7.4. It consisted of the following (Dulbecco and Vogt, 1954):

Compound	Final concentration (g/L)
Type A	
NaCl	8.0
KCl	0.2
Na_2HPO_4	0.2
KH_2PO_4	0.2
Type B (supplement)	
$CaCl_2$	0.1
$MgCl_2$	0.1

(iii) Triethanolamine-KOH

This was prepared by adding potassium hydroxide (final concentration 2 M) to a 0.5 M solution of triethanolamine.HCl buffer previously adjusted to pH 7.4.

(iv) Amino acid autoanalysis

Analysis of acidic, neutral and basic amino acids was carried out with an automated amino acid analyser (Model LKB 4400) using a Na-citrate buffer system. Samples were loaded with 0.2 M Na-citrate pH 2.2 Amino acids were eluted using the following b uffer system:

First buffer: pH 3.20 0.2 M Na-citrate

Second buffer: pH 4.25 0.2 M Na-citrate

Third buffer: pH 8.60 0.5 M Borate-citrate

Regeneration of the column was at 0.4 M NaOH. The buffers were obtained from LKB.

(v) Oleate: BSA complex preparation

A stock solution of this complex was prepared as follows: the organic solvent in the vial containing radiolabelled oleate (obtained commercially) was evaporated under a stream of N_2. Unlabelled sodium oleate is then added to the vial. The fatty acid is then dissolved in 10 mls of 10% (w/v) defatted BSA (Chen, 1967; and dialysed against 0.9% NaCl overnight) by incubating the mixture (flushed with N_2 and capped) overnight at 37°C. This stock solution had the following composition:

5 µCi/ml: [1-^{14}C]-oleate

6 mM: oleate

10% (w/v): B.S.A

The stock solution was kept at -20°C, and was incubated at 37°C for at least 4 h before use.

APPENDIX D

Materials

Animals

Female mice (12-16 week-old) of the C57 BL/6 strain were bred in the animal house of the Sir William Dunn School of Pathology, Oxford.

Male Wistar albino rats (160-180 g) were obtained from Batin and Kingman Ltd., Grimston, Hull, Yorks., HU11 4QU, U.K.

Chemicals and enzymes

All chemicals and enzymes were of an analytical grade and were obtained from Boehringer Mannheim, G.m.b.H., Lewes, East Sussex, BN7 1LG, U.K. except for the following: glucose and all inorganic reagents were obtained from Fisons Scientific Apparatus, Loughborough, Leics. LE11 ORG U.K.; Tes(2-/2-hydroxy-1-1-bis(hydroxymethyl)ethyl/-amino)-ethane-sulphonic acid) was obtained from Hopkin and Williams, Chadwell Heath, Essex. U.K.; hydrazine hydrate and sucrose were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K.; Thethanolamine.HCl, 2-mercaptoethanol, acetoacetyl-CoA, iodoacetamide, DL-isocitrate, diamino-ethanetetra-acetic acid, sodium oleate, asparaginase, L-glutamine, sodium butyrate, lithium citrate, bovine serum albumin (fraction V) 5,5'dithiobis-(2-nitrobenzoic acid), phenylmethylsulphonyl fluoride and all other protease inhibitors were obtained from Sigma Chemicals, Poole, Dorset, BH17 7NH, U.K.

All radiochemicals and tissue solubilizer were obtained from the Radiochemical Centre, Amersham, U.K., except for [3-^{14}C]-pyruvate which was obtained from New-England-Nuclear, Boston, MA0211, U.S.A.

Media

Eagle's minimum essential medium, trypsin solution and foetal bovine serum were obtained from Gibco-Biocult Ltd., Paisley, Scotland, PA3 4EF. Phosphosphate-buffered saline A and B were obtained from Oxoid Ltd., Basingstoke, Hants. RG24 OPW, U.K. Thioglycollate broth was obtained from Difco Laboratories, Detroit, MI48232, U.S.A. Hepes buffer and gentamiacin were obtained from Flow laboratories, Irvine, Scotland, KA12 8NB.

Miscellaneous

Tissue culture dishes and plates were obtained from Flow laboratories, Irvine, Scotland, KA12 8NB. Zymosan type A, trypan blue, Triton-X-100 and Ficoll (type 400) were obtained from Sigma Chemicals, Poole, Dorset, BH17 7NH, U.K. Repelcote was obtained from Hopkins and Williams, Chadwell Heath, Dorset, BH12 4NN, U.K. T.L.C. plates were obtained fromn Anachem, Luton, Beds. LU2 OEB, U.K. "Liquiscint" (aqueous) and "Betafluor" (non-aqueous) scintillation cocktails were obtained from National Diagnostics, Somerville, New Jersey, 08876, U.S.A. All organic solvents were obtained from Fisons Scientific Apparatus, Loughborough, Leics. LE11 ORg, U.K.; except

2-phenylethylamine and methanol which were obtained from BDH Chemicals, Poole, Dorset, BH12 4NN, U.K.

REFERENCES

REFERENCES

Albina, J.E., Shearer, J., Mastrofrancesco, B. & Caldwell, M.D. (1986) Am. J. Physiol. 250: E24.

Albina, J.E., Henry, W., King, P.A., Shearer, J., Mastrofrancesco, B., Goldstein, L. & Caldwell, M.D. (1987) Am. J. Physiol. 252: E49.

Alp, P.R., Newsholme, E.A. & Zammit, V.A. (1976) Biochem. J. 154: 689.

Aoki, T.T., Müller, W.A. & Cahill, G.F. (1971) Adv. Enz. Reg. 10: 145.

Ardawi, M.S.M. (1983) D. Phil Thesis Oxford University,.

Ardawi, M.S.M. & Newsholme, E.A. (1982) Biochem. J. 208: 743.

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

Ardawi, M.S.M. & Newsholme, E.A. (1984a) Biochem. J. 221: 255.

Ardawi, M.S.M. & Newsholme, E.A. (1984b) Biochem. J. 217: 289.

Ardawi, M.S.M. & Newsholme, E.A. (1985a) Essays Biochemistry 21: 1.

Ardawi, M.S.M. & Newsholme, E.A. (1985b) Biochem. J. 231: 713.

Ardawi, M.S.M. (1987) Clin. Sci. (in press).

Askanazi, J., Carpenter, Y.A., Micholsen, C.B., Elwyn, D.H., Furst, P., Kantrowitz, L.R., Gump, F.E. & Kinney, J.M. (1980) Ann. Surg. 192: 78.

Aulick, L.H. & Wilmore, D.S. (1979) Surgery 85: 560.

Baldwin, K,M., Winder, W.W. & Terjung, R.L. (1973) Am. J. Physiol. 225: 962.

Ballard, F.J. & Hanson, R.W. (1967) Biochem. J. 104: 866.

Beatty, C.H., Peterson, R.D., Bocek, R.M. & West, E.S. (1964) J. Biol. Chem. 239: 2106.

Beis, A., Zammit, V.A. & Newsholme, E.A. (1980) Eur. J. Biochem. 209: 209.

Bergmeyer, H.-U. & Bernt, E. (1974a) In: Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.) Academic Press, London & New York.

Bergmeyer, H.-U., Gawehn, K. & Grassl, M. (1974b) In: Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.) Academic Press, London & New York.

Bernt, E. & Bergmeyer, H.-U. (1974) In: Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.) Academic Press London & New York.

Berton, G. & Gordon, S. (1985) In: Mononuclear Phagocytes. Characteristics, Physiology and Function (Van Furth, R., ed.) Martinus Nijhoff, Dordrecht.

Bessman, S.P. , Magnes, J., Schwerin, P. & Waelsch, H. (1948) J. Biol. Chem. 175: 817.

Best, C.H., Dale, H.H., Hoet, J.P. & Marks, H.P. (1926a) Proc. Roy. Soc. B 100: 55.

Best, C.H., Hoet, J.P. & Marks, H.P. (1926b) Proc. Roy. Soc. B 100: 32.

Bettger, W.J. & McKeehan, W.L. (1986) Physiol. Rev. 66: 1.

Bieber, L.L., Abraham, T. & Helmarth, T. (1972) Anal. Biochem. 50: 509.

Bissell, M.J., Hatie, C. & Rubin, H. (1972) J. Natl. Cancer Inst. 49: 555.

Bradford, M.M. (1976) Anal. Biochem. 72: 248.

Brand, K. (1985) Biochem. J. 228: 353.

Brooks, B.J., Arch, J.R.S. & Newsholme, E.A. (1983) Biosc. Rep. 3: 263.

Bryant, J.C., Schilling, E.L. & Earle, W.R. (1958) J. Natl. Cancer 21: 349.

Budohoski, L., Chaliss, R.A.J. & Newsholme, E.A. (1982) Biochem. J. 206: 169.

Cahill, G.F. (1976) Clin. End. Metab. 5: 397.

Cahill, G.F. & Owen, O.E. (1968) In: Carbohydrate Metabolism and its Disorders (Dickens, F., Randle, P.J. & Whelan, W.J., eds.) Academic Press, London & New York.

Challiss, R.A.J., Lozeman, F.J., Leighton, B.L. & Newsholme, E.A. (1986) Biochem. J. 233: 377.

Chen, C. (1967) J. Biol. Chem. 243: 173.

Chiu, J.F. & Boeker, E.A. (1979) Arch. Biochem. Biophys. 196: 493.

Cohn, Z.A. (1974) Methods Enzymol. 32B: 758.

Cohn, Z.A. (1978) J. Immunol. 121: 813.

Cooney, G.J. & Newsholme, E.A. (1982a) Trends Biochem. Sci. 9: 303.

Cooney, G.J. & Newsholme, E.A. (1982b) FEBS Lett. 148: 198.

Cooney, G.J., Taegtmeyer, H. & Newsholme, E.A. (1981) Biochem. J. 200: 701.

Cooper, A.J.L. & Meister, A. (1972) Biochemistry 11: 661.

Cornblath, M., Randle, P.J., Parmeggiani, A. and Morgan,H.E. (1963) J. Biol. Chem. 238: 1592.

Crabtree, B. & Newshome, E.A. (1972a) Biochem. J. 126: 49.

Crabtree, B. & Newsholme, E.A. (1972b) Biochem. J. 130: 697.

Crabtree, B. & Newsholme, E.A. (1975) In: Insect Muscle (Usherwood, P.N.R., ed.) Academic Press, London & New York.

Crabtree, B. & Newsholme, E.A. (1978) Eur. J. Biochem. 89: 19.

Crabtree, B. & Newsholme, E.A. (1985) Curr. Top. Cell Reg. 25: 21.

Crabtree, G. & Taylor, D.J. (1978) In: Biochemical Thermodynamics (Jones, M.N., ed.) Elsevier/North Holland, Amsterdam.

Crabtree, B., Higgins, S.J. & Newsholme, E.A. (1972) Biochem. J. 130: 391.

Crabtree, B., Leech, A.R. & Newsholme, E.A. (1979) In: Techniques in Life Sciences (Pogson, C., ed.) Elsevier/North-Holland Amsterdam.

Crompton, M., McGivan, J.D. & Chappell, J.B. (1973) Biochem. J. 132: 27.

Cruickshank, E.W.H. & Kosterlitz, H.W. (1941) J. Physiol. (London) 99: 208.

Curi, R., Newsholme, P. & Newshome, E.A. (1986) Biochem. Biophys. Res. Commum. 138: 318.

Curi, R., Newsholme, P. & Newsholme, E.A. (1987) Biochem. J. (submitted)

Currie, G.A. (1978) Nature 273: 758.

Curthoys, M.P. & Kehlenschmidt, T. (1975) J. Biol. Chem. 250: 2099.

Curthoys, N.P. & Lowry, O.H. (1973) J. Biol. Chem. 248: 162.

Curthoys, N.P. & Weiss, R.F. (1974) J. Biol. Chem. 249: 3261.

Curthoys, N.P., Kuhlenschmidt, T. & Godfrey, S.S. (1976) Arch. Biochem. Biophys. 174: 82.

Curthoys, N.P., Shapiro, R.A. & Haser, N.G. (1984) In: Glutamine Metabolism in Mammalian Tissues (Häussinger, D. & Sies, H., ed.) Springer-Verlag, Berlin-Heidelberg-New York-Tokyo.

Czok, R. & Lamprecht, W. (1974) In: Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.) Academic Press, London & New York.

Damelly M. & Scheffer, I.E. (1976) J. Cell Physiol. 89: 39.

Denyer, G.S., Kerbey, A.L.& Randle, P.J. (1986) Biochem. J. 239: 347.

Donnelly, M. & Scheffler, I.E. (1976) J. Cell Physiol. 89: 39.

Drath, D.B. & Karnovsky, M.L. (1975) J. Exp. Med. 141: 257.

Drummond, G.I. (1967) Fortschr. Zool. 18: 359.

Dulbecco & Vogt (1954) J. Exp. Med. 99: 167.

Durum, S.K., Schmidt, J.A. & Oppenheim, J.J. (1985) Ann. Rev. Immunol. 3: 263.

Eagle, H. (1959) Science 130: 432.

Eagle, H., Oyama, V.I., Levy, M., Horton, C.L. & Fleischman, R. (1956) J. Biol. Chem. 218: 607.

Eagle, P.C. & Jones, J.B. (1978) Anal. Biochem. 88: 475.

Ekelund, L.-G. (1969) Ann. Rev. Physiol. 31: 85.

Evans, C.L. (1914) J. Physiol. (London) 47: 407.

Ezekowitz, R.A.B., Sim, R.B., Hill, M. & Gordon, S. (1983) J. Exp. Med. 159: 244.

Fay, J.P. & Farias, R.N. (1975) J. Gen. Microbiol. 91: 233.

Felig, P. (1975) Ann. Rev. Biochem. 44: 933.

Fletcher, W.M. & Hopkins, F.G. (1907) J. Physiol. 35: 247.

Fogelman, A.M., Van Lenten, B.J., Hchom, M., Seager, J., Wang, H., Navab, M., Shapiro, S., Haberland, M.E. & Eduards, P.A. (1985) In: Mononuclear Phagocytes. Characteristics, Physiology and Function (Van Furth, R., ed.) Martinus Nijhoff, Dordrecht.

Fowler, S., Shio, A. & Haley, N.J. (1979) Lab. Invest. 41: 372.

Fredrickson, D.S. & Gordon, R.S. (1958) Physiol. Rev. 38: 585.

Freese, E., Sheu, C.W. & Galliers, E. (1973) Nature 241: 321.

Garber, A.J., Karl, I.E. & Kipnis, D.M. (1976) J. Biol. Chem. 251: 826.

George, J.C. & Berger, A.J. (1966) Avian Myology Academic Press, London & New York.

Gerrity, R.G. (1981) Am. J. Pathol. 103: 181.

Godfrey, S.S., Kuhlenschmidt, T. & Curthoys, N.P. (1977) J. Biol. Chem. 252: 1927.

Goldberg, A.L., Demartino, G. & Chang, T.W. (1978) FEBS 11th Meeting-Copenhagen 42: 347.

Goldstein, L. (1966) Am. J. Physiol. 210: 661.

Goldstein, L. (1976) Int. Rev. Physiol. 11: 283.

Goldstein, L. & Newsholme, E.A. (1976) Biochem. J. 154: 555.

Gordon, S. (1986) J. Cell Sci. (Suppl.) 4: 267.

Graff, S., Moser, H., Kastner, O., Graff, A.M. & Tannenbaum, M. (1965) J. Natl. Cancer 34: 511.

Guha, S.R. (1961) Enzymologia 23: 94.

Hales, C.N., Luzio, J.P. & Siddle, K. (1978) Biochem. Soc. Symp. 43: 97.

Hammer, T.A. & Rannels, D.E. (1981) Biochem. J. 198: 53.

Hanson, P.J. & Carrington, J.M. (1981) Biochem. J. 200: 349.

Hanson, P.J. & Parsons, D.S. (1977) Biochem. J. 166: 509.

Hanson, P.J. & Parsons, D.S. (1980) Biochem. Soc. Trans. 8: 506.

Hard, G.C. (1970) Br. J. Exp. Path. 51: 97.

Harper, A.E. (1986) Fed. Proc. 2179.

Hartwig, J.H., Yin, H.L. & Stossel, T.P. (1980) In: Mononuclear Phagocytes. Functional Aspects (Van Furth, R., ed.) Martinus Nijhoff, The Hague-London-Boston.

Hawkins, R.A., Williamson, D.H. & Krebs, H.A. (1971) Biochem. J. 122: 13.

Hems, R., Stubbs, M. & Krebs, H.A. (1968) Biochem. J. 107: 807.

Hickman, P.E. & Weidmann, M.J. (1975) Biochem. J. 151: 327.

Hicks, R.M. & Kerly, M. (1960) J. Physiol. (London) 150: 621.

Hofman, A.F. (1962) J. Lip. Res. 3: 391.

Horowitz, M.L. & Knox, W.E. (1978) Enz. Biol. Clin. 9: 241.

Huang, Y.Z. & Knox, W.E. (1976) Enzyme 21: 408.

Hume, D.A., Radik, J.L., Ferber, E. & Weidmann, M.J. (1978) Biochem. J. 174: 703.

Jaworek, D., Gruber, W. & Bergmeyer, H. -U. (1974) In: Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.) Academic Press, London & New York.

Johnston, R.B., Godzik, C.A. & Cohn, Z.A. (1978) J. Exp. Med. 148: 115.

Kalra, J. & Brosnan, J.T. (1973) FEBS Lett. 37: 325.

Kalra, J. & Brosnan, J.T. (1974) J. Biol. Chem. 249: 3255.

Kampschmidt, R.F. (1984) J. Leuk. Biol. 36: 341.

Karnovsky, M.L., Simmons, S., Glass, E.A., Shafer, A.W. & D'Arcy Hart, P. (1970) In: Mononuclear Phagocytes (Van Furth, R., ed.) Blackwell Scientific Publications, Oxford & Edinburgh.

Karnovsky, M.L., Lazdins, J. & Simmons, S.R. (1975) In: Mononuclear Phagocytes in Immunity, Infection and Pathology (Van Furth, R., ed.) Blackwell Scientific Publications Oxford-Edinburgh-Melbourne.

Karnovsky, M.L. & Lazdins, J.K. (1978) J. Immunol. 121: 809.

Katunuma, N., Huzino, A. & Tomino, I. (1967) Adv. Enz. Regul. 5: 57.

Kerby, A.L., Randle, P.J., Cooper, R.H., Whitehouse, S., Park, H.K. & Denton, R.M. (1976) Biochem. J. 154: 327.

Kielian, M.C. & Cohn, Z.A. (1980) In: Mononuclear Phagocytes. Functional Aspects (Van Furth, R., ed.) Martinus Nijhoff, Dordrecht.

Kiyotaki, C., Peisach, J. & Bloom, B.R. (1984) J. Immunol. 132: 857.

Klein, J. (1979) Science 203: 516.

Knight, B.L., Patel, D.D. & Soutar, A.K. (1983) Biochem. J. 210: 523.

Knox, W.E. (1976) Enzyme Patterns in Fetal Adult and Neoplastic Rat Tissues S. Karger, Basel.

Kovacevic, Z. (1974) Cancer Res. 34: 3403.

Kovacevic, Z. & McGivan, J.D. (1983) Physiol. Rev. 63: 547.

Kovacevic, Z. & Morris, H.P. (1972) Cancer Res. 32: 326.

Krebs, H.A. (1935) Biochem. J. 29: 1951.

Krebs, H.A. (1972) In: Essays in Biochemistry (Campbell, P.N. & Dickens, F., eds.) Academic Press, London & New York.

Krebs, H.A. (1980) In: Glutamine: Metabolism, Enzymology & Regulation (Mora,J. & Palacius, R., eds.) Academic Press, New York.

Krebs, H.A. & Johnson, W.A. (1948) Tabulae Biologicae 19: 100.

Krebs, H.A., Williamson, D.H., Bates, M.W., Page, M.A. & Hawkins, R.A. (1971) Adv. Enzyme Regul. 9: 387.

Kung, J.T., Brooks, S.B., Jackway, J.P., Leonard, L.L. & Talmage, D.W. (1977) J. Exp. Med. 146: 665.

Lavietes, B.B., Regan, D.H. & Demopoulos, H.B. (1974) Proc. Natl. Acad. Sci. (USA) 71: 3993.

Lazo, P.A. (1981a) Eur. J. Biochem. 117: 19.

Lazo, P.A. (1981b) FEBS Letts. 135: 229.

Lee, S.-H., Starkey, P. & Gordon, S. (1985) J. Exp. Med. 161: 475.

Lehninger, A.L. (1975) Biochemistry Worth Publishers, Inc. New York.

Leighton, B.L., Budohoski, L., Lozeman, F. J., Challiss, R.A.J. & Newsholme, E.A. (1985) Biochem. J. 227: 337.

Loike, J.D., Kozler, V.F. & Silverstein, S.C. (1979) J. Biol. Chem. 254: 9558.

Lokesh, B.R. & Wrann, M. (1984) Biochim. Biophys. Acta. 792: 141.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) J. Biol. Chem. 193: 265.

Lucas, D.O. & Epstein, L.B. (1984) In: Physiology of the Reticuloendothelial System: A Comprehensive Treatise (Reichard, S.M. & Filkins, J.P., ed.) Plenum, New York.

Lund, P. (1980) FEBS Letts. 117: K86.

Lund, P. & Williamson, D.H. (1985) Br. Med. Bull. 41: 251.

Mahley, R.W. (1979) In: Atherosclerosis Rev. (Paoletti, R. & Gotto, A.M., Jr., ed.) Raven Press, New York.

Mahoney, E.M., Hammil, A.L., Scott, W.A. & Cohn, Z.A. (1977) Proc. Natl. Acad. Sci. (USA) 74: 4895.

Mahoney, E.M., Khoo, J.C., Witztum, J.L. & Steinberg, D. (1985) In: Mononuclear Phagocytes. Characteristics, Physiology & Function (Van Furth, R., ed.) Martinus Nijhoff, Dordrecht.

Maizels, E.Z., Ruderman, N.B., Goodman, M.N. & Lau, D. (1977) Biochem. J. 162: 557.

Martin, B.R. & Denton, R.M. (1970) Biochem. J. 117: 861.

Martin, C.E., Hiramitsu, K. & Kitajima, Y. (1976) Biochemistry 15: 5218.

McGarry, J.D., Leatherman, G.F. & Foster, D.W. (1978) J. Biol. Chem. 253: 4128.

Mckeehan, W.L. (1982) Cell Biol. Int. Rep. 6: 635.

Meister, A. (1956) Physiol. Rev. 36: 103.

Meister, A. (1965) Biochemistry of Amino Acids Academic Press, New York.

Meister, A. (1979) In: Glutamic Acid: Adv. in Biochem. & Physiol. (Filer, L.J., Garattini, S., Kare, M.R., Reynolds, W.A. & Wartman, R.J., eds.) Raven, New York.

Meister, A. (1980) In: Glutamine : Metabolism, Enzymology and Regulation (Mora, J. & Palacios, R., eds.) Academic Press, New York.

Mellamby, J. & Williamson, D.H. (1974) In: Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.) Academic Press, London & New York.

Metchnikoff, E. (1901) L'immunité dans les maladies infectieuses Masson, Paris.

Michl, J., Ohlbaum, D.J. & Silverstein, S.C. (1976a) J. Exp. Med. 144: 1415.

Michl, J., Ohlbaum, D.J. & Siverstein, S.C. (1976b) J. Exp. Med. 144: 1484.

Miller, L.L. (1962) In: Amino acids (Holden, J.T., ed.) Elsevier/North Holland, Amsterdam.

Mofena, M., Bosca, L. & Hue, L. (1985) Biochem. J. 232: 521.

Moolten, F.L., Moolten, D.N. & Capperell, N.J. (1977) J. Cell Biol. 93: 147.

Moreadith, R.W. & Lehninger, A.L. (1984) J. Biol. Chem. 259: 6215.

Morell, B. & Froesch, E.R. (1973) Eur. J. Clin. Invest. 3: 112.

Muller, W.A., Steinman, R.M. & Cohn, Z.A. (1980a) In: Mononuclear Phagocytes (Van Furth, R., ed.) Martinus Nijhoff, The Hague-Boston-London.

Muller, W.A., Steinnam, R.M., Cohn, Z.A. (1980b) J. Cell Biol. 86: 292.

Muntz, J.A. (1953) Arch. Biochem. Biophys. 42: 435.

Muntz, J.A. & Hurwitz, J. (1951) Arch. Biochem. Biophys. 32: 137.

Nathan, C.F. (1980) In: Mononuclear Phagocytes. Functional Aspects (Van Furth, R., ed.) Martinus Nijhoff, The Hague-Boston-London.

Needham, D.M. (1971) Mchina Carins Cambridge University Press.

Neptune, E.M., Jr. (1965) Am. J. Physiol. 209: 329.

Newsholme, E.A. (1976) Clin. Endocrinol. Metab. 5: 543.

Newsholme, E.A. (1977) Ann. N. Y. Acad. Sci. 301: 81.

Newsholme, E.A. (1978) Biochem. Soc. Symp. 43: 183.

Newsholme, E.A. & Crabtree, B. (1973) Symp. Soc. Exp. Biol. 26: 429.

Newsholme, E.A. & Crabtree, B. (1976) Biochem. Soc. Symp. 41: 61.

Newsholme, E.A. & Crabtree, B. (1979) J. Mol. Cell Cardiology 11: 839.

Newsholme, E.A. & Leech, A.R. (1983) Biochemistry for the Medical Sciences John Wiley & Sons, Chichester-New York-Brisbane-Toronto-Singapore.

Newsholme, E.A. & Paul, J. (1983) Symp. Soc. Exp. Biol. Semin. Ser. 17: 81.

Newsholme, E.A. & Start, C. (1973) Regulation in Metabolism John Wiley & Sons, London & New York.

Newsholme, E.A. & Taylor, K. (1969) Biochem. J. 112: 465.

Newsholme, E.A. & Randle, P.J. (1964) Biochem. J. 93: 641.

Newsholme, E.A. & Williams, T. (1978) Biochem. J. 176: 623.

Newsholme, E.A. , Crabtree, B. & Zammit, V.A. (1980) Ciba Found. Symp. 73: 245.

Newsholme, E.A., Lang, J. & Relman, A.R. (1982) Contrib. Nephrol. 31: 1.

Newsholme, E.A., Leighton, B., Challiss, R.A.J. & Lozeman, F.J. (1986) Biochem. J. 238: 621.

Newsholme, E.A., Zammit, V.A. & Crabtree, B. (1978) Biochem. Soc. Trans. 6: 512.

Newsholme, E.A., Brand, K., Lang, J., Stanley, J.C. & Williams, T. (1979) Biochem J. 182: 621.

North, R.J. (1978) J. Immunol. 121: 806.

O'Connor, J.A., Scott, R.L., Mellick, P.W. & Caldwell, M.D. (1981) Am. J. Physiol. 242: R570.

O'Donnell-Tormey, J., Nathan, C.F., Lanks, K., DeBoer, C.J. & De La Hasse, J. (1987) J. Exp. Med. 165: 500.

Odessey, R., Khairallah, E. & Goldberg, A.L. (1974) J. Biol. Chem. 249: 7623.

Olds, G.R., Ellner, J.J., Kearse, L.A., Jr., Kazura, J.W. & Mahmoud, A.A.F. (1980) J. Exp. Med. 151: 1557.

Opie, L.H. (1968) Am. Heart J. 76: 685.

Opie, L.H. & Newsholme, E.A. (1967) Biochem. J. 104: 353.

Owen, O.E., Morgan, A .P., Kemp, H.G. , Sullivan, J.N., Herrera, M.G. & Cahill, G.F. (1967) J. Clin. Invest. 46: 1589.

Owen, O.E., Felig, P., Morgan, P.A., Wahren, J. & Cahill, G.F. (1969) J. Clin. Invest. 48: 574.

Parnas, J. & Wagner, R. (1914) Biochem. Z. 61: 387.

Paul, J. (1979) D. Phil. Thesis Oxford University.

Pinkus, L.M. & Windmueller, H.G. (1977) Arch. Biochem. Biophys. 182: 506.

Pitts, R.F. (1973) In: Handbook of Physiology, section 8 (Orloff, J. & Berliner, R.W., eds.) Am. Physiol. Soc. Washington, D.C.

Playfair, J.H.L. (1979) Immunology at a glance Blackwell Scientific Publications Oxford-London-Edinburgh-Melbourne.

Prosser, F.H., Schmidt, C.J., Nichols, S.V. & Nichols, W.K. (1984) J. Cell Physiol. 120: 75.

Quastel, J.H. (1979) Essays Med. Biochem. 4: 1.

Randle, P.J., Denton, R.M. & England, P.J. (1968) Biochem. Soc. Symp. 27: 87.

Randle, P.J., Garland, P.B., Hales, C.N., Newsholme, E.A., Denton, R.M. & Pogson, C.I. (1966) Rec. Prog. Horm. Res. 22: 1.

Randle, P.J., Sugden, P.H., Kerbey, A.L., Radcliffe, P.M. & Hutson, N.J. (1978) Biochem. Soc. Symp. 43: 47.

Rapoport, S., Rost, J. & Schultze, M. (1971) Eur. J. Biochem. 23: 166.

Read, G., Crabtree, B. & Smith, G.H. (1977) Biochem. J. 164: 349.

Reitzer, L.J., Wice, B.M. & Kennel, D. (1979) J. Biol. Chem. 254: 2667.

Rheinwald, J.G. & Green, H. (1974) Cell 2: 287.

Richardson, H.B., Shorr, E. & Loebell, R.O. (1930) J. Biol. Chem. 85: 551.

Rider, C.C. (1983) Biochem. J. 214: 299.

Robinson, D.S. (1970) Comprehensive Biochemistry 18: 51.

Roediger, W.E.W. (1982) Gastroenterology 83: 424.

Roitt, I. (1980) Essential of Immunology Blackwell Scientific Publications.

Roos, D. & Loos, J.A. (1973) Expt. Cell Res. 77: 127.

Rose, W.C. (1938) Physiol. Rev. 18: 109.

Rossi, F. Bellavite, P., Dobrina, A., Dri, P. & Zabucchi, G. (1980) In: Mononuclear Phagocytes. Functional Aspects (Van Furth, R., ed.) Martinus Nijhoff, The Hague-Boston-London.

Rossi, R., Bellavite, P. Serra, M.C. & Papini, E. (1985) In: Mononuclear Phagocytes. Characteristics, Physiology and Function (Van Furth, R., ed.) Martinus Nijhoff, The Hague-Boston-London.

Rossi, F., Zabucchi, G. & Romeo, D. (1975) In: Mononuclear Phagocytes in Immunity, Infection and Pathology (Van Furth, R., ed.) Blackwell Scientific Puiblications, Oxford-Edinburgh-Melborne.

Ruderman, N.B. & Berger, M. (1974) J. Biol. Chem. 249: 5500.

Sacktor, B. (1965) In: Physiology of Insecta (Rockstein, M., ed.) Academic Press, London & New York.

Sayre, F.W. & Roberts, E. (1958) J. Biol. Chem. 233: 1128.

Schaffner, T., Taylor, K. & Bartucci, E.J. (1980) Am. J. Pathol. 100: 57.

Schwerin, P., Bessman, S.P. & Waelsch, H. (1950) J. Biol. Chem. 184: 37.

Shapiro, R.A., Morehouse, R.F. & Curthoys, N.P. (1982) Biochem. J. 207: 561.

Silverstein, S.C. & Loike, J.D. (1980) In: Mononuclear Phagocytes Functional Aspects (Van Furth, R. , ed.) Martinus Nijhoff, The Hague-Boston-London.

Simon, L.M., Robin, E.P., Phillips, J.R., Acevedo, J., Axline, S.G. & Theodore, J. (1977) J. Clin Invest. 59: 443.

Snell, K. (1980) Biochem. Soc. Trans. 8: 205.

Snell, K. (1984) Adv. Enz. Reg. 22: 325.

Snell, K. & Duff, D.A. (1977) Biochem. J. 162: 399.

Spector, A.A., John, K. & Fletcher, J.E. (1969) J. Lipid Res. 10: 56.

Stanley, J.C. & Williams, S.G. (1969) Anal. Biochem. 29: 381.

Steinman, R.M., Brodie, E.S. & Cohn, Z.A. (1976) J. Cell Biol. 68: 665.

Steinman, R.M., Mellman, I.S., Mueller, W.A., Cohn, Z.A. (1983) J. Cell Biol. 96: 1.

Stenson, W.F. & Parker, C.W. (1980) J. Immunol. 125: 1.

Stoner, G.D. & Merchant, D.J. (1972) In Vitro 7: 330.

Stubbs, M., Kuhner, A.V., Glass, E.R., David, J.R. & Kamovsky, M.L. (1973) J. Exp. Med. 137: 537.

Sugden, P.H. & Newsholme, E.A. (1975) Biochem. J. 150: 105.

Sumbilla,C.M., Zielke, C.L., Reed, W.D., Ozand, P.T. & Zielke, H.R. (1981) Biochim. Biophys. Acta. 675: 301.

Sung, S.-S.J. & Silverstein, S.C. (1985) Biochim. Biophys. Acta. 845: 204.

Suter, D. & Weidmann, M.J. (1975) Biochem. J. 148: 583.

Svenneby, G. (1970) J. Neurochem. 17: 1591.

Svenneby, G. (1972) J. Neurochem. 19: 165.

Takemura, R. & Werb, Z. (1984) Am. J. Physiol. 246: C1.

Traybum, P. & Van Heyningen, R. (1973) Expt. Eye Res. 17: 149.

Van Furth, R. (1980) Mononuclear Phagocytes. Functional Aspects Martinus Nijhoff The Hague-London-Boston.

Van Furth, R. (1985) Mononuclear Phagocytes. Characteristics, Physiology and Function Martinus Nijhoff, Dordrecht.

Vinay, P., Lemieux, G., Gougoux, A. & Watford, M. (1978) Proc. Int. Congr. Nephrol. 7th 199.

Von Hodenberg, E., Khou, J.C., Jensen, D., Witztum, J.L. & Steinberg, D. (1984) Atheriosclerosis 4: 630.

Wagenmakers, A.J.M., Schepens, J.T.G., Veldhuizen, J.A.M. & Veerkamp, J.H. (1984a) Biochem. J. 220: 273.

Wagenmakers, A.J.M., Schepens, J.T.G. & Veenkamp, J.H. (1984b) Biochem. J. 223: 815.

Warburg, O. (1926) Uber den Stoffwechsel des Tumoren Springer-Verlag Berlin.

Warburg, O. (1956a) Science 123: 309.

Warburg, O. (1956b) Science 124: 269.

Watford, M., Lund, P. & Krebs, H.A. (1979) Biochem. J. 178: 589.

Weber, G. (1977a) New Engl. J. Med. 296: 486.

Weber, G. (1977b) New Engl. J. Med. 296: 541.

Werb, Z. (1984) In: Basic and Clinical Immunology (Stites, D.P., Stobo, J.D., Fudenburg, H.H. & Wells, J.V., eds.) Lange Los Altos, California.

Werb, Z. & Cohn, Z.A. (1971) J. Exp. Med. 134: 1545.

Werb, Z. & Cohn, Z.A. (1972) J. Biol. Chem. 247: 2439.

Williamson, D.H. (1974) In: Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.) Academic Press, London & New York.

Williamson, D. H. & Hems, R. (1970) In: Essays in Cell Metabolism (Bartley, W., Kornberg, H.L., Quayle, J.R., eds.) Wiley, London.

Williamson, D.H. & Krebs, H.A. (1961) Biochem. J. 80: 540.

Williamson, D.H. & Mellamby, J. (1974) In: Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.) Academic Press. London & New York.

Williamson, D.H., Lund, P. & Krebs, H.A. (1967) Biochem. J. 103: 514.

Williamson, D.H., Bates, M.W., Page, M.A. & Krebs, H.A. (1971) Biochem. J. 121: 41.

Williamson, D.H., Krebs, H.A., Stubbs, M., Page, M.A., Morris, H.P. & Weber, G. (1970) Cancer Res. 30: 2049.

Windmueller, H.G. (1982) In: Advances in Enzymology (Meister, A., ed.) John Wiley & Sons, London.

Windmueller, H.G. (1984) In: Glutamine Metabolism in Mammalian Tissues (Häussinger, D. & Sies, H., ed.) Springer-Verlag, Berlin-Heidelberg-New York- Tokyo.

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

Windmueller, H.G. & Spaeth, A.E. (1975) Arch. Biochem. Biophys. 171: 662.

Windmueller, H.G. & Spaeth, A.E. (1978) J. Biol. Chem. 253: 69.

Zammit, V.A. & Newsholme, E.A. (1976) Biochem. J. 160: 447.

Zammit, V.A. , Beis, I. & Newsholme, E. A. (1978) Biochem. J. 174: 989.

Zammit, V.A. & Newsholme, E.A. (1979) Biochem. J. 184: 313.

Zielke, H.R., Zielke, C.L. & Ozand, P.T. (1976) Fed. Proc. Fed. Am. Soc. Exp. Biol. 43: 121.

Zielke, H.R., Ozand, P.T., Tildon, J.T., Serdalian, D.A. & Comblath, M. (1978) J.Cell Physiol. 95: 41.

Zielke, H.R., Ozand, P.T., Tildon, J.T., Serdalian, D.A. & Comblath, M. (1976a) Proc. Natl. Acad. Sci.(USA) 73: 4110.

Zielke, H.R., Sumbilla, C.M., Sevdalian, D.A., Hawkins, R.L. & Ozand, P.T. (1980) J. Cell Physiol. 104: 433.

Zielke, H.R., Sumbilla, C.M., Zielke, C.L., Tildon, J.T. & Ozand, P.T. (1984) In: Glutamine Metabolism in Mammalian Tissues (Häussinger, D. & Sies, H., eds.) Springer-Verlag, Berlin-Heidelberg-New York-Tokyo.