UTILISATION OF RESPIRATORY SUBSTRATES BY RESTING

AND CONCANAVALIN A-ACTIVATED RAT SPLENOCYTES

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ABSTRACT.

The in vitro culture of resting and Concan-
avalin A activated rat spleen lymphocytes has been
carried out with the aim of identifying respiratory
substrates used in the generation of additional energy
required for lymphocyte activation. Previous work in
this field had concentrated on the role of glucose but
very little is known of the contribution of alternative
substrates.

The long term (72h) and short term (24h)
culture of rat splenocytes necessitated a prior investi-
gation of the effect of empirical culture conditions on
mitogenic stimulation. Cell activation was assessed by
the incorporation of $[^3H]$-thymidine into DNA in long term
cultures, and by $[^3H]$-leucine incorporation into protein
in short term cultures.

Patterns of consumption of glucose, glutamine
acetoacetate and D β-hydroxybutyrate were assessed in
long term cultures; the uptake of glucose and glutamine
was shown to alter on mitogenic stimulation. In addition,
the presence of glutamine was shown to be obligatory to
long term activation. The ketone bodies showed very little
uptake at physiological concentrations; this was not
affected by Con A or by prior starvation of the cells.

The metabolism of glucose in 72h cultures was
shown to be profoundly affected by the prevailing culture
conditions. Conventional activation of the cells was
demonstrated under conditions which were associated with
both aerobic and partially anaerobic glucose metabolism;
the consumption of glucose by splenocytes could thus not
be used as an index of mitogenic stimulation.

The detailed examination of glucose and glutamine metabolism was carried out using radiolabelled substrates in 24h cultures. The complete oxidation of glucose may contribute up to 70% of glucose derived ATP and that of glutamine up to 65% of glutamine derived ATP.

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ABBREVIATIONS.

AcAcO	Acetoacetate
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
BSA	Bovine serum albumin
cAMP	Adenosine 3':5-phosphate
cGMP	Guanosine 3':5-phosphate
Con A	Concanavalin A
$\underline{D}$ β-HB	$\underline{D}$ β-Hydroxybutyrate
EDTA	Ethylenediaminetetraacetic acid
Glc	Glucose
Gln	Glutamine
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid
Lac	Lactate
LPS	Lipopolysaccharide
NAD^+	Oxidised β-nicotinamide-adenine dinucleotide
NADH	Reduced β-nicotinamide-adenine dinucleotide
$NADP^+$	Oxidised β-nicotinamide-adenine dinucleotide phosphate
NADPH	Reduced β-nicotinamide-adenine dinucleotide phosphate
PHA	Phytohaemagglutinin
PPO	2,5-diphenyloxazole
PWM	Pokeweed Mitogen
PBL	Peripheral Blood Lymphocytes
TCA	Trichloroacetic acid
Tdr	Thymidine
Tris	2-amino-2-hydroxymethyl propan-1,3-diol.

1. INTRODUCTION.

1.1. MITOGENIC LYMPHOCYTE STIMULATION

Lymphocyte stimulation is the process where-
by thymus derived T lymphocytes and bone-marrow derived B
lymphocytes (in mammals) are challenged by antigen and
subsequently undergo extensive morphological and metabolic
changes (Yoffey,1965; Douglas,1972; Wedner and Parker,
1976; Hume and Weidemann,1980). These changes result in
the formation of activated cells with distinct specialised
roles, and as such both T and B lymphocytes are of central
importance to the generation of an immune response. On
antigenic challenge B cells develop via activation, prol-
iferation and differentiation into antibody secreting
cells (Falkoff et al.,1982; Kishimoto,1985), while T cells
develop specialised effector roles. Suppressor and helper
T cells are responsible for the manipulation of the B cell
response via lymphokines (Howard and Paul, 1983) and
cytotoxic T cells participate in cell-mediated functions
such as graft rejection and the direct elimination of
infected cells (Nabholz and MacDonald,1983).

The sensitivity of the immune response is
based in the antigen specificity of lymphocyte activation.
This property renders activation by antigen extremely
difficult technically to study in vitro; the very small
proportion of a cell population which would be responsive
to a given antigen means that the biochemical and immuno-
logical changes occurring in responding cells would be
lost in a culture of predominantly non-responsive cells,
(Ada,1971). In addition, prior sensitisation of the host
with antigen is required to obtain detectable stimulation
in vitro, and the response may take four to seven days to
come into effect.

Although stimulation by specific antigen in vitro would be an ideal sytem in which to study the biochemistry of the activation process, it's usefulness is severely limited by the antigen specificity described and by the restraints inherent in the level of sensitivity of most biochemical techniques. This situation was improved when work by Nowell (1960) led to the fortuitous discovery of a now universal tool which goes some way toward alleviating these problems. Using the plant lectin phytohaemagglutinin (PHA) from the red kidney bean Phaseolus vulgaris to remove contaminating red blood cells from a leucocyte preparation, Nowell showed that cells prepared by this method were unusually mitotic in culture. Investigation of the culture components identified PHA as responsible for the initiation of mitosis in a large proportion of lymphocytes.

Since this finding many other plant lectins (Sharon and Lis,1972) and other classes of compounds have been shown to induce lymphocyte activation in vitro, regardless of their antigen specificity (Table 1). All mitogens have this property and it is undoubtedly a major reason for their use. As a result, a large proportion of cells are activated and biochemical changes can be detected more easily. In addition, the technique can be carried to completion in three days in vitro.

Mitogens thus have considerable technical advantages over antigen for the in vitro study of lymphocyte activation. Of considerable importance is that the metabolic changes induced by mitogenic stimulation should also be those which would occur in an antigen specific response. However, since mitogen binding to membranes probably involves binding to carbohydrate moieties on a

TABLE 1. Examples of lymphocyte mitogens.

MITOGEN	SOURCE	SPECIFICITY	REFERENCE
Lectins:			
Phytohaemagglutinin	Phaseolus vulgaris	T	Naspitz & Richter,1968
Concanavalin A	Canavalia ensiformis	T	Sharon & Lis,1972
Pokeweed Mitogen	Phytolacca americana	T B	Farnes et al.,1964
Bacterial Products:			
Lipopolysaccharide	Gram negative bacteria	B	Greaves & Janossy,1972
Protein A	Staph. aureus	T B	Sakane & Green,1978
Ca^{2+} Ionophore	Strep. sp.	T B	Reed & Lardy,1972; Jensen et al.,1977
Miscellaneous:			
Periodate		T B	Novogrodsky & Katchalski,1971
Galactose oxidase/			Novogrodsky & Katchalski,1973
Neurominidase		T B	
Heavy Metals	Zn^{2+} Hg^{2+}	T B	Kirchner & Ruhl,1970; Hutchinson et al.,1973
Monoclonal Antibodies	OKT3	T	Kung et al.,1979
	FMC-1	B	Brooks et al.,1980

number of surface molecules it is perhaps likely that some of the subsequent alterations in metabolism are irrelevant to the activation process.

After inital binding of mitogen, lymphocytes proceed through a complex series of metabolic reactions including enhanced protein, RNA and DNA synthesis which culminate in cell division (Hume and Weidemann,1980). The intermediary reactions will be considered in more detail in the following section, but it is the final increase in DNA synthesis which forms the conventional assay of mitogenic lymphocyte stimulation. Rapidity and ease of execution have led to the use of increased incorporation of radiolabelled thymidine into newly synthesised DNA as a sensitive index of activation (Dutton and Pearce, 1962).

Despite the fact that mitogens bypass the antigen specificity of lymphocyte stimulation, they do show some degree of subpopulation selectivity which ultimately depends on the mitogen employed. Con A and PHA are both T cell mitogens, PWM stimulates T and B cells in many species, while LPS is exclusively a B cell mitogen (Stobo,1972; Greaves and Jannosy,1972; Andersson et al., 1972a). However, responses seen in a mixed culture of T and B cells should not be solely attributed to one cell type as activated T cells, for example, can produce factors which may initiate activation of other cells in culture (Andersson et al.,1972b; Moller,1982).

The molecular basis for the activation of lymphocytes by mitogens is not clear but various lines of evidence suggest that, while binding of mitogen is a prerequisite to cell activation, it is not sufficient to elicit the entire response. Wheat germ agglutinin is a

lectin which binds to lymphocytes and causes a degree of aggregation also seen with other lectins, but it is not mitogenic (Reichert et al.,1972; Greene et al.,1976). Interestingly, this lectin also produces changes in intracellular free calcium concentration and phosphatidyl-inositol metabolism comparable to that caused by Con A, and yet does not elicit an increase in DNA synthesis in mouse thymocytes (Hesketh et al,1983). Con A and PHA both bind to B cells under precise conditions but do not activate them (Andersson et al.,1972a; Greaves and Janossy 1972; Greaves and Bauminger,1972). Since binding of the mitogen to lymphocyte membranes alone does not predict the outcome of the interaction, and as they bind to a number of surface molecules, it is probable that lectin binding may cause sufficient perturbation of the plasma membrane to cause responses not truly associated with lymphocyte activation. The recent elucidation of the protein nature of the T lymphocyte receptor for antigen has revealed the presence of a monomorphic protein and antigen specific heterodimer (McIntyre and Allison,1983; Meuer et al,1983; Samelson et al.,1983). It is likely that monoclonal antibodies raised against these proteins will aid in the dissection of true receptor-associated events on activation of heterogenous T cell populations and cloned lines, respectively.

The discovery of polyclonal mitogens has thus allowed the study, under controlled conditions, of some of the biochemical and immunological bases of lymphocyte activation. The "semi-specificity" of some mitogens has also allowed the definition of qualitative roles for T cells and their subsets. For example, it is known that PHA and Con A, although both are T cell mitogens, activate different subpopulations (Hume and Weidemann,1980). In

addition, it appears that Con A can activate both T-helper and T-suppressor cells at different doses (Persson et al., 1978). From a clinical point of view, mitogenic lymphocyte stimulation has been used to assess the function of lymphocytes from patients with a variety of immune-associated diseases. These include cancer (Tavadia et al., 1974); Yonkosky et al.,1978; Pluzanska, 1978), autoimmune diseases (Horwitz,1976; Haines and Hough,1978), immuno-deficiency disease (Carson et al.,1976) and infectious disease (Kirkpatrick,1978). Subtle changes in the ability of lymphocytes from these patients to respond normally to mitogens has given insight into the relationship between the disease and the activity of the immune system. Moreover, since the method provides an indication of the functional capacity of lymphocytes it has additional advantages over mere quantitation of T and B cells in clinical states.

Immunologically, in vitro lymphocyte activation has contributed to the understanding of the roles of accessory cells and lymphocyte subpopulations in the response. In general, the cell requirement for a mitogenic response depends on the mitogen; the removal of T cells from a spleen population, for example, suppresses the response to PWM (Weksler,1973). Macrophage depletion leads to a suppression of the responses of both T and B cells to mitogen (Persson et al.,1978). In addition, macrophages are more stringently required by the T cell response to Con A than PHA (Arala-Chaves et al.,1978). This may indicate that the activation signal delivered by the two mitogens is not identical.

Studies on the soluble products of activated lymphocytes have identified a number of lymphokines having a variety of roles in the modulation of the immune

response (Jacobsson and Blomgren,1977; Smith et al.,1979; Watson,1979; Larsson et al.,1980; Moller,1982). The ability to produce these factors in the quantities needed for the characterisation of physical and biochemical properties has been assisted by the large scale culture of mitogenically stimulated lymphocytes.

The biochemical interest in lymphocytes lies in the fundamental importance of the cells in the immune system as a whole, and in the use of their response to mitogens as a model for biochemical control of cell growth and proliferation. The understanding of lymphocyte metabolism in both resting and activated states will shed light on many clinical states; the value of this in potential therapeutic management is self evident.

The mechanism by which antigen binding to a lymphocyte receptor triggers the initiation of cell activation, and the subsequent translocation of that signal to the nucleus may be expected to occur in a manner similar to that which functions in other ligand-activated cell types. There is no reason why the intracellular regulation of activation in lymphocytes should be profoundly different to that in other cells, and thus knowledge gained may aid in the understanding of growth and proliferatory control in many mammalian cells. This is supported by the recent uncovering of the involvement of protein kinase C and inositol triphosphate in signal transduction in a variety of cell types which undergo stimulus-secretion coupling processes (Nishizuka,1984; Berridge,1984). These include liver in response to hormones (Burgess et al.,1984; Charest et al.,1985), platelets in response to thrombin (Lapetina,1985) and also in lymphocytes in response to mitogens (Taylor et al.,1984; Kaibuchi et al.,1985).

The continued study of the basic metabolism of

a cell with such a fundamentally important role is thus
justified. It is evident that there are drawbacks to the
use of non-specific mitogens as a model for the antigen-
specific situation, but these are likely to be overcome
by the future use of monoclonal antibodies directed at the
antigen receptor. It is undoubtedly true, however, that
the use of mitogens has considerably advanced knowledge
of the metabolism of activated lymphocytes.

1.2. BIOCHEMISTRY OF LYMPHOCYTE ACTIVATION

Following the incubation of resting lymphocytes
with a mitogen a considerable number of qualitative and
quantitative metabolic changes occur as a prelude to
eventual DNA synthesis and cell division (For reviews see
Wedner and Parker,1976; Hume and Weidemann,1980). As
would be expected, the earliest recorded changes in meta-
bolism have been those occurring at the membrane; these
include alterations in monovalent and divalent cation
fluxes, increased transport of small molecules such as
glucose and amino acids, and the turnover of membrane
components. Some of these are detailed in Table 2; changes
occurring in the cytoplasm and nucleus are given in Table
3. They are not an exhaustive documentation of such
changes but serve to illustate the variety of findings
with different mitogens and cells from various sources.

It is perhaps the area of plasma membrane-
associated events in lymphocyte activation which has been
the subject of the most intense activity, and this is
reflected in the lack of consistency particularly in the
time courses of putative alterations. The wealth of
literature available is a direct result of the search for
an event or combination of events which convey the initial
binding signal into the cell to trigger the activation
process. Ca^{2+} influx, in particular, is considered likely

Table 2. Plasma membrane-associated events on lymphocyte activation.

PROCESS	MITOGEN EFFECT	REFERENCE
1) Na$^+$ K$^+$ Transport	↑K$^+$ uptake within 1h	Quastel & Kaplan,1970
	↑K$^+$ uptake within 5min;	Averdunk & Lauf,1975
	↑Na$^+$ efflux	
	↑K$^+$ membrane channels	Hardy et al.,1984
2) (Na$^+$K$^+$) ATPase	↑activity of both	Pommier et al.,1975
(Na$^+$K$^+$) Indep.	↑activity of independent	Novogrodsky,1972
ATPase activities	process only	
	No change in activity	Negendank & Collier,1976
	of either enzyme	
3) Ca^{2+} Transport	↑uptake within 80min	Allwood et al.,1971
	↑over 60h;↓K$_m$ V$_{max}$ same	Whitney & Sutherland,1973
	↑uptake within seconds;	Freedman et al.,1975
	'gated' response	
	↑uptake corresponds to	Parker,1974
	mitogenicity	

TABLE 2 (CONTINUED)

PROCESS	MITOGEN EFFECT	REFERENCE
	↑uptake corresponds with supra-optimal mitogen dose	Hesketh et al.,1977
	Non-mitogenic lectin WGA also elicits ↑ in free Ca^{2+}	Hesketh et al.,1983
	↑Ca^{2+} channels at membrane	Birx et al.,1984
4) Amino acid Transport	↑within 1h	Peters and Hausen,1971a
	↑rate but no change in affinity of transport	Van den Berg and Betel,1973 1974
	↑rate of natural and ASC transport systems	Borghetti et al.,1979;1981
5) Glucose Transport	↑rate using 3-O-MeGlc, facilitated diffusion	Peters and Hausen,1971b
	↑V_{max}, K_m remain unchanged	Reeves,1975
	↑within 10min with A23187	Whitesell et al.,1977 Yasmeen et al.,1977

TABLE 2. (CONTINUED)

PROCESS	MITOGEN EFFECT	REFERENCE
6) Phospholipid Metabolism	↑ turnover PI	Crumpton et al.,1976
	↑ ^{32}P incorporation within 5min	Fisher and Mueller,1971 Allan and Michell,1977
	↑ phosphorylation	Taylor et al.,1984

NOTE: ↑ increase; ↓ decrease; 3-O-MeGlc 3-O-Methyl glucose; A23187 calcium ionophore; PI phosphatidylinositol.

Table 3. Cytoplasmic and nuclear events on lymphocyte activation.

PROCESS	MITOGEN EFFECT	REFERENCE
1) Protein Metabolism	↑Protein synthesis	Fillingame & Morris,1973
		Wettenhall & London,1974
	↑Protein phosphorylation	Wang et al.,1981
		Ramanadham et al.,1984
	↑Protein turnover	Tanaka & Ichihara,1976
2) Cyclic nucleotide Metabolism	↑cAMP 50x within 15min	Lönroth & Lönroth,1977
	↑cAMP at supraoptimal mitogen doses only	Lyle & Parker,1974
	↑cAMP at supraoptimal doses, no change at mitogenic doses	Weber & Goldberg,1976
	↑and ↓cAMP required for DNA synthesis	Wang et al.,1978
	.↑cGMP only	Haddox et al.,1976;
		Hadden et al.,1972,1976

Table 3. cont.

PROCESS	MITOGEN EFFECT	REFERENCE
	No change cAMP or AC activity	Novogrodsky & Katchalski, 1970
	↑cGMP dependent on Ca^{2+} uptake via GC activity	Katagiri et al., 1976
4) RNA Metabolism	↑RNA polymerase activity	Handmaker & Graaf, 1970
	↑activity from 4h	Cooke & Kay, 1973
	↑rate of polyadenylation	Hauser et al., 1978
	↑rate of transport from nucleus to cytoplasm	Mitchell et al., 1978
	↑RNA synthesis within 1h	Rubin & Cooper, 1965
5) DNA Metabolism	↑DNA polymerase activity	Tyrsted et al., 1973
	↑DNA Pol activity correlates with ↑Tdr incorporation	Loeb et al., 1968
	↑dATP and dTTP pool size parallels DNA synthesis	Munch-Petersen et al., 1973
	↑thymidine kinase, dcytidine kinase	Pegararo & Bernengo, 1971
	↑pool size of dGTP and dCTP	Tyrsted, 1975

↑ Increase ↓ Decrease   AC Adenyl Cyclase   GC Guanylate cyclase.

to be involved in this. An increase in intracellular free Ca^{2+} has consistently been noted as an early manifestation of activation (Table 2); Ca^{2+} must be present in the medium for stimulation to occur (Maino et al.,1974) and the Ca^{2+} ionophores ionomycin (Lyall et al. 1980) and A23187 (Luckasen et al.,1974) are both mitogenic. These observations are not without controversy, however, as an increase in intracellular free Ca^{2+} also occurs in rat thymocytes on binding of the lectin wheat germ agglutinin, but this does not result in long term activation (Hesketh et al.,1983). It is perhaps not surprising that the measurement of early membrane related phenomena are accompanied by such inconsistencies, as the changes occurring are often small and transient. Many additional problems in the analysis of data can result from the variety of methods employed. Examples of this may be the mitogen used and the dose required to elicit 'optimal' stimulation; the heterogeneous nature of the cell population under study; species differences and the method of cell preparation and subsequent culture.

Differences in the method of cell preparation and the purity of the resulting lymphocytes have been cited as possible reasons for the discrepancies in recorded changes in the levels of cyclic nucleotides. There are two schools of thought with respect to the increase in the intracellular concentration of cGMP; increases have been reported within minutes of mitogen binding to lymphocytes (Hadden et al.,1972; 1976; Haddox et al.,1976; Katagiri et al.,1976). In contrast, no significant increase could be detected by workers using a variety of species and organs as lymphocyte sources (Wedner et al.,1975; Weber and Goldberg-1976; Derubertis and Zenser,1976; Greene et al.,1976). Species differences,

accessory cell contamination and the method of cyclic
nucleotide extraction have all been suggested to be
responsible for these discrepancies.

Evidence is also available for a mitogen
induced increase in cAMP levels in lymphocytes (Lyle and
Parker,1974; Lönroth and Lönroth,1977). In general, how-
ever, most documented evidence concurs on the fact that
increased levels of cAMP are generally found to be assoc-
iated with the use of supra-optimal mitogen doses (Weber
and Goldberg,1976; Hadden et al.,1976; Watson,1976; Haddox
et al.,1976). In view of the general anti-anabolic role
of cAMP it is possible that, if an increase in the level
does occur on activation, it is involved in the prevent-
ion of a continued triggering signal after the initial
event has occurred at the cell membrane.

There is perhaps no reason why the use of
different mitogens and cell populations should not result
in confliction of results, in quantitative terms, in early
membrane changes and subsequent intracellular reactions.
However, if a putative change is truly associated with
lymphocyte activation it would surely be expected to
occur in all activated lymphocytes. Thus, in qualitative
terms the same response would occur in different cell
populations. A mixed culture of T and B cells from the
spleen, for example, may respond differently to a T cell
mitogen such as Con A than to a T and B cell mitogen like
PWM, but only in quantitative levels. The presence of
accessory cells in a lymphocyte population may also
influence the recorded changes in response to an activa-
ting agent. Depletion of macrophages from a human periph-
eral blood cell preparation results in long term inhibition
of the response to PHA (Kaibuchi et al.,1985). Since these
cells supply the lymphokine interleukin-1, which is needed

for activation, their absence would be expected to affect
the changes occurring in the lymphocyte population.

Thus, comparison of results obtained using a
variety of mitogens and cell populations is made more
difficult in the absence of clearly defined preparative
techniques for lymphocytes and subsequent cell culture. In
addition, since the nature of the signal delivered by
different mitogens is not known, it is also possible that
they differ in the reactions they elicit by virtue of
non-specific membrane binding.

Very high doses of mitogen are routinely
employed in the analysis of data from short term lymphocyte
culture, in order to elicit a response in a large proport-
ion of cells. By contrast, long term cultures use much
lower doses as the optimal response is measured at a much
later incubation time. A major complication of very short
term lymphocyte incubations is that at least some of the
biochemical changes occurring prior to commitment of the
cells to division atapproximately 24h may not be truly
associated with the activation process. Furthermore, such
changes may be erroneously considered to be an integral
part of growth, when they may actually be accompanied by
the return of the cell to a resting state or high dose
inhibition of the response. Unless short term cultures
are accompanied by concomitant assays of protein or RNA
synthesis to confirm the stimulated state of the cells,
changes may be assigned to the activation sequence merely
because they occur in response to a mitogen, without
considering the metabolic state of the cells. Since it is
not yet known which early events are an integral part of
the process by which cells become committed to cell
division, it may be argued that all short term incubations

should be accompanied by cultures which are allowed to continue to a stage where enhanced protein or RNA synthesis can be measured. Short term incubations are also complicated by the possibility of recorded alterations being due to membrane perturbation by mitogen binding; an example of this may be the activation of intrinsic membrane proteins such as adenylate cyclase leading to an increase in the levels of cAMP. In such cases it may be impossible to distinguish between those changes which are truly activation associated and those which are a direct result of non-specific membrane perturbation.

The designation of 'optimal' and 'supra-optimal' mitogen doses in short term cultures becomes arbitrary if they are based on the amplitude of the reaction being investigated. If the 'optimal' dose is considered to be that eliciting the highest response, then this should also be shown to be capable of activation as a long term event. In this respect, it is equally misleading when such results are presented but have been carried out under an entirely different set of culture conditions from those on which the original response was measured. This confirms the capacity of the cells to be stimulated, but it does not necessarily follow that the short term cultures would have resulted in this.

It is undoubtedly true that knowledge derived from short term lymphocyte incubations is essential to the complete understanding of the activation process, but the complications described above could well be lessened by assays of activation which are performed at the time of sampling. Both enhanced protein and RNA synthesis can be detected within hours of mitogen binding to lymphocytes.

Many of the points discussed here will be

further established with respect to results obtained in
the present study, but they serve here to illustrate the
potential complexity of interpretation of data from
studies using a variety of methods. The need to fully
investigate the prevailing culture conditions is evident.
The exact protocol being followed will depend on the aim
of the study, and it is therefore pertinent to question
whether mitogenic lymphocyte stimulation should be viewed
as an immunological system which lends itself to bio-
chemical studies, or as a model of the activation and
proliferation of mammalian cells which has inherent
immunological interests. This must be subjective but it
will surely influence the order of priority given to
the maintenance of certain culture conditions. For example
it is usual to express the viability of freshly prepared
lymphocytes, but reports of cell death throughout the
subsequent culture period are sparse. This may not be
important if all one is measuring is the relative
response of a cell population to different mitogens, in
which case it may be assumed that the cells are subject
to the same conditions, but cell viability becomes more
important if quantitative estimations of metabolism are
made. The importance of viability and aerobic culture
conditions will be more relevent to metabolic studies
than to purely immunological investigations, but in both
situations priority should be given to obtaining data
which confirms the stimulated state of the cells. Thus, a
study on an intracellular event which occurs within hours
of contact with mitogen may ignore the need to show that
this correlates with stimulation as a long term event.

There is a definite need to strike a balance
between obtaining a good immunological model of lymphocyte
stimulation in vitro, while remaining aware of the

possible repercussions of conditions so imposed on the
metabolic capabilities of the cells. This is particularly
important as cells in culture undoubtedly have certain
idiosyncratic responses which may not occur in vivo.

1.3. METABOLISM OF RESPIRATORY SUBSTRATES BY LYMPHOCYTES.

Despite the fact that considerable attention
has been paid to many aspects of the metabolism of activ-
ated lymphocytes, the pathways by which the increased
energy demands for activation are satisfied remain large-
ly unresolved. Glucose has, understandably, commanded the
most attention as a potential source of ATP, but the
observation that a large proportion of consumed glucose
is metabolised only as far as lactate by activated lympho-
cytes has made the role of enhanced glycolysis in these
cells a source of controversy (Hume and Weidemann,1979).
This will be discussed in greater detail in the following
section, but the propensity of activated lymphocytes to
metabolise glucose by this pathway may have detracted from
the possible involvement of other substrates in the
supply of energy. It is reasonable to assume that lympho-
cytes, in common with other mammalian cells, will require
alternative energy sources at times of low carbohydrate
availability, and it is therefore of interest to assess
the contribution of those substrates which are known to
be mobilised under these conditions in vivo.

Conditions such as insulin deficiency or
insensitivity, starvation and high lipid diets are known
to be associated with an increase in the circulating
concentrations of the ketone bodies. In humans the normal
plasma concentration of total ketone bodies (both aceto-
acetate and D β-hydroxybutyrate) is less than 0.01mM; this
rises to approximately 2.9mM after 96h starvation (Cahill

et al.,1966). The corresponding levels in the rat are
0.2mM in the fed state, rising to approximately 2.8mM
after 48h starvation (Berry et al.,1965; Newsholme and
Start,1973). The major importance of the ketone bodies
is that they represent an alternative oxidisable fuel
to glucose at times of low carbohydrate availability.

The possibility that the amino acid L-
glutamine has a role in energy metabolism in cultured
cells is a relatively recent proposal. Work by Windmue-
ller and Spaeth (1974;1975;1978;1980) has shown that
glutamine is a major respiratory substrate for the iso-
lated rat small intestine. Of total CO_2 evolved from this
organ in the presence of glucose, ketone bodies and glu-
tamine, approximately a third was derived from glutamine
oxidation. Thus, the amino acid was the preferred fuel
of respiration in this tissue; this was also shown to be
the case in vivo (Windmueller and Spaeth,1978;1980).
Confirmation that glutamine can also be oxidised by
cultured cells came with the observation that dividing fibro-
blasts could oxidise various respiratory substrates in
addition to glucose; glutamine was oxidised at a greater
rate than the ketone bodies, free fatty acids and glucose
(Sumbilla et al.,1981).

In optimal culture media for in vitro study of
mammalian tissues and cells, glucose has traditionally
been considered to be the energy source available to the
cells, and glutamine a supply for biosynthetic purposes.
The fact that other respiratory substrates are not
routinely added to the media, and are not obligatory
to the maintenance of cell viability does not necessarily
preclude a role for them in the energy metabolism of the
cells. Their contribution to lymphocyte metabolism, and

in particular their possible roles on mitogenic lymphocyte
stimulation is therefore of interest.

1.3.1. GLUCOSE

Glucose has long been considered to be the
major energy providing substrate for a variety of
cultured cells (Paul,1965). The observation that cultur-
ed leucocytes show high glycolytic activity, even under
aerobic conditions (Warburg,1938) initiated a wealth of
work on the role of glycolysis in the energy metabolism
of malignant and non-malignant cells (Paul,1965; Morell
and Froesch,1973; Morgan and Faik,1981). The term aerobic
glycolysis has consistently been used to describe the
tendency of such cells to metabolise glucose to lactate
despite the presence of functionally intact mitochondria
and concomitant oxygen consumption; this is also true of
the activated lymphocyte (Wang et al.,1976; Hume et al.,
1978). Enhanced aerobic glycolysis occurring in mitogen
activated lymphocytes, however, has also been suggested
to have its basis in biosynthesis by maintaining an
elevated level of glycolytic intermediates, rather than
for energy production (Hume et al.,1978; Hume and
Weidemann.1979). In view of this proposal, it is more
surprising that the examination of alternative pathways
of energy production in lymphocytes has received relative-
ly little attention.

Increased aerobic glycolysis has been demon-
strates in lymphocytes from a variety of sources using
a number of different mitogens; some examples are given
in Table 4. Resting lymphocytes show very little tend-
ency to metabolise glucose by this route, but on activ-
ation the transfer in metabolism is immediate. The
stoichiometric conversion of glucose to lactate induced by PHA
in human thoracic duct lymphocytes was one of the

first instances in which this was shown. In this case,
the result was attributed to anaerobiosis as the cells
settled to the base of the culture vessel (Cooper et al.,
1963). Table 4 illustrates the variation in the extent to
which the increase in metabolism of glucose to lactate
occurs after mitogen activation, but this is a quantit-
ative, rather than qualitative variation.

Several trends may be noted between these
parameters and the experimental conditions prevailing.
Lymphocyte cultures lasting only a few hours tend to
show slightly more consistency in the levels of glucose
consumed and the increase in lactate production, compared
with longer incubations. This may be due to the greater
variety of conditions employed in the latter studies, and
the range of donor cell sources. Also, the high initial
glucose concentrations used in long term lymphocyte cult-
ures (11m$\underline{M}$) may increase the absolute glucose consumption
over that seen at the more physiological concentrations
used for short term incubations (Hedeskov and Esmann,1967).
Lymphocyte preparations vary in their contamination with
red blood cells and since these cells are highly glycoly-
tic their presence may affect the absolute glucose uptake
and degree of lactate production in lymphocyte cultures.
An estimation of the contribution of red blood cells to
glycolysis can be made, assuming a red blood cell rate of
glucose consumption of approximately 1nmol/min/10^8cells
(Rapoport,1969). The equivalent rate by lymphocytes,
based on the range of rates found in the studies cited in
Table 4, is between 4.6 and 85.7 nmol/min/10^8cells
(Culvenor and Weidemann,1976; Sagone et al.,1974). Thus,
a lymphocyte preparation which is 5% contaminated by red
blood cells would exhibit a rate of glucose consumption
of which less than 1% could be attributed to the contam-

TABLE 4. VARIATION IN GLUCOSE UPTAKE AND LACTATE PRODUCTION

BY MITOGEN ACTIVATED LYMPHOCYTES

MITOGEN	CELL SOURCE	CHANGE WITH MITOGEN	REFERENCE
PHA	Human thoracic duct	↑Glc to Lac 100%	Cooper et al.,1963
PHA	Human PBL	↑Glc to Lac x2 at 26h, x3 at 72h.	Parenti et al.,1966
PHA	Horse PBL	↑Glc to Lac x4	Pachman,1967
PHA	Human PBL	↑Lac produced x3 at 24h, x 13 at 96h.	Polgar et al.,1968
PHA	Human PBL	↑Glc to Lac x2 at 2.5h, x5 at 72h.	Rabinowitz et al,1968
PHA	Human PBL	↑Glc to Lac x2.	Hedeskov,1968
PHA	Human PBL	↑Glc to Lac x4 at 4h, x4-7 at 24-72h.	Roos and Loos,1970 1973a,b
PHA	Human PBL	↑Glc consumption x3 at 0-24h, x9 at 24-48h, x8 at 48-68h.	Sagone et al.,1974

TABLE 4. CONTINUED

MITOGEN	CELL SOURCE	CHANGE WITH MITOGEN	REFERENCE
Con A	Mouse splenocytes	Small ↑ in Lac production up to 20h, then ↑ x20 at 50-60h.	Wang et al.,1976
Con A PHA	Rat thymocytes	↑Glc uptake x2; Lac produced x2.6 with PHA. Glc uptake ↑ x2.4; Lac produced x3.6 with Con A, 8h.	Culvenor and Weidemann, 1976
Con A	Mouse thymocytes	↑in Lac output linear x2. 95% Glc to Lac, 24h.	Lengle et al.,1978
Con A	Rat thymocytes	↑Glc uptake x2, Lac x3, 3h.	Hume et al.,1978
Con A	Mouse splenocytes	↑Lac 1.5x after 1h	Nista et al.,1980
Con A	Rat mesenteric lymph nodes	↑Glc uptake x1.5, Lac x1.8 after 1h.	Ardawi and Newsholme, 1984
Con A	Rat thymocytes	↑Glc uptake x1.9, Lac x2.7 after 3h.	Brand et al.,1984

inating cells. Red blood cell contamination would thus
not appear to be a major source of error in the case of
activated rates of lymphocyte glucose consumption, but
as these calculations are based on the activated rate it
is evident that the proportion of glucose consumption
attributable to red blood cells in resting lymphocyte
culture would be much greater as the absolute of
glucose uptake in the resting state is very much less
than in the activated state.

While the measured rates of conversion of
glucose to lactate by cells in culture are an indication
of the degree of aerobicity of the culture environment
at least in the case of resting lymphocytes, the cell
densities and culture vessels used in the study of
glucose metabolism often imply that a certain degree of
anaerobiosis may exist within the cellular microenviron-
ment. The extent to which this may affect the studies
cited in Table 4 is not known, but many of the early
studies in particular appear to have been carried out
under conditions which would result in the formation of
a cell button, and consequent anaerobiosis. If this is
the case, it is probable that any effect would be more
pronounced in activated cultures as the absolute glucose
consumption is higher.

In general, approximately 50% of consumed
glucose is converted to lactate by activated lymphocytes.
The net yield of ATP on a molar basis from this pathway
is only 2, compared with 38 from the complete oxidation
of glucose to CO_2. This strongly supports the view that,
if aerobic glycolysis is a physiological response, it is
unlikely to have its basis in energy provision alone.

The fate of the balance of consumed glucose should also be considered; possible oxidative pathways include the tricarboxylic cycle and the hexose monophosphate shunt. In addition, glucose carbon may also be incorporated into macromolecules such as glycogen and lipids, in which case it would contribute incompletely or not at all to energy production.

Polgar et al.(1968) could find no evidence for an increase in oxygen consumption on PHA stimulation of human peripheral blood lymphocytes, implying that an increase in respiration does not occur on mitogen activation. By contrast, Roos and Loos (1973a,b) using the same system, found a two-fold increase in oxygen uptake after 24h and a three-fold increase after 72h. Rat thymocytes stimulated with Con A and PHA showed a 10-15% increase in oxygen uptake compared with resting cells (Culvenor and Weidemann,1976). Resting peripheral blood lymphocytes were found by Hedeskov and Esmann (1967) to metabolise only 1% of consumed glucose through the TCA cycle. On stimulation with PHA these cells were found to evolve 30-40% more CO_2 than in the resting state, indicating an increase in the activity of both the TCA cycle and pyruvate dehydrogenase complex (Hedeskov,1968). Thus, the evidence suggests that a combination of increased CO_2 evolution and oxygen consumption is a result of enhanced oxidative activity in mitogenic stimulation.

Both enhanced glycolysis and TCA cycle activity have been shown to be essential to three day in vitro stimulation of lymphocytes; either pathway alone could not support long term activation of human peripheral blood lymphocytes, but was able to initiate activation (Roos and Loos,1973b). Calculations of glucose-derived

energy production showed that 70% of consumed glucose was converted to lactate, producing 15% of total ATP, and the remaining 30% of consumed glucose was fully oxidised, producing up to 85% of glucose-derived ATP by oxidative phosphorylation. Thus, even though glycolysis is quantitatively the major fate of glucose, its relative contribution to energy production is small. This was also found to be the case for mouse thymocytes stimulated with Con A (Lengle et al.,1978). In this study 23% of ATP produced from glucose by resting cells was derived from glycolysis and the balance from complete oxidation; in Con A stimulated cells the proportion derived from glycolysis was slightly greater.

The hexose monophosphate shunt is an important route for the production of ribose moieties from glucose; in view of the requirement for this pathway in nucleic acid synthesis, it is surprising that it is not of major importance in the metabolism of activated lymphocytes.In resting human lymphocytes only 1.4% of consumed glucose is metabolised by this route; this proportion decreases slightly on activation to 0.8% of consumed glucose (Hedeskov,1968). Similarly, mouse thymocytes stimulated with Con A showed no change in the percentage contribution of the hexose monophosphate shunt to glucose metabolism, and constituted only 1% (Lengle et al.,1978). A recent report on the metabolism of glucose by rat thymocytes has concluded that, although both the TCA cycle and glycolysis are activated by Con A, the activity of the hexose monophosphate shunt remains unaltered (Brand et al.,1984). By contrast, Sagone et al. (1974) have detected a three-fold increase in the activity of this pathway in PHA stimulated human peripheral blood lymphocytes cultured for 72h. In total, however,

the TCA cycle and the hexose monophosphate shunt together
only accounted for 5% of metabolised glucose. It is
possible that the explanation for the apparent discrepancy
in the activity of the hexose monophosphate shunt on
lymphocyte activation may lie in the length of incubation
time used. Since activation of nucleic acid synthesis may
not occur until some hours after contact of the cells with
mitogen, short term incubations may genuinely be unable
to .show = any increase in the proportion of glucose being
metabolised by the hexose monophosphate shunt. This may
explain why Sagone et al.(1974) could show an increase
at the longer incubation time.

It thus appears that, although the TCA cycle
and hexose monophosphate shunt are not quantitatively
major fates of glucose in activated lymphocytes, complete
oxidation of the sugar does supply the majority of ATP,
with glycolysis representing only a minor source of energy.

The increase in glycolytic rate which occurs
in activated lymphocytes is accompanied by an increase in
activation, and a change in the isoenzyme expression of
associated enzymes. Interestingly, it has been noted that
the measurement of such changes can be influenced by the
culture conditions. Both the depth of medium and the
oxygen tension have been shown to affect the result of
measuring total activity and isoenzyme composition of
lactate dehydrogenase in lymphocytes (Rabinowitz,1967;
Hellung-Larssen and Anderssen,1969). The effect of these
two parameters are undoubtedly related, as an increase in
the depth of culture medium will influence the oxygen
tension in the microenvironment of the cells in a static
culture.

Measurement of the activities of all the

glycolytic enzymes in lymphocytes have been made, as have
the enzymes from other glucose metabolising pathways. An
increase in activity of 200-300% has been recorded in all
the glycolytic enzymes (except phosphofructokinase; not
studied) of human peripheral blood lymphocytes stimulated
for 72h with PHA and PWM (Kester et al.,1977). The indivi-
dual enzymes, however, showed a number of qualitative
differences between resting and stimulated states. Hexo-
kinase, glucose phosphate isomerase, phosphoglucomutase
and glyceraldehyde 3-phosphate dehydrogenase all remained
as in the unstimulated state. Aldolase showed a change in
the relative content of two isoenzymes; three new forms
of lactate dehydrogenase appeared; pyruvate kinase showed
two further forms and phosphoglycerate kinase one further
isoenzyme. Comparable increases in enzyme activity were
reported in a similar study (Rogers et al.,1980) but here
a further form of enolase and aldolase were also seen,but
no change was detected in the expression of either pyruvate
kinase or phosphoglycerate kinase. The basis for the
discrepancies between these two studies is difficult to
isolate as both systems used human peripheral blood cells
stimulated with PHA. The study by Kester et al. (1977)
however, employed both PHA and PWM combined to stimulate,
and it is possible that the qualitative differences seen
is based in a direct effect of PWM on B lymphocytes. This
is supported by the findings of Diaz-Espada and Lopez-
Alarcon (1982) who found differences in the activities of
pyruvate kinase and lactate dehydrogenase of mouse spleen
lymphocytes, depending on whether the mitogen used to
activate was Con A or LPS. Since the preparation contain-
ed both T and B lymphocytes this was assumed to be a
reflection of differential responses to the mitogens.

A study of the maximal activities of the TCA

cycle and glycolytic enzymes in freshly isolated rat
mesenteric lymphocytes has been carried out (Ardawi and
Newsholme,1982). This has indicated the importance of
both pathways in the metabolism of glucose in lymphocytes.
The maximal activities of hexokinase, pyruvate kinase,
lactate dehydrogenase and citrate synthase were studied
after an in vivo graft-versus-host reaction; all the
activities showed an increase, indicating the possible
increased importance of the two routes of glucose metab-
olism on immunological challenge. Measurements of maximal
enzyme activities can provide an indication of potentially
important fluxes, but they do not shed light on the
possible influence of the culture conditions on the state
of the cells during activation. In addition it is possible
that the demands made on individual pathways may also
alter at different stages of the activation sequence.

An interesting observation in this respect
is that conditions that were found to enhance the growth
of chick embryo fibroblasts such as the presence of serum
and high external pH, have been shown to directly affect
the activity of phosphofructokinase in these cells (Fodge
and Rubin,1973). This effect has also been noted in
leucocyte preparations (Halperin et al.,1969); since
this enzyme has a central regulatory role in glycolysis
such effects could also influence overall glucose metabol-
ism. This may be particularly important to the comparison
of changes occurring in short and long term lymphocyte
cultures. Serum is often ommitted from very short term
cultures but it is essential to the maintenance of long
term cell viability. It is likely that the routine
replacement of serum with defined growth-supporting
factors in culture will minimise such effects (Yen and
Duigon,1983).

Amongst the other metabolic fates of glucose are the synthesis of glycogen and lipids. The maximal enzyme activity of glycogen phosphorylase in rat mesenteric lymphocytes, however, has been shown to be considerably less than that of hexokinase (Ardawi and Newsholme,1982). This would imply that the mobilisation of glycogen is unlikely to be of major importance in the provision of energy in lymphocytes. Conflicting evidence is also available, however, which maintains that glycogen is actively synthesised on activation of lymphocytes. Pachman (1967) has shown a 98% increase in the glycogen content of PHA stimulated horse lymphocytes, from 8.5 to $16.8\mu g/10^7$ cells between 24 and 72h incubation. Resting cells also showed a transient increase in glycogen content between 24 and 48h, but this decreased again during the final 24h of incubation. Mouse spleen lymphocytes have also been shown to synthesise glycogen after stimulation by LPS (Monos et al.,1984). In this study too, resting cells were shown to increase their glycogen content from 7.6 to 128.3 nmoles/10^8cells (content expressed as glucose equivalents). On activation this level reached 167.1 nmoles/10^8cells, but the fact that such a large increase was also detected in resting cells implies that this is at least partly due to the prevailing culture conditions. Glycogen synthase activity was increased in the mouse lymphocytes on activation, but this was balanced by a steady increase in glycogen phosphorylase activity in the latter stages of the incubation. The authors suggest that this correlated with glycogen mobilisation for ATP provision during enhanced synthetic activity late in the incubation period. Activation of glycogen phosphorylase has also been noted as early as 2.5-5min after contact of pig lymphocytes with Con A, PHA or trypsin (Ruf and Gella,

1982). The significance of this remains unclear as, although the authors suggest a role in early energy metabolism, it has been shown that stimulation of glucose transport can also occur within 1-5min of addition of mitogen to lymphocytes (Reeves,1977; Yasmeen et al.,1977).

It should be noted that the degradation and synthesis of glycogen in mammalian tissues is under strict hormonal control. As serum is a potential source of insulin and glucagon it is unclear as to whether, for lymphocytes derived from certain species, the presence of these hormones may influence the long term metabolism of glucose, and glycogen in serum containing media.

In all these studies the important point is whether glycogen synthesis on activation of lymphocytes represents a quantitatively major fate of consumed glucose. The variation in expression of results, and the fact that the number of residues contained in, and hence the molecular weight of glycogen is extremely variable, make an exact estimate impossible. However, in studies where a complete, or nearly complete carbon balance for consumed glucose can be deduced, glycogen has been shown to become a less important fate on activation of the cells (Hedeskov 1968; Hume et al.,1978). Interestingly, a recent report on rat thymocyte glucose metabolism was unable to detect any synthesis of glycogen, despite the fact that glucose consumption was activated 53-fold by the addition of Con A and interleukin-1 (Brand,1985).

Glucose may also be incorporated into lipid, but this has been reported to occur at very low levels in activated lymphocytes. Human peripheral blood lymphocytes stimulated with PHA incorporate less than 1% of consumed

glucose into lipids (Hedeskov,1968), and rat thymocytes activated by Con A metabolise only 2% of glucose to this fate (Hume et al.,1978).

The major fate of consumed glucose in activated lymphocytes therefore appears to be lactate, with the complete oxidation via the TCA cycle and incorporation into macromolecules representing a quantitatively minor fate. In the study of energy metabolism of resting and activated lymphocytes the high rate of aerobic glycolysis which is routinely observed has led to a disproportionate emphasis being placed on it as a means of supplying ATP. It is undoubtedly true that this propensity for glycolysis does exist in culture, but it is also possible that the situation would not be the same in vivo. This may be a direct result of the development of an in vitro model for lymphocyte activation which invariably requires the static culture of the cells for prolonged periods; by contrast lymphocytes in vivo are in a state of dynamic equilibrium between the circulation and the lymphoid organs. The presence of functional mitochondria in lymphocytes supports the view that there is no metabolic defect associated with their tendency toward glycolysis, indeed, the in vitro observations imply an inherent adaptability of the cells to the prevailing conditions in order to maintain the ability to respond to a stimulus. There may be situations in vivo in which activation is required in conditions which are unfavourable to oxidative metabolism; in such cases the ability to shift the relative activities of the TCA cycle and glycolysis would be necessary. If the high rates of aerobic glycolysis seen on lymphocyte activation are induced solely by the culture conditions then not only is the emphasis placed on it as an energy producing route misguided, but it may also have detracted from alternative

substrates in the oxidative metabolism of lymphocytes.

1.3.2 L-GLUTAMINE

Glutamine is a five carbon amino acid, the
most important source of which in mammals is skeletal
muscle, where it is the major end product of amino acid
catabolism (Marliss et al.,1971). Glutamine is of central
importance in the metabolism of the amino sugars, purines
and pyrimidines and hence in the overall metabolism of
protein and nucleic acids, but its role in the animal is
generally organ-dependent (Lund,1980; Windmueller,1982;
Sies,1983; Kovačević and McGivan,1983). In the kidney, for
example, it is used in the generation of NH_4^+ for mainten-
ance of normal acid-base balance (Pitts and Pilkington,
1966); in brain it is a precursor of glutamate and its
metabolite γ-aminobutyric acid, both of which are import-
ant neurotransmitters (Bradford and Ward,1976).

Interest in the role of glutamine in the
metabolism of cultured cells has been a result of the
high requirement for the amino acid in normal culture
media (2mM) compared with other amino acids (0.02-1.1mM).
In addition, glutamine is by far the most abundant amino
acid in plasma at approximately 0.6mM (Meister,1956). The
central role of glutamine in protein and nucleic acid
synthesis has been noted in cultured HeLa cells (Levintow
et al.,1957; Saltzman et al.,1958), but the high medium
requirement was also thought to be due to the inherent
instability and spontaneous decomposition of glutamine
(Tritsch and Moore,1962).

The presence of glutamine in the culture
medium has been shown to be essential for the mitogenic
stimulation of lymphocytes. In a study of human peripher-

al blood lymphocytes, cells in the presence of PHA failed
to incorporate radiolabelled leucine above that shown by
resting cells unless glutamine was also added. Readdition
of the amino acid restored the stimulated rates of protein
synthesis (Waithe et al.,1975). Taudou et al. (1977) have
noted a similar effect of glutamine deprivation on the
uptake of thymidine into guinea-pig DNA after stimulation
of lymphocytes with Con A. Thus, glutamine is essential to
the generation of a response to mitogens.

Interest in the role of glutamine as a resp-
iratory substrate for mammalian tissues has been raised
as a direct result of the observations of Windmueller and
Spaeth (1974;1975;1978;1982). These workers have shown
that the amino acid is a significant substrate for oxida-
tion by rat small intestine; furthermore, it is the
preferred fuel of respiration for the tissue both in vivo
and in vitro, even in the presence of glucose and the
ketone bodies. These observations opened up the way for
an investigation of such a role in other mammalian tissues.
The high requirement for glutamine which had previously
been noted in cell culture led to interest in the role of
the amino acid in various types of cultured cells. In
consequence, a number of different cell types have been
shown to utilise the amino acid, and in some cases this
far exceeds utilisation expected based solely on the role
of glutamine in biosynthesis (Table 5). Interestingly,
many of the cell types which have been shown to utilise
glutamine as a respiratory substrate have potential for
rapid proliferation in culture; it is also noteworthy
that many also exhibit varying degrees of aerobic glycol-
ysis in culture. The presence of a high rate of aerobic
glycolysis does thus not preclude the oxidation of sub-
strates other than glucose. The finding that the uptake

Table 5. Glutamine utilisation by cultured cells.

Cell Type	Observation	Reference
Rabbit reticulocytes	Glutamine provides 15-20% evolved CO_2; added glucose has no effect.	Rapoport et al.,1971
Fibroblasts	Glutamine used in excess of biosynthetic requirements; 56% evolved as CO_2. Contributes up to 30% of total energy requirements.	Stoner & Merchant,1972
Fibroblasts	Grow and proliferate in $17\mu M$ glucose if glutamine, hypoxanthine, thymidine and uridine are present. Reciprocal control of glucose and glutamine oxidation.	Zielke et al.,1976;1978
Fibroblasts	Glutamine is used in preference to other TCA cycle substrates. Physiological concentrations of KB or FFA have no effect on glutamine oxidation.	Sumbilla et al.,1981

Table 5 cont.

Cell Type	Observation	Reference
Rabbit Oocytes	Glutamine supplies energy required for growth and development in the absence of carbohydrates.	Bae & Foote,1975
Ascites tumor Cells	Glutamine supplies 19% ATP and is oxidised at 20x the rate of glucose, but cannot replace glucose as main energy source.	Lazo,1981
Rat Bone cells	Glutamine acts as an amino acid precursor but also gives rise to CO_2.	Biltz et al.,1983
Chick Pigment cells	Glucose and glutamine are both required for long term growth, glutamine alone is inadequate.	Barbehenn et al., 1984
Dissociated brain cells	Glutamine competes with glucose for oxidation, also depresses oxidation of β-HB.	Roeder et al.,1984

of glucose is reciprocally regulated by glutamine in fibroblasts supports this (Zielke et al.,1978). In this study, the uptake of glutamine was suppressed by 30% by the addition of glucose at physiological levels (5.5m$\underline{M}$) compared to its consumption in the presence of trace (70$\mu\underline{M}$) glucose. Similarly, glucose oxidation was decreased in the presence of glutamine. These authors have calculated that glutamine can supply up to a third of the total energy demands of proliferating fibroblasts when both glucose and glutamine are present at physiological levels (Zielke et al.,1984). This proportion increases when glucose levels fall below 95$\mu\underline{M}$, since the K_i for glucose inhibition of glutamine uptake has been shown in fibroblasts to be approximately 95$\mu\underline{M}$ (Sumbilla et al.,1981).

Studies on other cultured cells have thus indicated a role for glutamine in the provision of energy as well as its compulsory role in biosynthesis. This, in addition to the fact that there is an absolute requirement for the amino acid in mitogenic activation of DNA synthesis in lymphocytes, makes a similar role for glutamine in energy provision a compelling possibility.

Prior to the initiation of the present study no information was available on the metabolism of glutamine in lymphocytes; during the course of this work, however, two reports have emerged which together support the idea that glutamine functions in energy production in these cells. An investigation of glutamine utilisation by rat mesenteric lymphocytes stimulated with Con A for 1h has shown that the amino acid was consumed at a rate five times that of glucose when either substrate was present alone, at either 2m$\underline{M}$ glutamine or 5m$\underline{M}$ glucose (Ardawi and Newsholme,1983). The major end products of glutamine

metabolism were found to be ammonia, glutamate and aspartate. Small quantities of lactate, pyruvate, alanine and asparagine were also detected when glutamine was the sole substrate. This observation may explain, at least in part, why estimations of lactate production by cells cultured in glutamine containing media often result in an apparent conversion of glucose to lactate of over 100%. In this study no data was presented on the production of CO_2 from glutamine which would indicate the extent to which glutamine was fully oxidised, but the authors suggest it may contribute up to 30% to respiration (Ardawi and Newsholme,1983). In the presence of Con A rat mesenteric lymphocytes showed an increase in glutamine consumption of 51%, and more glutamine was metabolised past the stage of glutamate than in resting cells. Thus, although the rate of production of glutamate remained similar, more aspartate was produced in the activated state. When glucose was added to the incubations at 5mM, resting cells showed an increase in the rate of uptake of both substrates over that observed when either glucose or glutamine were present alone. However, the proportion of glucose and glutamine which contributed to respiration was not altered, as all the increased glucose used was converted to lactate, and all the increased glutamine could be accounted for by production of aspartate and glutamate. Hence, the presence of glucose did not affect the oxidation of glutamine. No data was provided on the effect of Con A on glutamine metabolism in the presence of glucose.

The metabolism of glutamine by rat thymocytes stimulated with Con A for 3h showed both similarities and differences to the observations in rat mesenteric lymphocytes. Incubations performed in the presence of

4m$\underline{M}$ alone showed rates of glutamine consumption only
20% of those seen by Ardawi and Newsholme (1983), even
though the concentration of the amino acid was double that
of the earlier study (Brand $\underline{et}$ $\underline{al}$.,1984). The major end
products were again glutamate and aspartate (61 and 25% of
glutamine carbon, respectively); this was similar to that
exhibited by rat mesenteric lymphocytes. The balance of
consumed glutamine carbon was metabolised by rat thymo-
cytes to CO_2, indicating a significant contribution to
respiration. In contrast to mesenteric lymphocytes,
thymocytes metabolised virtually no glutamine to lactate.
Incubations of rat thymocytes with Con A increased the
consumption of glutamine and the production of aspartate;
glutamine carbon metabolised beyond glutamate was thus
also increased by Con A. The production of CO_2, as a
proportion of consumed glutamine, remained the same in
resting and activated cells. These findings, in the
presence of glutamine alone are very similar to those
shown by Ardawi and Newsholme,(1983) on rat mesenteric
lymphocytes, but thymocytes were found to differ consid-
erably in their metabolism of glutamine in the presence
of glucose. When both substrates were present at 4m$\underline{M}$,
resting thymocytes showed a decrease in glutamine consum-
ption by 36% and more glutamate but less aspartate was
produced. The addition of Con A to these incubations
further decreased glutamine utilisation to a level lower
than that seen in the presence of glutamine alone. This
contrasts with the situation in mesenteric lymphocytes in
which the presence of glucose enhanced glutamine uptake.

The marked differences between the findings
of these two groups, on the metabolism of glutamine in
the presence of glucose, led Brand $\underline{et}$ $\underline{al}$.(1984) to suggest

the explanation lay in the use of immature versus mature
T cells. It may also be suggested, however, that the
conflict may have arisen from the differences in the
concentrations of substrates used. Brand et al. (1984)
used both glucose and glutamine at 4mM, while Ardawi and
Newsholme (1983) employed 2mM glutamine and 5mM glucose.
It has yet to be shown whether glutamine uptake in
lymphocytes is concentration dependent, but it may also
be the case that the ratio of the two substrates to each
other can influence their respective metabolism; indeed,
this has been shown to be the case for fibroblasts
(Zielke et al.,1978).

Once glutamine has been taken up into the
cell and deaminated to glutamate, it enters the TCA cycle
as α-ketoglutarate. Two energy yielding steps are then
involved in the conversion of succinyl Co A to fumarate
(Fig 1). The conversion of malate to pyruvate may be
achieved by a number of routes, all of which would result
in the generation of approximately equal ATP equivalents.
The production of small quantities of alanine and lactate
from glutamine by rat mesenteric lymphocytes (Ardawi and
Newsholme,1983) implies the existence of routes D, E and
F in Fig.2, and the very small levels of pyruvate result-
ing make it likely that decarboxylation of pyruvate to
acetyl CoA followed by complete oxidation occurs. Interest-
ingly, the phosphoenolpyruvate ᶜᴷ(inhibitor, 3-mercaptopico-
linate was shown by Ardawi and Newsholme (1983) to result
in suppression of glutamine uptake by rat mesenteric
lymphocytes by 28%. Glutamate production was also decreased
but the proportion of aspartate, lactate and malate releas-
ed was increased. These observations imply that, in theory
all of the potential routes for the conversion of malate
to pyruvate may be operational in lymphocytes, (Fig.2).

FIG.1. <u>PATHWAYS OF L-GLUTAMINE OXIDATION</u>

CO_2

(8) →LAC

PYR

(9)

GLU →ALA

KG

(5)

(6) (7) CO_2

PEP

CO_2 ACETYL CoA

(3) CO_2 (4)

TCA CYCLE

CIT

ASP← OAA

(1)

(2)

CO_2

MAL ← KG

CO_2

(1) OAA

GLU
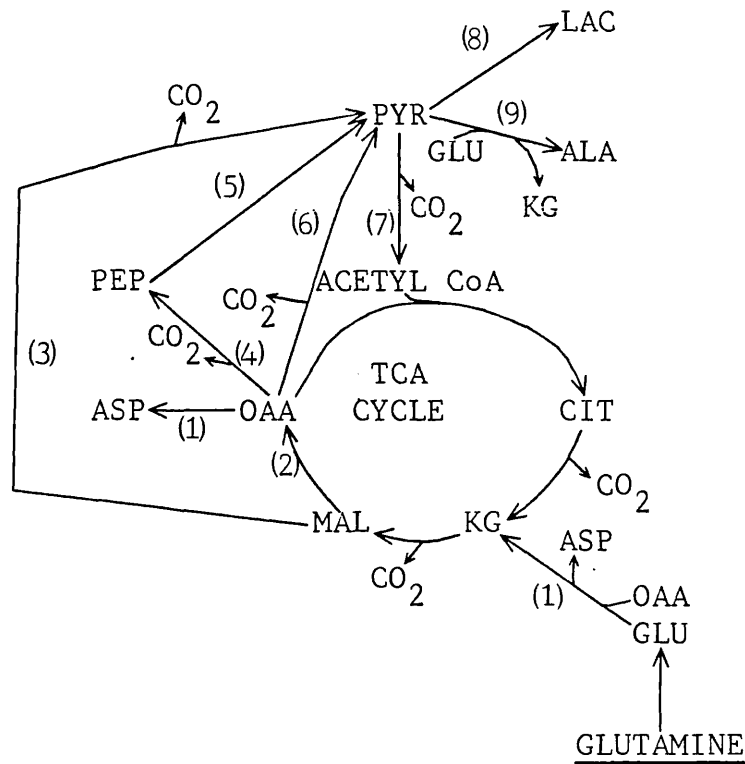
<u>GLUTAMINE</u>

<u>ENZYMES</u>	<u>SUBSTRATES</u>	
1. Aspartate aminotransferase	GLU	Glutamate
2. Malate dehydrogenase	KG	α-ketoglutarate
3. 'Malic' Enzyme	MAL	Malate
4. Phosphoenolpyruvate carboxykinase	OAA	Oxaloacetate
	CIT	Citrate
6. Oxaloacetate decarboxylase	ASP	Aspartate
7. Pyruvate dehydrogenase	PYR	Pyruvate
8. Lactate dehydrogenase	LAC	Lactate
9. Alanine aminotransferase	ALA	Alanine

FIG.2. <u>POTENTIAL ENERGY YIELDS FROM GLUTAMINE OXIDATION</u>

GLUTAMINE
↓
GLU
1 ↓ 7   3ATP
α-KG
↓   3ATP
SUC. CoA
↓   GTP
SUC
↓   2ATP
FUM
↓
MAL

_ _ _ _ A _ _ _ _ _ B _ _ _ _ _ C _ _ _ _ D _ _ _ E _ _ _ F _ _

A	B	C	D	E	F
MAL	MAL	MAL	MAL	MAL	MAL
3ATP ↓2	3ATP ↓2	3ATP ↓2			
OAA	OAA	OAA	3ATP ↓3	↓	↓
	↓4			3ATP	
↓1	PEP	↓6		PYR	PYR
	ATP ↓5			↓8	↓9
ASP	PYR	PYR	PYR	LAC	ALA

_ _

PYR $\xrightarrow{\text{TCA CYCLE}}$ 3 CO_2 15 ATP

Route	ATP Equivalents (mol/mol)
A	9-12 (enzyme 1 or 7)
B	25-28
C	24-27
D	24-27
E	10-13
F	10-13
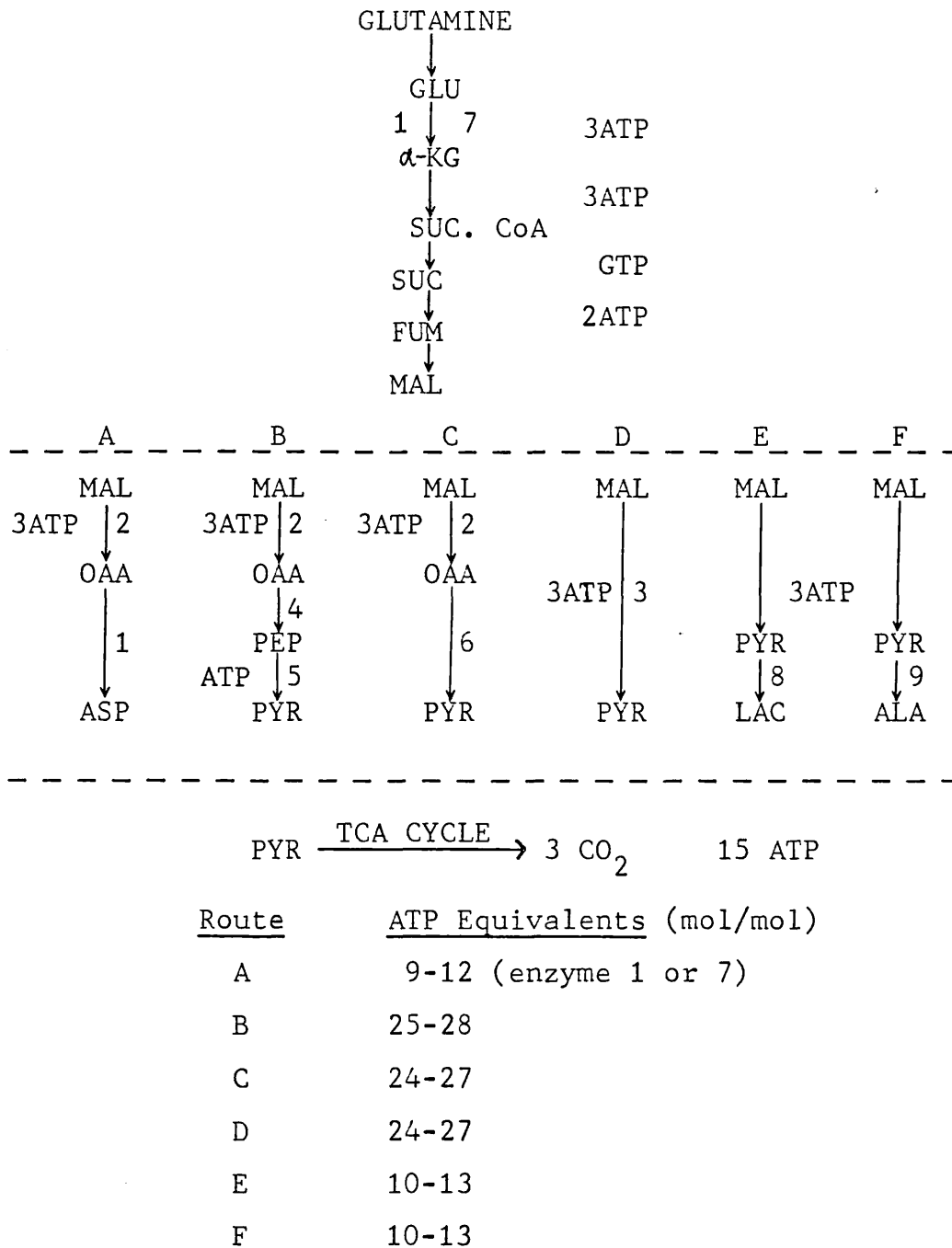
Enzymes are numbered as in Fig.1. except number 7 which is glutamate dehydrogenase.

The ATP yields resulting from complete or
partial oxidation of glutamine (Fig.2) illustrate the
fact that, even if the amino acid is not completely
metabolised to CO_2 in lymphocytes, its potential to
produce ATP far exceeds that of aerobic glycolysis of
glucose, on a mole to mole basis. Since the consumption
of glutamine has so far only been analysed on lymphocytes
cultured for between 1-3h, the importance of glutamine in
quantitative terms, to energy metabolism over the entire
mitogenic response remains to be seen. It is surely
significant, however, that the two studies on lymphocyte
glutamine metabolism discussed here have both shown an
undoubted ability of lymphocytes to consume and metabol-
ise the amino acid to a stage where ATP production results.
Thus, glutamine may be at least partially oxidised in
lymphocytes to supply energy as well as having a role in
the metabolism of protein and nucleic acid.

The study of energy metabolism in activated
lymphocytes may shed light on the reasons for the propen-
sity of activated lymphocytes to display aerobic glycol-
ysis of glucose in culture. It is evident that glutamine
has a role in energy production in other cell types that
show a similar tendency; the two reports discussed here
also support such a role for the amino acid in lymphocytes.
In view of the essential role glutamine has in biosynth-
esis it is possible that the two substrates may act in
parallel to the benefit of the cell, allowing both the
external conditions and the metabolic requirements of the
cell to dictate the relative consumption and degree of
oxidation of both glucose and glutamine. In this respect,
the utilisation of an amino acid which can supply both
biosynthetic precursors and carbon for energy production

would be of considerable advantage to a cell which must undergo proliferation and differentiation in order to fulfil its role in the immune system.

1.3.3. KETONE BODIES

The ketone bodies D β-hydroxybutyrate and acetoacetate are formed in the liver from acetyl CoA via the hydroxymethylglutaryl CoA pathway (Lynen et al.,1958). In extrahepatic tissues the ketone bodies are metabolised via acetyl CoA through the TCA cycle for the production of ATP, or for lipogenesis (Fig.3). Tissues which have been shown to metabolise the ketone bodies to lipid products include adipose tissue (Rous and Favarger,1973); nervous tissue (Edmond,1974; Ramsay,1976; Webber and Edmond,1977) and mammary gland (Hawkins and Williamson, 1972; Robinson and Williamson,1978).

The activities of the enzymes responsible for the conversion of the ketone bodies into acetyl CoA for further metabolism do not alter dramatically under physiological conditions associated with enhanced ketone body utilisation, except in neonates (Dierks-Ventling and Cone,1971; Williamson et al.,1971). This has indicated that the control of ketone body utilisation by peripheral tissues lies in their concentration in the circulating plasma (Bates et al.,1968; Reichard et al.,1974). The major importance of the ketone bodies is that they provide alternative respiratory substrates to glucose under conditions of low carbohydrate availability; as such they also regulate the intracellular metabolism of glucose. Acetoacetate and D β-hydroxybutyrate have been shown to suppress glucose utilisation in vitro in the small intestine (Hanson and Parsons,1978), heart (Garland et al

FIG.3. <u>METABOLISM OF KETONE BODIES IN EXTRAHEPATIC</u>
<u>TISSUES.</u>

1) $CH_3CHOHCH_2COOH$ $\underset{\longleftarrow}{\overset{NAD^+ \quad NADH_2}{\longrightarrow}}$ CH_3COCH_2COOH

β-Hydroxybutyrate $\qquad$ Acetoacetate

2) CH_3COCH_2COOH $\quad + \quad$ $COOH(CH_2)_2COSCoA$

Acetoacetate $\qquad$ Succinyl CoA

$CH_3COCH_2COSCoA$ $\quad + \quad$ $\begin{array}{l}CH_2COOH \\ | \\ CH_2COOH\end{array}$

Acetoacetyl CoA $\qquad$ Succinic acid

3) $CH_3COCH_2COSCoA \underset{\longleftarrow}{\longrightarrow} 2\ CH_3COSCoA$

Acetoacetyl CoA $\qquad$ Acetyl CoA
$\quad$ + CoA

LIPOGENESIS $\qquad$ OXIDATION
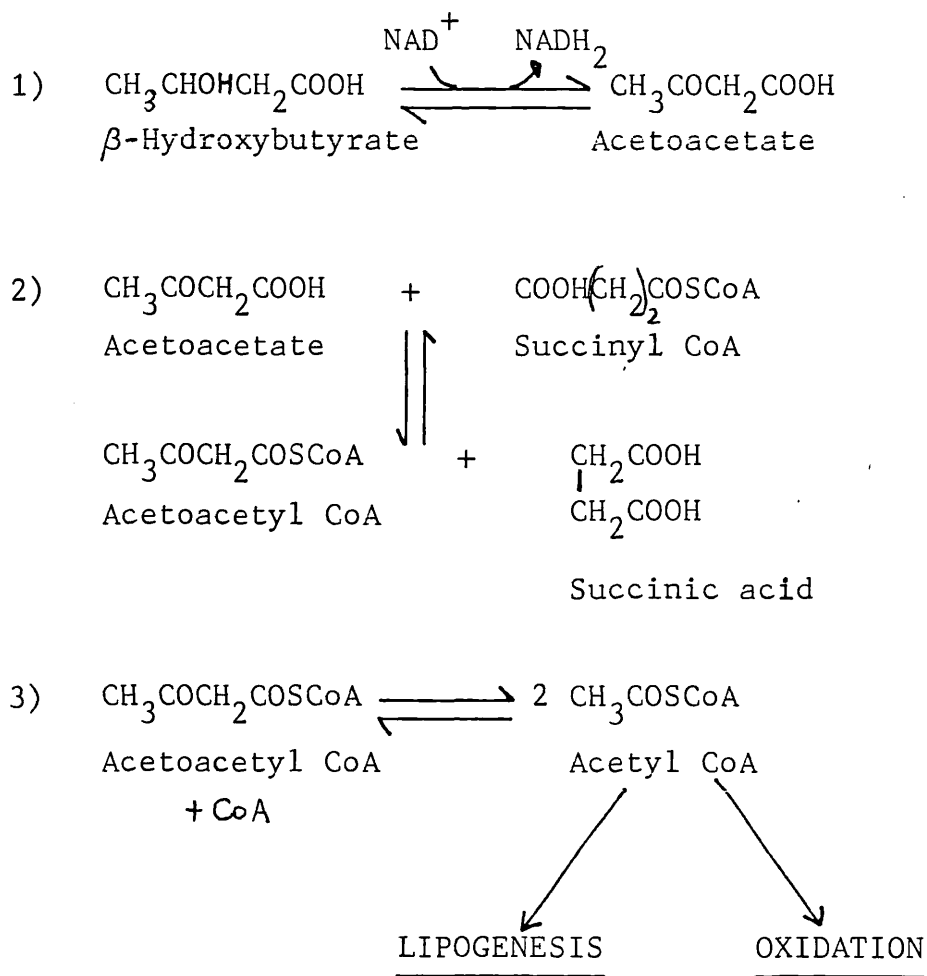
Enzymes catalysing reactions:

1) <u>D</u> β-hydroxybutyrate dehydrogenase

2) 3-oxoacid CoA transferase

3) Acetoacetyl CoA thiolase.

1964; Randle et al.,1964), mammary gland (Williamson et al.,1975) and brain (Rolleston and Newsholme, 1967; Ruderman et al.,1974). There are differences, however, in the tissue-specific site for glucose inhibition. In brain and small intestine, for example, glucose oxidation is suppressed but overall glucose uptake remains the same; thus, more lactate is produced (Ide et al.,1969; Itoh and Quastel, 1970; Hanson and Parsons,1978). The sites of ketone body inhibition of glucose uptake and oxidation have been elucidated by Randle and coworkers (Randle et al 1964;1970; Newsholme and Randle,1964). They have shown that the metabolism of acetoacetate and $\underline{D}$ β-hydroxybutyrate in rat heart and diaphragm resulted in an increase in levels of acetyl CoA and citrate. The latter metabolite inhibited phosphofructokinase activity, resulting in an increase in the levels of fructose 6-phosphate and glucose 6-phosphate. This in turn inhibited hexokinase activityleading to suppression of glucose uptake. Similarly, the increase in the ratio of tissue acetyl CoA to CoA results in inhibition of pyruvate dehydrogenase activity with concomitant suppression of pyruvate oxidation by the TCA cycle.

The metabolism of the ketone bodies by cultured mammalian cells is not well documented. Fibroblasts showed very little oxidation of either acetoacetate or $\underline{D}$ β-hydroxybutyrate at physiological concentrations (0.05 and 0.15mM respectively in the fed state; 0.5 and 1.5mM after starvation). Although a small level of uptake and oxidation was detected this was not greater than the oxidation of glucose or glutamine at physiological levels (Sumbilla et al.,1981).

To the authors knowledge, there is no documented evidence that conditions associated with an increase

in ketone body concentration and low carbohydrate avail-
ability result in the loss of ability to mount an immune
response in vivo. It would therefore be of interest to
assess the likelihood of the ketone bodies acting as
respiratory substrates in resting and mitogen activated
lymphocytes.

Acetoacetate at 5mM has been shown to be
utilised by rat thymocytes (Hume et al.,1978) and also
resulted in the suppression of glucose oxidation, but the
addition of Con A did not significantly alter its metabo-
lism. The maximal enzyme activities of the ketone body
utilising enzymes were found to be much lower than those
of hexokinase and phosphofructokinase in rat mesenteric
lymphocytes (Ardawi and Newsholme,1982). This implies
that the ketone bodies may not be important substrates
in activated lymphocytes, although in view of the fact
that the ketone body metabolising enzymes catalyse near
equilibrium reactions in vivo, measurement of their
maximal enzyme activities cannot be used to indicate
fluxes through the pathway. In addition, the requirement
for energy during lymphocyte activation may well alter
at different stages of the process. Thus, the uptake of
ketone bodies may not be detectable in significant
quantities in very short term cultures.

A recent study by Ardawi and Newsholme (1984)
of rat mesenteric lymphocyte ketone body metabolism has
shown that neither acetoacetate nor D β-hydroxybutyrate
alone at 2mM could support Con A activation, as judged
by radiolabelled thymidine incorporation. Both the ketone
bodies were consumed by lymphocytes at similar rates but
interestingly, starvation of the donor animal for 48h
prior to sacrifice did not affect utilisation.

Acetoacetate was shown to suppress glucose uptake and pyruvate oxidation. Since acetoacetate increased the levels of fructose 6-phosphate and citrate, it is evident that the classical effect of ketone body inhibition of glucose metabolism is occurring in lymphocytes. In common with the findings of Hume et al. (1978) Con A had no effect on acetoacetate metabolism in rat mesenteric lymphocytes (Ardawi and Newsholme,1984).

It therefore appears that, while lymphocytes can utilise ketone bodies as respiratory substrates, and their metabolism results in the suppression of glucose uptake, they do not play an additional role on mitogen activation. This, together with the lack of effect of starvation on the utilisation of acetoacetate or D β-hydroxybutyrate, implies that the ketone bodies are not major sources of energy for lymphocytes. However, both the studies cited here were performed on cultures lasting only a few hours; Hume et al. (1978) used 3h cultures, whilst Ardawi and Newsholme (1984) studied the response for only 1h. It is possible that the demands for respiratory substrates may alter on the long term transformation of lymphocytes. The exact ability of resting and mitogen activated lymphocytes to metabolise the ketone bodies is thus far from clear.

1.4. AIMS OF THE STUDY

The use of polyclonal mitogens to activate lymphocytes from a variety of species and organs has allowed the detailed examination of metabolic changes occurring on lymphocyte activation. There is a wealth of literature available on the early changes in intermediary

reactions which are involved in the generation of differentiated lymphocytes, but the pathways by which the cell satisfies the increase in energy demands for these processes has received comparable attention only with respect to glucose. It is evident from the preceding discussion that the underlying metabolic basis for the propensity of lymphocytes to display aerobic glycolysis on activation is still the subject of some controversy. Since a small proportion of consumed glucose is fully oxidised it is reasonable to assume that other respiratory substrates may also be consumed and metabolised on activation. Alternative methods of ATP production in lymphocytes have, however, received relatively little attention.

The large quantitative increase in glucose consumption which occurs on activation is undoubtedly a major reason for the attention focussed on its metabolism, but this does not mean that alternative routes are not operational in the production of ATP; indeed they may be masked by the very fact that there is such a difference between the metabolism of glucose in resting and activated lymphocytes. All previous studies on the utilisation of respiratory substrates, other than glucose, by lymphocytes have been performed on very short term incubations (1-3h). A major aim of the present study was therefore to establish the utilisation of the ketone bodies, glutamine and glucose in incubations of rat spleen lymphocytes which had been cultured over a 72h period. Furthermore, any alteration in metabolism induced by mitogen activation will be correlated with conventional assays of stimulation; thus, important changes can be unequivocally be correlated with the state of stimulation of the cells. In this way, pathways of energy metabolism

considered important on activation in long term studies
will be further investigated by tracing the metabolic
fate of radiolabelled substrates in 24h cultures. Thus,
a detailed analysis of events in energy metabolism can
be performed between the initial time of contact with
mitogen through to the enhanced synthesis of protein and
finally, DNA.

Although a variety of experimental data is
available on the metabolic alterations associated with
lymphocyte activation, a general lack of uniformity in
preparative and culture methods has been responsible, at
least in part, for many of the discrepancies arising.
It is therefore an aim of the present study to investigate
some of the empirical culture conditions used to activate
rat spleen lymphocytes with Con A. This may be found to
aid in the interpretation of data, but of particular
importance is the need to ensure that conditions under
which metabolic changes are measured also allow stimula-
tion to be confirmed by conventional assessment of
enhanced protein and DNA synthesis.

The overall aims of the study are therefore
to produce a system which allows the controlled stimulat-
ion of rat spleen lymphocytes and which is also conducive
to the detailed examination of consumption and metabolism
of respiratory substrates. It is of importance that the
system allows a good model of lymphocyte activation to
be achieved whilst remaining aware of the possible
effects of the conditions on the intracellular reactions
of the cells. In this way it is hoped to further present
knowledge with respect to energy metabolism in lymphocytes
by studying a variety of potential substrates over the
entire course of the mitogenic response.

2. MATERIALS AND METHODS.

2.1. General Materials.

All general chemicals, substrates and enzymes were obtained from Sigma Chemical Co. Ltd (Poole,UK) or BDH Ltd (Poole,UK) except glucose 6-phosphate dehydrogenase which was from Boehringer Corporation (London) Ltd (Lewes, Sussex,UK). Culture media, Foetal bovine sera, lymphocyte separation medium, balanced salt solution, glutamine and HEPES were purchased as sterile solutions from Flow Labs, (Irvine,Scotland). All sterile plasticware and microtitre plates were obtained from Gibco Ltd (Uxbridge,UK) and glass incubation vials from Pierce Chemical Co. Ltd (Rockford,USA). Radiolabelled compounds were from Amersham International PLC (Bucks,UK).

All solutions were prepared in distilled, deionised water and were sterilised where necessary by autoclaving. Glassware was siliconised and autoclaved before use for culture procedures.

2.2. Animals.

Male or female Wistar rats and Balb/c mice were obtained from the departmental animal house and in all cases were aged 6-8 weeks. Animals were maintained on a 12h light/dark cycle and were allowed standard PRD laboratory diet ad lib. In experiments requiring starvation of the donor animals food was removed 48h prior to sacrifice, but free access to water was allowed.

2.3 Preparation of Spleen White Blood Cell fraction.

All techniques involving preparation of cells for subsequent cell culture were carried out aseptically in a laminar flow cabinet, at room temperature.

Rats were killed by cervical dislocation and

the spleen immediately removed and placed in approx. 20ml
Hanks' Balanced Salt solution (HBSS) at pH 7.2. A single
cell suspension was produced by pressing the roughly chopped
spleen through a wire mesh with the barrel of a 5ml disposa-
ble plastic syringe, followed by gentle pipetting. The
resulting cell suspension was stood for 5min to allow any
remaining clumps to settle. 8ml of cells were then gently
layered on to 3ml of lymphocyte separation medium (LSM) and
centrifuged at 2000g for 25min in an MSE minor bench
centrifuge. The white blood cell fraction occurring at the
HBSS/LSM interface was removed, mixed with 10ml HBSS and
recentrifuged at 400g for 10min. Where the pelleted white
blood cell fraction contained red blood cell contamination
visual by microscopic examination, it was mixed with five
volumes of 0.87% (w/v) NH_4Cl, pH 7.2 and allowed to stand
for 5min. After lysis treatment, where necessary, the cell
suspension was diluted to 15ml with HBSS, pelleted and
washed three times more with 10ml HBSS as described.

The washed white blood cell fraction was then
resuspended in a known volume of complete, supplemented
medium (Section 2.5.1) as appropriate to experimental
requirements. A sample of cell suspension was diluted with
white blood cell counting fluid (2% (v/v) glacial acetic
acid containing crystal violet to colour). This was allowed
to stand for 10min and the white blood cells then enumer-
ated in a haemocytometer. Cells were finally resuspended
in an appropriate volume of complete, supplemented medium
for subsequent culture.

Enumeration of lymphocytes was periodically
carried out on the purified white blood cell fraction, using
Leishman's stain. The pelleted cells were resuspended in a
small volume of foetal bovine serum and a thin film smear
produced on a microscope slide. Preliminary investigations

in which cells were resuspended in various combinations of foetal bovine serum and HBSS failed to provide an even smear. After air drying the slide was covered with approx. 7ml undiluted stain and left for 20-60sec. This was then quickly drained and replaced with a solution of diluted stain (1:2 (v/v) stain:buffer pH 6.8) and left for 10min. After this time the slide was repeatedly washed with distilled water. The slide was then air dried vertically.

Under microscopic examination four separate 100-cell counts were made and classified into lymphocytes, red blood cells and non-lymphocytic white blood cells. Typical figures for contamination of the lymphocyte preparation with red blood cells and morphologically-recognisable leucocytes are given in Table 6. below.

Table 6.   PURITY OF SPLEEN LYMPHOCYTE FRACTION

Results are Mean +/- SEM for three separate preparations, each performed on four 100-cell counts.

Preparation	Lymphocytes	Other WBC	RBC
1	93	2	5
2	90	1	9
3	96	3	1
MEAN +/-SEM	93+/-3	2+/-0.3	5+/- 2.3

2.4. Determination of lymphocyte viability.

2.4.1 Nigrosin Exclusion.

A 1% (w/v) solution of nigrosin was diluted 1 in 10 with 5% (w/v) BSA-HBSS just prior to use. The cell suspension was mixed with the 0.1% nigrosin solution to give a final ratio of dye to cells of 1:5 (v/v). After 10

min the percentage viability was determined by enumeration on a haemocytometer; viable cells exclude the dye.

Viability was routinely determined immediately prior to cell culture, and then as described in Section 3.

2.4.2. Linearity of Protein Synthesis.

The incorporation of radiolabelled leucine into acid-precipitable material, and the extent to which this remained linear with increasing incubation time was used as an indication of viability in short term lymphocyte culture.

1μCi L-$[4,5-^3H]$ Leucine (S.A. 55.1Ci/mmol) was added to each culture at zero time. At various times throughout the 24h culture period cells were harvested onto glass fibre filter discs (Whatman GF/C; 2.5cm diam.). High molecular weight material was precipitated by repeated washing with 5% (w/v) trichloroacetic acid and finally rinsing with methanol. The discs were air dried and counted for $[^3H]$ leucine incorporation in 10ml Toluene/PPO (5gPPO/ L Toluene) in a Beckman LS2500 liquid scintillation counter.

2.4.3. Extracellular Lactate Dehydrogenase Activity.

Cells and medium were harvested throughout the culture period and separation achieved by rapid centrifug- ation (1min, bench microcentrifuge). Cells were resuspended in 800μl extraction buffer, pH 7.5 containing 500m$\underline{M}$ triethanolamine hydrochloride; 1m$\underline{M}$ EDTA; 2m$\underline{M}$ magnesium chloride and 30m$\underline{M}$ 2-mercaptoethanol. The cell suspension was sonicated using a microprobe in an MSE sonicator for 2x20sec, at 6μm amplitude and 0°C. Cell debris was removed by centrifugation (5min, bench microcentrifuge) and the supernatant used in the LDH assay. The samples of medium were kept on ice and used directly in the assay without further treatment.

LDH was assayed by the following method;

The assay components were potassium phosphate buffer $0.1\underline{M}$, pH 7 (made by mixing equimolar solutions of KH_2PO_4 and K_2HPO_4); $23m\underline{M}$ sodium pyruvate and $12m\underline{M}$ NADH. After 5min preincubation at $25°C$, $20-50\mu l$ cell extract or medium was added, and the reaction followed for 10min at 340nm.

2.5. Culture Techniques.

2.5.1. Long Term Cultures.

Lymphocytes were cultured in round- or flat-bottomed microtitre plates depending on experimental requirement, in a final volume of $200\mu l$. Concanavalin A or medium for control cultures was added in $50\mu l$ and cells in a volume of $150\mu l$. Complete, supplemented medium was made up as required by adding 10% (v/v) heat inactivated foetal bovine serum ($56°C;30min$); 1% (v/v) Penicillin/ Streptomycin (6mg &10mg/ml, respectively) and 1% (v/v) L-glutamine ($200m\underline{M}$). Details of the culture components in RPMI 1640 (DM) and αMEM can be found in Appendix 5.

Cells were placed in a humidified CO_2 incubator at $37°C$ for the duration of the experiment. CO_2 tensions of 2 or 10% CO_2 in air were employed according to experimental requirements. Flat plates were used unless specified.

Assessment of mitogenic stimulation in long term cultures was usually made between 64 and 72h culture except where specified. $10\mu l$ of medium containing $1\mu Ci$ [methyl-^{3}H] Thymidine (S.A. 47 Ci/mmol) was added to each well, and at the end of the incubation (72h) the cells were harvested on a semi-automatic cell harvester (Ilacon Ltd,UK). High molecular weight material was precipitated onto glass fibre filter discs (Whatman GF/C, 1cm diam.) using three consecutive washes each of 5% (w/v) trichloroacetic acid and methanol. Discs were placed directly into scintillation

vials, air dried overnight and counted in 10ml Toluene
/PPO (5gPPO/L Toluene) as described in Section 2.4.2.

2.5.2. Short Term Cultures.

Short term cultures were performed in 25ml glass
incubation vials, i.d. 2.5cm. For normal incubation proced-
ures 2.5cm diameter, $0.22\mu m$ pore size cellulose acetate
membrane filters were placed in the open caps and these
fitted onto the vials. This was found to allow ample circ-
ulation of the CO_2 in air gas phase whilst preventing
bacterial contamination. When incubations were performed
with added radiolabelled substrates the membrane filter
was replaced with a teflon septum to render the vials
completely airtight.

The final incubation volume was 1ml; Con A was
added in $100\mu l$ and cells in $900\mu l$ complete, supplemented
medium. Medium used in short term cultures was αMEM
containing either $24m\underline{M}$ NaHCO$_3$ (αMEM 1) or $5m\underline{M}$ NaHCO$_3$ and
$20m\underline{M}$ HEPES (αMEM 2). Activation of protein synthesis by
Con A was used as an index of stimulation in short term
cultures and was assessed by the incorporation of radio-
labelled leucine into high molecular weight material. $1\mu Ci$
L-$[4,5-^3H]$ leucine was added to each vial in $10\mu l$ medium
and the vial left for the appropriate pulse period. At the
end of this time cells were harvested and $[^3H]$ leucine
incorporation assessed as described in Section 2.4.2.

2.6. Deproteinisation of Samples.

Throughout both long and short term incubations
samples of medium were removed and prepared for substrate
assays. In long term cultures the $200\mu l$ well volume was
removed and cells were spun down in a microcentrifuge. The
medium was placed directly into an equal volume of cold
perchloric acid (5% (w/v)). Protein was spun down by 5min in

a microcentrifuge. The supernatant was removed and neutra-
lised to pH 7-7.5 with 20% (w/v) KOH. Precipitated $KClO_4$
was removed by centrifugation as above. The neutralised
supernatant was then used for substrate assays either
immediately or after storage at $-20°C$.

Short term medium samples were treated in the
same way, except that $HClO_4$ was added as 100μl of a
25% (w/v) solution to avoid unnecessary dilution of substr-
ates.

2.7. Substrate Assays.

All substrate assays were carried out on medium
samples deproteinised as described above. They were
performed on a Beckman 3600 or LKB Biochrom Ultrospec 4501
spectrophotometer at controlled temperature of $25°C$ unless
stated.

Assays were carried out by linking the reaction
to an NADH consuming or producing dehydrogenase reaction,
which was then monitored at 340nm. Each assay was initially
standardised using a substrate solution in medium which had
been deproteinised as described above. Comparison with
results obtained using substrate solutions in water ensured
against possible interference in the assay by medium
components or perchloric acid. Reagent blanks were run with
each batch of assays.

2.7.1. Glucose.

Glucose was initially assayed by the glucose
oxidase method (Fleming & Pegler,1963; Catley,1967).Although
this was satisfactory when assaying standard glucose
solutions, when standards in medium or test sample were
assayed a strong inhibition was noted. Pachman (1967) had
previously noted the interference of phenol red pH indicator
in this assay. Since the media used in this study contained

phenol red, it was considered likely that the same phenom-
enon was occurring here. The source of the interference was
not pursued further, but the assay was discontinued.

The enzymatic method of Bergmeyer et al. (1974)
was used as published, except that glucose 6-phosphate
dehydrogenase from Leuconostoc mesenteroides was used and
therefore NADP$^+$ was replaced with NAD$^+$. In a final volume
of 1ml at pH 7.4 the assay contained 0.27mM triethanolamine
hydrochloride; 2.7mM magnesium sulphate; 1mM ATP; 0.84mM
NAD$^+$ and deproteinised sample. The reaction was initiated
by the addition of 1 unit each of Yeast hexokinase and
glucose 6-phosphate dehydrogenase from Leuconostoc
mesenteroides. The reaction was followed at 37°C for 25min.

2.7.2. Lactate.

Lactate was assayed by the method of Gutmann &
Wahlefeld (1974). During the initial standardisation of the
assay a severe background drift was noted to the extent
that an endpoint could not be obtained. Since this occurred
even in the presence of buffer and NAD$^+$ alone, it was assumed
that a complex had been formed between NAD$^+$ and hydrazine
which absorbs at 340nm; this had also been noted for the
assay of glutamate (Bernt & Bergmeyer,1974). The problem
was found to be considerably alleviated by substituting the
hydrazine hydrate with the sulphate salt, and under these
conditions the small remaining drift could be corrected for
by carefully timed control incubations.

In a final volume of 1ml the modified assay
contained 0.35M hydrazine sulphate and 0.44M glycine buffer,
pH 9.5; 2.5mM NAD$^+$ and deproteinised sample. The reaction
was initiated by the addition of 15 units of lactate
dehydrogenase from Rabbit muscle, and was continued for
30min.

2.7.3. Pyruvate.

Pyruvate was assayed by the method of Czok &
Lamprecht (1974) without further modification. In a final
volume of 1ml and at pH 7.6 the assay contained 0.3$\underline{M}$
triethanolamine hydrochloride; 3m$\underline{M}$ EDTA; 0.1m$\underline{M}$ NADH and
deproteinised sample. The reaction was initiated by the
addition of 3 units lactate dehydrogenase from rabbit
muscle and was continued for 30min.

2.7.4. Acetoacetate.

Acetoacetate was assayed by the method of
Mellanby & Williamson (1974). In a final volume of 1ml and
at pH 6.8 the assay contained 33m$\underline{M}$ phosphate buffer (made
by mixing equimolar solutions of KH_2PO_4 and K_2HPO_4); 0.2m$\underline{M}$
NADH and deproteinised sample. The reaction was initiated
by the addition of 50 munits $\underline{D}$ β-hydroxybutyrate dehydrog-
enase from Pseudomonas lemoignei and was continued for
20min.

2.7.5. D β-Hydroxybutyrate.

D β-hydroxybutyrate was assayed according to
the method of Williamson & Mellanby (1974) with some
modifications. Hydrazine hydrate was again replaced with
the sulphate, as described for lactate measurement. In
addition, it was noted that the reaction was completed
satisfactorily in the complete absence of hydrazine, if the
pH was at least 8.5. The interference of hydrazine in the
production of an endpoint in this assay has been the sub-
ject of two studies by Bach et al. (1971) and Brashear &
Cook (1983). Both studies found that hydrazine was not
obligatory to the reaction. It is probable that the high
pH is sufficient to shift the equilibrium in favour of
acetoacetate formation. In view of the fact that the back-
ground reaction could be adequately controlled by the use of

the sulphate salt of hydrazine and careful timing of the reaction time, the use of hydrazine was continued.

The assay, in a final volume of 1ml and at pH 8.5, contained 74m$\underline{M}$ Tris; 0.74m$\underline{M}$ hydrazine sulphate; 0.4m$\underline{M}$ NAD$^+$ and deproteinised sample. The reaction was initiated by the addition of 50 munits $\underline{D}$ β-hydroxybutyrate dehydrogenase from Pseudomonas lemoignei and was continued for 30min. EDTA was omitted from the original assay protocol as this has been shown to inhibit the enzyme from this source (Sigma Chemical Co.Ltd).

2.7.6. Glutamate.

Glutamate was assayed according to the method of Bernt & Bergmeyer (1974). Hydrazine hydrate in the buffer system was compared with the sulphate for interference. In this case the absorbance drift was found to be small compared with that seen during the assay of lactate or $\underline{D}$ β-hydroxybutyrate. The reason for this discrepancy is not known as the concentration of hydrazine here is much greater than that in the $\underline{D}$ β-hydroxybutyrate assay, and the ratio of hydrazine to NAD$^+$ is approx. that in the assay of lactate. Thus, neither the absolute concentration of NAD$^+$ or hydrazine, nor the ratio of the two components to each other provides an explanation.

In a final volume of 1ml and at pH 9 the assay consisted of 300m$\underline{M}$ glycine; 250m$\underline{M}$ hydrazine hydrate; 1m$\underline{M}$ ADP; 1.6m$\underline{M}$ NAD$^+$ and deproteinised sample. The reaction was initiated by the addition of 5 units glutamate dehydrogenase from ox liver and was continued for 30min.

2.7.7. Glutamine.

Glutamine was assayed in a two part system according to the method of Lund (1974). Part one consisted of the enzymatic conversion of glutamine to glutamate; part

two involved the assay of glutamate according to Section
2.7.6. Free glutamate was subtracted to give total glutamine.

The conversion of glutamine to glutamate was
performed in a volume of 500μl at pH 5 and consisted of
250mM sodium acetate, 50 munits glutaminase from
Escherichia coli, and deproteinised sample containing up
to 0.2 mM glutamine. This was incubated for 1h at 37°C
then cooled on ice. 150-200μl was removed and assayed for
glutamate without further deproteinisation.

2.8. Incubations with labelled substrates.

2.8.1. Incubation Method.

All labelling experiments were carried out on
short term cultures which were initially set up as
described in Section 2.5.2. At various times throughout
the incubation replicate vials were removed for a 2h
pulse period in the presence of the appropriate radio-
labelled substrate. A small sample (50μl) of medium was
first removed and deproteinised for determination of
initial substrate concentration. The labelled substrate
was added to the remaining 950μl in a volume of 10μl.
Vial contents were mixed and a small glass tube suspend-
ed into the air space using a parafilm strip. The vial
was sealed by replacing the membrane filter with a teflon
seal. The vials were then gassed for 30sec with 5% CO_2 in
air and left undisturbed for 2h. All medium used in the
labelling experiments was αMEM 2 in order to maintain the
pH over the pulse period.

At the end of this time 100μl 25% (w/v) $HClO_4$
was injected into the vial with a Hamilton syringe, to
stop metabolism and release dissolved CO_2 into the gas
phase. 0.2ml CO_2 trapping agent (2-methoxyethanol; ethanol-
amine 2:1(v/v)) was injected into the suspended well, and
the vials shaken at 37°C for a further hour (Jeffay &

Alvarez,1961). Trapped $[^{14}CO_2]$ was measured by placing the glass tube directly into 10ml Toluene PPO/ 2-methoxy-ethanol (2:1 (v/v)) and counting as described.

The medium/$HClO_4$ mixture was neutralised with 20% (w/v) KOH after removal of protein, and the supernatant stored at $-20^{\circ}C$ for separation of labelled metabolites. Precipitated protein was washed three times by alternate resuspension and reprecipitation with $HClO_4$. The washed sample was finally suspended in 100μl 0.1M NaOH and placed in 10ml Toluene PPO/Triton X-100 (2:1, (v/v)) for scintillation counting.

The efficiency of $[^{14}CO_2]$ trapping by the 2-methoxyethanol: ethanolamine mixture was determined by incubating standard $NaH^{14}CO_3$ solutions and quantitatively releasing $[^{14}CO_2]$ by acidification with $HClO_4$. In addition, the effect of the initial gassing procedure on trapping efficiency was also determined by this method. The results of these determinations are given in Fig.4.

2.8.2. Separation of labelled metabolites by ion-exchange chromatography.

(a) Dowex Cl^-.

Dowex 1-X8 Cl^- resin was washed five times in three volumes distilled, deionised water, followed by three volumes of 0.1M HCl and left overnight in the latter solution. The resin was then rinsed in water and packed into columns of dimensions 1.5cm i.d. x 10cm. A sample of deproteinised, neutralised medium was applied to the column and 3ml fractions collected immediately. Glucose was washed through the column with 30ml water, lactate was eluted with 30ml 0.02M HCl and pyruvate with 30ml 0.06M HCl. Elutions were initially carried out with stepwise additions of water, 0.02, 0.04, 0.06, 0.08 and
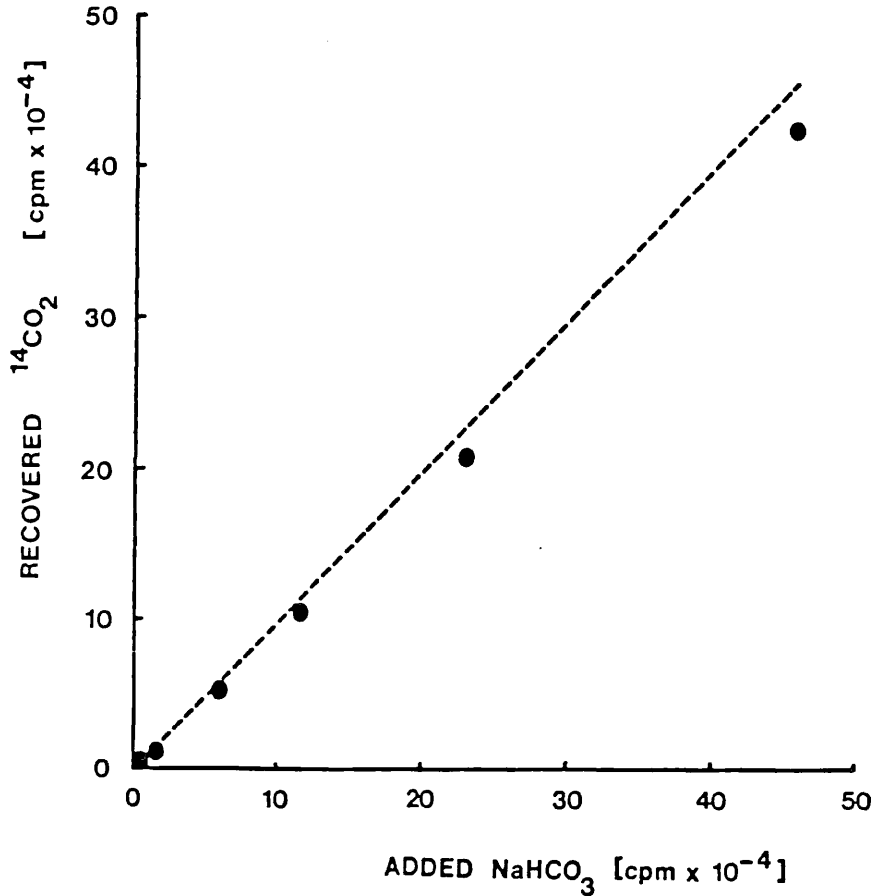
Figure 4. Efficiency of $^{14}CO_2$ Trapping

Known quantities of $NaH^{14}CO_3$ were incubated in $9m\underline{M}$ Tris, $^{14}CO_2$ released with $HClO_4$ and trapped by 2-methoxyethanol: ethanolamine according to Materials and Methods. Points are the mean of triplicates. The dashed line indicates theoretical trapping of $^{14}CO_2$ of 100%. Prior gassing of the vials with CO_2 for 30sec had no effect on trapping.

0.1$\underline{M}$ HCI , and glucose, lactate and pyruvate assayed in each fraction. Having ascertained the elution profile, thereafter the concentrations of HCl described were used. Duplicate samples from each fraction were assayed for radioactivity by counting in 10ml Toluene PPO/Triton X-100 (2:1 (v/v)). An illustrative elution profile is given in Fig.5.

(b) Dowex CH_3COO^-.

Dowex 1-X8 Cl^- resin was converted to the acetate form by the method of Berl $\underline{et}$ $\underline{al}$.(1961). The resin was washed sequentially with three x five volumes of 2$\underline{M}$ NaOH, 2$\underline{M}$ CH_3COOH and water. The conversion was confirmed by testing the eluate with acidified $AgNO_3$ until no precipitate was formed on addition of the nitrate to the eluate. Columns were then packed with resin as described for Dowex Cl^-. Glutamine was washed through the column with 20ml water; glutamate was eluted with 20ml 0.5$\underline{M}$ sodium acetate ; aspartate with 20ml 1.5$\underline{M}$ sodium acetate and lactate with 35ml 3$\underline{M}$ sodium acetate. After confirmation of the elution profile by thin-layer chromatography of the amino-acids and assay of lactate, 200μl samples of each fraction were counted as described for Dowex Cl^-. An illustrative elution profile is given in Fig.6.

Figure 5. <u>Elution profile of ^{14}C from Dowex Cl$^-$ ion</u>

<u>exchange resin:[^{14}C] Metabolites from [^{14}C] Glucose.</u>

Sample was applied to the column according to Materials
and Methods. Glucose, lactate and pyruvate were eluted
with A water, B 0.02M HCl and C 0.04M HCl. 3ml fractions
were collected immediately and assayed for substrates
and radioactivity according to Section 2.

RADIOACTIVITY / FRACTION (cpm x 10^{-3})
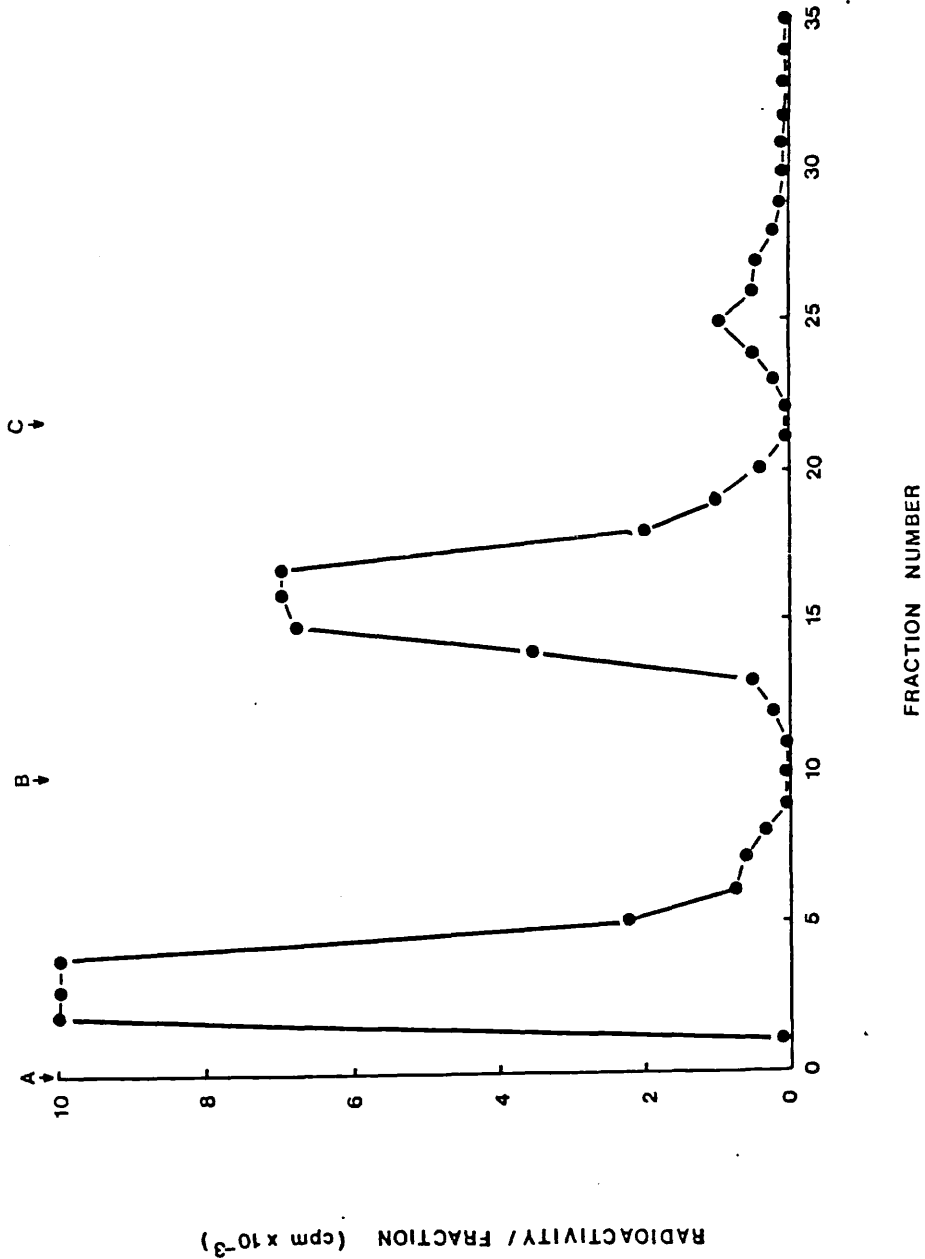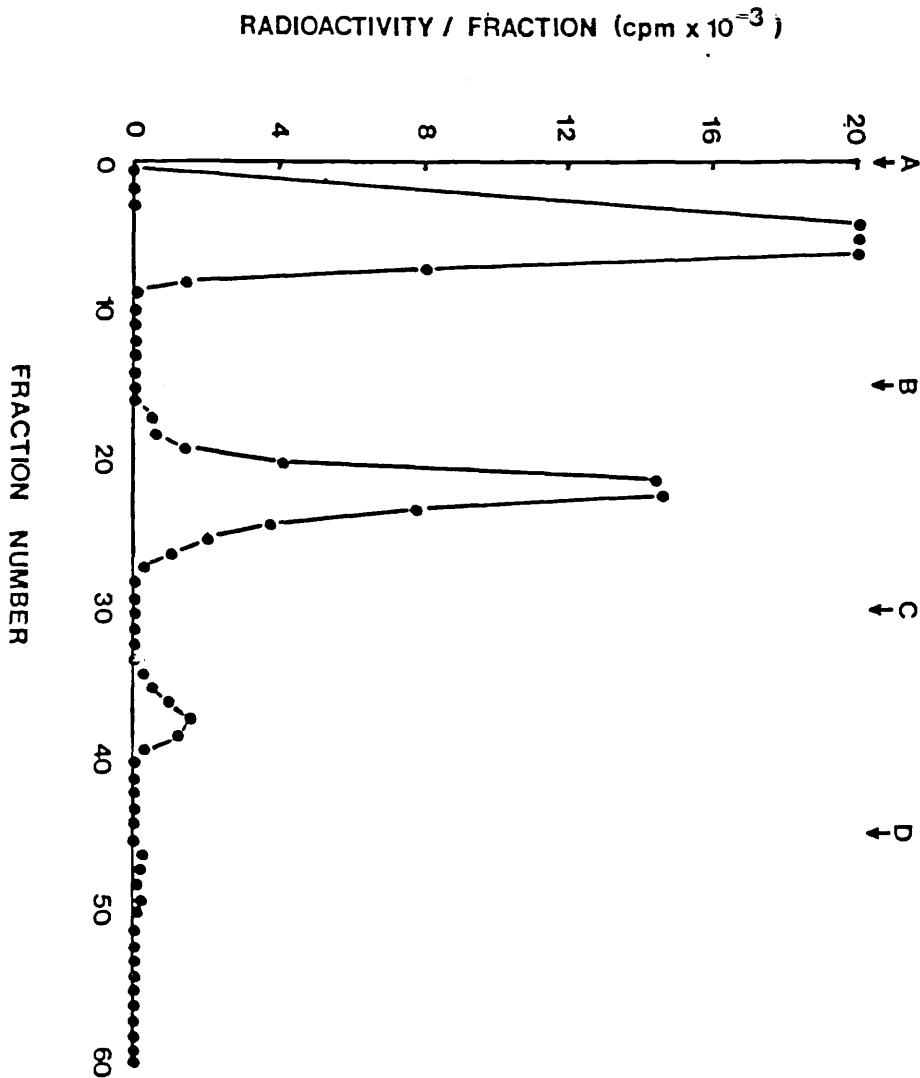
Figure 6. Elution profile of ^{14}C from Dowex CH$_3$COO$^-$ ion exchange resin: [^{14}C] Metabolites from [^{14}C] Glutamine.

Sample was applied to the column according to Materials and Methods. Glutamine, glutamate, aspartate and lactate were eluted with A water, B 0.5M, C 1.5M and D 3M sodium acetate. 2ml fractions were collected and assayed for radioactivity as described in Section 2.

3. RESULTS AND DISCUSSION.

3.1 LONG TERM CULTURES

3.1.1. EFFECT OF CULTURE CONDITIONS ON MITOGENIC STIMULATION

The conventional method of measurement of
lymphocyte activation by mitogen and antigen in long term
cultures is the incorporation of radiolabelled thymidine
into DNA (Dutton and Pearce,1962; Michalowski,1963). This
is conventionally carried out on the third or fourth day
of incubation with mitogen, and a number of parameters
may influence its outcome. These include the mitogen
concentration, cell density and incubation time (Knight,
1980). The ultimate aim of the study of such variables is
to derive a single set of culture conditions which will
provide an optimal system for further studies of lymphocyte
activation. Since, as will be shown in the present study,
the perturbation of a particular condition can considerably
affect the incorporation of $[^3H]$-Thymidine, it is essential
to understand the way this may be occurring, and the extent
to which it may be controlled. Mitogenic lymphocyte
stimulation is here being used to investigate the utilisa-
tion of potential respiratory substrates, and valuable
information may be missed if the effect of culture condit-
ions on their uptake is not taken into account. This is
particularly pertinent here since mouse and human cells
are routinely used in immunological studies of this kind,
with rat lymphocytes primarily used for metabolic studies.
Thus, the extent to which rat cells are subject to the
same influences as mouse or human lymphocytes, in terms of
the conditions òf culture, is less well documented. This
is a further justification for the need to evaluate some
of the culture conditions which may affect both activation

and concomitant metabolic changes.

(a) Cell Density.

The investigation of cell density of rat splenocytes stimulated with Con A utilised concentrations of 10^4, 10^5 and 10^6 cells/well in round-bottomed microtitre plates. Fig. 7 illustrates that increasing cell density leads to a greater stimulation index (S.I.=cpm incorporated by activated cells/ cpm incorporated by resting cells). There is an approximate doubling of the optimal S.I. with a tenfold increase in cell density. The rise in the level of $[^3H]$-thymidine incorporation into resting cells at 10^6 cells/culture effectively decreases the S.I. seen; cpm incorporated by controls in both 10^4 and 10^5 cells/well are very similar.

The dose of Con A required for optimal stimulation also depends on the cell density. For 10^6 cells /well this is achieved at 0.6 and 1.2μg Con A. A larger dose is required by 10^5 cells/well (2.5μg) and for 10^4 cells /well (1.2 and 2.5μg). Although the shift is not clear cut there is a general trend that increasing cell density requires less Con A to achieve optimal stimulation. This is also supported by the fact that 0.3μg Con A effects activation of 10^6 cells/well but not of 10^5 or 10^4 cells/ well. Concentrations of Con A greater than 2.5μg cause a decreased response in all three cell densities; complete inhibition of the response is only seen at the highest mitogen concentration and lowest cell density.

Optimal mitogenesis occurs in lymphocytes with binding of a certain number of mitogen molecules to the cell membrane. Sub- or supra-optimal responses occur in the absence of this and would be reflected in the ability to incorporate $[^3H]$-thymidine into DNA. Fig. 7

Figure 7. The effect of cell density on [³H] -Thymidine

incorporation by rat splenocytes cultured in

round-bottomed microtitre plates.

Cells were cultured according to Materials and Methods for

90h, the final 18h in the presence of 1μCi/well [³H] -Tdr.

Cpm incorporated by Con A containing cultures were

compared with that by resting cells and analysed by the

Student t-test. Cpm incorporated by resting cells were

10^4/well (●) 57 +/- 12; 10^5/well (▲) 79 +/- 25; 10^6/well

(o) 631 +/- 181. P<0.1 *;<0.05 **;<0.02 ***. Points are

mean +/- SEM of triplicates. Pvalues for cpm +or- ConA .

shows an increasing stimulation index with increasing
cell density; thus it appears that stimulation is en-
hanced in an environment that promotes cell-cell contact.
Observation of round-bottomed plate cultures revealed the
formation of a tight button of cells at the base of the
well after approximately 24h incubation. It is therefore
of interest to compare the pattern of stimulation seen
in round-bottomed plates with similar measurements per-
formed in flat-bottomed plates, as the latter wells
provide a greater surface area over which the cells can
settle.

In flat plates, Fig. 8(A) shows that 10^6 cells
/well, which gave high stimulation indices in round
plates gave very low levels of stimulation. An increase
in cell density to 5.10^6 cells/well resulted in a three-
fold increase in $[^3H]$ -thymidine incorporation over that
shown by 10^6 cells/well, but a further increase in cell
density to 10^7 cells/well abolished this increase. The
transient nature of the density-dependent increase in the
level of stimulation , and the low absolute levels of
$[^3H]$ -thymidine incorporation in the presence of mitogen
implies the optimal cell density may lie between 10^6 and
10^7 cells/well in flat-bottomed microtitre plates. This was
confirmed by studying smaller incremental changes in cell
density between these limits (Fig.8(B)). It can be seen
that the cell densities of 2 and $2.5.10^6$ cells/well
resulted in very high stimulation indices, far exceeding
those occurring in round-bottomed plate cultures, within
the range studied. An increase to 3.10^6 cells /well led
to a decrease in stimulation, but this level was still
comparable to that seen in round plate cultures. Thus,
in flat-bottomed plate cultures, a range of stimulation
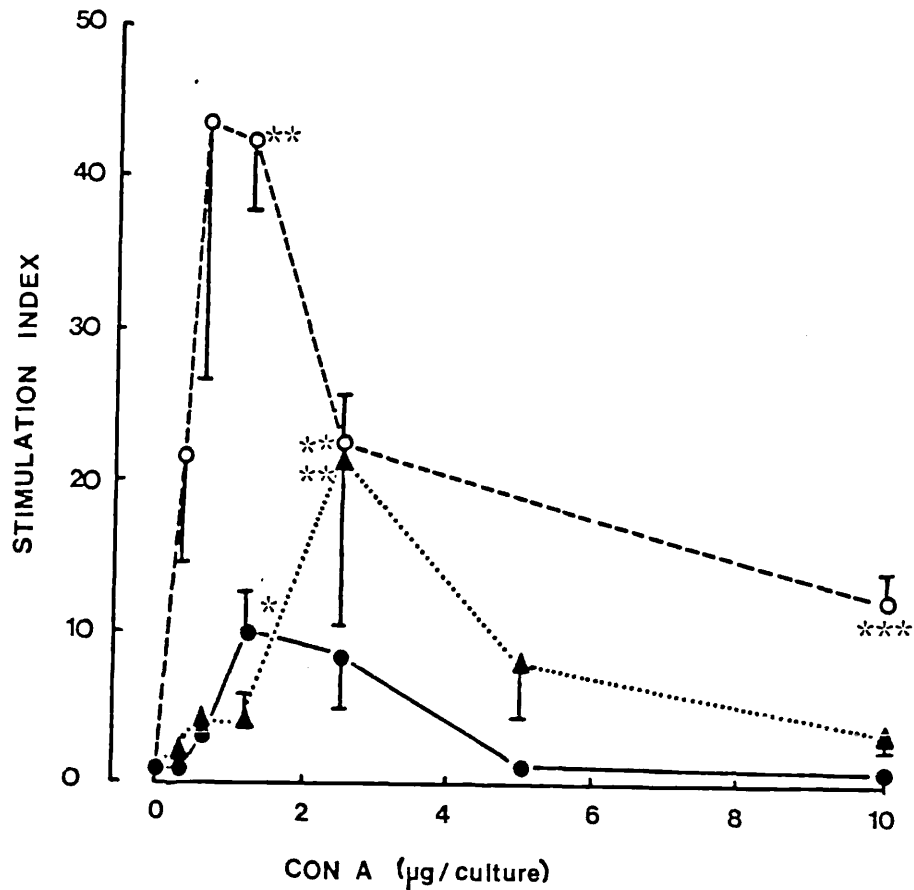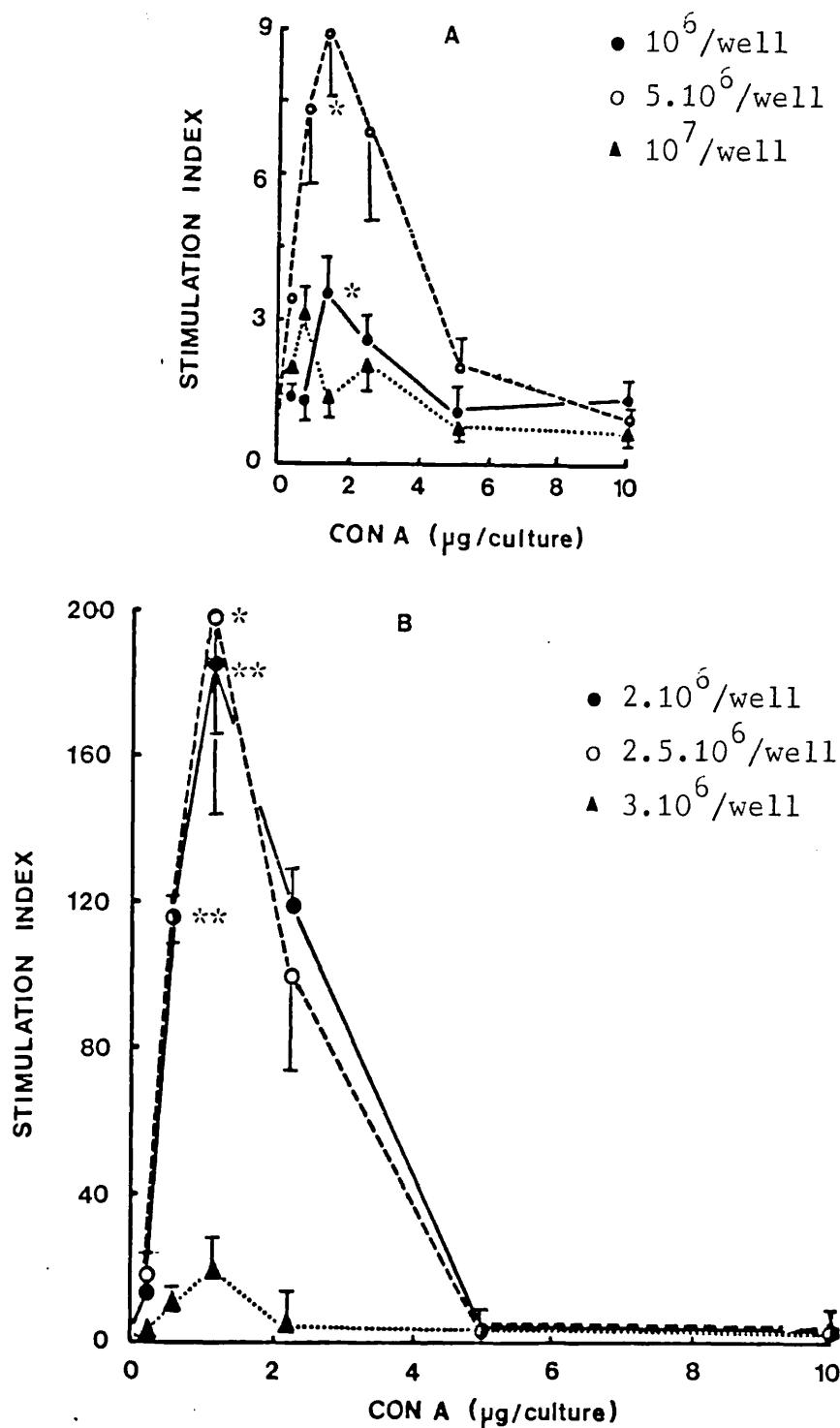
Figure 8. The effect of cell density on $[^3H]$-Thymidine incorporation by rat splenocytes cultured in flat-bottomed microtitre plates.

Cells were cultured for 94h, the final 20h in the presence of 1μCi/well Tdr. Points are mean +/- SEM. P <0.1 *<0.01**. Resting cell cpm: A ● 151+/-37 o 221+/-78 ▲ 345+/-120: B ● 195+/-12 o 261+/-34 ▲ 201+/-27. P compares cpm +/- Con A.

indices was encompassed by a ten-fold increase in cell density (10^6-10^7 cells/well). The peak stimulation indices were also very much higher than those observed in round-bottomed plate cultures although intermediate cell densities were not studied in the latter system.

The optimal Con A concentration for the response in flat-bottomed plates did not change with small changes in cell density in that 10^6, 2.10^6, $2.5.10^6$, 3.10^6 and 5.10^6 cells/well all had optimal stimulation at $1.2\mu g$ Con A. At a density of 10^7 cells/well, however, $0.6\mu g$ Con A gave optimal stimulation; it is thus possible that the same trend in decreasing mitogen concentrations needed with an increase in cell density may exist here as in round plate cultures. Confirmation of this would require the study of a much greater range of cell densities than was used in the present study.

The shape of the dose-response curves to Con A seen in Figs. 7 and 8 indicate an inhibition of stimulation at very high doses of mitogen. This phenomenon has been considered to be due to cell surface binding site interactions when supra-optimal numbers of mitogen molecules are bound to the membrane (McClain and Edelman,1976). It has been shown that high dose inhibition of $[^3H]$-thymidine incorporation is reversible by removal of excess mitogen (Lindahl-Kiessling,1972). Recently, an indication of a possible explanation of high dose inhibition has shown that lymphocytes become resistant to the effects of interleukin-2 during high dose inhibition; this presumably prevents continued progression of the cell through the cell cycle (Ravid et al.,1983). This would explain the observations that high dose inhibition does not prevent early (IL-2 independent?) events on lymphocyte

contact with mitogen, and also that removal of excess mitogen by competing saccharide can release cells from this inhibition (IL-2 susceptibility re-established ?).

Activation of lymphocytes appears to be dependent on cell interactions afforded by vessel geometry. Moorhead et al. (1967) have shown that culture conditions which decrease cell contact adversely affect the response of human lymphocytes to lectins. Thurman et al.(1973), using mixed lymphocyte cultures noted that for a fixed cell density of stimulator and responder cells, flat plate cultures routinely gave lower levels of $[^3H]$-thymidine incorporation than cultures in round plates; these in turn gave lower results than V-shaped well cultures. Similarly, mouse lymph node cells stimulated with PHA and Con A have been shown to require lower cell densities for optimal stimulation in round-bottomed microtitre plates than flat plates (Thorpe and Knight,1974).

Whilst the range of cell densities employed in the present study is not broad enough to be as unequivocal as the above studies, it is evident that, in common with the findings with rat splenocytes, culture conditions that enhance the degree of cell contact promote activation. In the absence of this (flat-bottomed plates) a higher cell density is required to achieve activation. Thus, both the absolute cell density and the degree of cell contact afforded by vessel geometry influence the mitogen stimulation of lymphocytes.

(b) Incubation Time.

For DNA synthesis to occur in lymphocytes in response to a mitogen, the mitogen must be in contact with the cell membrane for fixed periods of time until

a point of commitment is reached beyond which the mito-
gen is unnecessary. This normally takes place at approx-
imately 24h, but the mitogen has been shown to be required
at 1-6h and 18-24h to deliver a first activating signal,
and then to commit the cells to cell division (Lindahl-
Kiessling,1972; Weber et al.,1974; Toyoshima et al.,1976).
The exact time at which the cells begin to synthesise DNA
varies considerably and it has been shown that the length
of G_1 in mitogen activated lymphocytes varies with the
dose of mitogen (Steffen and Stolzman,1969). In addition,
Stenzel et al.(1978) have shown that in human lymphocytes
stimulated with Con A and then treated with α-methylmanno-
side to remove Con A from the cell surface, cells become
commited to DNA synthesis within a few hours at supra-
optimal Con A concentrations; at approximately 24h with
optimal Con A doses and around 40h at sub-optimal doses.
It is thus evident that a single Con A concentration will
elicit DNA synthesis in a responding cell population at
a defined time of incubation. Pulses of [^{3}H]-thymidine
carried out before or after this time will result in :
lower stimulation indices. From this it follows that any
alteration of the incubation time and pulse period will
result in a change in observed stimulation and mitogen
dose required to achieve optimal stimulation. The effect
of incubation time on the stimulation of rat splenocytes
by Con A was investigated in round-bottomed microtitre
plates (Fig 9), in which dose response curves for [^{3}H]-
thymidine incorporation were performed at the times shown.
Although the length of the pulse periods varied it has
been shown that incorporation of thymidine into lymphocyte
DNA is approximately linear for pulse periods of up to
but this requires thymidine to be present at a minimum concentration.
24h (Schellekens and Eijsvoogel,1968). In addition, the
recorded stimulation indices are a function of the level

Figure 9. The effect of incubation time on $[^3H]$-Thymidine incorporation by rat splenocytes cultured in ·round-bottomed microtitre plates.

10^6cells/well were cultured for A 50h, B 64h, C 70h or D 88h, the final 4, 12, 5 or 16h in the presence of 1μCi/well$[^3H]$-Tdr. Points are mean +/-SEM for triplicates. P <0.05* or<0.01** for Tdr incorporation +/- Con A.

of thymidine incorporation by resting cells for the same
period and can therefore be compared.

After 50h incubation it can be seen that
increasing Con A doses gave increasing stimulation indices,
with 10μg achieving optimal activation of DNA synthesis.
At 64h the curve has been shifted left to give optimal
stimulation by 2.5μg Con A and relative suppression at
5 and 10μg. This pattern remained at 70 and 88h incubation
although at 70h there is a transient increase in the level
of incorporation at 10μg Con A. At 88h the dose response
curve is broader with similar stimulation indices seen
with 1.2, 2.5 and 5μg Con A. It is possible that the long-
er pulse period used here has encompassed asynchronous
levels of DNA synthesis.

Patterns of stimulation in flat plates were
routinely found to be similar to those in Fig.9. However,
occasional cultures were seen in which an interesting
difference from the round plate cultures was noted (Fig.10).
In all cultures of duration greater than 50h a dual peak
of stimulation was noted. One of these peaks was consis-
tently observed at 10μg Con A, but the dose of mitogen
eliciting the second peak was found to alter with time.
At 64 and 70h the peak appeared at 2.5μg; by 88h this
occurred at 1.2μg Con A. Thus, with increasing incubation
time lower doses of Con A reveal an emerging stimulation
peak in addition to the consistent peak at 10μg Con A.
Interestingly, this may also explain the transient peak
seen at 70h with 10μg Con A in Fig.9.

The presence of dual peaks of [^{3}H]-thymidine
incorporation in Fig.10 suggests the presence within the
splenocyte fraction of two separate subpopulations of cells
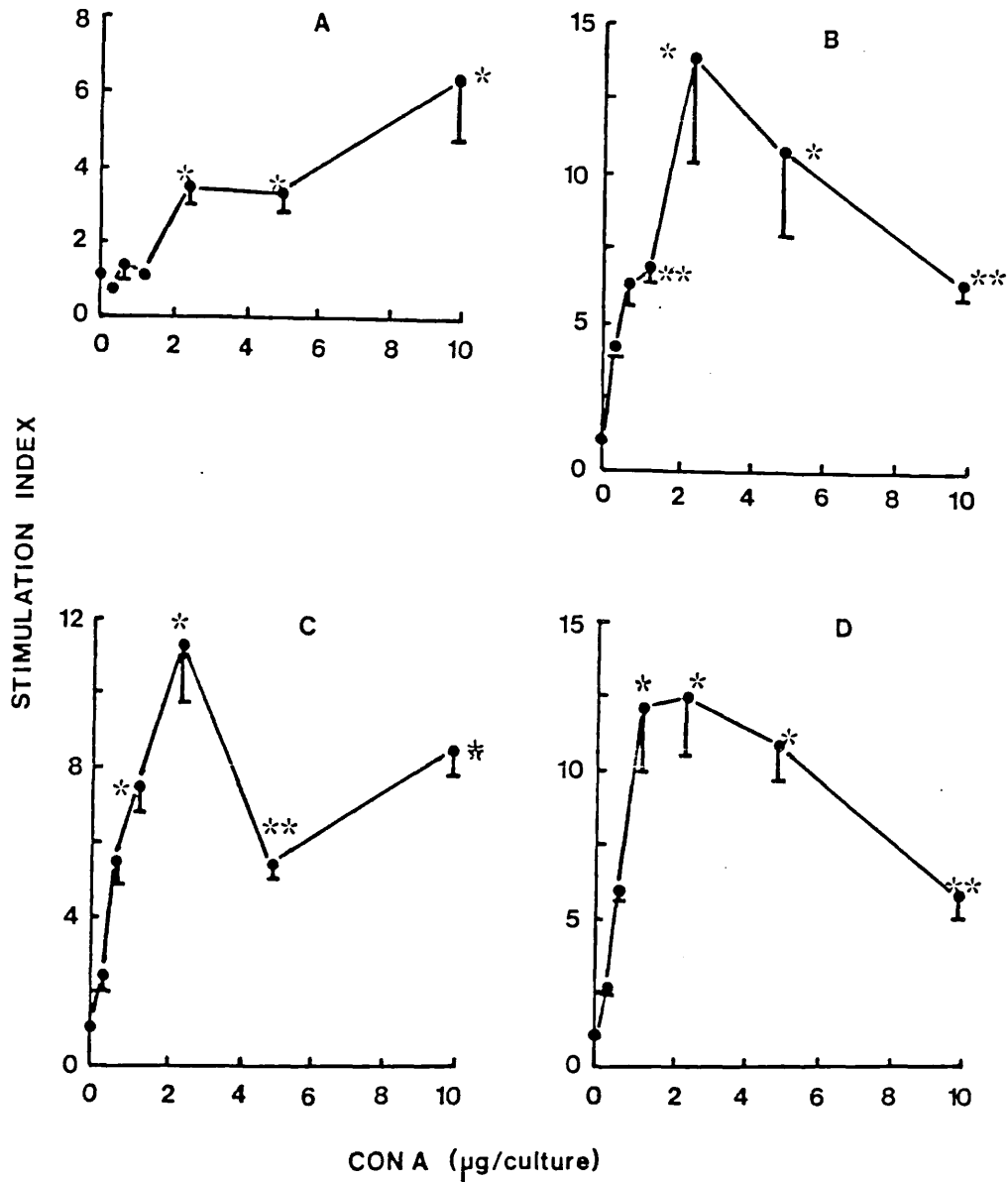
CON A (µg/culture)
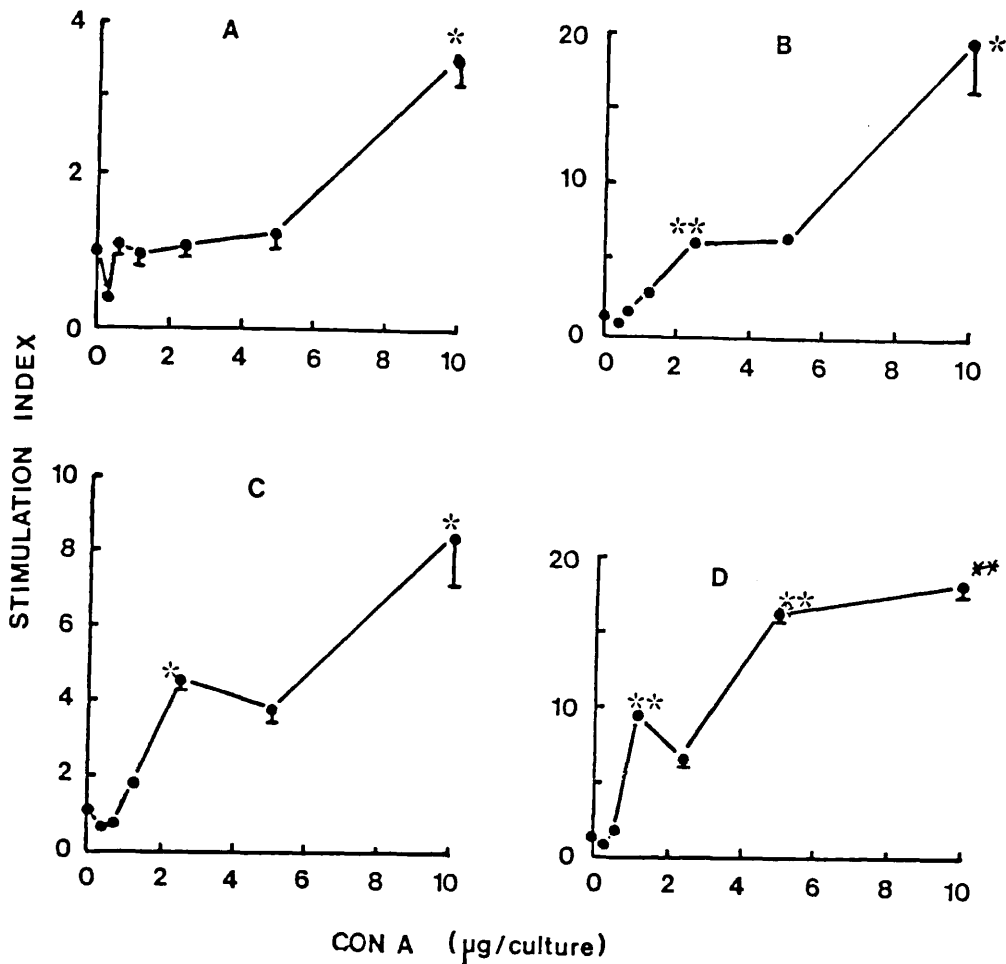
Figure 10. The effect of incubation time on [^{3}H]-Thymidine incorporation by rat splenocytes cultured in flat-bottomed microtitre plates.

2.10^6 cells/well were cultured for A 50h, B 64h, C 70h or D 88h, the final 4, 12, 5 or 16h with 1µCi/well [^{3}H] -Tdr. Points are mean +/-SEM of triplicates. P<0.05* or<0.01**, for cpm +/- ConA.

responsive to different doses of Con A. In support of this
it has been shown that rabbit spleen contains two sub-
populations of T lymphocytes responsive to different
Con A doses (Hardt and Panijel,1976a,b). Mouse spleen
cells also show this phenomenon (Persson et al.,1978); in
addition the response to a particular dose of Con A had
different accessory cell requirements. With respect to
dual stimulation it was suggested , in the latter study,
that macrophages may act by absorbing excess Con A which
would normally result in high dose inhibition of the
response. If a similar phenomenon is occurring in the
present study, it is evident that cell contact is an
important factor, since the dual peak was normally seen
only in flat plates.

It should also be emphasised that the spleen
lymphocyte population used in the present study contains
both T and B lymphocytes, and no attempt has been made
to study their response to the mitogens separately. B cells
are known to enhance the response of rabbit spleen T cells
to Con A and PHA but are not themselves stimulated by
the mitogens (Cavaillon et al,1972). A further possibility
is that activated T cells may initiate a response in B
cells by soluble factors. In this respect, it has recently
been shown that mouse B lymphocytes can be activated in a
T dependent manner in response to Con A (Ratcliffe and
Julius,1984). The response was abolished by competing αMM
saccharide, but culture supernatants were ineffective in
replacing the T cell requirement which implies that the
effect was not mediated by lymphokines alone.

It is evident from Fig. 9 and 10 that a
given Con A concentration will elicit an optimal response
in a cell population at a precise incubation time. This

means that the designation of Con A concentrations as sub- or supra-optimal will only apply at a given time of incubation; it therefore seems of paramount importance in investigations of this kind that a range of mitogen doses be studied in addition to incubation times to produce an optimal system. Thus, an alteration in incubation time will also require a simultaneous change in the dose of mitogen used to maintain optimal stimulation.

(c) CO_2 Tension.

Bicarbonate buffered media are routinely employed in mammalian cell culture, especially those involving long term culture. The major drawback associated with the use of bicarbonate buffered media is the re-quirement for CO_2 in the gas phase in order to control medium pH. A bicarbonate concentration requiring an external CO_2 tension of 5-10% in air is usual for the culture of lymphocytes, but in the present study initial culture of rat and mouse spleen lymphocytes using 10% CO_2 in air revealed an interesting effect on Con A stimulation (Fig. 11). Mouse splenocytes showed very high levels of [^{3}H]-thymidine incorporation in response to Con A over that observed in resting cells, but no response was seen by rat splenocytes. In contrast, cells from both sources were capable of responding to Con A at the lower CO_2 tension of 2% in air. This shows a fundamental difference in the susceptibility of the cells from the two species to the prevailing pH. On examination of the cultures, it was observed that rapid acidification of the medium occurred in both rat and mouse splenocyte cultures. (as judged by the colour change of phenol red indicator), but the significance of this was not immediately realised

A] 2% CO$_2$ IN AIR

STIMULATION INDEX

B] 10% CO$_2$ IN AIR

STIMULATION INDEX

MOUSE

RAT

CON A (µg /culture)
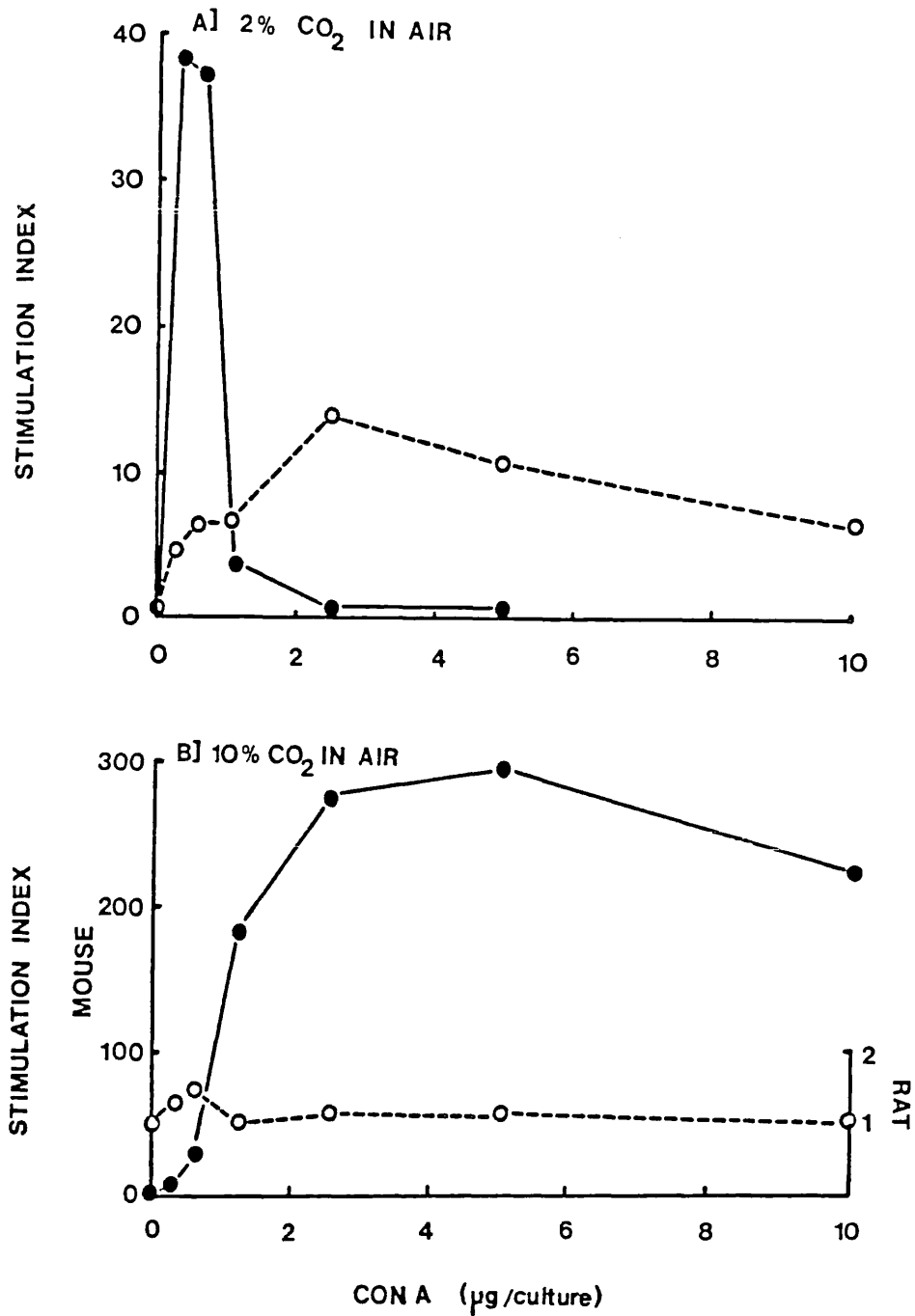
Figure 11. The effect of CO_2 tension on [^{3}H] -Thymidine incorporation by rat and mouse splenocytes in round-bottomed microtitre plates.

10^6 cells/well were cultured for A 64h and B 90h, the final 16h with 1µCi/well [^{3}H]-Tdr. Points are mean of 3-6 replicates. ● Mouse cells o Rat cells.

as mouse spleen lymphocytes responded well to Con A under these conditions. At 2% CO_2 in air both mouse and rat splenocyte cultures maintained media pH's of between 7.2-7.4 and stimulation resulted in response to Con A in both cases (Fig.11). The explanation for this lies in the presence of HEPES buffer in RPMI 1640 (Dutch Modification) medium, thereby reducing the CO_2 tension required to maintain optimal medium pH. It is evident that mitogen stimulation of mouse splenocytes is more resistant to fluctuations in pH than that of rat splenocytes.

While rat splenocytes were subsequently cultured in an external CO_2 tension of 2% in air with successful stimulation, it was noted that wells situated at the periphery of the microtitre plate routinely showed evidence of acidification whilst medium in central wells maintained pH 7.2-7.4. This, again, was shown to have a profound effect on the response of the cells to Con A (Fig.12). In this experiment the position of replicate resting and Con A activated cultures was altered at the different incubation times shown; in 48h and 64h cultures 0.3, 1.2 and 5μg Con A wells were situated in the peripheral rows. At 89h this was altered so that 0.6, 2.5 and 10 μg Con A containing cultures were in these positions. A comparison of the resulting dose response curves of stimulation reveals that the effect of the pH change in these wells was independent of both the Con A concentration present and the incubation time. Not only was the response to Con A in the peripheral wells effectively abolished by the acidification of the medium, but resting cells also showed suppressed incorporation of [^{3}H]-thymidine by comparison with resting cells cultured in central wells. A low level of stimulation is still seen in peripheral wells (Fig.12) since the stimulation index has been

Figure 12. <u>The effect of culture position on $[^3H]$-
Thymidine incorporation by rat splenocytes
cultured in flat-bottomed microtitre
plates.</u>

2.10^6 cells/well were cultured for the times shown,
the final 6h with 1μCi/well $[^3H]$-Tdr. Arrows show
replicate cultures postioned at the periphery of the
culture plate. Points are mean+/-SEM of triplicates.
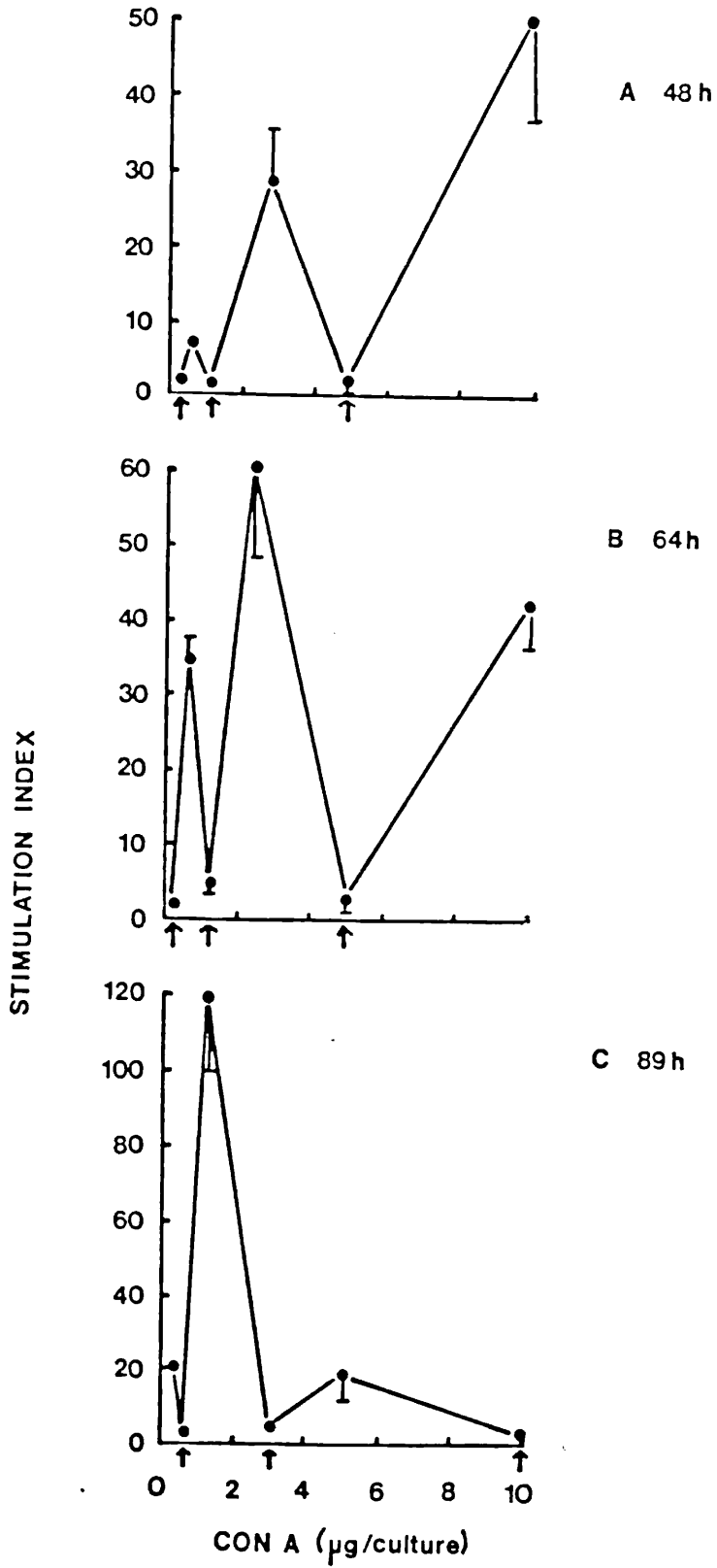
calculated using the appropriate resting cell cpm as the denominator.

The 'edge effect' observed here was routinely seen in both flat and round bottomed microtitre plate cultures. Replacement of the peripheral well cultures with sterile water or medium alone merely displaced the effect to the next row of wells, thereby reducing the number of wells available for analysis of results. For this reason subsequent cultures were performed with intact peripheral cultures but the results were not analysed.

The susceptibility of lymphocytes to small changes in medium pH has been noted previously. Thorpe and Knight (1974) have noted the depressed response of mouse lymphocytes when cultured at the the edge of the plate; thus stimulation of mouse cells also appears to be affected by the pH although this was not evident in the present study. Rat peripheral blood lymphocytes stimulated with PHA showed inhibition of thymidine incorporation ; this was attributed to minor variations in medium pH by the authors (Keller et al.,1982).

It is quite probable that the observations reported here are commonly encounted in lymphocyte culture, but in view of the increasing use of rat cells in metabolic studies of lymphocyte activation, effects such as these should be noted. Mouse splenocytes have been shown here to respond to Con A under conditions which almost completely abolished the response by rat cells; it is thus highly possible that such suppression would also have repercussions on the changes in metabolism associated with activation.

(d) Cell Viability.

Cell viability in long term cultures of lymphocytes is routinely assessed by the exclusion of a dye by living cells. Cells were harvested at various times throughout the incubation and the ability of the cells to exclude the dye nigrosin analysed. Estimations in Con A containing cultures were made difficult by the inherent clumping of mitogen activated cells, but it is evident from Fig.13 that in flat plate cultures both resting and Con A activated cells maintain greater than 60% viability for the whole of the 72h culture period. Similar rates of cell death were observed for the initial 2ȝh of incubation, thereafter Con A containing cultures continue to die while resting cells maintain a continued level of viability. In round plates resting cells appear to lose viability at the same rate as Con A containing cultures throughout the incubation; this may be influenced by the degree of pelleting which occurs in these plates.

The reporting of levels of viability in long term cultures of lymphocytes is scant and this may be a reflection of the difficulty in assessing stained cells within clumps of mitogen activated cells. However, where reports do exist the use of dyes which are excluded by living cells is common. Dyes such as trypan blue (Hoskins et al.,1956) and nigrosin (Kaltenback et al.,1958) have a major advantage over alternative methods since they provide quantitative measurements of viability. A criticism of the method is that it relies on the relative permeability of the cells to the dye, and therefore it is a direct reflection only of the membrane integrity, and does not supply information on the metabolic competence of the cells.
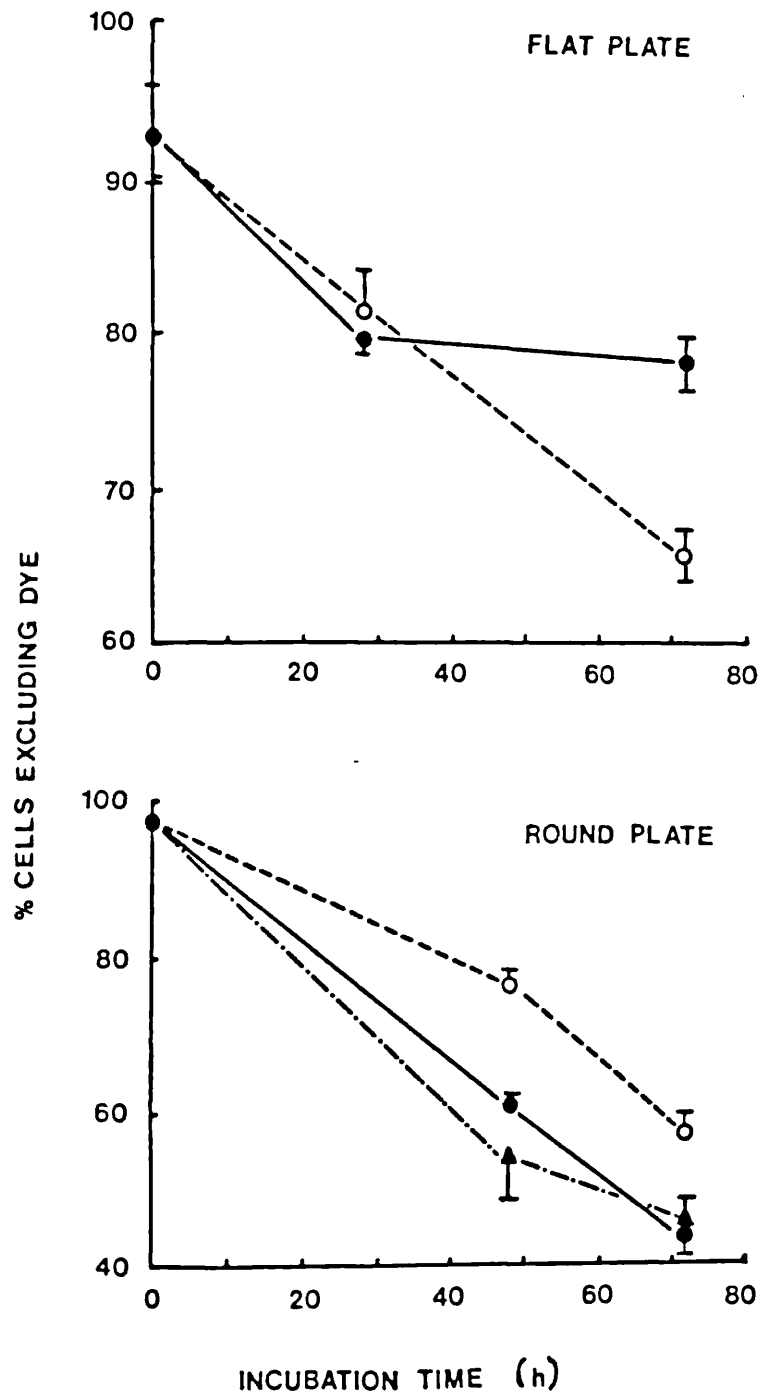
Figure 13. <u>Viability of rat splenocytes in long term</u>
         <u>culture</u>.

2.10^6 cells/well (flat) or 10^6 cells/well (round) were
incubated for 72h in the absence (●) or presence   of   (o)
1.25μg and (▲) 5μg Con A. Points are mean +/-SEM of 4
replicates. Total viable cell number at 72h : Flat -ConA 1.29×10^6, +ConA
9.86×10^5 ; Round -ConA 8.7×10^4 $1.25 \mu g$ ConA 1.12×10^4 $5 \mu g$ ConA 6.55×10^4.

Literature reports of long term viability of lymphocytes show in general a high retention of viability by resting cells. Horse peripheral blood cells show 81, 79 and 71% viability on day 1, 2 and 3 of incubation (Pachman,1967). Human peripheral blood lymphocytes do not drop below 94% viability during a three day culture (Roos and Loos,1973). In general, mitogen activated cells show lower viabilites than resting cells, especially after two to three days in culture. For example, human peripheral blood lymphocytes activated with PHA are only 53% viable after 72h (Roos and Loos,1973), and Lengle et al. (1978) report a drop in viability from 80 to 10% between 24 and 48h incubation of young mouse thymocytes with Con A.

Whilst the argument against the use of exclusion dyes as a measure of cell viability is justified, it continues to be a usual method of assessment, but the use of alternative methods such as metabolite incorporation and enzyme leakage will be discussed with respect to short term cultures of rat splenocytes in Section 3.2.

3.1.2. THE METABOLISM OF EXOGENOUS RESPIRATORY SUBSTRATES

The large quantitative change in medium glucose concentration that accompanies mitogenic lymphocyte stimulation in culture has, understandably, led to considerable attention being paid to the role of this substrate in the provision of energy for transformation. Whilst it has been shown that glucose is essential to the stimulation process (Kay,1976), the involvement of other potential energy producing substrates has received relatively little attention. It is likely that the study of the capacity of lymphocytes to utilise respiratory substrates other than glucose, such as the ketone bodies, free fatty acids and amino acids will also aid in the understanding of the role of aerobic glycolysis in lymphocyte transformation.

Only in recent years has this approach to the study of alternative respiratory substrates been applied to lymphocytes (Ardawi and Newsholme,1983;1984; Brand et al.,1984; Brand,1985). In all these instances experiments were carried out on very short term incubations of lymphocytes with mitogen (1-3h), and whilst this has undoubted advantages in terms of ease of experimentation, it also lacks the ability to detect any qualitative or quantitative changes in substrate utilisation which may be occurring at later stages of the transformation event. It is certainly true that cells studied immediately after harvest from the host animal will maintain high viability and will not be subjected to effects caused by nutrient depletion, accumulation of toxic waste materials etc, but there are also arguments for at least establishing patterns of substrate consumption over the entire length of the mitogen stimulated culture. As an example, it has been

shown that there is a temporal correlation between DNA synthesis and enhanced aerobic glycolysis by Con A activated mouse splenocytes (Wang et al.,1976). This type of association has been cited as evidence for the importance of aerobic glycolysis on lymphocyte transformation, and also emphasises the need to extend current studies on early changes in metabolism to the long term model.

In the following sections the consumption of glucose, acetoacetate, D β-hydroxybutyrate and L-glutamine by rat splenocytes in various combinations will be investgated with a view to focussing on relevant substrate metabolism in 24h cultures in the subsequent sections. In this way it is envisaged that substrates which represent a viable alternative to glucose as energy providing compounds will be identified; their change in metabolism on lymphocyte activation can then be studied in detail.

(a) GLUCOSE

The uptake of glucose by rat splenocytes was measured in cultures of cells incubated with 0-10µg Con A in round-bottomed microtitre plates (Fig.14). Surprisingly, there is no significant difference between the rates of glucose consumption by resting and Con A activated cells, nor is there a difference between the overall change in medium glucose concentration over the entire length of the culture. This occurs despite the confirmation of significant stimulation as judged by enhanced [^{3}H]-thymidine incorporation. Concentrations of Con A between 0.3 and 5µg elicited parallel profiles of glucose consumption to those shown in Fig.14, but have been ommitted for clarity. The uptake of glucose at all concentrations of Con A is between three and six times slower in the first 44 hours than in
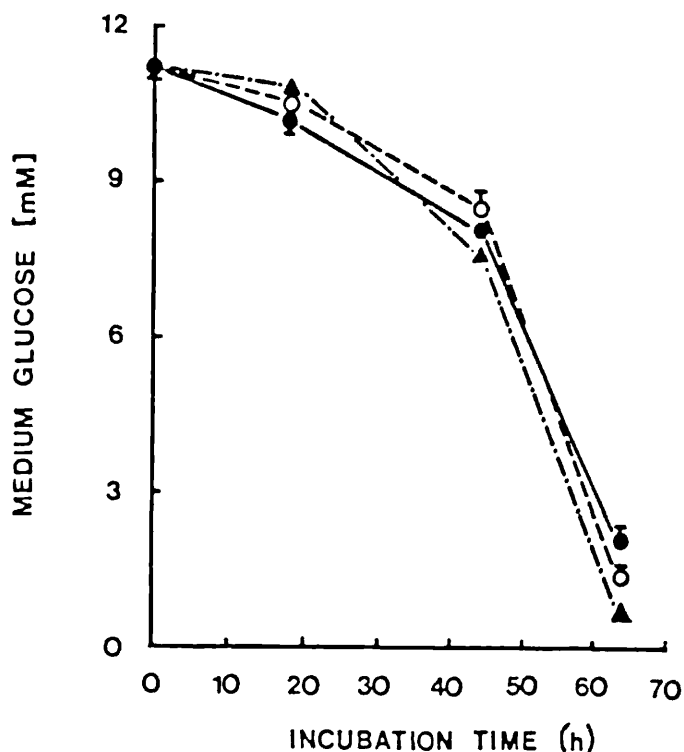
Figure 14. The uptake of glucose by rat splenocytes
            cultured in round-bottomed microtitre plates.

10^6 cells/well were cultured for 64h in the absence (●)
or presence of (o) 2.5µg and (▲) 10µg Con A. Medium
glucose concentrations were assayed at the times shown
according to Materials and Methods. Stimulation indices
measured over the final 6h of culture are 2.5µg Con A:
10.4+/- 0.8 and 10µg Con A: 0.83+/-0.2. Points are mean
+/-SEM of triplicates except where this is smaller than
the symbol size.

the final 20 hours; thus both control and mitogen activated rat splenocytes consumed the majority of glucose in the final third of the incubation period (Table 7).

Table 7. <u>Rates of glucose consumption by rat splenocytes cultured in round-bottomed plates.</u>

Con A (μg)	Glucose uptake (μmol/10^{10} cells/h)			S.I +/- SEM
	0-44h	44-64h	0-64h	* P$\leq$.001
0	141 $\pm$ 15.9	635 $\pm$ 35	295.6 $\pm$ 11	
.3	150 10.0	563 13.2	279.1 1.8	4.9 $\pm$ .5*
.6	186 9.6	523 12.3	291.6 1.2	14.8 1.9*
1.2	171 12.8	627 16.3	314.1 .9	12.3 1.3*
2.5	128 16.3	715 20.9	311.5 1.0	10.4 .8*
5.0	179 8.2	700 10.4	341.9 0.4	11.8 .9*
10.0	176 3.6	676 16.2	331.9 0.7	0.8 .2

Results are mean +/- SEM of triplicates. SI determined 58-64h.

It is notable from Table 7 that the ability of the cells to respond to Con A, in terms of [^{3}H]-thymidine incorporation is not reflected in their glucose consumption. The results conflict with literature findings on lymphocyte glucose consumption on mitogen activation in two main ways; the addition of Con A to resting cells does not alter the rate of glucose consumption, and the quantitative uptake of glucose by resting cells is very high. Table 8 gives a representative sample of the literature rates of glucose uptake by a variety of resting and mitogen activated lymphocytes. Comparison of the data with that presented in Table 7 indicates that the rates found in the present study are generally much higher than those found by other workers, particularly with respect to

Table 8. Rates of glucose consumption by mitogen stimulated lymphocytes.

Rates have been recalculated or estimated from graphs where necessary to provide a uniform denominator.

Cell Source and Mitogen	Vessel (Cells/ml)	Glucose Consumption $\mu mol/10^8$ cells/h (Incubation Time)	Reference
Horse PBL + PHA	Tubes $6.5.10^6$	Control 16.3(24h)16.3(48h)15.4(72h) PHA 58.3 59.6 65.7	Pachman,1967
Human PBL + PHA	Tubes $1-2.10^6$	Aerobic: Control 60.2(2.5h)60(24h)69(48h)66(72h) PHA 102 240 264 340 N_2 Flushed: Control 84(2.5h)138(24h)144(48h)168(72h) PHA 174 480 480 480	Rabinowitz et al. 1968
Human PBL + PHA	Flasks $1.9.10^7$	PHA 156(24h) 426(72h)	Roos & Loos,1973
Human PBL + PHA	Flasks 1.10^6	Control 68(24h)62(48h)64(68h) PHA 202 539 514	Sagone et al. 1974
Rat thymocytes + Con A	Flasks $1.3.10^8$	Control 13.9(1h) Con A 32.6 PHA 27.5	Culvenor & Weidemann,1976

Table 8. cont.

Cell Source and Mitogen	Vessel	(Cells/ml)	Glucose Consumption μmol/10^{10}cells/h (Incubation Time)	Reference
Rat thymocytes + Con A	Flasks	1.3.10^8	Control 24.8 (1h) Con A 52.0	Yasmeen et al.,1977
Rat thymocytes + Con A	Flasks	1.3.10^8	Control 24.2 (3h) Con A 47.1	Hume et al.,1978
Rat mesenteric + Con A	Flasks	1-2.10^8	Control 28.7 (1h) Con A 44.2	Ardawi & Newsholme, 1984
Rat thymocytes + Con A	Vials	1-5.10^8	Control 24.5 (3h) Con A 45.4	Brand et al.,1984
Rat thymocytes + Con A	Vials	1-5.10^8	Control 17.0 (1-3h) Con A 38.0 Con A + IL-2 893 (60h prior incubation)	Brand,1985

resting cells. Interestingly, the long term experiments
(68-72h) show average rates of consumption of glucose
which are higher than those of much shorter cultures. It
is possible that this may be due to a species difference
as all the short term experiments in Table 8 employed rat
lymphocytes. An alternative explanation may be the culture
vessel used; the settling of cells to the base of the vessel
is perhaps more likely to result in overcrowding in the
long term studies which used tubes rather than flasks which
have a greater surface area over which the cells may settle.
In addition, it is more common for short term cultures to
be accompanied by gassing with oxygen and concomitant
shaking; by contrast long term studies invariably remain
static. With respect to this, it is relevant to note that
an early study by Cooper et al. (1963) recorded a stoichio-
metric conversion of glucose to lactate by human thoracic
duct lymphocytes incubated with PHA; this was attributed
to anaerobiosis induced by the settling of the cells to
the base of the culture tube.

The rates of glucose uptake by lymphocytes
in which the cultures were first flushed with nitrogen
(Rabinowitz et al.,1968) show similarity to the present
data (Table 7). Even so, PHA was still shown to induce a
two to three fold increase in glucose consumption over
resting cells; this was not seen with rat splenocytes and
Con A. Three points which may aid in the explanation of
the lack of correlation of results presented in Table 7
with literature findings are that (i) visual examination
of the round-bottomed plate cultures revealed the forma-
tion of tight cell 'buttons' at the base of the well after
approximately 24h of culture; (ii) resting splenocytes
consume vast quantities of glucose and (iii) the majority
of glucose consumed by both resting and Con A activated

splenocytes is taken up during the final 20h of culture.
These observations are consistent with the hypothesis
that the cells are initially able to metabolise glucose
aerobically, but become essentially anaerobic after the
settling of the cells to the base of the well. In order to
test this hypothesis, the cell density was decreased;
based on the rationale that the transition of the cells to
anaerobic glucose metabolism should be delayed. Supra-
optimal Con A concentrations were also employed and were
observed to lead to extensive aggregation of the cells
with subsequent dispersal over the base of the wells.
This was shown to result in the high dose inhibition of
$[^3H]$-thymidine incorporation at all Con A concentrations
used. An interesting effect of the dispersal on glucose
consumption was also noted (Fig.15). Resting cells, which
pelleted normally in the absence of Con A showed rates of
glucose consumption of 65.7 μmol/10^{10}cells/h over the first
4$\leq$h of culture. This rate increased to 666 μmol/10^{10}cells
/h during the final 20h. By contrast, the consumption of
glucose by Con A containing cultures was inversely related
to the mitogen concentration. The most extensive dispersal
of cells was seen at 25μg Con A and correspondingly less
aggregation at the lower mitogen concentrations. That the
effect on glucose uptake was not a direct result of toxicity
of increasing concentrations of Con A is shown by the fact
that, where significant uptake of glucose does occur, it
takes place during the final stages of the incubation; this
was also noted in Fig.14.

Taken together, therefore, Figs.14 and 15 lead
to the suggestion that dissolved oxygen in the medium is
adequate to support aerobic metabolism of glucose under
normal conditions. However, pelleting of the cells results
in a partially anaerobic microenvironment with the concom-
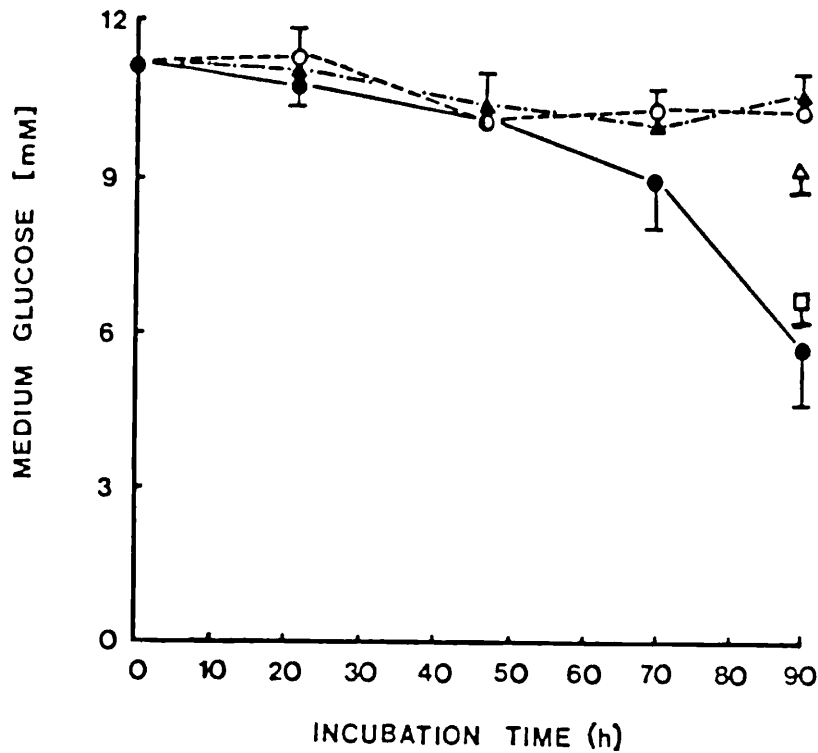
Figure 15. The effect of supra-optimal Con A concentration on glucose uptake by rat splenocytes cultured in round-bottomed microtitre plates.

5.10^5 cells/well were cultured for 90h in the absence (●) or presence of (□) 3µg (▲) 6µg (o) 12µg or (▲) 25µg Con A. Medium glucose concentrations were assayed at the times shown. Points are mean +/-SEM of triplicates.

itant alteration in glucose consumption. At supra-optimal
Con A concentrations less glucose is consumed since oxygen
is available to the cells in the severely dispersed state.
An alternative view is that the transition from an aerobic
to an essentially anaerobic metabolic state is a normal
part of the stimulation process; this cannot be confirmed
here, since in round-bottomed plates resting splenocytes
show an identical pattern of glucose uptake (Fig.14).

If the pelleting of the splenocytes induced
by the well geometry is responsible for the high rates of
glucose consumption observed in Fig. 14 and 15 it follows
that flat-bottomed microtitre plates should present a
sufficiently large surface area for the cells to maintain
relatively aerobic glucose metabolism. Fig.16(A) shows the
uptake of glucose under these conditions. Medium contain-
ing 11m$\underline{M}$ glucose is routinely used in long term lymphocyte
cultures; in this medium resting splenocytes consume 17.6
+/- 3.4 μmol/10^{10}cells/h and Con A activated cells 51.9 +/-
5.3 μmol/10^{10}cells/h. These rates correlate well with the
rates found by other workers in Table 8. Interestingly,
very similar rates were seen when resting splenocytes were
cultured in the more physiological concentration of 5.5m$\underline{M}$
glucose (Fig.16(B)); 19.3+/-1.8 μmol/10^{10}cells/h, but the
slightly higher rate of 69.2 +/- 1.7 μmol/10^{10}cells/h was
observed in the activated cultures in this medium.

These results confirm the view that rat splen-
ocytes cultured in round-bottomed plates were apparently
forced into an essentially anaerobic environment by the
well geometry. It is relevant to note that the ability of
the cells to respond to Con A in terms of $[^3H]$-thymidine
incorporation was not affected by this. Therefore, at least
in round plates, there is no direct correlation between

glucose consumption and enhanced DNA synthesis on mitogen activation. Moreover, these results suggest an inherent metabolic adaptability of the cells to the prevailing culture conditions whilst maintaining a 'conventional' response to the mitogen. The latter point is particularly interesting, as it implies that the emphasis laid on the propensity of activated lymphocytes for aerobic glycolysis may be a direct result of the conditions employed, as is the case in the present work.

Confirmation of the relatively aerobic state of the cells cultured in flat-bottomed microtitre plates is afforded by the measurement of lactate production in the absence of Con A (Fig.16). Net lactate production by resting lymphocytes cultured in 11mM glucose was 20.3 +/- 6.4 μmol/10^{10}cells/h and by Con A activated cells 52.2 +/- 5.6 μmol/10^{10}cells/h ; this is equivalent to 58 and 50% conversion of glucose to lactate respectively. In 5.5mM glucose medium, lactate production is 5.2 +/- 3.9 and 73.8 +/- 5.3 μmol/10^{10}cells/h by resting and Con A activated cells, respectively, which is equivalent to 14 and 53% conversion of consumed glucose to lactate. Thus, there is an increase in the proportion of consumed glucose to lactate in 5.5mM glucose in the presence of Con A. Hume et al.(1978) showed a 38% and 58% conversion rate of glucose to lactate by resting and Con A activated rat thymocytes. Similarly, Brand et al.(1984) found that 40% of glucose consumed by resting rat thymocytes and 57% of that consumed by Con A activated cells was converted to lactate. However, in both these studies the total incubation time was only 3h long. In the long term study of Rabinowitz et al.(1968), human peripheral blood lymphocytes showed a 50% conversion rate and this increased to 60% in the presence of PHA. Interestingly, resting horse lymphocytes show a higher glycolytic
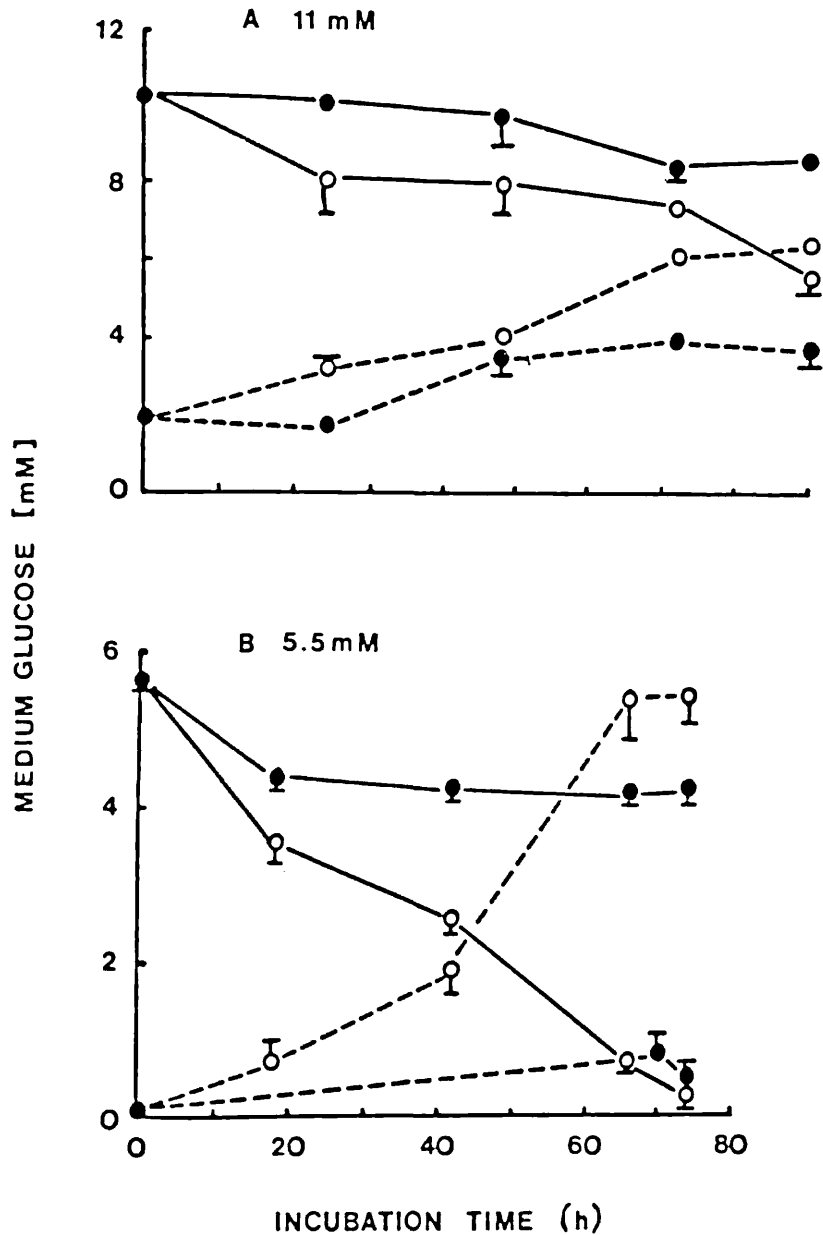
Figure 16.   Glucose consumption and lactate production
             by rat splenocytes cultured in flat wells.

2.10^6cells/well were cultured in A 11mM or B 5.5mM glucose
medium in the absence or presence of 1μg Con A. Stimulation
indices measured over the final 7h of incubation were
A: 24.3+/-12 and B: 38.6+/-0.9. Points are mean +/-SEM of
triplicates except where this is smaller than the symbol
size. (●—●) Glc -Con A, (o—o) Glc +Con A, (●- -●) Lac
-Con A and (o--o) Lac +Con A.

rate (94% conversion) than in the activated state (75%)
during a 72 hour incubation. It would thus appear that
the duration of the incubation affects the proportion of
glucose converted to lactate; it is not known to what
extent this is influenced by the physical culture condit-
ions rather than being due to an integral metabolic trans-
ition at a stage late in the incubation. From results
presented here it is evident that Con A activation of rat
splenocytes can occur without a major quantitative
difference in glucose uptake between resting and activated
cells (Fig.14) and without a large quantitative change in
terms of the proportion of lactate produced from consumed
glucose (Fig.16A), as well as with the conventional
increase in both absolute glucose consumption and aerobic
glycolysis (Fig.16B).

The absolute amount of glucose consumed and
the proportion of this metabolised to lactate by rat
splenocytes cultured in round plates was also found to be
affected by the initial glucose concentration (Fig.17).
As before, the rates of glucose uptake by resting and Con A
activated cells are very similar, despite the presence of
significant stimulation of $[^3H]$-thymidine incorporation.
However, the quantitative rates of uptake are increased
when cultures are performed in 11mM glucose, compared
with the rates observed in 5.5mM glucose; resting cell
consumptions were found to be 122.3 +/- 4.2 $\mu mol/10^{10}$cells
/h in 5.5mM and 184.2 +/- 13.3 $\mu mol/10^{10}$cells/h in 11mM
glucose. Con A activated cells consumed 130.3 +/- 0.4 μmol
/10^{10}cells/h in 5.5mM glucose and 208.9 +/- 8.3 $\mu mol/10^{10}$
cells/h in 11mM glucose. Although the supraphysiological
initial glucose concentration was found to increase the
absolute glucose uptake by cells cultured in round plates

Figure 17. <u>Glucose consumption and lactate product-</u>
<u>ion by rat splenocytes cultured in round</u>
<u>wells.</u>

10^6 cells/well were cultured in (A) 11mM or (B) 5.5mM glucose medium in the absence or presence of 1μg Con A. Stimulation indices for thymidine incorporation during the final 7h of culture were (A) 8.5+/-1.1 and (B) 7.0+/-0.7. Points are mean+/-SEM of triplicates except where this is smaller than the symbol size. (●—●) Glc -Con A, (o—o) Glc + Con A, (●--●) Lac- Con A and (o--o) Lac + Con A.
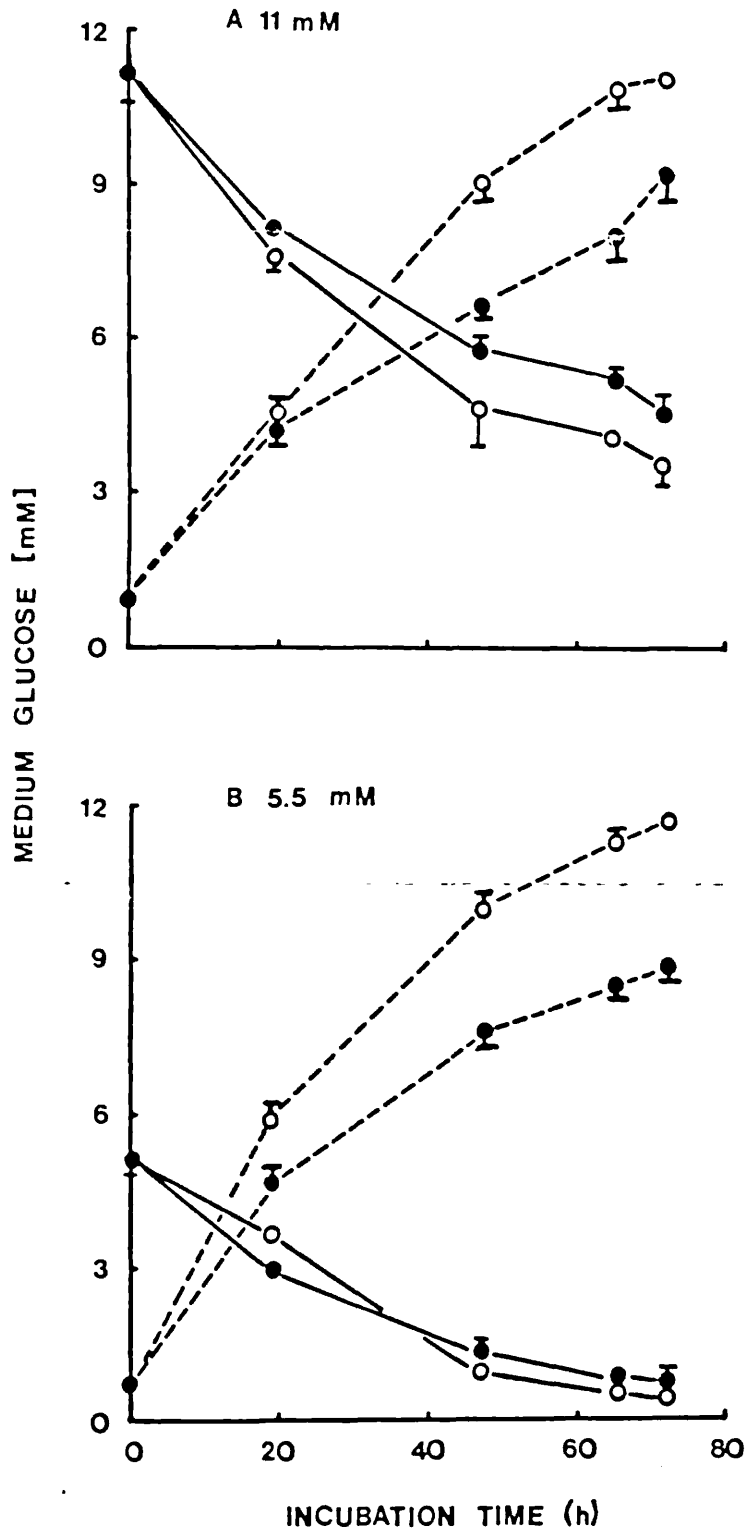
a smaller proportion of consumed glucose was converted to lactate than by cells cultured in physiologicalglucose. Thus, 83% and 116% of consumed glucose was converted to lactate by resting and activated cells respectively at 5.5 m$\underline{M}$ glucose; this was reduced to 62 and 70% at the higher glucose concentration.

The effect of high initial glucose concentrations on the proportion of consumed glucose to lactate by lymphocytes has not been previously proposed; this is likely to be a result of the tendency for metabolic studies on lymphocytes to confine themselves to physiological glucose concentrations. There is some support for the view that glucose metabolism may be altered by the length of incubation of lymphocytes since it is noticeable from Table 8 that the long term studies of Pachman (1967), Rabinowitz et al. (1968) and Roos and Loos (1973a,b) show higher mitogen stimulated rates of conversion to lactate (90,60 and 85% respectively) than the short term incubations. The latter studies by Hume et al.(1978), Ardawi and Newsholme (1983), Brand et al.(1984) and Brand (1985) all show percentage conversions between 50-57% for mitogen activated cells. This occurs despite the fact that cell densities employed in the shorter studies were much larger and would therefore be expected to predispose the cells to relatively anaerobic conditions. It is therefore possible that a transition to a more anaerobic glucose metabolism may occur in cells cultured for more than a few hours.

In the present study it has been shown that both the absolute rate of glucose consumption and the degree of glycolysis resulting are affected by the vessel geometry and initial glucose concentrations for both resting and Con A activated splenocytes. Perhaps the most

important conclusion from this is not related to the absolute quantitative glucose consumption, but is the fact that a variety of results have been obtained which show different metabolic responses by the cells to the culture conditions whilst maintaining the ability to mount a response to the mitogen. Since conventional activation has been shown to occur in the present system with aerobic and at least partially anaerobic glucose metabolism, the results lead to the suggestion that, in this system, the metabolism of glucose inresponse to Con A activation is largely dictated by the prevailing culture conditions. By inference, it may be suggested in addition that this could also be the case for many other observations of lymphocyte glucose metabolism.

In view of the fact that glucose consumption by rat splenocytes cultured in round-bottomed plates was shown to have no correlation with the extent of stimulation as judged by $[^3H]$-thymidine incorporation (Fig.14), it is of interest to establish whether such a relationship exists in flat-bottomed plates. Table 9 gives the results for a representative group of experiments in which a range of stimulation indices were obtained after pulsing with $[^3H]$-thymidine. Overall rates of glucose uptake were measured throughout the incubation and these were analysed in the light of the stimulation indices; although high stimulation was associated with a large increase in Con A activated glucose consumption, it can be seen that there is no direct relationship between the two parameters. This effectively suggests that there is no qualitative association between enhanced glucose uptake induced by Con A and the mitogen activated increase in DNA synthesis which results. This, together with the results obtained with round plate cultures

TABLE 9. LACK OF CORRELATION BETWEEN GLUCOSE UPTAKE AND
STIMULATION INDEX BY FLAT PLATE CULTURED RAT
SPLENOCYTES.

Results are mean +/-SEM of triplicate cultures.

GLUCOSE CONSUMPTION μmol/10^{10}cells/h		%INCREASE + CON A	SI +/- SEM	
Control	+CON A			
32.3+/-2.9	50.3+/-2.0	156	3.4+/-0.9	
24.2 1.9	69.5 0.3	287	18.2	0.7
32.9 3.2	59.9 6.2	182	22.8	6.0
19.3 1.8	69.2 1.7	359	38.6	0.9
17.5 6.3	57.4 2.0	328	237.0	16.0

also imply that the measurement of enhanced glucose uptake
cannot be used as an alternative method of assessing
mitogenic lymphocyte activation, as has been suggested for
human peripheral blood lymphocytes (De Cock et al,1980).

Since it is apparent thus far that, at least
when cultured in flat plates approximately 40% glucose
carbon cannot be accounted for as lactate, it is of interest
to examine the fate of the remaining carbon. One possible
fate is pyruvate, which may then be removed into the medium
as was the case with lactate. Levels of medium pyruvate
were measured in rat splenocyte cultures performed in both
flat and round bottomed microtitre plates, in the presence
and absence of Con A (Fig.18). The absolute level of
pyruvate present at the start of the incubation is low,
even so, it can be seen that a small amount of pyruvate is
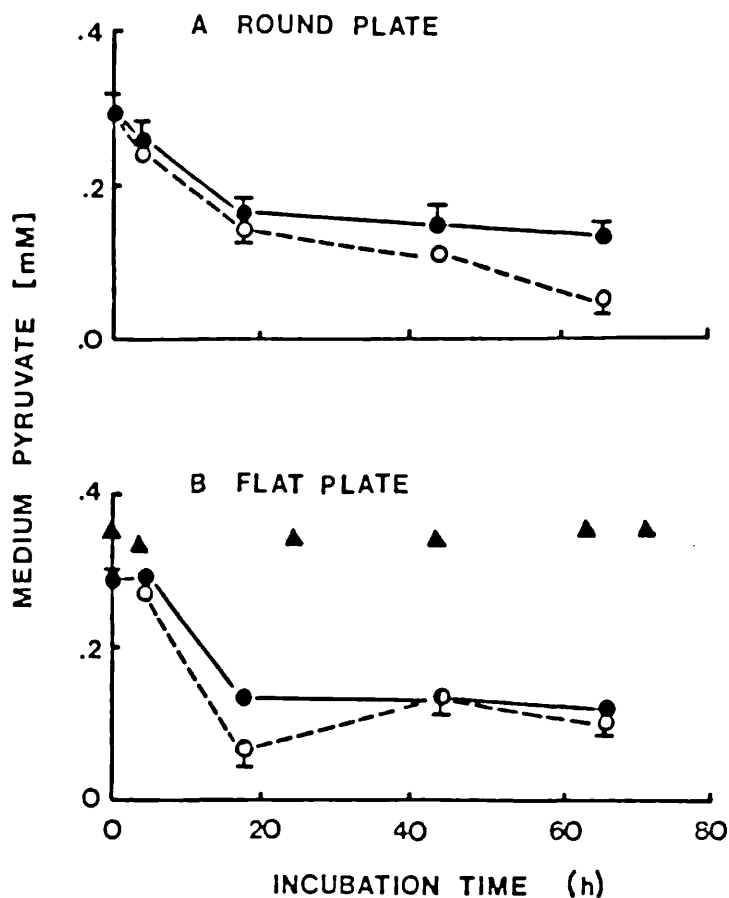
Figure 18. Pyruvate consumption by rat splenocytes.

10^6 cells (A) or 2.10^5 cells (B) were cultured for 66h in the absence (●) or the presence (o) of 1µg Con A, and pyruvate in the medium assayed at the times shown. Points are mean +/-SEM of triplicates, except where this is smaller than the symbol size. Pyruvate concentration in the medium alone are also given (▲). Glucose concentration was 5.5mM.

consumed under aerobic and partially anaerobic culture
conditions. In both culture vessels the majority of con-
sumed pyruvate is taken up during the first 20h of incub-
ation; in round plates this is 82% of total pyruvate
consumed by control cells and 65% by activated cells. In
flat plates approximately the same proportion of consumed
pyruvate is taken up by resting cells within 20h, but in
the presence of Con A there is apparent output of pyruvate
after 20h (Fig.18). In both flat and round plate cultures
resting cells do not consume significant amounts of pyruvate
after 20 hours incubation, but the Con A activated uptake
continues after this time in round plates. It is possible
that, while the net amount consumed is small, this may be
contributing to lactate levels in round plates; this is
also consistent with theview that cells cultured in round
bottomed plates are under partially anaerobic conditions.

It is evident from the foregoing discussion
that the consumption of glucose by rat splenocytes in
response to activation by Con A is far from straight-
forward. Both the qualitative and quantitative changes
in glucose metabolism are not functions solely of the
mitogenic stimulation of the cells; the physical culture
conditions have here been shown to strongly influence
the effect of activation on glucose metabolism.

Whilst enhanced glucose consumption by mitogen
activated lymphocytes has been well documented, the
extent to which alternative factors may affect this has
not been previously discussed. There exists an almost
universal view that the enhanced consumption of glucose
by activated lymphocytes is a direct result of an increase
in energy demands for the activation process. Results
presented here clearly imply that the increased uptake of
glucose in this manner only occurs when prevailing culture

conditions allow it. Furthermore, conventional activation
of splenocytes is not affected by artificially imposed
anaerobiosis, nor by the manipulation of media glucose
concentrations. By contrast, the metabolism of glucose
is strongly affected by these parameters.

The results of the present study lead to the
conclusion that the relationship between conventional
mitogen activation of lymphocytes and corresponding
changes in glucose metabolism is not merely a question
of aerobic glycolysis. Rather, there exists a complex
range of potential responses to activation in terms of
quantitative and qualitative glucose metabolism. When
all physical culture conditions allow, activation of
aerobic glycolysis does indeed occur. However, the ability
of the cells to adapt metabolically to changes in the
conditions and consequently alter the demands made on
the glycolytic pathway is of equal importance. It may
be suggested that such metabolic adaptation is to be
expected from the role of the cells in vivo. It is quite
possible that as a consequence of the interest in aerobic
glycolysis the potential utilisation of other respirat-
ory substrates has been neglected.

(b) KETONE BODIES.

The ketone bodies acetoacetate and $\underline{D}$ β-hydroxybutyrate have been shown to be oxidisable substrates for a number of peripheral tissues such as adipose tissue, brain, kidney and small intestine (For review see Robinson and Williamson,1980). Lymphoid tissue, however, has not been investigated in any detail in this respect and thus the possible contribution of the ketone bodies to energy production in either resting or mitogen activated lymphocytes is unknown. Prior to the initiation of the present study the only study on this subject was performed on 3h incubations of rat thymocytes with acetoacetate at non-physiological concentrations. It is therefore relevant to investigate the possible uptake of the ketone bodies by rat splenocytes in both the fed state and after 48h starvation of the donor animal, in which rat blood ketone body concentrations would be expected to rise from 0.3mM to approximately 2mM (Hawkins et al.,1971).

It is first of interest to investigate any potential effects of the presence of the ketone bodies on the incorporation of [^{3}H]-thymidine in response to Con A activation. Fig.19 shows the results of incubating rat splenocytes from fed rats with fed ketone body concentrations, and splenocytes from 48h starved rats with starved ketone body concentrations. Both conditions resulted in a normal stimulatory response to Con A; 2μg Con A resulted in slightly lower stimulation indices but this was also seen in the absence of ketone bodies. There is no significant difference between fed and starved cell stimulation, in the presence or absence of ketone bodies at any Con A concentration. This is to be expected, since the immune system must be capable of mounting a response under

Figure 19. <u>The effect of the ketone bodies on $[^3H]$-</u>

          <u>Thymidine incorporation by rat splenocytes.</u>

2.10^6 cells/well were incubated with Con A; (A) in the presence (o) or absence (●) of 0.12m<u>M</u> AcAcO and 0.08m<u>M</u> <u>D</u> β-HB; (B) in the presence (o) or absence (●) of 0.5 m<u>M</u> AcAcO and 1.8m<u>M</u> <u>D</u> β-HB. In (C) fed (o) or 48h pre-starved cells (●) were incubated with2m<u>M</u> AcAcO and 3m<u>M</u> <u>D</u> β-HB. Points are mean +/-SEM of triplicates except where this is smaller than the symbol size. Thymidine incorporation was measured in the final 8h of a 72h incubation.
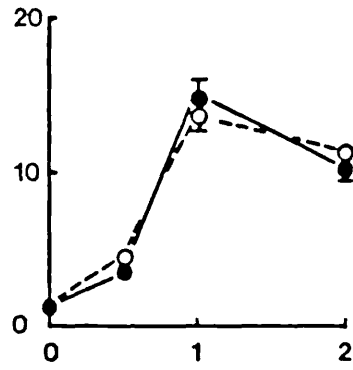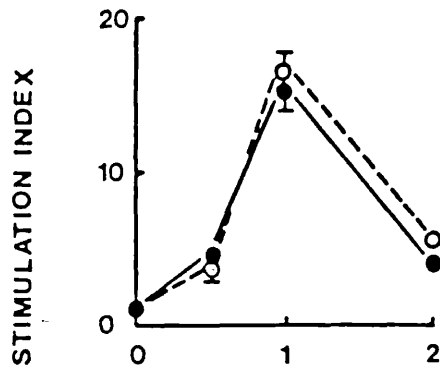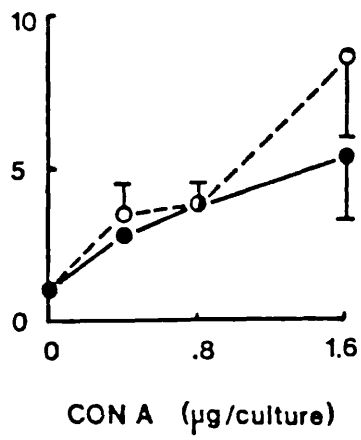
various conditions including physiological ketosis; this was confirmed by Hume et al. (1978) who noted that the addition of ß-hydroxybutyrate to rat thymocyte incubations did not affect their response to Con A in the presence of various concentrations of glucose. Rat mesenteric lymphocytes stimulated with Con A could not incorporate $[^3H]$-thymidine over that incorporated by controls in the absence of glucose, even in the presence of 2mM acetoacetate or D ß-hydroxybutyrate (Ardawi and Newsholme,1984). This, however, indicates that the ketone bodies alone could not support stimulation and does not provide information on stimulation in the presence of glucose or when the ketone bodies are added together at physiological concentrations.

During starvation, when the blood ketone body concentrations rise, the ketone bodies become alternative respiratory substrates to glucose for many peripheral tissues, especially brain (Owen et al.,1967). To investigate the potential contribution of the ketone bodies to lymphocyte energy metabolism the consumption of the substrates was measured in 72h incubations of rat splenocytes. Fig.20 shows that the rates of uptake and the total amount consumed are similar in both the fed and starved states; prior starvation of the donor animal rather surprisingly does not alter the consumption of either ketone body. The rate of consumption of D ß-hydroxybutyrate is approximately double that of acetoacetate, although the proportion of total available acetoacetate used is greater than that of the reduced compound (Table 10).

The rates of uptake over the 72h incubation are significantly lower than those observed by Hume et al. (1978) in a 3h incubation of rat thymocytes with Con A. The uptake of acetoacetate by resting thymocytes was 30 μmol/
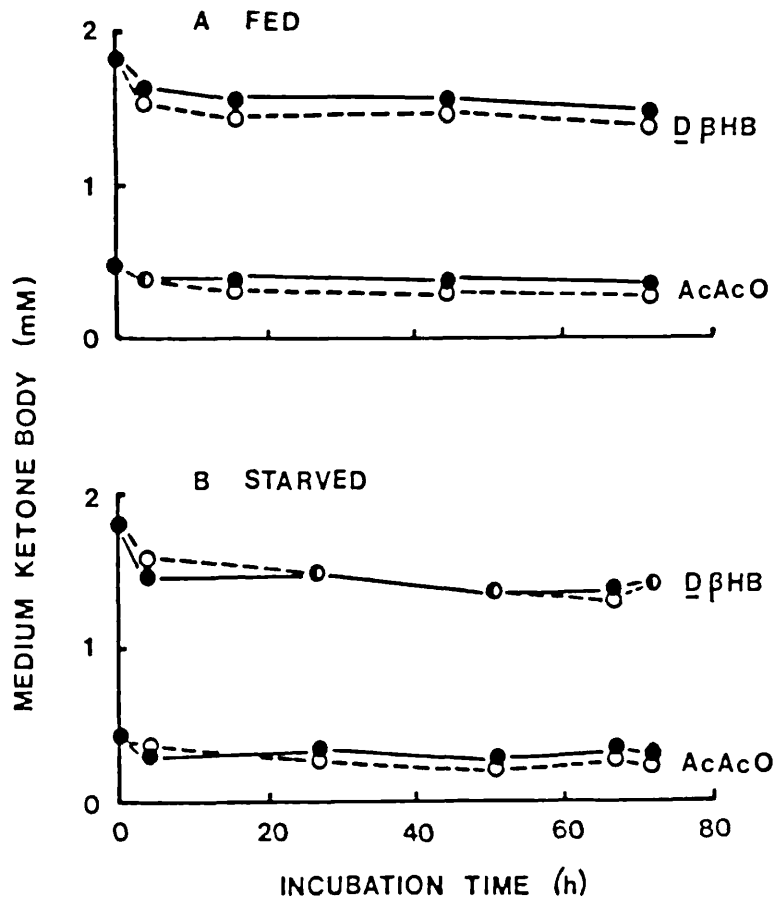
Figure 20. The uptake of ketone bodies by fed and starved rat splenocytes.

Fed or 48h starved cells were incubated at 2.10^6 cells/ well in the presence (o) or absence (•) of 1.2µg Con A for 72h. Points are the mean of triplicates. In all cases the SEM values are smaller than the symbol sizes.

TABLE 10. THE CONSUMPTION OF KETONE BODIES BY RAT SPLENOCYTES.

TREATMENT	KETONE BODY UPTAKE (μmol/10^{10} cells/h)		% TOTAL CONSUMED
	0-4h	0-72h	
Acetoacetate 0.5mM			
Fed: Control	48.5+/-8.0	4.4+/-0.1	31.4
Con A	50.5 10.5	5.1 0.3	37.0
Starved:			
Control	55.5 8.0	3.8 0.3	32.9
Con A	51.5 14.5	4.8 0.2	41.7
D β-hydroxybutyrate 1.8mM			
Fed: Control	103.0 10.5	10.1 0.3	19.8
Con A	136.0 5.0	11.8 0.2	23.3
Starved:			
Control	180.0 20.0	10.6 0.8	20.8
Con A	110.0 25.0	10.6 1.1	20.8

2.10^6 cells/well were incubated for 72h in flat wells. Experimental details are described in Section 2.

10^{10}cells/h and for Con A activated cells 27 μmol/10^{10}

cells/h. In thak study $\underline{D}$ β-hydroxybutyrate was produced

from acetoacetate metabolism, and Fig.20 shows that,.

whilst it cannot be ruled out that this may be occurring

in the present study, there is net uptake of $\underline{D}$ β-hydroxy-

butyrate overall. In a 1h study of ketone body consumption

by rat mesenteric lymphocytes (Ardawi and Newsholme,1984),

acetoacetate uptake was found to be 25.9 μmol/10^{10}cells/h

by control cells and 31.7 μmol/10^{10}cells/h by Con A

activated cells. In this study the acetoacetate concentrat-

ion was 3m$\underline{M}$ and the addition of 5m$\underline{M}$ glucose did not alter

significantly the rates of consumption.

In both the above studies the addition of
either Con A or glucose did not alter the rates of aceto-
acetate consumption, and in both cases the rates of its
uptake were 6-8 times greater than the overall rates of
uptake by rat splenocytes. Analysis of the present data
for uptake during the first 4 hours of incubation of rat
splenocytes explains this apparent anomaly (Table 10).
Acetoacetate consumption during this period is in fact
greater than those rates seen by Hume $\underline{et}$ $\underline{al}$.,(1978) or
Ardawi and Newsholme(1984), and it is thus evident that
the rates of ketone body consumption by rat splenocytes
after the first few hours of incubation are very low.

The fact that starvation does not affect the
consumption of ketone bodies was also noted by Ardawi and
Newsholme (1984), working on rat mesenteric lymphocytes.
This was taken as further evidence that the ketone bodies
are not major respiratory fuels for lymphocytes.

Acetoacetate uptake as a percentage of total
available to the cells is greater than that for $\underline{D}$ β-
hydroxybutyrate, despite the fact that the reduced form

of the ketone body is present in the medium at more than three times the concentration of acetoacetate. This trend holds whether the cells are incubated in the presence of Con A, or are pre-starved. The apparent preference of the cells for acetoacetate has been noted for other ketone body consuming tissues. Rat hindquarter muscle (Ruderman and Goodman,1973) and rat brain (Yeh et al.,1977) also exhibit this phenomenon and this has been confirmed for both tissues in vivo by measurement of arteriovenous differences in ketone body concentrations (Hawkins et al., 1971; Ruderman et al.,1971). This observation has not previously been noted for lymphocytes.

The utilisation of ketone bodies by peripheral tissues in vivo is apparently controlled predominantly by their concentration in plasma (Nelson et al.,1941; Bates 1968; 1972) and this explains the lack of effect of starvation alone on the rates of uptake by rat splenocytes (Table 10). In order to investigate the possibility that uptake of ketone bodies is controlled in a similar manner in lymphocytes, the cells were incubated with increasing concentrations of the ketone bodies in the ratios in which they would be present in the starved state in vivo. Fig.21 shows the patterns of ketone body uptake in the presence and absence of Con A. Again, it can be seen that the majority of acetoacetate and D β-hydroxybutyrate consumption occurs in the early stages of the incubation; approximately 50% of consumed aceto-acetate and 75% of D β-hydroxybutyrate is consumed in the first 19 hours of incubation irrespective of the initial concentration or the presence of Con A. The quantitative rates of uptake occurring in the early stages of culture are given in Table 11 overleaf.
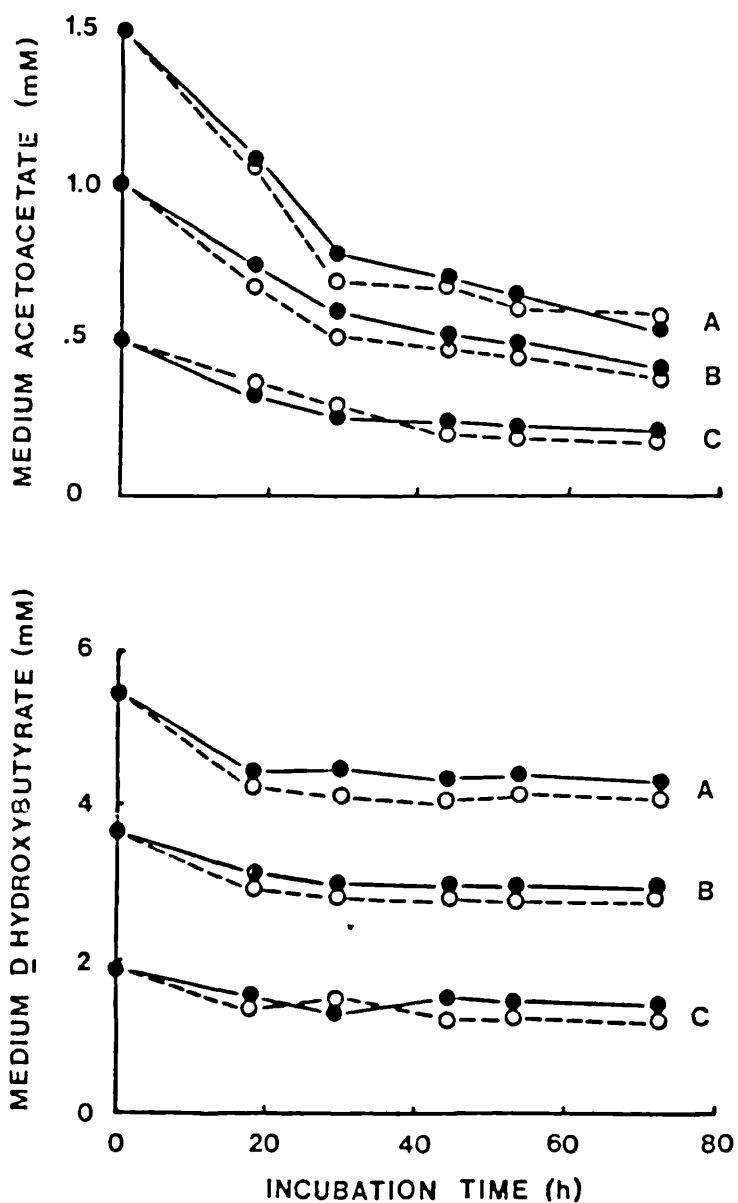
Figure 21. The effect of concentration on rat splenocyte
ketone body consumption.

2.10^6 cells/well were incubated in the absence (●) or
presence (o) of 1.2µg Con A for 72h. Concentrations of
ketone bodies in the incubation were (A) 0.5m$\underline{M}$ AcAcO +
1.8m$\underline{M}$ $\underline{D}$ β-HB; (B) 1.0m$\underline{M}$ AcAcO + 3.6m$\underline{M}$ $\underline{D}$ β-HB and (C)
1.5m$\underline{M}$ AcAcO + 5.4m$\underline{M}$ $\underline{D}$ β-HB. Stimulation indices are
(A) 7.17+/-0.92 (B) 6.90+/-0.38 (C) 21.4+/-5.39.

Points are mean of triplicate cultures.

TABLE 11. THE EFFECT OF CONCENTRATION ON KETONE BODY
CONSUMPTION BY RAT SPLENOCYTES.

INITIAL KB LEVEL (mM)	CON A (1µg)	KETONE BODY UPTAKE (µmol /10^10 cells/h: 0-18.5h)			
		AcAcO		D β-HB	
0.5 AcAcO	-	10.3+/-3.0		18.3+/-6.5	
1.8 β-HB	+	9.8	1.6	25.5	6.5
1.0 AcAcO	-	14.1	2.2	28.1	8.2
3.6 β-HB	+	15.9	3.3	36.0	7.5
1.5 AcAcO	-	26.8	4.3	53.2	11.4
5.4 β-HB	+	27.9	6.5	61.3	4.9

Results are mean +/- SEM of triplicate cultures.

The initial rates of acetoacetate utilisation
by rat splenocytes at 1mM are approximately half of
those seen in short term incubations by Hume et al.(1978)
and Ardawi and Newsholme, (1984), but the rates at 1.5mM
are the same as those seen in the latter study with 3mM
acetoacetate. There could be a tissue specific difference
operating here as the former study used thymocytes, the
latter mesenteric lymphocytes and the present study used
splenocytes. Alternatively, the presence of D β-hydroxy-
butyrate may be stimulating the uptake of acetoacetate by
rat splenocytes, or a plateau may have been reached in the
relationship between the concentration of acetoacetate and
its uptake.

Of major importance in the results presented
in Fig.21 and Table 11 with respect to rat splenocytes is
the observation that no significant difference is seen

TABLE 12. THE EFFECT OF KETONE BODIES ON GLUCOSE AND LACTATE METABOLISM

BY RAT SPLENOCYTES.

KETONE BODY CONCENTRATION (mM)	CON A (1μg)	GLUCOSE UPTAKE / LACTATE PRODUCTION (μmol/10^{10}cells/h 0-72h)		% GLUCOSE TO LACTATE
0	-	26.1+/-4.3	36.1+/-4.3	69.2
0	+	55.4 1.3	81.1 7.9	73.2
0.5 AcAcO	-	17.8 1.5	26.4 2.8	74.2
1.8 B-HB+	+	37.2 2.4*	82.5 5.7	110.8
1.0 AcAcO	-	16.7 2.5	42.8 5.6	128.4
3.0 B-HB+	+	42.2 2.9*	79.2 5.6	93.8
1.5 AcAcO	-	11.6 5.0	31.4 5.0	136.1
5.4 B-HB+	+	29.9 1.3*	50.2 3.1	83.9

2.10^6 cells/well were incubated for 72h, according to Section 2. Points are mean +/-SEM of triplicate cultures. Significance between cultures +/- KB: P<0.01 *.

between uptake of either acetoacetate or <u>D</u> β-hydroxybut-
yrate in the presence of Con A. Bearing in mind the large
quantitative difference in glucose consumption of mitogen
stimulated lymphocytes compared with resting cells, this
implies that, whilst the ketone bodies may contribute to
energy provision of lymphocytes, they do not become more
important on activation; they are therefore unlikely to
be a major source of energy for the activation processes.

A major metabolic effect of the ketone bodies
in many peripheral tissues is the suppression of glucose
uptake and/or pyruvate oxidation (See Robinson and
Williamson,1980). The exact result of ketone body metab-
olism is tissue-specific; in kidney and muscle both
glucose uptake and oxidation are suppressed, while in
brain and small intestine suppression of glucose oxidat-
ion occurs without decreasing its consumption (Rolleston
and Newsholme,1967; Hanson and Parsons,1978). The effect
of ketone bodies on uptake and oxidation of glucose by
rat splenocytes can be inferred from their effects on
the production of lactate (Table 12).

The increase in glucose consumption and
lactate production which is elicited by Con A is signif-
icant irrespective of the presence of the ketone bodies
($P < 0.5-.001$). The presence of ketone bodies causes a
significant decrease in glucose consumption which is
related to the concentration of the ketone bodies, but
this is more marked for mitogen activated incubations.
Despite the effect on glucose consumption, the ketone
bodies only affect lactate production at the highest
concentration used; this effectively means that the con-
version of glucose to lactate is increased by the presence
of the ketone bodies. Interestingly this effect is

inversely related to the ketone body concentration for Con A containing cultures. The present observation that Con A does not significantly affect the uptake of either ketone body is difficult to reconcile with this differential effect on the conversion of glucose to lactate (Table 12), but is supported by the results of other workers. Rat thymocytes showed no alteration in glucose consumption in the presence of 5mM acetoacetate by either resting or Con A activated cells, but glucose conversion to lactate was increased in both cases (Hume et al.,1978). In rat mesenteric lymphocyte incubations, however, acetoacetate at 3mM was shown to significantly decrease glucose uptake but increased the proportion of lactate derived from glucose in response to Con A as well as in resting cells (Ardawi and Newsholme,1984). In the latter study, the presence of acetoacetate was found to increase the intracellular concentrations of glucose 6-phosphate and fructose 6-phosphate, as well as of citrate. This was consistent with the inhibition of phosphofructokinase by citrate and glucose 6-phosphate inhibition of hexokinase as a consequence (Newsholme and Randle,1964; Randle et al., 1964).

If the ketone bodies were to of importance in the provision of energy for lymphocyte activation it would be expected that a quantitative difference in consumption would exist between resting and activated cells. Fig.20 and Table 10, however, clearly show that there is no significant difference between the rates of consumption of either acetoacetate or D β-hydroxybutyrate by resting or Con A activated splenocytes. Furthermore, this is apparent over the entire length of a 72h culture, during which time a sigmificant change in the metabolism of glucose occurs in response to Con A.

It may be argued, in view of the important role which the ketone bodies play as respiratory subst-rates during starvation, that their enhanced metabolism by lymphocytes may only be apparent during starvation. Starvation of the donor animals prior to sacrifice has shown that this is not the case; there is no significant difference in the rates of consumption of either ketone body by cells from fed or starved animals (Table 10).

In common with affects noted for other cell types, rat splenocytes show a concentration dependence of ketone body consumption, and in addition a preference was noted for the uptake of acetoacetate. The presence of the ketone bodies increases the proportion of glucose metabolised to lactate by both resting and Con A activated cells (Table 12), despite the apparent low quantitative consumption of the substrates. It is therefore evident that rat splenocytes do consume both acetoacetate and $\underline{D}$ β-hydroxybutyrate at low rates, and in consequence show a change in glucose metabolism. Furthermore the preference of rat splenocytes for acetoacetate and the concentration dependence of uptake was previously unknown.

More importantly, the present study reveals that no significant difference exists in the consumption of either ketone body in the presence of Con A. It is therefore concluded that the ketone bodies offer a potential source of energy for rat splenocytes but do not play an additional role on cell activation; they are therefore unlikely to be involved in the provision of energy for the transformation process.

(c) <u>L-GLUTAMINE</u>.

The possibility that glutamine behaves as a respiratory substrate for cultured cells, in addition to its essential involvement in nitrogen metabolism and biosynthesis (Meister,1956) was raised by observations that glutamine was used in excess of its biosynthetic requirements, and that a proportion of it was metabolised to CO_2. This has been shown in a number of proliferating cells, including rabbit reticulocytes (Rapoport <u>et al</u>.,1971), rabbit oocyte (Bae and Foote,1975), ascites tumour cells (Kvamme and Svenneby,1961; Lazo,1981) and fibroblasts (Morell and Froesch,1973; Donnelly and Scheffler,1976; Zielke <u>et al</u>.,1976;1978;1984). In addition, the activity of glutaminase has been shown in rat neoplasms, hepatomas and fibroblasts to correlate with the growth of the cells (Knox <u>et al</u>.,1969; Kovacevic and Morris,1972; Sevdalian <u>et al</u>.,1980).

Since glutamine is routinely added to culture media at $2m\underline{M}$ for long term lymphocyte stimulation studies, it is of interest to investigate the effect of this amino-acid on the ability of rat splenocytes to respond to Con A. Fig.22 indicates that resting cells show a small increase in the levels of $[^3H]$-thymidine incorporation with increasing glutamine concentration. The absence of glutamine completely abolished mitogen stimulation, but significant stimulation by Con A was seen in all cultures containing glutamine; peak stimulation was seen at $2m\underline{M}$ glutamine.

The requirement for glutamine to achieve stimulation by Con A has also been shown in guinea-pig lymphocytes (Taudou <u>et al</u>.,1977). Cells in glutamine free medium incorporated only 1.3% $[^3H]$-thymidine compared with cells cultured in $2m\underline{M}$ glutamine; this compares with 1.5%
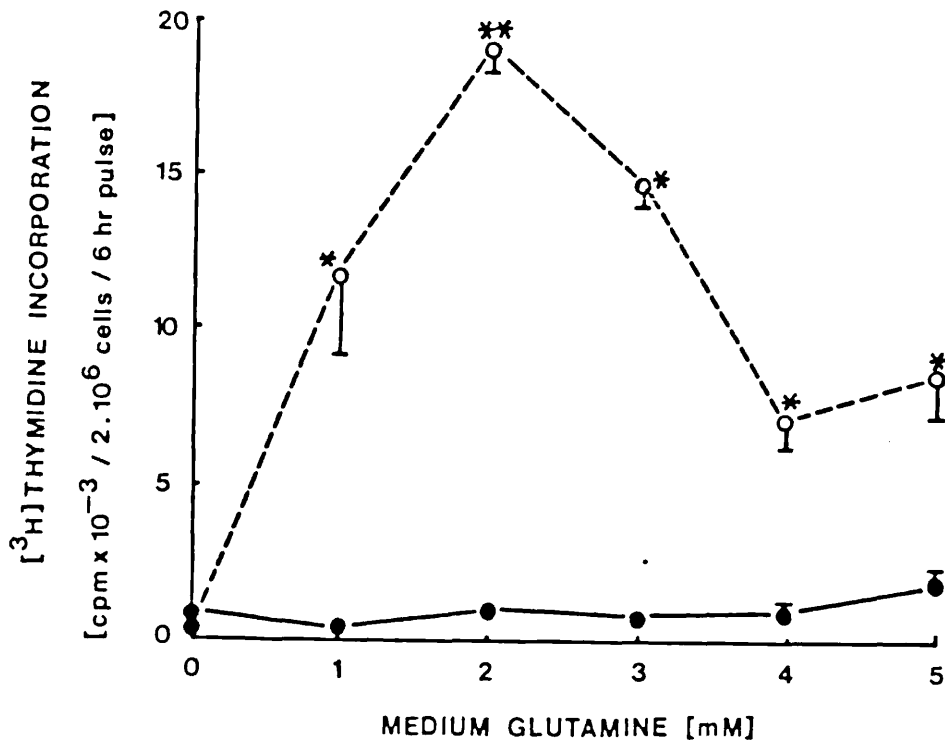
Figure 22. The effect of Glutamine concentration on
$[^3\text{H}]$-Thymidine incorporation by rat
splenocytes.

2.10^6 cells/well were incubated in the absence (●) or
presence (o) of 1µg Con A, the final 6h of a 72h incub-
ation with 1µCi/well $[^3\text{H}]$-Tdr. Points are the mean +/-
SEM of triplicates except where this is smaller than
the symbol size. *P<.01   ** P<0.001 for cpm +/- ConA .

at the same concentrations in the present study. Ardawi
and Newsholme (1983), using a range of glutamine concent-
rations showed that maximal incorporation of radiolabelled
thymidine by rat mesenteric lymphocytes in response to
Con A was achieved at 0.3mM glutamine. The presence of
only 1μM glutamine caused a four fold increase in thymidine
incorporation. While concentrations of less than 1mM were
not employed in the present study, it is evident that since
the resting cells' incorporation increases with increasing
glutamine concentration, peak stimulation , as a ratio of
test to control cpm, occurs at 1mM glutamine, while peak
incorporation of absolute cpm occurs at 2mM glutamine. In
fact, stimulation indices obtained with 1, 2 and 3mM
glutamine are very similar, and are four to five fold lower
at 4 and 5mM glutamine.

Glutamine has thus been shown to be essential
to Con A induced activation of rat splenocytes, but it is
unclear as to why 4 and 5mM glutamine should result in
suppression of thymidine incorporation. It is possible
that the concentration dependent effects on DNA synthesis
may be related to the consumption of glutamine. Fig.23 and
Table 13 illustrate the rate of glutamine consumption as
overall rates throughout the incubation, and as a percent-
age of available substrate consumed.

The rates of uptake by Con A activated cells
are greater overall than by resting cells, thus confirming
the requirement for glutamine on stimulation, but the per-
centage increase in consumption does not correlate with
stimulation indices. The greatest difference between Con A
activated and resting cell consumption is seen at 5mM
glutamine, which also has the lowest stimulation index
(Fig.22). At this concentration, controls exhibit a faster
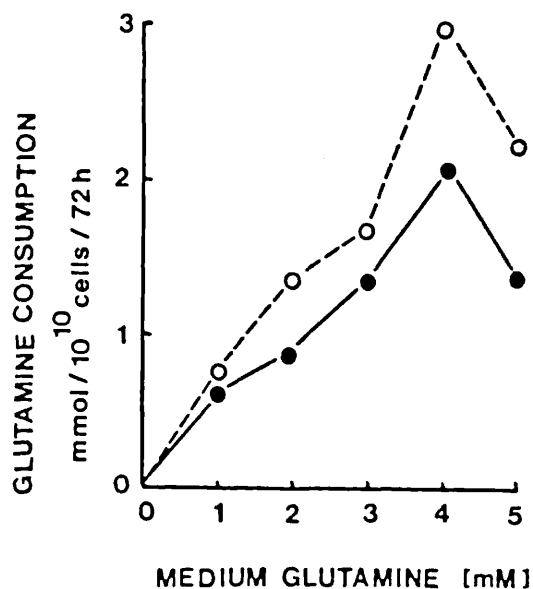
Figure 23. The concentration dependence of rat splenocyte
glutamine consumption.

2.10^6 cells/well were incubated in the absence(●) or
presence (o) of 1μg Con A for 72h. Medium glutamine
concentrations were assayed throughout the incubation.
Rates of consumption are calaculated from data shown in
Table 13.

TABLE 13. <u>L-GLUTAMINE CONSUMPTION BY RAT SPLENOCYTES.</u>

INITIAL GLUTAMINE (mM)	CON A (1μg)	GLUTAMINE UPTAKE ($\mu mol/10^{10}$ cells/h)			% INITIAL GLN CONSUMED
		0-30h	30-72h	0-72h	
1	-	0.47+/-1.9	14.1+/-1.4	8.4+/-0.8	58.0
	+	4.8 1.3	14.2 1.0	10.3 0.6	70.9
2	-	15.6 3.0	10.3 1.4	12.5 0.6**	41.3
	+	20.5 2.3	17.2 1.0	18.5 0.3	61.4
3	-	15.1 4.7	21.8 2.9	19.0 1.7	44.5
	+	17.1 3.3	27.6 2.6	23.2 1.5	54.5
4	-	10.2 4.6	42.5 7.7	29.0 2.6*	50.8
	+	13.8 3.0	60.9 6.2	41.3 1.5	72.3
5	-	36.6 5.3	5.3 2.1	18.3 1.3	26.4
	+	33.1 3.3	28.9 3.3	30.7 2.0*	44.2

2.10^6 cells/well were incubated for 72h and glutamine uptake assayed according to Section 2. Significance of glutamine uptake +/- Con A : * P<0.01 ** P<0.001. Results are mean +/-SEM of triplicate cultures.

rate of uptake than the Con A containing cultures over the
first 30h; thereafter a much slower consumption is seen in
resting cells. Glutamine consumption increases in a concen-
tration dependent manner up to $4mM$, with lower rates at $5mM$
glutamine. In comparison with other values for glutamine
consumption by lymphocytes (Ardawi and Newsholme,1983; Brand
et al.,1984), the rates seen here are lower, especially rates
observed between 0 and 30h. One explanation for this may be
that all other incubations were studied for only 1-3 hours
and thus the consumption in this study may also have shown
greater rates during the initial few hours of incubation.
Rat mesenteric lymphocytes incubated with $2mM$ glutamine
for 1h showed rates of glutamine consumption of 132 and 199
$\mu mol/10^{10}$cells/h in the absence and presence of Con A, resp-
ectively (Ardawi and Newsholme,1983). By contrast, rat
thymocytes consumed the amino acid at 35 and 45 $\mu mol/10^{10}$
cells/h (controls and Con A activated cells, respectively)
when cultured with $4mM$ glutamine for 3h (Brand et al.,1984).
When lymphocytes were cultured with glucose and glutamine
the findings by these two groups differed considerably.
The addition of $5mM$ glucose to cultures of rat mesenteric
lymphocytes resulted in a 39% increase in glutamine uptake
(Ardawi and Newsholme,1983); by contrast, rat thymocytes
cultured with $4mM$ glucose showed an inhibition of glutamine
uptake and the mitogen induced increase in glutamine
consumption was completely abolished by the addition of
glucose (Brand et al.,1984).

The rates of glutamine consumption by rat
mesenteric lymphocytes in the presence of glucose are
approximately fifteen times greater than those seen here
with rat splenocytes (Table 13). However, rat thymocytes
show rates of consumption smaller than in the present study:
consumption by the latter cells is 1.3 times that of

controls and two times that of the rates seen with rat
thymocytes (Brand et al.,1984). It is possible that some
of these discrepancies may be based in the concentration
of glutamine presented to the cells, or the ratio of
glucose to glutamine. As an example, Brand et al.(1984)
utilise concentrations of 4mM glucose and 4mM glutamine
and report a suppression of glutamine uptake on addition
of Con A. Interestingly, Table 13 shows that a mitogen
induced decrease in glutamine consumption by rat splenoc-
ytes occurs in the first 30h of incubation when glucose
and glutamine are present at approximately equal levels
of 5.5mM and 5mM respectively. This, however, does not
explain the large differences seen in glutamine consump-
tion rates by rat mesenteric lymphocytes compared with
thymocytes or splenocytes; it must be assumed that a tissue
specific difference is operating here.

Whilst Table 13 and Fig.22 show that, in rat
splenocytes glutamine consumption is not related to the
extent of activation in terms of $[^3H]$-thymidine incorpor-
ation, it has recently been shown that major differences
in glutamine consumption exist between mitogen activated
and proliferating cells (Brand,1985). Con A activated rat
thymocytes showed a 1.4 fold increase in glutamine cons-
umption compared with resting cells in a 3h incubation.
By contrast, cells which had been cultured with Con A and
interleukin-2 for 60h and were in the S-phase of the cell
cycle utilised glutamine at 8 times the rate shown by
resting cells and at approximately 6 times the rate of
cells activated by Con A alone. Thus, cells which were in
a commited, proliferating state had a much greater demand
for glutamine than cells which had been activated by Con A
and were therefore in the initial stages of the transfor-

mation process. Although these incubations were performed in the absence of glucose, there is a similarity in the trend between the conclusions of Brand (1985) and the long term culture of rat splenocytes (Table 13.). The uptake of glutamine between 30 and 72h incubation is either greater than, or shows insignificant differences to, that which occurs in the presence of Con A during the first part of the incubation.The requirement for the amino acid at this stage of the incubation is presumably related to its central role in nucleic acid synthesis, as well as its putative role in energy provision.

Since a significant increase in the consumption of glutamine in the presence of Con A has been shown, it is pertinent to examine the effects on glucose metabolism in long term cultures. Fig.24 shows the patterns of glucose consumption in a 72h incubation in which the cells were cultured with optimal Con A doses and various concentrations of glutamine. The uptake of glucose by resting cells in the presence of glutamine is not signif-icantly different to that seen in its absence, irrespect-ive of the glutamine concentration present. By contrast, in the presence of Con A, all glutamine containing cultures show a significant increase in glucose consumpt-ion, but this occurs irrespective of the concentration of the amino acid present. It is of interest that, in the absence of added glutamine the mitogen induced increase in the rate of glucose consumption still occurs despite the absence of conventional stimulation as judged by $[^3H]$-thymidine incorporation. This is further evidence in support of the view, presented in Section 3.1.2. (a) that the consumption of glucose by mitogen-activated lymphocytes cannot be used as an alternative index of stimulation. The quantitative effects of

Figure 24. <u>The effect of glutamine concentration on</u>

<u>glucose consumption by rat splenocytes.</u>

2.10^6 cells/well were incubated in the absence (———)

or presence (- - -) of 1μg Con A for 72h, in the

absence or presence of different amounts of glutamine.

The final 6h of culture was accompanied by the add-

ition of 1uCi/well $[^3H]$-Tdr. Stimulation indices for

Con A containing cultures are:

0mM Gln 0.64+/-.02; 1mM : 24.6+/-5.5 2mM: 18.2+/-.7

3mM: 21.3+/-1.3 4mM: 6.8+/-.7 and 5mM 4.3+/-.8.
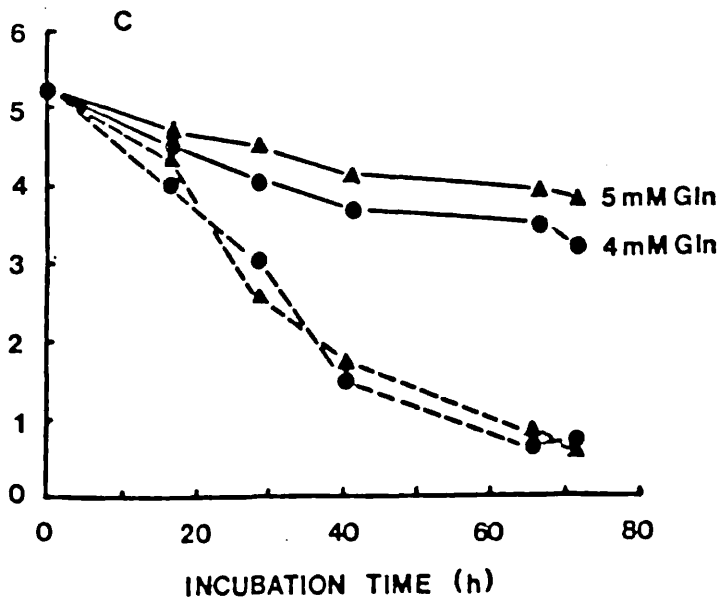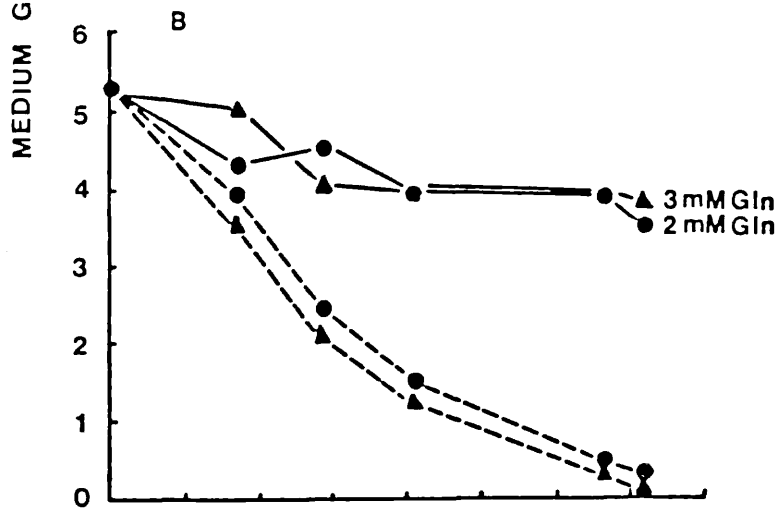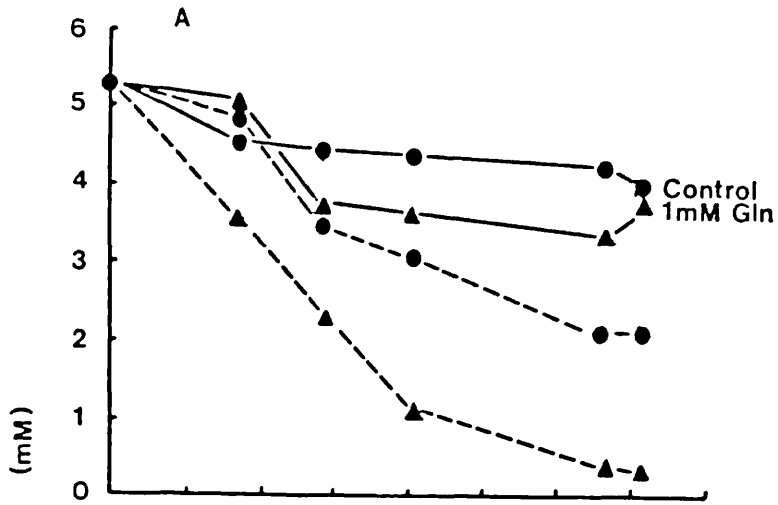
Con A on glucose consumption are shown in Table 14. Overall rates of glucose uptake in Con A activated and resting cells alike are greater in the first 30h of culture than in the final 42h; this is the opposite to the pattern of glutamine uptake, where more was consumed in the latter half of the incubation (Table 13).

In rat mesenteric lymphocytes, the addition of 2mM glutamine was shown to increase the glucose consumption of resting cells from 30 to 50 μmol/10^{10}cells/h (Ardawi and Newsholme,1983). Rat thymocytes showed no change in the consumption of glucose in the presence of 4mM glutamine (Brand et al.,1984). However, in the latter study, the addition of glutamine to glucose in Con A containing cultures had no effect on glucose uptake; Table 14 shows that, in rat splenocyte cultures, the addition of 1-5mM glutamine considerably increases glucose uptake by Con A activated cultures.

Thus, in rat mesenteric lymphocytes, glutamine enhances the uptake of glucose by resting cells; in rat thymocytes the amino acid does not affect glucose consumption by either resting or Con A activated cells, and in the present study the addition of glutamine considerably affects glucose uptake in activated cells, but this is independent of the concentration of the amino acid. As this is the case, it is of interest to see whether the addition of glutamine also affects the metabolic fate of glucose in these cells. Table 15 shows that lactate production is significantly increased by incubation with Con A, but the proportion of consumed glucose reappearing as lactate varies with the added glutamine concentration. For resting cell incubations, the presence of glutamine affects slightly the overall lactate production, but in all cases the levels of

TABLE 14. THE EFFECT OF L-GLUTAMINE ON GLUCOSE CONSUMPTION BY RAT SPLENOCYTES.

(a)* P<.01 ** P<.001 for difference between +/- Con A : (b) P<.01 ** for +/- Gln.

INITIAL GLN (mM)	CON A (1μg)	GLUCOSE UPTAKE ($\mu mol/10^{10}$ cells/h)			%TOTAL CONSUMED	%INCREASE BY CON A
		0-30h	30-72h	0-72h		
0	-	28.0+/-4.3	10.4+/-3.3	(a)17.7+/-1.9	(b)24.3	
	+	60.0 5.0	32.9 8.5	*44.2 4.9	60.5	150
1	-	52.3 4.4	0.0 0.6	21.4 0.8	29.3	
	+	98.4 6.6	47.9 2.9	**68.9 1.0	94.5	223
2	-	23.7 7.4	24.5 3.3	24.2 3.3	33.1	
	+	94.4 2.0	51.7 0.8	**69.5 0.2	95.2	187
3	-	41.3 3.0	4.8 1.2	20.0 0.7	27.4	
	+	103.8 5.7	45.1 2.4	**69.6 0.4	95.3	249
4	-	40.7 3.4	19.3 1.2	28.2 0.7	38.7	
	+	74.0 7.0	54.8 3.0	**62.8 0.7	86.0	122
5	-	22.4 6.0	18.0 2.9	20.0 1.5	27.2	
	+	90.3 3.0	46.2 1.8	**64.6 0.5	88.5	225

2.10^6 cells were incubated for 72h as described in Section 2. Results are mean +/-sem of triplicates.

TABLE 15. THE EFFECT OF L-GLUTAMINE ON LACTATE PRODUCTION BY RAT SPLENOCYTES.

(a) * P<.01 ** P<.001 for difference between +/- Con A:(b)* P<.10 ** P<0.01 +/- Gln.

INITIAL GLN (mM)	CON A (1μg)	LACTATE PRODUCTION (μmol/10^{10} cells/h)			% GLC TO LAC	%↑LAC BY CON A
		0-30h	30-72h	0-72h		
0	-	10.4+/-9.0	28.7+/-6.9	27.7+/-4.0	78.3	
	+	56.3 11.3	79.5 11.7	(b) 76.5 6.8* (a)	86.6	176
1	-	57.5 8.3	15.5 10.3	39.7 5.9	92.9	
	+	203.8 23.0	40.8 4.8	*115.4 2.7***	83.6	191
2	-	62.3 4.6	1.8 6.4	33.6 3.8	69.6	
	+	152.2 12.3	61.2 15.5	105.8 9.0*	76.1	215
3	-	29.5 13.0	13.3 5.7	26.7 3.3	66.9	
	+	131.2 12.7	69.4 14.7	*101.8 8.6*	73.2	281
4	-	37.7 4.3	17.9 2.3	32.8 1.4	58.0	
	+	183.3 12.0	58.9 1.7	**117.4 0.9***	93.5	258
5	-	31.2 10.3	2.9 6.7	21.4 3.9	53.8	
	+	140.5 16.6	56.5 15.5	*98.1 9.1*	76.0	358

statistical evaluation are not significant. The presence
of Con A significantly increases the production of lactate,
and this occurs irrespective of the presence of glutamine.
However, the absolute level of glutamine in the culture
does not affect the amount of lactate produced, nor the
percentage conversion of glucose to lactate. Thus, there
appears to be an all-or-nothing relationship between the
the effects of glutamine on the metabolism of glucose, at
the concentrations studied here. The presence of glutamine
increases the absolute glucose consumption and lactate
production, but the effect is not related to the glutamine
concentration, even though the uptake of glutamine appears
to be concentration dependent.

Table 15 also indicates that the metabolism
of glucose to lactate is greater in the first 30h of incub-
ation than in the final 42h in both resting and Con A
cultures. It is interesting to note that, in addition,
the consumption of glutamine was increased in the latter
portion of the incubation, when the amino acid was present
at between 1 and 4mM (Table 13). It is possible that the
metabolism of the amino acid is affecting the proportion
of glucose metabolised to lactate, as in the absence of
glutamine lactate production increases during 30-72h of
incubation; by contrast in the presence of glutamine lactate
production declines dramatically in the second half of the
incubation.

It thus seems that, when glucose is the sole
fuel, there is a switch from aerobic to more anaerobic
metabolism in the latter half of the incubation by both
control and Con A activated cells. In the presence of
glutamine, however, the pattern is less precise. For Con A
activated cells, however, there is a general trend that

the cells become less glycolytic during the latter half of the incubation.

In other studies it has been noted that the presence of glutamine increases glucose consumption and the proportion metabolised to lactate; in rat mesenteric lymphocytes the proportion of glucose converted to lactate was increased from 55 to 79% by the addition of 2mM glutamine (Ardawi and Newsholme,1983). Rat thymocytes, however, showed no significant difference in lactate production from glucose in the presence or absence of 4mM (Brand et al.,1984). In a comparative study of resting, Con A activated and Con A + IL-2 activated rat thymocytes, Brand (1985) noted that the conversion of glucose to lactate by these cells was 56, 68 and 90% respectively. Since the Con A activated incubations were of only 3h duration, it is perhaps more accurate to compare the long term incubations of rat splenocytes with the proliferating rat thymocytes. It is evident that rat thymocytes undergo a switch in glucose metabolism with activation to a more anaerobic pathway; this was also seen in the absence of glutamine in rat splenocytes (Table 15). This observation supports the view that long term incubations of lymphocytes with mitogen are worthwhile since an alteration in the metabolism of the cells may reflect the different requirements at separate stages of cell activation.

The first step in glutamine metabolism in the mitochondria is deamination by glutaminase to glutamate. Ardawi and Newsholme (1983) have shown that exogenously added glutamate is not capable of removing the glutamine requirement for mitogen activation of lymphocyte DNA synthesis; this is thought to be due to the relative difficulty in transport of glutamate compared with the

parent amino acid. If glutamine is deaminated to glutamate by lymphocytes and transported out of the cell, this effectively decreases the available carbon for metabolism. Equally if glutamate is consumed from the medium this may also influence subsequent glucose metabolism. Fig.25. shows medium glutamate levels throughout the incubation of resting and Con A activated splenocytes. Although there is fluctuation in the production and consumption of glutamate, most notably in the presence of high glutamine concentrations, there is no net glutamate consumption irrespective of the presence of Con A or of the glutamine concentration.

The essential role of glutamine in lymphocyte transformation has been shown here to occur in rat splenocytes; this alone does not implicate glutamine as a respiratory substrate since it has an obligatory role in nucleic acid synthesis. However, there is a significant increase in glutamine consumption in the presence of Con A (Table 13.) and, in addition, the uptake of glutamine is accompanied by an activation of glucose uptake in the presence of mitogen.

Clearly, it is not possible to be unequivocal about the role of glutamine as an additional energy source on activation, but the effects of glutamine consumption on glucose metabolism and its change of uptake on mitogen activation warrant investigation of the detailed metabolism of the amino acid in rat splenocytes in short term cultures.
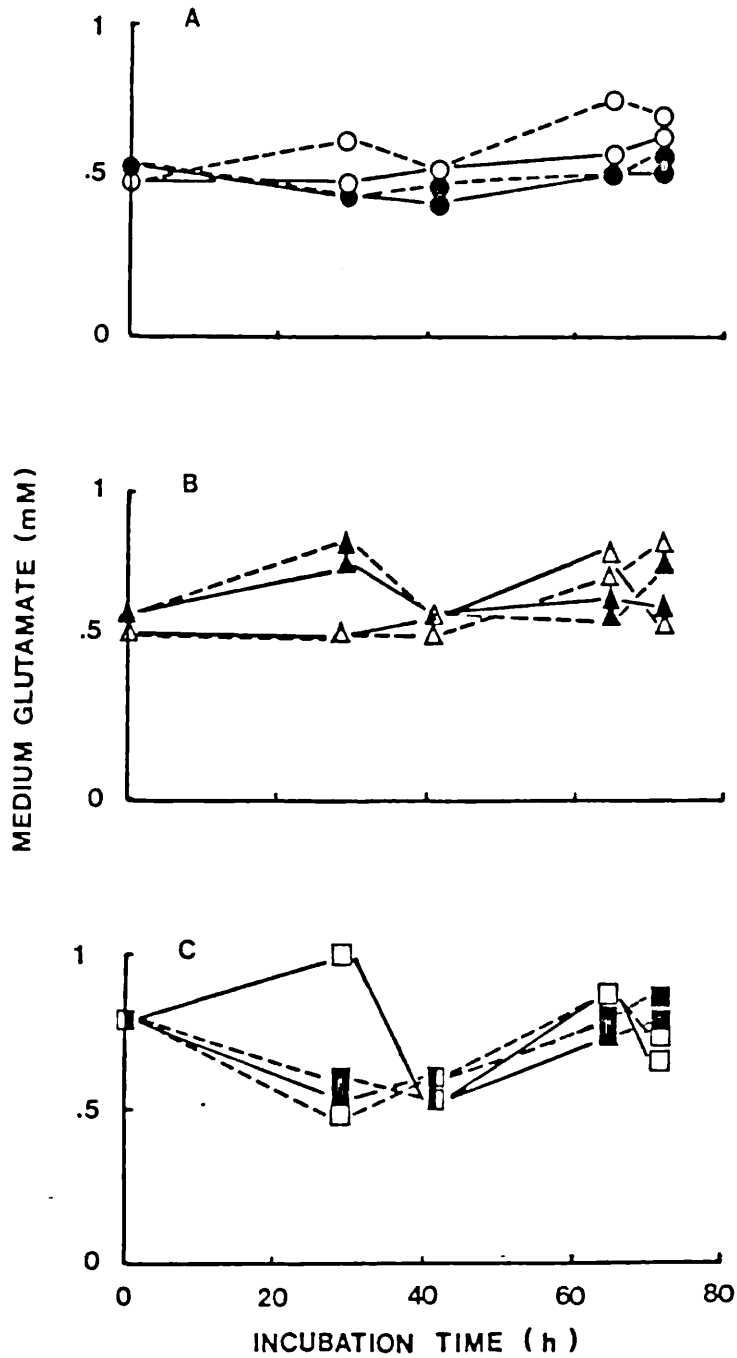
Figure 25. <u>Glutamate consumption by rat splenocytes.</u>
2.10^6 cells/well were incubated in the absence —— or
presence --- of Con A for 72h, in the absence or pres-
ence of varying concentrations of glutamine. (A) (●)
0m$\underline{M}$ Gln (o) 1m$\underline{M}$ Gln, (B) (▲) 2m$\underline{M}$ Gln (Δ) 3m$\underline{M}$ Gln, (C)
(■) 4m$\underline{M}$ Gln and (□) 5m$\underline{M}$ Gln. Points are mean of triplicates.

General Conclusions.

The foregoing section has investigated the
behaviour of rat splenocytes in response to Con A
activation under a variety of physical and metabolic
conditions. The empirical study of the culture condit-
ions required to achieve conventional activation of the
cells has revealed a hitherto unknown susceptibility of
the cells to fluctuations in medium pH. In addition,
conditions which were found to be optimal for activation
were shown to be a function of the culture vessel
geometry. Whilst the effect of mitogen concentration,
cell density and incubation time on lymphocyte activation
have been well documented with respect to lymphocytes
from a variety of species and organs, it is perhaps the
effect of the physical culture conditions on the metabolism
of glucose which presented the major confliction with
the literature.

The ability of rat splenocytes to undergo
conventional mitogen activation in the absence of a
quantitative or qualitative change in glucose consumption
(round wells) raised the possibility that, under these
conditions, the metabolism of glucose was largely dictated
not by the presence or absence of mitogen, but rather by
the conditions of culture. This was indeed confirmed
in the presence of supra-optimal Con A concentrations
in which mitogen stimulation was abolished despite a
quantitative change in glucose consumption between
resting and Con A containing cultures. Since, under
favourable conditions (flat wells) the 'classic' response
of enhanced aerobic glycolysis in response to activation
was obtained, it is evident that the metabolism of glucose
by rat splenocytes in culture is extremely susceptible

to the physical culture conditions. More importantly, the results presented here reveal an inherent metabolic adaptability of the cell to conditions so imposed whilst preserving the ability to mount a response to mitogen. A major conclusion is therefore that the consumption of glucose by mitogen activated lymphocytes cannot be used as an alternative method of assessment of stimulation. (Table 16).

The consumption of the ketone bodies by rat splenocytes was shown to be unaffected by the prior starvation of the donor animals, or by the presence of Con A. Whilst a concentration dependence of uptake and a preference for acetoacetate consumption was revealed, the lack of a quantitative difference in uptake between resting and mitogen activated cells led to the conclusion that the ketone bodies are not involved in the provision of energy for the splenocyte activation process.

The presence of glutamine was shown to be essential to increased rat splenocyte $[^3H]$-thymidine incorporation in response to Con A. In addition, the consumption of glutamine was significantly increased during activation, and in turn this led to an increase in the consumption of glucose, (Table 17).

The detailed examination of rat splenocyte glucose and glutamine metabolism in 24h cultures will therefore be undertaken with a view to quantitating the possible importance of each substrate in the provision of energy during rat splenocyte mitogen activation.

Table 16. Effect of culture vessel and initial glucose concentration on glucose metabolism in long term cultures of rat splenocytes.

Significance between (a) +/- Con A * P<.05 ** P<0.001

(b) Round and flat * P<0.001

(c) 11mM and 5.5mM * P<0.05 ** P<0.001

Initial Glucose	Glucose Consumption (μmol/10^{10}cells/h)			
Plate:	FLAT		ROUND	
Con A:	-	+	-	+
5.5mM	26+/-2	57+/-2 (a)**	122+/-4 (b)*	130+/-0.4 (b)*
(% Glc → Lac)	(64)	(63)	(93)	(116)
11mM	18+/-3	52+/-5 (a)*	184+/-13 (b)*(c)*	208+/-8 (b)*(c)**
(% Glc → Lac)	(58)	(50)	(62)	(70)

Table 17. <u>Effect of respiratory substrates on glucose metabolism in long term cultures of splenocytes.</u>

[Gln] m$\underline{M}$	[AcAcO] m$\underline{M}$	[Dβ-HB] m$\underline{M}$	Con A	Glucose Uptake μmol/10^{10}cells/h		%Glc→ Lac
-	-	-	-	18+/-2.0		78
-	-	-	+	44	5.0	87
2	0.5	1.8	-	18	1.5	74
2	0.5	1.8	+	38	2.0*[b]	111
2	1.0	3.6	-	17	2.5	128
2	1.0	3.6	+	42	3.0*[b]	94
2	1.5	5.4	-	12	5.0	136
2	1.5	5.4	+	30	1.3*[b]	84
1	-	-	-	21	1.0	93
1	-	-	+	69	1.0*[a,b]	84
2	-	-	-	24	0.2	70
2	-	-	+	70	0.2*[a,b]	76
3	-	-	-	20	0.7	67
3	-	-	+	70	0.4*[a,b]	73
4	-	-	-	28	0.7	58
4	-	-	+	63	0.7*[a,b]	94
5	-	-	-	20	1.5	53
5	-	-	+	65	0.5*[a,b]	76

Results are taken from Section 3.2. All are based on mean +/- SEM of at least triplicate cultures. $P<0.01$ * for difference in glucose consumption + Con A compared with uptake in the absence of added substrates;(a),or Con A;(b).

3.2. SHORT TERM CULTURES.

3.2.1. THE EFFECT OF CULTURE CONDITIONS ON MITOGENIC STIMULATION.

Section 3.1. has shown that long term culture of rat splenocytes can be used to study glucose, glutamine and ketone body consumption measured in the light of a conventionally acceptable method of mitogen activation. This system has shown that the uptake of a potential respiratory fuel is not uniform throughout the incubation time. The major drawback inherent in the use of the long term microtitre system is its unsuitability for detailed analysis of the fate of consumed substrates; the inability to trap CO_2 evolved and the small cell densities contribute to this. As was shown in Section 3.1. the major fluctuation in the rate of substrate consumption by both resting and Con A activated cells occurred in the first 24h of culture. For this reason a short term culture system was developed which would allow the detailed analysis of radiolabelled substrate metabolism to be made. Since small alterations in the conditions of long term cultures have been shown here to affect both the quantitative evaluation of splenocyte stimulation by Con A and subsequent glucose metabolism, a major aim of such a short term culture system was that it allows metabolic measurements to be made on a cell population which had not been subjected to multiple manipulations which could result in unseen repurcussions affecting the metabolism of the cells.

After preliminary studies the short term system adopted used 25ml glass incubation vials in which resting and Con A stimulated splenocytes could be given radiolabelled substrates at various incubation times without the need to transfer them to a new environment.

Assessment of mitogenic stimulation could thus be made
under the same conditions as prevailed during metabolic
studies. This is particularly important as an important
aim of the present work has been to separate those changes
which occur as an integral part of the mitogenic response
from those which may have been induced by the culture
conditions. An important qualification of such studies
should therefore be that a conventional method of measure-
ment of mitogenic activation be applied to the system
during the time course of, and under the same conditions
as metabolic response measurements.

Whilst enhanced DNA synthesis in Con A activ-
ated rat splenocytes was used effectively as an index of
stimulation in long term studies, it is inappropriate to
24h cultures, as the activation of this process begins at
approximately 24h after the addition of mitogen (Stenzel
et al.,1978). More commonly used indices of activation
for short term cultures are the incorporation of radio-
labelled amino acids or uridine into protein or RNA
respectively, since these processes are activated at an
earlier time (Kay,1976; Van Rooijen et al.,1983). In the
present study the enhanced incorporation of $[^3H]$-leucine
into acid-precipitable material was employed, and this was
initially tested in the microtitre system used for long
term cultures (Fig.26). Cells incubated in the absence or
presence of Con A were pulsed with $[^3H]$-leucine between
12-16, 16-20 or 20-24h culture. It is apparent that whilst
Con A has stimulated the incorporation of the amino acid
into protein in terms of cpm recovered, the relative
difference between incorporation by resting and activated
cells (i.e. stimulation index) is much smaller for protein
synthesis than was seen for DNA synthesis in long term

Figure 26. <u>The effect of incubation time on</u>
$[^3H]$<u>-Leucine incorporation by rat</u>
<u>splenocytes.</u>

2.10^6 cells/well were incubated with varying
amounts of Con A for (a) 16h, (b) 20h and (c)
24h; the final 4h in the presence of $1\mu Ci$/well
$[^3H]$-leucine. Points are the mean +/-SEM of 6
replicates. * P<0.01 and ** P<0.001 for the diff-
erence between incorporation +/- Con A.
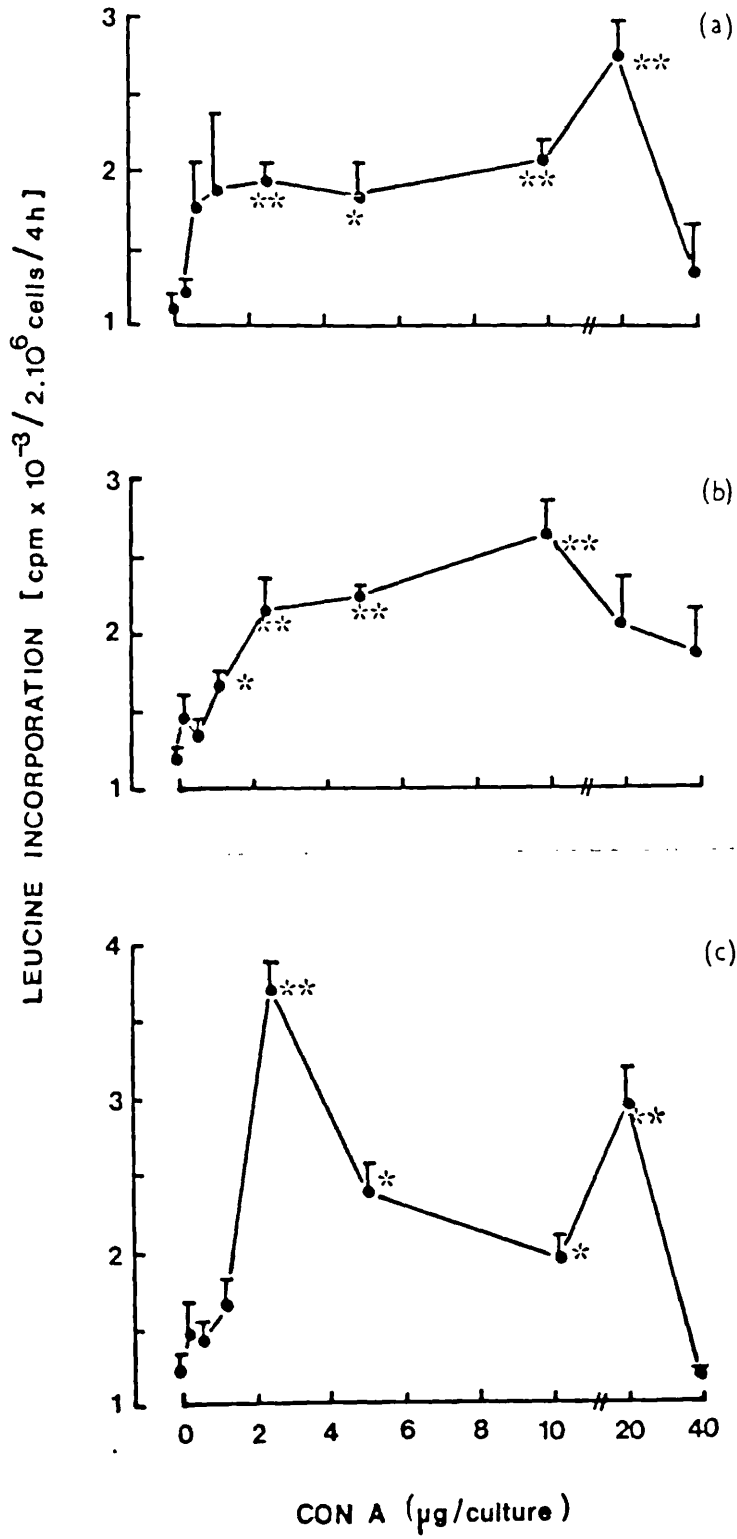
cultures. This is a reflection of the fact that protein synthesis is an ongoing process in resting lymphocytes; by contrast, in the absence of a mitogenic signal DNA synthesis proceeds at a relatively low rate. This low rate gives DNA synthesis great sensitivity as an index of lymphocyte stimulation; using protein synthesis results in much lower stimulation indices. Despite this, the smaller range of cpm in replicates found with the lower absolute level of incorporation during protein synthesis means that the results obtained here are as statistically significant as those for DNA synthesis. Fig. 26 also indicates that optimal stimulation of protein synthesis occurs at progressively lower Con A concentrations with increasing incubation time, as was the case for DNA synthesis. Interestingly,a dual stimulation peak is seen at 20-24h pulse period; the presence of rat splenocyte populations responsive to different Con A concentrations was also noted with respect to long term cultures (Fig.10).

It is thus apparent that the incorporation of $[^3H]$ -leucine can be used effectively to indicate mitogen stimulation for short term splenocyte cultures. Long term cultures have indicated that the alteration in cell density, mitogen concentration, incubation time and culture vessel all affect the response to Con A (Section 3.1.1.). It was thus anticipated that the transfer of cultures to larger scale incubation vials would necessitate a reevaluation of the optimal conditions for culture. Table 18 shows that a relatively narrow range of cell densities allowed a measurable response in terms of increased protein synthesis. A cell density of 10^7/ml showed a classic dose response curve to Con A, but cell densities of $7.5.10^6$/ml or lower showed no stimulation

Table 18. Variation in leucine incorporation and glucose uptake with cell density in short term culture of rat splenocytes.

Cell Density (x/ml)	Con A (µg/ml)	$[^{3}H]$-Leucine Incorporation above control values	Increased Glucose uptake
10^{6}	5 10 15	No	No
5×10^{6}	5-30	No	No
7.5×10^{6}	5-30	No	At 25 and 30µg
10^{7}	5-20	Dose responsive	nm
10^{7}	5-15	Peak at 12.5 & 15	nm
10^{7}	15	Peak at 15µg	100% Glc → Lac
8.5×10^{6}	5-30	Peak at 5-15µg	nm
8.5×10^{6}	5-15	Peak at 10µg	50% Glc - Lac

nm: not measured

at the Con A concentrations tested. The increased consumption of glucose and its conversion to lactate on Con A activation were also monitored in this system as an additional criteria. for the designation of optimal cell density; for the purposes of this study it was essential to ensure that the cell environment allowed the potential metabolism of substrates by aerobic pathways. Table 18 shows that at a cell density of 10^7/ml splenocytes exhibit high glucose consumption but this is totally converted to lactate. At this cell density it was therefore assumed the cells were in an essentially anaerobic environment. By contrast, a compromise cell density of 8.5×10^6 cells/ml showed both a dose responsiveness to Con A and the ability to metabolise glucose by at least partially aerobic pathways. This was therefore adopted as an optimal cell density for short term splenocyte cultures.

The larger cell number used here compared with long term cultures, and the shorter incubation time at which increased protein synthesis was measured (20-24h) led to an optimal Con A concentration of 10-15µg Con A/well. Although this is 10-15 times the concentration found to be optimal for 2×10^6 cells/well in long term cultures, a much greater surface area exists in the incubation vials. This may explain the lack of a simple numerical correlation between the Con A dose and cell number in microtitre plate and incubation vial cultures.

At the optimal cell density of 8.5×10^6 cells/ml used, a dual peak dose-response curve to Con A was routinely seen (Fig.27). Since in long term cultures this phenomenon was not seen in round-bottomed microtitre plates and was occasionally seen in flat-bottomed plate cultures, it is possible that the ability of a rat splenocyte
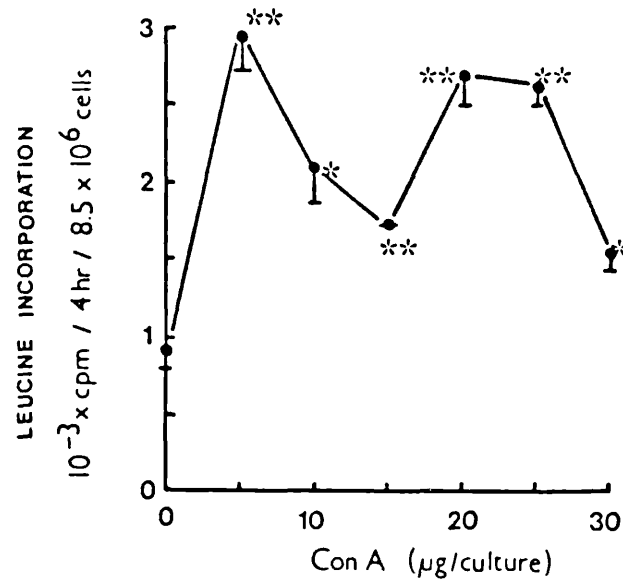
Figure 27. The effect of Con A on $[^3H]$-Leucine
            incorporation by rat splenocytes.

$8.5.10^6$ cells were incubated in 1ml final volume in the presence of varying concentrations of Con A for 24h, the final 4h in the presence of $1\mu Ci/well$ $[^3H]$-Leucine. Points are mean +/-SEM of 4 replicates. *P<0.05 **P<0.01, for cpm +/-ConA.

population to respond to a low and high Con A dose simultaneously may depend on the degree of cell contact prevailing in the culture. Whilst the lack of further investigation precludes anything other than speculation, it appears that a cell density of 10^6cells/200μl and a high degree of cell contact (round microtitre wells) leads to an absence of the dual peak; at 2×10^6cells/200μl and a greater surface area (flat microtitre wells) the dual peak was often seen and at 8.5×10^6cells/ml and a very high surface area (incubation vials), the dual peak was routinely seen. This trend implies that the lower the degree of cell contact induced in the culture the more likely the appearence of more than one optimal Con A concentration. In flat-bottomed plates and incubation vials the effective cell density was very similar (10^7 and 8.5×10^6cells/ml, respectively), but in the latter system the surface area available to the settled cells is approximately 13 times greater ($0.39 \mathrm{cm}^2$ and $4.9 \mathrm{cm}^2$, respectively).

The results shown in Table 18 illustrate the importance of investigating a range of culture conditions. In the present study, for example, both 10^7 and 8.5×10^6cell /ml cultures showed very similar levels of activation of $[^3\mathrm{H}]$-leucine incorporation into protein. Thus, it may be suggested that a similar response would also be observed in a number of metabolic parameters; the difference in glucose consumption and the proportion metabolised to lactate at the two cell densities illustrates that this is not the case. The radiolabelled pulse period employed in all studies using incubation vials was between 20-24h. Preceding metabolic changes which occur in mitogen activated cells can, within reason, then be assumed to form an integral part of the complex series of events which culminate in the conventionally acceptable demonstration of

mitogenic lymphocyte stimulation. The majority of litera-
ture on energy metabolism of lymphocytes has employed
cultures of 1-3h duration and are invariably unaccompanied
by data in which cultures are allowed to continue to a
time at which quantitative measurements of mitogen stimul-
ation can be applied. Equally, studies in which these
measurements are carried out under entirely different
experimental conditions from those of metabolic data
are also misleading. Whilst under these conditions the
cell population is shown to be capable of increased
protein, RNA or DNA synthesis in response to a mitogen,
it may be argued that this would not necessarily occur
under the conditions employed for metabolic studies. An
example of this is that high concentrations of mitogen are
often used in very short term cultures, solely with the
justification that they elicit the highest recordable
response in the parameter being investigated. Since cAMP
has been shown to increase at Con A concentrations that
result in later high dose inhibition of DNA synthesis
(Lyle and Parker,1974), such responses cannot be assumed
to be an integral part of the activation process merely
on the basis that they are induced by the mitogen. Until
such time that biochemical events occurring between initial
binding of antigen or mitogen to the lymphocyte membrane
and later cell division and proliferation are shown to be
a necessary accompaniment to activation, reports of such
changes in metabolism should surely be accompanied by a
conventionally acceptable method of measurement of activ-
ation. In the present study, therefore, all experiments
on the metabolism of rat splenocytes in 24h cultures were
carried out under conditions which were unaltered through-
out the incubation. In all cases the final 4h of culture

Figure 28. <u>The viability of rat splenocytes in short</u>

<u>term culture.</u>

8.5×10^6 cells were cultured in a final

volume of 1ml for 24h, and viability

measured by three different methods acco-

rding to section 2. Cells were cultured

in the absence (•) or presence (o) of

10µg Con A/ml. Points are means of 6

replicates. Total viable cell number at 24h (A):

Control 7.02×10^6

ConA 5.17×10^6.

A. NIGROSIN
EXCLUSION
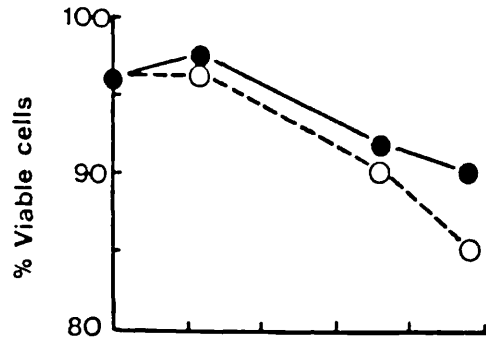
B. LEUCINE
INCORPORATION

C. LDH LEAKAGE

INCUBATION TIME (h)
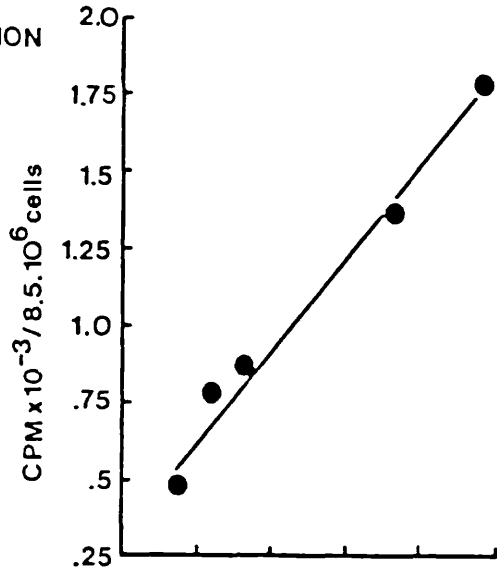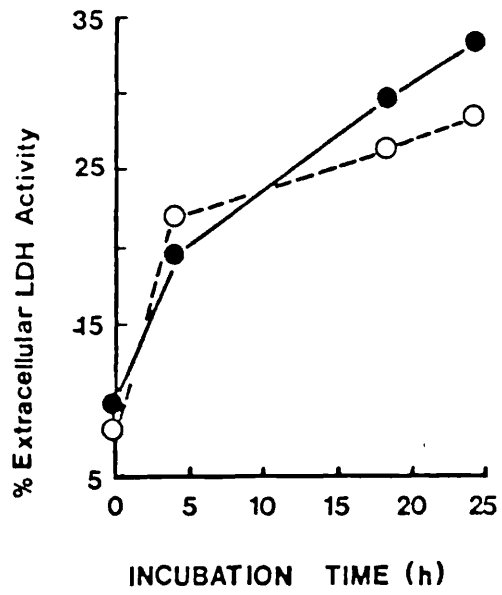
was accompanied by identical replicates which were used to assess mitogen activated $[^3H]$-leucine incorporation into protein.

Lymphocyte cultures performed for only a few hours would be expected to maintain a very high degree of viability. Since an increased loss of viability is often cited as an argument against the use of long term metabolic studies on lymphocytes, it is important to establish the viability of rat splenocytes under the present conditions. Fig.28 illustrates the results of testing this parameter by three alternative methods. Nigrosin exclusion, as used in long term cultures, remained the usual method of viability measurement as it allows an absolute quantitation in terms of cell number, whereas most other methods merely report the qualitative change. Fig.28(a) shows that, over the 24h incubation period the viability of resting cells remains above 90%, and that of Con A stimulated cells remains above 85%. Thus, in spite of a small drop under both conditions a very high proportion of cells remain in a viable state for 24h. Reports of viability data concomitant with metabolic data on short term lymphocyte cultures are scarce. Mouse thymocytes have been reported to exhibit a drop in viability from 80% to 10% between 24 and 48h as judged by trypan blue exclusion (Lengle et al.,1978). As long term cultures of rat splenocytes have shown that 65% viability remains at 72h (Fig.13), it is evident that the culture conditions can dictate the maintenance of viability and this is a further reason for either the reporting of levels of viability at various incubation times or the expression of results in terms of viable cell number.

Other methods of estimation of cell viability are useful for showing trends in loss of viability but

usually cannot be expressed in terms of absolute viable
cell numbers. One such method is the linearity of protein
synthesis. Based on the fact that protein synthesis
requires the collaboration of both nuclear and cytoplasmic
pathways and is thus a more accurate reflection of cell
integrity than membrane permeability, a linear increase
in protein synthesis with time is a reflection of favour-
able culture conditions. Fig.28(b) shows the results of
adding radiolabelled leucine to rat splenocyte cultures
at the beginning of the incubation and harvesting at the
times shown. Since, at 24h, the cells show no evidence of
a decrease in the rate of protein synthesis, this is
further evidence of the maintenance of metabolic capabil-
ity under these culture conditions.

A further method of measurement of the
viability of cultured cells is the leakage of the enzyme
lactate dehydrogenase from the cytoplasm into the external
medium. Fig.28(c) shows that this method conflicts severe-
ly with the results obtained by nigrosin exclusion and
the linearity of protein synthesis by rat splenocytes.
Both resting and Con A activated cells show a rapid rise
inreleased lactate dehydrogenase in the first 4h of incub-
ation, and a steady but decreased rate of leakage thereafter.
It is also notable that by 24h culture the loss of enzyme
by resting cells is greater than that seen with Con A
treated cells, despite the fact that total lactate dehydr-
ogenase activity in activated cells was 25% greater than
in control cells. By this method, up to a third of total
LDH activity is seen extracellularly, yet nigrosin exclus-
ion shows only 5-10% cell death at 24h. The reason for the
inconsistency of the two methods is unknown. Despite this
conflict, perhaps the most compelling evidence of favour-
able culture conditions is the linearity of protein

synthesis. If a third of the incubated cells were in a poor metabolic state, as indicated by LDH leakage, it would be expected to be reflected in at least a decreased ability to maintain protein synthesis at the rate seen at the beginning of the incubation.

Since the metabolism of glucose will be investigated in the following section in the absence of added glutamine, it is pertinent to examine the effect of glutamine deprivation on the viability of short term rat splenocyte cultures. Table 19 compares the data for nigrosin exclusion and protein synthesis presented in Fig.23 with measurements made in the absence of 2m$\underline{M}$ glutamine. Whilst cultures deprived of the amino acid do show a small decrease in viability this is not apparent until 24h incubation; similarly the lack of glutamine does not affect the linearity of protein synthesis nor the absolute incorporation of $[^3H]$-leucine into acid-precipitable material. It is relevant to point out the possibility that a source of glutamine other than exogenously added amino acid is the bovine foetal serum with which all culture media used in the present study was supplemented. Less than 0.1m$\underline{M}$ glutamine was detected in this source; this may have been due to the repeated freezing and thawing of the serum over a period of time. Whilst it should therefore be noted that0.1m$\underline{M}$ glutamine is thus present in cultures that have no exogenously added glutamine, it is possible that this may be capable of supporting protein synthesis in short term cultures.

The culture system thus developed has the ability to maintain rat splenocyte viability over 24h; to allow a measureable increase in Con A activated $[^3H]$-leucine incorporation, and allows the addition of radio-labelled substrates to the system with concomitant $^{14}CO_2$

Table 19. The effect of glutamine on rat splenocyte viability in short term culture.

(a) Nigrosin exclusion

Time (h)	% Cells excluding dye			
	- Glutamine		+ 2mM Glutamine	
Con A:	-	+	-	+
0	97.1		96.7	
7	98.1	97.1	98.1	97.0
19	91.3	89.5	90.4	90.5
24	86.6	83.4	90.5	86.2

(b) Linearity of protein synthesis

Time (h)	$[^3H]$-Leucine incorporation (cpm/8.5.10^{6}cells)			
	- Glutamine		+ 2mM Glutamine	
4	521+/-24		495+/-0.4	
6	785	54	792	6.0
8	840	4.0	861	50
18	1268	41	1364	105
24	1878	72	1791	162

Details of experimental procedure are given in Section 2. Results are means (a) or means +/-SEM (b) of 6 replicate cultures.

trapping without the need to drastically alter the cell environment. This minimises the possiblity that multiple manipulations of the cells will affect their metabolism.

In the following section the new culture system will be used to examine the detailed metabolism of glucose and glutamine by both resting and Con A activated rat splenocytes. In this way it is anticipated that the repective importance of both substrates to energy production on activation can be estimated.

3.2.2. METABOLISM OF EXOGENOUS RESPIRATORY SUBSTRATES.

(a) GLUCOSE

Long term cultures have shown that glutamine is essential to the transformation process of rat splenocytes (Fig.22). Whilst suppressing the uptake of glucose by the cells, the absence of glutamine from the culture medium does not prevent the mitogen induced increase in glucose consumption. In short term cultures, however, the absence of glutamine does not affect the ability of the cells to incorporate the amino acid $[^3H]$-leucine into protein in resting splenocytes (Table 19). It is therefore of interest to examine the effect of glutamine deprivation on the metabolism of glucose in short term cultures.

Fig.29 shows the glucose consumption and production of lactate by rat splenocytes in the absence of added glutamine. The amount of glucose consumed by both resting and Con A activated cells, as a proportion of the initial concentration available, is similar to that consumed during long term culture incubations. 15% of total glucose is consumed by resting cells and 51% by activated cells in 24h cultures; this compares with 24% and 60% respectively in 72h incubations (Fig. 16). An increased rate of glucose consumption thus results from short term culture; in addition the transfer to incubation vials has resulted in an increased effect of Con A on glucose consumption from $44+/-5 \mu mol/10^{10}$ cells/h in 72h cultures to $131+/-14 \ \mu mol/10^{10}$ cells/h in 24h incubations (Table 20).

The patterns of lactate production exhibited by splenocytes in short term culture show an interesting similarity to that seen in long term cultures in the presence of glutamine. During the first few hours there
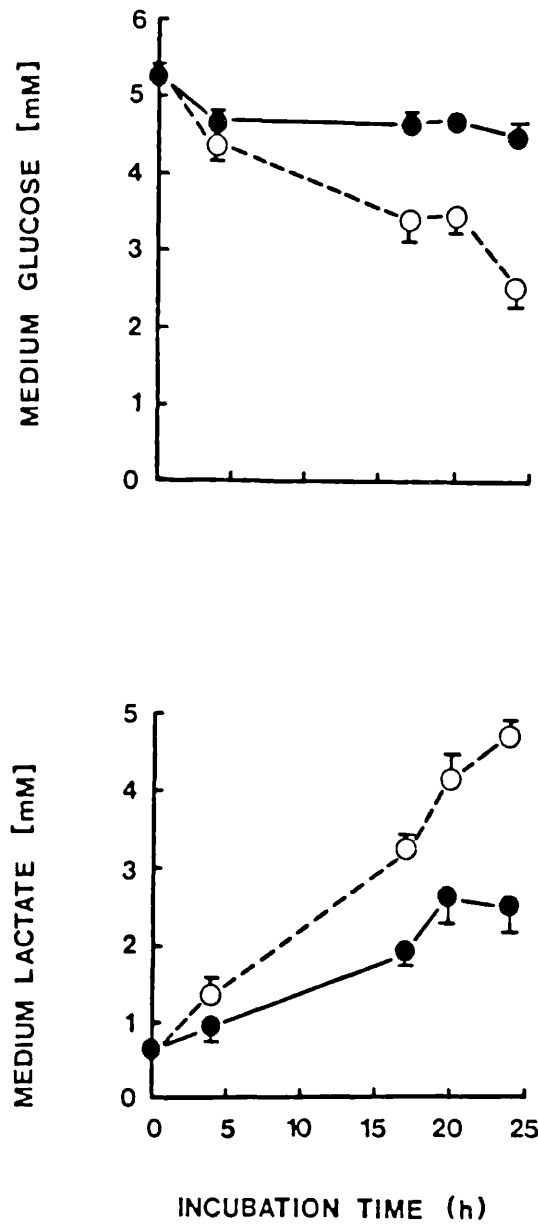
Figure 29. Glucose consumption and lactate production
           by rat splenocytes in the absence of
           glutamine.

$8.5.10^6$ cells /ml were cultured in the absence (•) or
presence (o) of 10μg Con A for 24h. Points are mean +/-
SEM of 4 experiments, each with triplicate cultures.

is rapid uptake of glucose, but less than 50% is con-
verted to lactate by either resting or activated cells.
During the middle of the culture period, however, splen-
ocytes continue to produce large quantities of lactate
despite the fact that glucose consumption decreases
dramatically (Table 20). This is particularly notable
for resting cells.

Table 20. Rates of glucose consumption and lactate
           production by rat splenocytes in the absence
           of glutamine.

$8.5.10^6$ cells/ml were incubated in the absence of L-Gln
for 24h in the presence or absence of 10µg Con A, as
described in Section 2. Results are mean +/-SEM of 3-5
experiments, each with duplicate cultures.

Time (h)	Con A (10µg)	Glucose consumption Lactate production μmol/10^{10} cells/h		% Glc → Lac
		Glucose	Lactate	
0-4	-	164 ± 21.2	102 ± 10.5	31
	+	235 20.6	206 23.5	44
4-20	-	0.96 3.2	124 28.7	6474
	+	70.5 12.5	210 19.8	149
20-24	-	67.4 10.2	-270 32.7	0
	+	270 79.4	168 21.5	31

The interpretation of these data cannot be made merely on the basis of apparent aerobic or anaerobic glycolysis. For example, during the middle period of culture the very high rate of lactate production in the presence of low glucose consumption implies the use of an alternative substrate, possibly mobilisation of an endogenous glycogen source. It is possible that the initial rapid uptake of glucose by both resting and Con A activated cells has resulted in the deposition of glycogen stores which could then be remobilised during the period of low glucose consumption. However, a significant increase in glycogen could not be detected in splenocytes cultured under these conditions despite the use of two methods of estimation. It was therefore concluded that if glycogen was indeed present in the cell population the levels were below the limits of detection of the assays used (10μg),(Hassid and Abraham,1957; Keppler and Decker,1974).

Glycogen is not considered to be a quantitatively important fuel for rat mesenteric lymphocyte activation, based on the maximal activity of phosphorylase (Ardawi and Newsholme,1982). Rat thymocytes stimulated to proliferate with Con A and interleukin-2 show a transition in glucose metabolism from partially aerobic to almost completely anaerobic when cultured for 60h; under these conditions no significant glycogen synthesis could be detected despite a very large increase in absolute glucose consumption (Brand,1985).

Whilst other authors have studied in detail glucose metabolism of lymphocytes in the first few hours following contact with mitogen, detailed studies of prolonged incubations are scarce. The very high rates of glucose consumption exhibited by splenocytes in 24h

cultures are not supported by observations with cells from other sources. Table 8 shows that rat thymocytes cultured for 1-3h at higher cell densities than those employed in the present study all exhibit initial rates of glucose consumption between 6 and 12 times lower than those of rat splenocytes. It is also notable that in those studies in which longer incubations were performed resting cells show no evidence of an alteration in the rate of glucose consumption at different periods of the culture. Mitogen stimulated cells uniformly show an increase in glucose uptake with increasing incubation time. Interestingly, all the studies cited in Table 8 utilise cells cultured in phosphate buffered media; the media employed in the present study were buffered using either HCO_3^- alone or a combination of HCO_3^- and HEPES for radio-labelling experiments. It has previously been suggested that the presence of HCO_3^- may result in an increased rate of glucose consumption by lymphocytes (Hedeskov and Esmann, 1967). Furthermore, the rates of rat splenocyte glucose consumption shown here are very similar to those exhibited by human peripheral blood lymphocytes cultured in HCO_3^- buffered medium (Hedeskov and Esmann, 1967).

The apparent qualitative change in rat splenocyte glucose metabolism with incubation time may be further analysed by the examination of the fate of radiolabelled exogenous glucose under the same conditions.
Table 21 illustrate the results of such experiments in which the cells received 2h pulses of radiolabelled glucose at various times throughout the 24h incubation.

The initial rates of glucose consumption in the radiolabelling experiments were consistently found to be lower than those previously. Whilst the reason for this is unclear, it is probable that the decrease in concentr-

Table 21. The effect of Con A on the metabolism of $[^{14}C]$-Glucose by rat splenocytes in the absence of glutamine.

Time (h)	Con A (10µg)	µmol consumed or produced/10^{10} viable cells/h				% Glc→Lac	% Glc→CO$_2$
		Glucose	$[^{14}C]$in Lac	$[^{14}C]$in Pyr	$[^{14}C]$in CO$_2$		
0	-	96.9±17.1	46.9±9.5	27.4±12.1	9.7±0.4	48.4	10.0
	+	125.8 14.3	66.4 10.3	15.4 0.5	13.3 1.0	52.8	10.6
4	-	68.6 10.8	39.8 3.6	4.9 0.4	4.6 1.1	57.9	6.8
	+	97.0 15.8	60.8 6.7	6.1 0.3	11.7 2.2	62.7	12.1
15	-	81.2 9.7	50.1 4.1	7.3 0.6	7.4 0.6	61.6	9.1
	+	111.3 20.4	61.9 4.5	9.3 0.7	11.1 0.6	55.6	10.0
22	-	43.6 5.3	30.0 2.7	3.1 0.2	5.5 0.6	68.7	12.6
	+	97.1 11.7	70.6 5.5	5.4 0.7	11.2 0.9	72.7	11.5

$8.5.10^6$ cells/ml were incubated in the presence of $[U-^{14}C]$ glucose for 2h at the times shown. Labelled products were isolated as described in Section 2. Results are mean +/- SEM of 2 experiments, each with duplicate cultures.

ation of HCO_3^- from 24m$\underline{M}$ to 4m$\underline{M}$, or the presence of HEPES as an additional buffer may have influenced this. This change was obligatory to retain adequate medium pH during the 2h pulse with labelled substrate. Furthermore, the massive increase in lactate production which was shown in the middle stage of the incubation does not occur here, (Table 21). This may be explained in two ways. It is possible that the change in buffering conditions may have altered the metabolism of glucose to lactate, in a similar fashion to the way in which the absolute uptake of glucose has been decreased. Alternatively, the mixing of the radiolabel pulse required at the beginning of the pulse period may have resulted in an increase in dissolved O_2, thus resulting in a decrease in glucose uptake and subsequent conversion of glucose to lactate.

The proportion of glucose converted to lactate by both resting and Con A activated cells increases throughout the incubation period. By contrast, the production of CO_2 by resting cells decreases afterthe first 4h of incubation, and then increases to a stage where the proportion of CO_2 produced by resting cells is slightly higher than that produced by activated cells. The proportion of CO_2 produced by activated cells is maintained throughout the incubation. Thus, whilst the absolute amount of glucose fully oxidised by Con A activated cells increases compared to resting cells, the relative amount is not significantly different at 0 or 22h incubation. This implies that the importance of glucose oxidation on cell activation only occurs by virtue of the increase in absolute glucose consumption which accompanies mitogen stimulation.

Initially, the proportion of glucose metabolised

to lactate is approximately 50% for both resting and
Con A activated cells; thereafter the proportion in-
creases with increasing incubation time. Conversely,
the proportion of glucose metabolised to pyruvate is
initially higher in control incubations than in the
activated cells. With increasing incubation time, how-
ever, a much lower and more consistent level of pyruvate
is produced in both cell states. Thus, the proportion
of glucose metabolised to lactate and pyruvate is
similar throughout the incubation; it is the relative
proportion of the 3-carbon products which alters.

The rates of glucose consumption are higher in
the present study than those exhibited by resting and
Con A activated rat thymocytes (Brand et al,1984). In the
latter study glucose carbon metabolised to lactate during
a 3h incubation accounted for 40 and 57% of glucose
consumed by resting and activated cells, respectively;
to pyruvate 5 and 3% and complete oxidation to CO_2
accounted for 19 and 15%. It is thus evident that, in the
present study more glucose was unaccounted for than in
the study of rat thymocytes. In a later study using the
same cell system Brand (1985) showed that 56 and 68% of
consumed glucose was metabolised to lactate, with 27 and
18% oxidised to CO_2 by resting and stimulated cells,
respectively. Thus, despite apparently identical culture
conditions the proportion of glucose metabolised to the
two major products varies between the two studies. Of
interest in the latter study is the finding that rat
thymocytes stimulated to proliferate by culturing with
Con A and interleukin-2 show a 90% conversion of glucose
to lactate and only 1.1% to CO_2. Thus, rat thymocytes
show a change in the fate of consumed glucose with the
transition from initial activation to long term prolif-

eration.

The major difference between the study of
Brand (1985) and results presented here is that rat
splenocytes metabolise a greater proportion of glucose
initially consumed to pyruvate, and less to CO_2. Glucose
carbon unaccounted for is of the same order, except that
in the present study less label is recovered in Con A
activated cells.

It is of interest to examine the potential
energy production from glucose metabolism by resting and
Con A activated splenocytes with respect to incubation
time. Table 22 shows the estimated ATP production from
consumed glucose based on calculations from results
presented in Table 21. This indicates the possible
importance of glycolysis and respiration to energy prod-
uction; it is acknowledged that absolute conclusions
cannot be drawn from data which has omitted the contri-
bution from other substrates , both endogenous and exo-
genous, Such calculations would require exhaustive
examination of the intermediary metabolism of the cells
and is beyond the scope of the present study. Rather,
Table 22 is an attempt to evaluate the relative contrib-
ution of glycolysis and oxidation to ATP production.
It can be seen that, despite the fact that CO_2 is a
quantitatively minor fate of consumed glucose compared
with lactate in both resting and activated cells, much
greater potential ATP production results from complete
oxidation. Between 66 and 75% of total ATP is derived
from CO_2 evolution irrespective of the incubation time
or the presence of Con A. Results presented here there-
fore support the view that the large quantitative
increase in glucose consumption and aerobic glycolysis
which accompanies the mitogenic activation of lymphocytes

Table 22. ATP formation from rat splenocyte glucose metabolism in the absence of glutamine.

Time (h)	Con A (10µg)	ATP production (μmol/10^{10}cells/h)				% ATP from CO_2 production	% Increase in ATP by Con A
		Glc→Lac	Glc→Pyr	Glc→CO_2	Total		
0	-	93.8	54.8	348.8	497.4	70.1	
	+	132.8	30.9	480.0	643.7	74.6	29
4	-	79.4	5.0	167.9	252.3	66.5	
	+	121.6	6.0	422.5	550.1	76.8	118
15	-	100.0	7.3	266.0	373.3	71.3	
	+	123.8	9.2	400.7	533.7	75.1	43
22	-	59.9	3.1	197.8	260.8	75.8	
	+	141.2	5.4	402.0	548.6	73.3	110

Data calculated from results presented in Table 21.

should not automatically be equated with an increase
in energy supply for activation (Hume et al,1978; Hume
and Weidemann,1979).

The presence of glutamine was shown in long
term cultures to affect glucose metabolism by rat spleno-
cytes, (Tables 14 and 15). It is therefore pertinent to
examine this with respect to short term glucose metabolism
and the potential effect this may have on glucose-
derived energy production. In the presence of 5.5mM
glucose and 2mM glutamine, long term cultures had shown
that cells cultured in round or flat wells showed no
correlation between Con A induced glucose consumption
and increased $[^3H]$-thymidine incorporation. This was
also found to be true for short term cultured cells,
despite the change in culture conditions and the method
of assessment of mitogenic stimulation (Fig.30). The
increase in glucose consumption by stimulated splenocytes
over the 24h incubation period ranges from 97% with
5µg Con A to 139% with 30µg Con A; increased incorpor-
ation of leucine ranges from 93% with 15µg Con A to 221%
with 5µg Con A, compared with resting cells. Thus, glucose
consumption by rat splenocytes cannot be used as an index
of stimulation under the conditions employed in the
present study. Neither 72h cultured cells in the micro-
titre system, nor the 24h cultured cells in vials show
any correlation between the extent of stimulation as
judged by conventional precursor incorporation, and the
quantitative increase in glucose consumption.

In the absence of added glutamine Fig. 29 had
shown that the transfer to incubation vials and the
inherent alteration of culture conditions had resulted
in an increased rate of glucose consumption by splenocytes
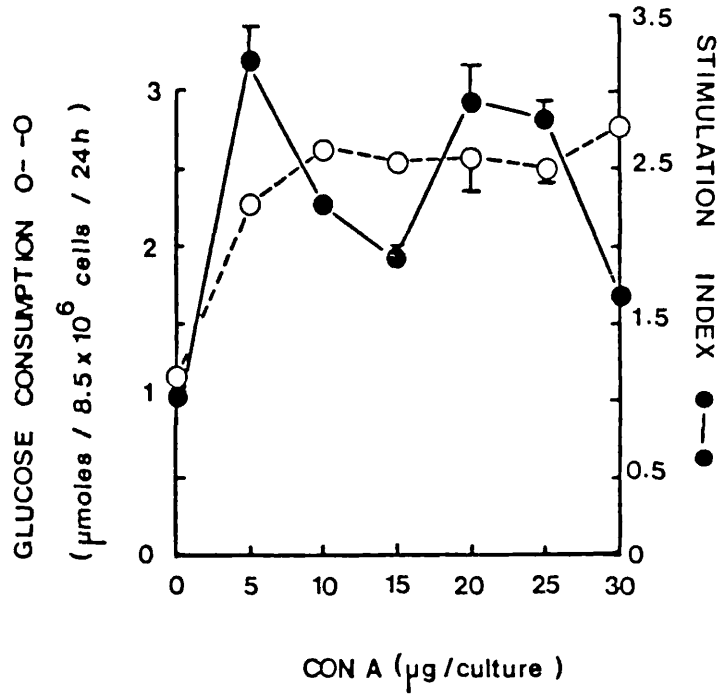
Figure 30. Lack of correlation between enhanced glucose consumption and stimulation index by rat splenocytes.

$8.5.10^6$ cells/ml were cultured in incubation vials for 24h in the presence of varying Con A concentrations. Stimulation indices were measured after the addition of $1\mu Ci$/vial $[^3H]$-Leu for the final 4h of culture. Points are mean +/- SEM for triplicates.

compared with long term cultures. Fig. 31 shows that this is also the case in the presence of 2mM glutamine. Furthermore, the pattern shown in Fig. 29 in which high initial rates of glucose uptake subsequently decrease and are accompanied by very high rates of lactate production is also seen here (Table 23).

Table 23. Rates of glucose consumption and lactate production by rat splenocytes in the presence of 5.5mM glucose and 2mM glutamine.

$8.5.10^6$cells/ml were cultured in the presence or absence of 10µg Con A for 24h, as described in Section 2. Results are mean +/- SEM for 5 experiments, each with duplicate cultures.

Time (h)	Con A (10µg)	Glucose consumption / Lactate production µmol/ 10^{10}cells/h		% Glc → Lac
		Glucose	Lactate	
0-4	-	190 ± 20.6	181 ± 20.7	48
	+	320 25.8	274 23.8	43
4-20	-	13 3.4	126 25.9	499
	+	140 16.7	404 40.3	144
20-24	-	214 25.8	-81 10.9	0
	+	329 29.3	-413 56.7	0

Whilst there still exists the very high rate of lactate production in the middle of the culture period the major difference compared with the results presented in Table 20 is that between 20 and 24h of culture both resting and activated cells exhibit apparent reutilisation of lactate.

INCUBATION TIME (h)

Figure 31. Glucose consumption and lactate production by rat splenocytes in the presence of 2mM glutamine.

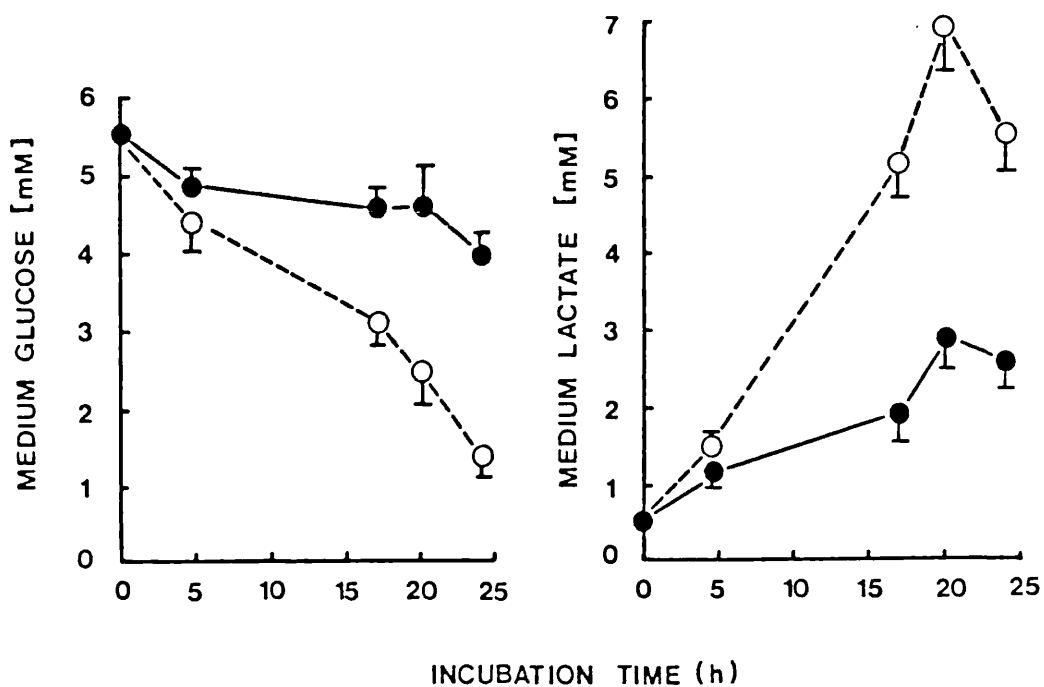$8.5.10^6$ cells/ml were cultured in incubation vials for 24h in the absence (●) or presence (o) of 10µg Con A. Points are mean +/-SEM of 8 experiments, each with duplicate cultures.

The increase in glucose consumption associated with the addition of glutamine is consistent with the findings of Ardawi and Newsholme(1983) in which rat mesenteric lymphocytes show the same effect. By contrast, rat thymocytes show no increased glucose consumption in the presence of 4m$\underline{M}$ glutamine (Brand $\underline{et}$ $\underline{al}$,1984). The use in the present study of radiolabelled glucose as described previously was found to support the finding that the presence of the amino acid increases the uptake of glucose (Table 24). Interestingly, the proportion of glucose metabolised to CO_2 in the presence of Con A decreases slightly with increasing incubation time, but the percentage glucose metabolised to CO_2 has remained similar to that seen in the absence of glutamine (Table 21). Thus, although glutamine has increased the absolute consumption of glucose by splenocytes, it has no effect on the proportion which is fully oxidised. The major qualitative difference between incubations in the presence and absence of glutamine concerns the proportion of glucose metabolised to pyruvate. In the presence of both glutamine and Con A the proportion metabolised to pyruvate decreases with increasing incubation time; conversely, resting cells show a steady increase in pyruvate production to 19h incubation. In the absence of added glutamine both resting and Con A activated cells show a steady decrease in pyruvate production with incubation time.

Thus, rat splenocytes incubated with glutamine show an increase in glucose consumption, lactate production and CO_2 evolution in the presence of Con A. In addition, the presence of glutamine has increased the production of pyruvate in the presence of Con A whilst decreasing that produced by resting cells. By contrast, Ardawi and Newsholme (1983) have shown that the addition

Table 24. The effect of Con A on the metabolism of [14C]-Glucose by rat splenocytes in the presence of 2mM glutamine.

Time (h)	Con A (10µg)	µmol consumed or produced/10^{10} viable cells/h				% Glc→Lac	% Glc→CO$_2$
		Glucose	[14C]in Lac	[14C]in Pyr	[14C]in CO$_2$		
0	-	187.9±44	125.7±25	2.5±0.5	19.1±2.9	66.9	10.1
	+	253.8 40	164.7 19	21.4 2.8	24.0 2.2	64.9	9.5
4	-	93.3 19	64.4 11	5.0 1.8	12.8 1.8	69.0	13.7
	+	165.3 15	82.1 14	14.2 4.3	18.8 2.2	49.6	11.4
15	-	99.5 7	59.8 1	16.5 7.5	7.5 0.4	60.1	7.5
	+	146.3 13	118.2 14	7.4 3.2	15.5 1.7	80.8	10.6
19	-	112.5 17	68.6 12	26.4 3.1	7.4 0.9	60.9	6.6
	+	161.5 13	86.7 23	9.1 0.5	15.1 3.1	53.7	9.4
22	-	73.4 10	50.3 8	8.7 2.6	8.2 1.7	68.4	11.2
	+	133.0 20	91.4 10	2.9 0.7	13.9 3.2	68.7	5.2

$8.5.10^6$ cells/ml were incubated with [U-^{14}C] Glucose for 2h at the times shown. Radiolabelled products were isolated as described in Section 2. Results are mean +/- SEM of 2 experiments, each with duplicate cultures.

of 2m$\underline{M}$ glutamine to 1h incubations of rat mesenteric lymphocytes also increases glucose consumption, but in this case all the additional glucose could be accounted for by lactate. Rat thymocytes incubated with 4m$\underline{M}$ glutamine showed no change in the metabolism of glucose in the resting or Con A activated state, in 3h incubations (Brand et al.,1984). Clearly, in the case of the first few hours of incubation all three studies conflict, although in the study of Ardawi and Newsholme (1983) no data is available on the production of CO_2 from glucose.

The complete oxidation of glucose to CO_2 by rat splenocytes incubated in the absence of glutamine has been estimated to be responsible for the provision of approximately 70% of glucose derived ATP (Table 22). In the case of incubations in the presence of 2m$\underline{M}$ glutamine, it can be seen that this proportion remains the same (Table 25). The major difference in ATP production in the presence of glutamine lies in the pyruvate and lactate derived ATP in the initial stage of the incubation. Interestingly, a comparison of Tables 22 and 25 also reveals a difference in the increase in absolute glucose derived ATP in the presence of Con A at different stages in the incubation. It can be seen that whilst, in the absence of glutamine Con A causes a large increase at the final stage of the incubation, this does not occur in the presence of the amino acid.

Tables 22 and 25 together lead to the conclusion that the complete oxidation of glucose in the presence or absence of added glutamine is quantitatively the major source of glucose derived ATP, despite the fact that only 10% of consumed glucose is metabolised by this route.

Table 25. ATP formation from rat splenocyte glucose metabolism in the presence of 2mM glutamine.

Time (h)	Con A (10µg)	ATP production (μmol/10^{10}cells/h)				% ATP from CO_2 production	% Increase in ATP by Con A
		Glc→Lac	Glc→Pyr	Glc→CO_2	Total		
0	-	251.0	4.9	684.6	940.5	72.8	
	+	329.4	42.6	863.4	1235.4	69.9	31
4	-	128.8	9.7	459.1	597.6	76.8	
	+	163.0	28.4	678.4	870.7	77.9	41
15	-	119.6	33.0	269.7	422.3	63.9	
	+	236.4	14.6	557.2	808.2	68.9	91
19	-	137.0	52.7	266.9	456.6	58.5	
	+	173.5	18.1	543.6	735.2	73.9	61
22	-	100.4	17.3	294.6	412.3	71.5	
	+	182.7	5.9	249.5	438.1	57.0	6

Data calculated from results presented in Table 24.

Long term incubation⁻ of rat splenocytes in supra-physiological glucose concentrations was shown to result in an increase in the overall rates of glucose consumption by both resting and Con A activated cells, and a decrease in the proportion metabolised to lactate (Fig.16). Similarly, splenocytes cultured in 5.5, 9 or 11mM glucose and 2mM glutamine for 24h show the same phenomenon (Fig. 32). Increased glucose consumption in incubations of resting splenocytes is of the order of 220% at 9mM and 315% at 11mM glucose, compared with rates in 5.5mM glucose. Con A activated cells also show an increase in the rate of glucose uptake by 148% in 9mM and by 161% in 11mM glucose; thus, the increase in glucose concentration affects resting cell metabolism to a greater extent than mitogen activated cells. By far the greatest rate of glucose consumption occurs in both resting and activated cells during the first few hours of incubation. This has been a consistent occurrence in short term incubations. In addition, increasing the initial glucose concentration has resulted· in a decrease in the proportion of glucose metabolised to lactate; this was also noted in long term cultures. The apparent reutilisation of lactate which occurs in short term cultures at the final stage of the incubation is seen to a lesser extent at 9mM glucose (Fig. 32) but is not seen in 11mM glucose.

It seems apparent that the concentration of glucose employed in lymphocyte cultures profoundly affects the individual rates of recorded glucose uptake and lactate production (Table 26). Since this also occurs during the first few hours of culture, which is the time period most usually chosen for detailed studies of lymphocyte metabolism, this may be a reason for the

Table 26. The effect of initial glucose concentration on the rate of glucose consumption by rat splenocytes.

Initial Glc mM	Con A (10µg)	Glucose consumption (μmol/10^{10}cells/h)			
		0-4h	4-17h	17-24h	0-24h
5.5	-	189.7±20	26.2±3	102.0±13	75.5±15.7
	+	320.3 29	117.3 14	290.8 31	201.7 10.8
9	-	880.0 114	70.6 21	25.9 6	167.2 19.1
	+	1266.0 130	127.1 40	204.6 47	298.5 17.2
11	-	631.2 59	247.1 45	0.0	238.7 8.8
	+	1359.4 71	158.2 28	21.8 3.5	324.3 3.0

Results are means +/-SEM of 2 experiments, each with triplicate cultures.
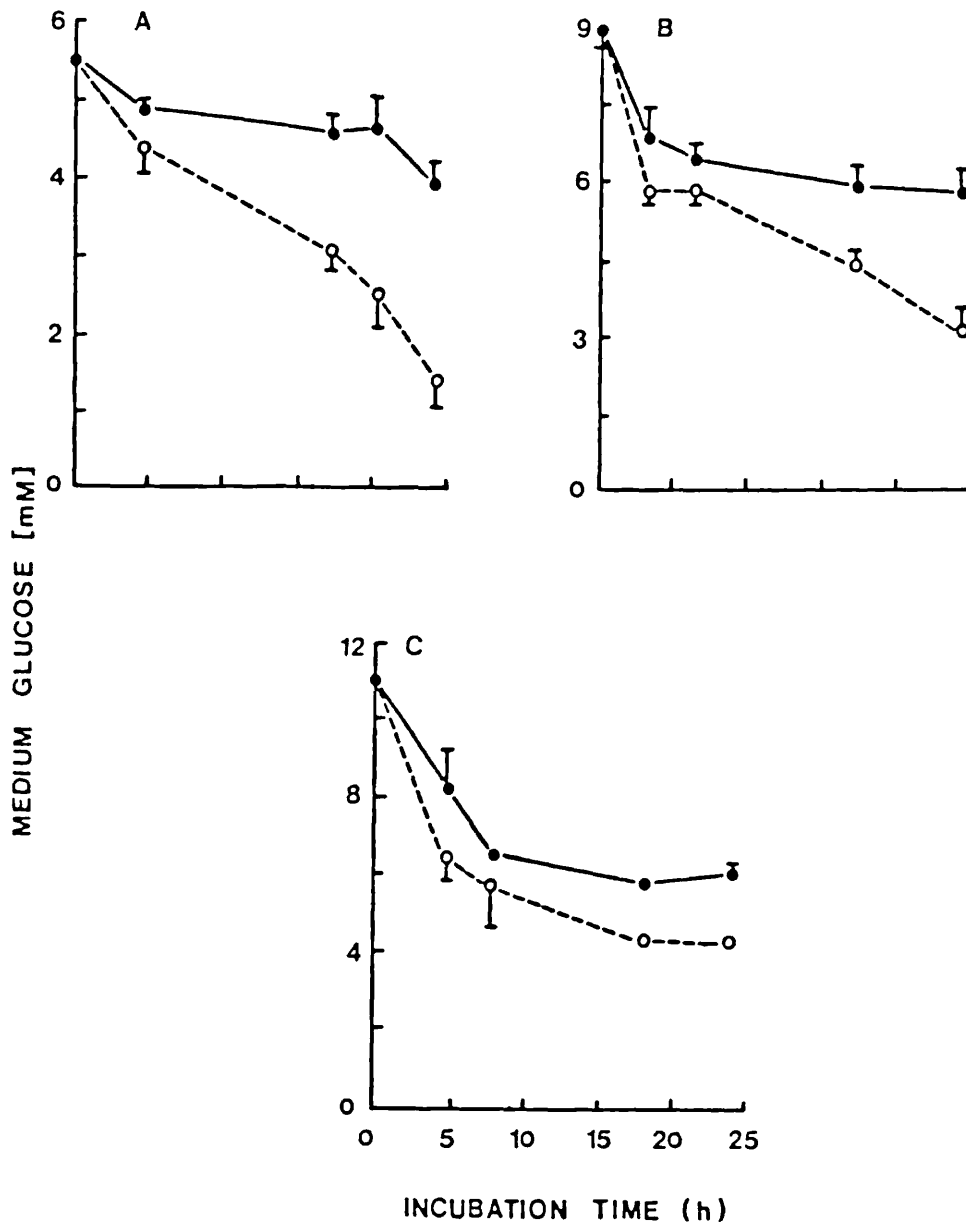
Figure 32. The effect of initial glucose concentration
on glucose uptake and lactate production by
rat splenocytes in short term culture.

$8.5.10^6$ cells/ml were cultured in incubation vials for
24h in the absence (●) or presence (o) of 10μg Con A.
Glucose consumption (above) and lactate production
(over) were measured in the presence of A 5.5mM,
B 9mM and C 11mM glucose. Points are mean +/- SEM of
triplicates.
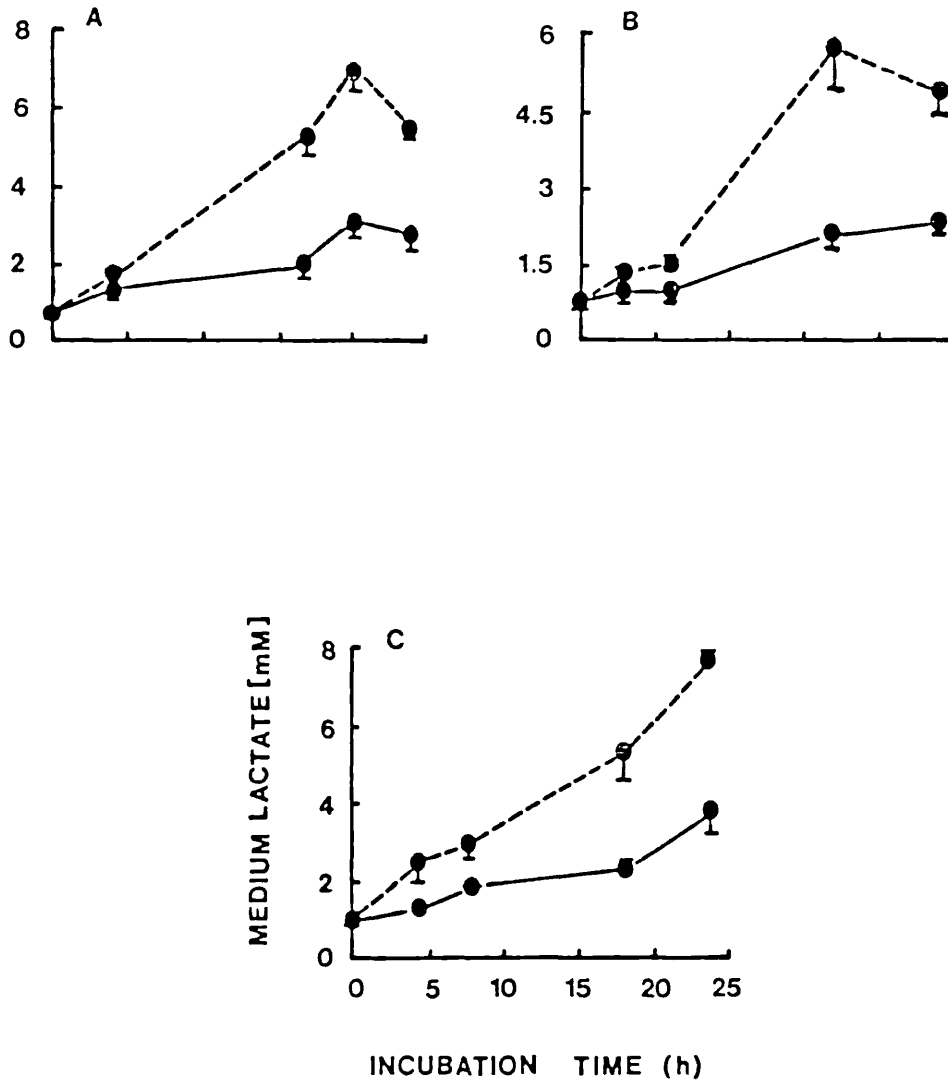
Figure 32. (cont).

variety of different rates of glucose consumption seen in a number of studies (Table 8). In addition, this section has shown that the detailed metabolism of glucose by rat splenocytes is considerably affected by the addition of Con A, glutamine and additional buffer to the culture medium. It is therefore perhaps not surprising that seemingly similar studies on different lymphocytes result in significant differences in the metabolism of glucose. Of overriding importance. is the fact that, despite these differences the ability of the lymphocyte population to respond to Con A in a predictable fashion remains. It is for this reason that the differences in metabolism induced, not by mitogen but by the physical culture conditions, may be underestimated. It is evident that similar metabolic properties cannot be assumed in cells that show similar responses to a given mitogen; the contribution of external factors must always be allowed for.

(b) GLUTAMINE.

Glutamine has been shown in long term cultures of rat splenocytes to affect the metabolism of glucose by both resting and Con A activated cells (Tables 14 and 15). The study of very short term glutamine metabolism has recently received increasing attention as a result of the finding that the amino acid may serve in an energy producing capacity in other types of cultured cells in addition to its role in the provision of nucleic acid precursors for cell division (Zielke et al.,1984). The finding in long term cultures, in the present work, that glutamine consumption has a concentration dependence and affects the proportion of glucose metabolised to lactate has been further supported by short term culture results in which glutamine increased the consumption of glucose and the production of lactate by rat splenocytes (Figs. 29 and 31). The detailed metabolism of glutamine can thus now be investigated with a view to quantitating its contribution to energy production by both resting and stimulated splenocytes.

Since the concentration of initial medium glucose was shown in the previous section to affect the consumption of glucose, it is of interest to see whether a similar effect results with respect to glutamine uptake. Fig. 33 shows that in the first 4h of a 24h incubation rat splenocytes consumed glutamine at a high rate compared with the rest of the incubation period. In addition, the Con A induced increase in uptake was affected by the initial glucose concentration (Table 27). A mitogen induced increase of 37, 45 and 53% was shown by cells cultured in 5.5, 9 and 11mM glucose, respectively. During the remainder of the incubation, however, this trend does not continue; during the period 4-24h glutamine

Figure 33. The effect of initial glucose concentration
on glutamine consumption by rat splenocytes.
8.5.10⁶cells/well were cultured for 24h in the absence
(●) or presence (o) of 10µg Con A. Glutamine consumption
was measured in medium containing A 5.5mM, B 9mM and
C 11mM glucose. Points are mean +/- SEM of triplicates.

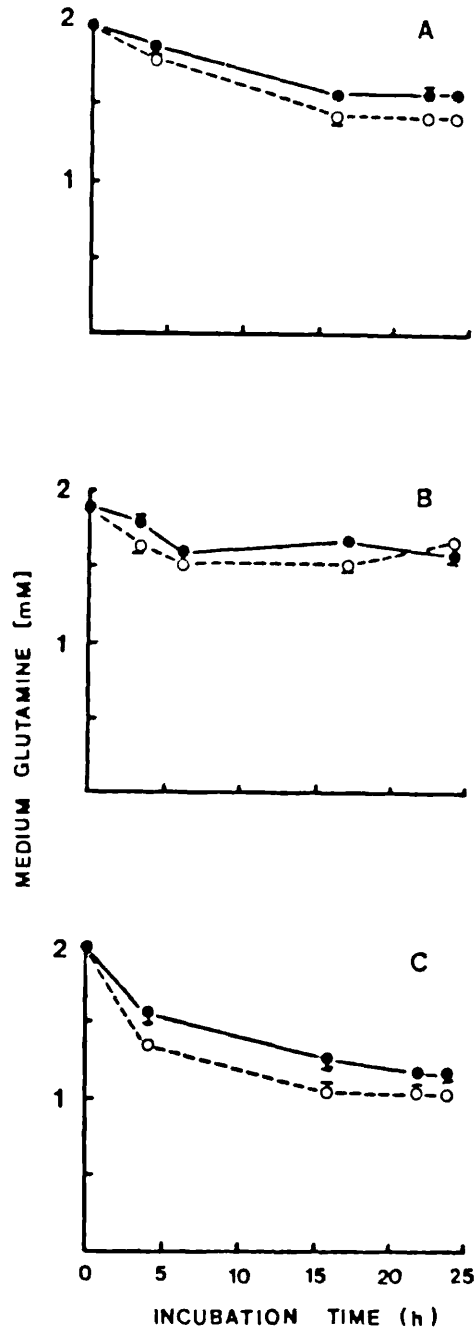consumption decreases dramatically at all glucose
concentrations, irrespective of the presence of mitogen.
In the presence of 5.5mM glucose, the initial rates of
glutamine consumption by rat splenocytes are low compared
with rat mesenteric lymphocytes cultured in the presence
of 5mM glucose and 2mM glutamine (Ardawi and Newsholme,1983).
In the latter study glutamine was consumed by resting
cells at approximately 181μmol/10^{10} cells/h; this compares
with a rate of 41.8 μmol/10^{10} cells/h by resting rat
splenocytes. Whilst Con A increased glutamine consumption
by rat mesenteric lymphocytes by 51% in the absence of
glucose, no data was provided for incubations containing
both glucose and Con A. In the present study the addition
of Con A to cultures containing glucose and glutamine
increased the early consumption of glutamine by 37%.
Incubations of rat thymocytes for 3h with 4mM glutamine
resulted in consumption of the amino acid at 35.4 μmol/
10^{10} cells/ (Brand et al.,1984). On the addition of 4mM
glucose glutamine consumption was decreased to 22.7 μmol/
10^{10} cells/h. The quantitative consumption of glutamine
therefore differs considerably with cell type on the
first few hours of culture; glutamine consumption by rat
splenocytes was approximately a quarter of that seen by
mesenteric lymphocytes and approximately double that
shown by thymocytes when all cells were incubated with
both glucose and glutamine. In the presence of Con A rat
splenocyte glutamine consumption is increased by 37%
during the first 4h of incubation; by contrast the addition
of Con A to incubation of rat thymocytes containing
glucose and glutamine resulted in a decrease in glutamine
consumption by 10%.

The addition of 5mM glucose to incubations of
rat mesenteric lymphocytes increased the rate of glutamine

consumption by 39% compared with incubations with glutamine alone (Ardawi and Newsholme,1983). Rat thymocytes, however, showed a decrease in glutamine consumption by the addition of 4mM glucose by 36% in the absence and by 55% in the presence of Con A. Whilst incubations in the absence of glucose were not performed in the present study, Table 27 shows that an increase in initial glucose concentration results in an increase in consumption of the amino acid during the first 4h of incubation by both resting and activated cells; by extrapolation it may be suggested that the addition of glucose to rat splenocytes would be likely to result in an activation of glutamine uptake. Furthermore, the Con A induced increase in glutamine consumption continues in the presence of increasing glucose concentrations.

It is thus evident that there are fundamental differences in the quantitative consumption of glutamine and the effect of glucose and mitogen on this, between rat mesenteric lymphocytes, thymocytes and splenocytes. The metabolism of glutamine may be further investigated by examination of the products formed from the metabolism of radiolabelled substrate by rat splenocytes.

Tables 28 and 29 present the results of incubations of rat splenocytes with 2mM glutamine and 5.5mM glucose in the presence and absence of Con A. The products of $[U-^{14}C]$-glutamine present in both medium and cells combined after 2h pulses throughout the incubation were then compared. The initial rates of glutamine consumption by resting and Con A activated cells are 42.9 and 61.4 μmol/ 10^{10} cells/h respectively; this is very similar to those presented inTable 27 showing the consistency of glutamine consumption as measured by enzymatic and radioactive assays. As seen previously, consumption of the

Table 27. <u>The effect of glucose on the rate of glutamine consumption by rat splenocytes.</u>

Initial Glucose mM	Con A (10μg)	Glutamine consumption (μmol/10^{10}cells/h)		
		0-4h	4-24h	0-24h
5.5	-	41.8 ± 5.0	16.2 ± 2.1	20.5 ± 0.8
	+	57.1 7.2	21.8 2.2	27.7 0.9
9.0	-	65.3 10.4	5.6 0.9	15.5 1.4
	+	94.7 8.6	0.0 0.7	11.4 0.8
11.0	-	133.2 12.1	21.8 2.6	40.3 3.3
	+	204.4 25.1	13.7 1.7	45.5 5.1

$8.5.10^6$ cells/ml were incubated in the presence of 5.5, 9 or 11m$\underline{M}$ glucose + 2m$\underline{M}$ glutamine. Experimental details according to Section 2.

Table 28. The effect of incubation time and Con A on [U-^{14}C]Glutamine metabolism by rat splenocytes: Formation of products.

Results are mean +/- SEM of triplicate cultures.

Time (h)	Con A (10μg)	μmol/10^{10} viable cells/h					[^{14}C Gln] in Products %
		Gln Consumed	[^{14}C Gln] in Glu	[^{14}C Gln] in Asp	[^{14}C Gln] in CO_2	[^{14}C Gln] in protein	
0	-	42.9±1.4	27.9±1.4	8.4±0.3	4.6±0.4	2.5±0.1	101.0
	+	61.4 1.9	34.2 1.9	14.5 0.4	7.0 0.5	2.7 0.2	95.2
4	-	29.8 2.7	19.3 1.2	5.8 0.6	2.5 0.2	2.2 0.2	99.7
	+	32.9 1.2	17.9 0.3	7.4 1.0	3.9 0.1	3.0 0.2	97.7
16	-	12.7 0.4	8.1 0.4	2.4 0.1	1.2 0.2	0.6 .02	96.8
	+	27.5 1.5	15.5 0.7	6.1 0.6	3.4 0.2	2.1 .03	98.4
22	-	11.6 0.7	7.6 0.5	2.0 0.2	0.6 .07	0.5 .04	92.7
	+	17.8 1.2	9.7 0.8	4.0 0.6	2.0 0.4	1.6 0.4	97.1

8.5.10^6 splenocytes were incubated in the presence of 5.5mM glucose and 2mM glutamine +/- Con A. At times shown [U-^{14}C]Gln was added for 2h, as described in Section 2.

Table 29. The effect of incubation time and Con A on $[U-^{14}C]$-Glutamine metabolism by rat splenocytes: percentage glutamine carbon to products.

Time (h)	Con A (10µg)	Percentage glutamine carbon to:				Of glutamine past glutamate	
		Glutamate	Aspartate	CO_2	Protein	% to Asp	% to CO_2
0	-	65.0	19.6	10.6	5.9	56.0	30.4
	+	55.7	23.6	11.4	4.4	53.3	25.7
4	-	64.7	19.4	8.4	7.5	55.3	23.7
	+	54.3	22.5	11.8	9.2	49.3	25.8
16	-	63.9	18.7	9.8	4.4	52.4	27.1
	+	56.3	22.4	12.2	7.5	51.0	28.0
22	-	65.3	17.7	5.5	4.1	50.4	16.1
	+	54.4	22.6	11.1	8.9	49.2	24.4

$8.5.10^6$ cells/ml were incubated with $[U-^{14}C]$-Glutamine for 2h in the presence or absence of Con A at the times shown. Experimental details according to Section 2.

amino acid decreases with increasing incubation time, but at all stages of the incubation mitogen activated cells show higher rates of consumption than resting cells. The major end products of glutamine metabolism by rat splenocytes were glutamate (54-65%), aspartate (17-23%) and CO_2 (5-12%), (Table 29). Glutamine carbon was also found in high molecular weight material. Of glutamine metabolised past the stage of glutamate approximately 50% was recovered as aspartate and 25-30% as CO_2.

Approximately 10% more glutamine was metabolised past the stage of glutamate by Con A activated cells than by resting cells; this is reflected by the small increases in the percentage glutamine carbon found in aspartate and CO_2 in Con A activated cells. The percentage glutamine carbon incorporated into CO_2 increases in the presence of Con A and this is also more noted with increasing incubation time. Thus, the production of CO_2 from glutamine is increased in the later stages of the incubation. Of glutamine carbon incorporated into high molecular weight material, there is a Con A induced increase in the relative proportion incorporated with increasing incubation time. 35% of glutamine consumed is metabolised past the stage of glutamate by resting cells; this increases to 45% by the addition of Con A. It is therefore evident that more glutamine is further metabolised by the TCA cycle in the activated state. The metabolism of glutamine by rat splenocytes shows a number of discrepancies with the findings of other authors working with rat mesenteric lymphocytes and rat thymocytes.

When incubated with glutamine as the sole substrate Ardawi and Newsholme (1983) showed that rat

mesenteric lymphocytes metabolised glutamine to glutamate, aspartate, asparagine, alanine, lactate and pyruvate. By contrast, in preliminary experiments with rat splenocytes, no lactate, pyruvate or alanine could be detected as products of glutamine metabolism. Of glutamine metabolised past glutamate, rat mesenteric lymphocytes metabolised approximately 70% to aspartate; in the present work this was 50-56%. In the presence of glucose mesenteric lymphocytes increased the uptake of glutamine but all the increase could be accounted for as glutamate and aspartate. Rat thymocytes incubated with glutamine alone were shown to produce glutamate (61%), aspartate (25%) and CO_2 (21%), (Brand et al.,1984). The level of CO_2 production here is greater than that shown by rat splenocytes. However, when both glucose and glutamine were present at 4m$\underline{M}$, rat thymocytes exhibited a decrease in glutamine utilisation and more glutamate (72%) and less aspartate (13%) were produced. Interestingly, Con A caused a decrease in the proportion of glutamate formed; this was also noted to occur with rat splenocytes. In addition, although the absolute production of CO_2 was increased by the presence of Con A, the relative proportion of CO_2 produced by thymocytes remained at 21%. This was also noted for splenocytes in the first 2h of culture (Table 29) but a greater difference between resting and Con A induced CO_2 production was shown at later incubation times.

Interestingly, incubations of thymocytes with glucose, glutamine and Con A resulted in rates of glutamine consumption below that observed with glucose and glutamine alone (Brand et al.,1984); this contrasts with the Con A induced increase exhibited by splenocytes.

Equivalent incubations on rat mesenteric cells

were not performed by Ardawi and Newsholme (1983), but it is evident that there exist major differences in the effect of Con A on cells incubated with both substrates. In the present work glutamine uptake was increased and its subsequent metabolism to products thus affected. Since more glutamine was metabolised past the stage of glutamate by Con A activated cells, the mitogen has resulted in an increase in the production of CO_2 and aspartate. By contrast, rat thymocytes showed a decrease in glutamine metabolism in the presence of Con A (Brand et al 1984).

In common with rat thymocytes, no lactate could be detected as a product of splenocyte glutamine metabolism; it is therefore apparent that a fundamental difference exists between the pathways of glutamine metabolism in these three types of lymphocytes. It is possible that this is due to an inherent difference in the use of mature T cells (mesenteric lymphocytes), immature T cells (thymocytes) and mature T and B cells (splenocytes).

It is evident from Tables 28 and 29 that glutamine contributes to CO_2 production from rat splenocytes, and therefore to energy production. In addition, Con A increased the absolute uptake of the amino acid and the proportion metabolised past glutamate. On a theoretical basis, the approximate ATP yield from glutamine metabolism by rat splenocytes under these conditions can be estimated, as was the case with glucose in the previous section. Based on the results presented in Table 28 it can be seen that approximately 60% of glutamine derived ATP is produced by the complete oxidation of the amino acid to CO_2 (Table 30). At all stages of the incubation Con A caused an increase in glutamine derived

Table 30. ATP formation from glutamine metabolism by rat splenocytes.

Time (h)	Con A (10µg)	ATP production (μmol/10^{10}cells/h) Gln→Asp	Gln→CO_2	Total	% ATP from CO_2 production	% increase in ATP by Con A
0	-	75.7	136.4	212.1	64.3	
	+	130.4	209.9	340.3	61.7	60.4
4	-	52.0	75.1	127.1	59.1	
	+	66.6	116.5	183.1	63.6	44.1
16	-	21.3	37.2	58.5	63.6	
	+	55.3	100.5	155.8	64.5	166.3
22	-	18.4	19.1	37.5	50.9	
	+	36.3	59.4	95.7	62.1	155.2

Data calculated from results presented in Table 29.

ATP, particularly at the later stages of the incubation when absolute glutamine consumption was considerably reduced. Also of interest is the fact that, in incubations performed in the absence of glutamine, the Con A induced production of glucose derived ATP increased throughout the incubation (Table 22). In the presence of glutamine, however, a drop in Con A induced ATP was seen at later stages of the incubation (Table 25). It is possible that this decrease may be related to the increase in ATP formed from glutamine at later stages in the culture period.

The major discrepancies between the findings of Ardawi and Newsholme (1983) and Brand et al.(1984) involve the metabolism of glutamine in the presence and absence of glucose. Since, in the present study no incubations were performed in the absence of added glucose it is perhaps of interest to examine the effect of high glucose concentrations on the metabolism of glutamine. Tables 31 and 32 give the corresponding results for the metabolism of 2mM glutamine by rat splenocytes in the presence of 11mM glucose. Immediately apparent is the increase in absolute glutamine consumption caused by the higher glucose concentration. A three-fold increase in the rate of glutamine consumption occurs compared with that seen with 5.5mM incubations. Glutamate is again the major product of glutamine metabolism, but in the initial stage of the incubation slightly more glutamate is produced than in incubations containing 5.5mM glucose. Glutamine metabolised to CO_2 is slightly higher at the increased glucose concentration, although this is more notable for resting cells. Interestingly, the increase in glucose has resulted in a greater proportion of glutamine metabolised past glutamate reappearing as CO_2 (Table 32).

Table 31. The effect of high glucose concentration, incubation time and Con A on [U-^{14}C]-Glutamine metabolism by rat splenocytes: formation of products.

Results are mean +/- SEM of triplicate cultures.

Time (h)	Con A (10μg)	μmol/10^{10} viable cells/h					
		Gln Consumed	[^{14}C Gln] in Glu	[^{14}C Gln] in Asp	[^{14}C Gln] in CO$_2$	[^{14}C Gln] in protein	[^{14}C Gln] in Products %
0	-	117.5±1.9	89.1±2.9	14.4±0.4	14.2±1.3	3.1±0.2	103.0
	+	193.9 3.8	121.4 4.9	42.5 2.5	22.0 1.7	3.0 0.2	97.4
4	-	30.9 2.3	18.2 1.6	3.2 0.4	2.5 .19	2.2 .08	84.3
	+	28.5 1.2	15.3 0.6	5.8 0.2	4.9 .29	2.6 .05	100.6
16	-	12.4 2.0	6.8 1.1	2.0 0.3	2.0 0.5	2.0 0.3	104.0
	+	22.0 0.8	13.9 1.0	2.2 0.2	2.7 0.3	2.4 0.2	98.7
22	-	6.9 1.3	4.4 0.9	0.9 0.1	0.8 0.2	0.3 .07	92.0
	+	9.0 1.2	5.0 0.8	1.6 0.2	1.1 0.2	1.0 0.2	98.0

8.5.10^6 splenocytes were incubated with 11mM glucose and 2mM glutamine +/- Con A. At the times shown [U-^{14}C]glutamine was added for 2h., as described in Section 2.

Table 32. The effect of incubation time, high glucose concentration and Con A on [U-^{14}C] Glutamine metabolism by rat splenocytes: percentage glutamine carbon to products.

Time (h)	Con A (10µg)	Percentage glutamine carbon to:				Of glutamine past glu	
		Glutamate	Aspartate	CO_2	Protein	% to Asp	% to CO_2
0	-	75.8	12.2	12.1	2.6	50.6	50.0
	+	62.6	21.9	11.4	1.6	58.6	30.4
4	-	58.9	10.3	8.0	7.1	25.1	19.4
	+	53.8	20.4	17.3	9.2	44.1	37.4
16	-	55.2	16.2	16.6	16.5	36.1	37.0
	+	63.5	10.3	12.1	10.3	28.1	33.0
22	-	64.1	12.6	11.0	4.1	35.0	30.5
	+	56.7	18.2	12.7	11.2	41.0	28.5

8.5.10^6 cells/ml were incubated with [U-^{14}C]Glutamine for 2h pulses in the presence or absence of Con A at the times shown. Experimental details according to Section 2.

Ardawi and Newsholme (1983) noted that the addition of glucose to incubations of rat mesenteric lymphocytes with glutamine caused an increase in absolute glutamine consumption. Although this also occurs with high glucose concentrations in the present work, 11mM glucose has caused an increase in glutamine metabolised to CO_2 and decreased aspartate production; by contrast all the increased glutamine consumed by mesenteric cells could be accounted for as glutamate. The addition of glucose to incubations of rat thymocytes not only caused a suppression of glutamine uptake but obliterated the Con A induced increase to below that seen by control incubations (Brand et al.,1984). It is clear that the effect of glucose on glutamine metabolism by rat splenocytes has more in common with that seen with mesenteric lymphocytes than with thymocytes; it is possible that the maturity of the cells has an affect on this.

The increase in glucose concentration has been shown to result in a greater proportion of consumed glutamine being metabolised to CO_2 (Table 32). Evidently, this has resulted in a greater proportion of glutamine derived ATP resulting from complete oxidation; 70% compared with 60% in the presence of 5.5mM glucose (Table 33). In addition, it can be seen that the Con A induced increase in ATP production is greater in the first half of the incubation than in the second; the reverse was true for incubations performed with 5.5mM glucose.

It has thus been possible to make an estimation of the production of ATP by different routes of the metabolism of both glucose andglutamine. By combining the data obtained from radiolabelling experiments in which 5.5mM glucose and 2mM glutamine were employed it is possible to obtain an overall picture of the importance

Table 33. ATP formation from glutamine metabolism by rat splenocytes in the presence of 11mM glucose.

Time (h)	Con A (10µg)	ATP production ($\mu moi/10^{10}$ cells/h)			% ATP from CO_2 production	% Increase in ATP by Con A
		Gln→Asp	Gln→CO_2	Total		
0	-	129.0	424.8	553.8	76.7	
	+	382.2	663.1	1045.3	63.4	89
4	-	28.6	74.1	102.7	72.2	
	+	52.3	147.6	199.9	73.8	95
16	-	18.0	61.6	79.6	77.4	
	+	20.4	79.6	100.0	79.4	26
22	-	7.8	22.6	30.4	74.3	
	+	14.8	34.3	49.1	69.9	62

Data calculated from results presented in Table 32.

of both substrates in relative ATP production. Tables 25 and 30 have given the estimated glucose and glutamine derived yields respectively; since these were based on experiments under identical conditions it is possible to combine the two to indicate the apparent relative proportion of ATP which may be derived from each substrate when presented simultaneously. Table 34 shows that, under these circumstances approximately 80% of ATP would be derived from glucose metabolism and approximately 20% from glutamine. By far the majority of energy derived from both substrates would result from the complete oxidation despite the fact that this is a quantitatively minor fate of glucose and glutamine.

Whilst the theoretical yield of ATP from glutamine is only a quarter of that from glucose when both substrates are present, it is evident that the amino acid serves in an energy producing capacity for both resting and mitogen stimulated splenocytes. In addition, this confirms the view that the inherent tendency of rat splenocytes to metabolise glucose to lactate is not based in energy production for activation.

The use of short term incubations of rat splenocytes has thus allowed the detailed metabolism of glucose and glutamine to be investigated. The uptake and subsequent metabolism of glucose has been shown to be influenced, not only by Con A, but also by the concentration of glucose and by the presence of glutamine. Similarly, the same factors considerably altered the metabolism of glutamine, indicating that the conditions of culture are of equal importance to the presence of mitogen in the outcome of such experiments. This has been evident in the attempts to explain discrepancies in the present work with the findings of other workers; these discrepancies

Table 34. ATP production from glutamine and glucose metabolism by rat splenocytes.

Time (h)	Con A (10µg)	Total ATP from Gln + Glc (μmol/10^{10}cells/h)	% ATP derived from Glucose	Glutamine
0	-	1152.6	81.6	18.4
	+	1575.7	78.4	21.6
4	-	724.7	82.5	17.5
	+	1053.8	82.6	17.4
16	-	480.8	87.8	12.2
	+	964.0	83.8	16.2
22	-	449.8	91.7	8.3
	+	533.8	82.1	17.9

Data is calculated from Tables 25 and 30.

are unlikely to be solely due to inherent metabolic differences in cells from different sources.

Whilst the calculations of potential ATP yields from glucose and glutamine carried out in this section are, by necessity, theoretical, they serve to show that glutamine may be an important source of energy for both resting and activated splenocytes. Furthermore, these calculations have confirmed the suggestion that the metabolism of glucose to lactate by splenocytes is relatively unimportant in energy production compared with that obtained from complete oxidation.

CONCLUSIONS.

The effect of the physical culture conditions on the Con A activation of rat splenocytes has shown that mitogen induced activation is a function of cell density, incubation time, mitogen concentration and culture vessel geometry. In addition, the susceptibility of rat splenocytes to fluctuation in CO_2 tension was revealed as a major influence on cell activation. Whilst many of these parameters have been previously shown to affect lymphocyte activation, it has been in the study of the metabolism of glucose, glutamine and the ketone bodies that previously unrecorded results have been obtained. In particular it is evident that the metabolism of glucose is profoundly affected by the physical culture conditions. The increase inaerobic glycolysis which occurs in lymphocytes inresponse to mitogenic activation has been well documented, but results presented here also show that Con A activation of rat splenocytes can occur under conditions which are accompanied by aerobic and partially anaerobic glycolysis. Furthermore, Con A activation has been shown to occur without a change in glucose consumption compared with resting cells. The consumption of glucose has also been shown to be affected by the concentration of both glucose and glutamine, but in all cases the ability of the cells to respond to Con A was unaltered. It is therefore a major conclusion of the present study that the consumption of glucose by rat splenocytes is considerably affected by the culture conditions in both the resting and activated states. For this reason the use of glucose consumption as an alternative index of stimulation to enhanced $[^3H]$-thymidine incorporation by lymphocytes cannot be supported.

The major end product of glucose metabolism
by rat splenocytes was found to be lactate. Despite this
measurements based on the use of radiolabelled glucose
consumption revealed that this was a quantitatively
minor source of glucose derived ATP. Although only
10% of consumed glucose was completely oxidised by rat
splenocytes, this provided up to 70% of glucose derived
ATP. The conclusion is therefore made that the production
of large quantities of lactate by mitogen activated
splenocytes does not have its basis in the increased
production of energy for activation.

The consumption of the ketone bodies by rat
splenocytes was shown to be concentration dependent in
both the resting and Con A activated states. In addition,
there exists a preference by splenocytes to consume
acetoacetate rather than D β-hydroxybutyrate; the reason
for this is unclear. Although the ketone bodies may be
considered to be additional respiratory substrates in the
starved state, this did not affect the consumption of
the ketone bodies by splenocytes. There was no significant
change in the consumption of the ketone bodies by rat
splenocytes on the addition of Con A; for this reason
although they represent a source of energy for the cells
the ketone bodies are not considered to be of any
additional importance on cell activation, and are therefore
not involved in the provision of energy for lymphocyte
transformation.

Glutamine has been shown to be essential to
the long term increase in $[^3H]$-thymidine incorporation
by splenocytes inresponse to Con A. In addition, the
amino acid was consumed in a concentration dependent
manner up to 4mM. Glutamine was also shown to affect the
metabolism of glucose by splenocytes. The major products

of glutamine metabolism were glutamate, aspartate and CO_2; the proportion of each was affected by both Con A and the concentration of glucose in culture. In a similar manner to the metabolism of glucose, the complete oxidation of glutamine accounted for only 5-15% of consumed glutamine. However, glutamine metabolised by this route supplied approximately 60% of glutamine derived ATP. It is evident that, although splenocytes metabolise both glucose and glutamine predominantly by non-oxidative routes, the production of ATP by these pathways is quantitatively minor compared with that produced by the complete oxidation of both substrates. When presented to splenocytes together with glutamine, glucose may produce up to 80% of ATP resulting from the metabolism of both substrates; the production of ATP from glutamine metabolism, although only 20% of the total, still implies a role for the amino acid in the production of energy for cell activation.

It is thus apparent that there is interplay between the metabolism of glucose and glutamine in rat splenocytes, and further work is required to clarify this. The short term labelling system thus developed has the capacity to monitor aspects of substrate consumption and metabolism throughout the 24h incubation. Of great interest would be the extension of the time course of study to include the period during which DNA synthesis occurs in activated cells; by necessity 24h culture periods do not cover this. The use of glucose depleted culture media would allow the selective addition of controlled amounts of substrates in various combinations; in this way the threshold concentrations of glucose and glutamine for activation could be found;

the detailed study of the metabolism of the substrate
would then be more likely to indicate the exact role
of each substrate. Of interest is whether the addition
of glutamine or other respiratory substrates can remove
the requirement for glucose on activation. If longer
incubations could be performed it is likely that any
additional requirement for energy during periods of
high synthetic activity would be identified.

It is evident from this work that the study of
the metabolism of lymphocytes on mitogen activation is
considerably complicated by the need to allow for the
effect of the prevailing conditions on metabolism; it
has often been difficult to distinguish those effects
which are inherent to the cell and those which are
elicited, or accentuated by the conditions. It seems
clear that any change of the culture system would demand
a reappraisal of the influence of such conditions on
the intermediary metabolism of the cells. Of particular
interest is the effects on glucose metabolism; this seems
to be particularly susceptible to the alteration of
the conditions. A thorough investigation of this aspect
of lymphocyte metabolism may go some way towards the
explanation of the propensity of activated lymphocytes
to metabolise glucose in a partially anaerobic manner.

5. APPENDIX.

5.1. The composition of RPMI 1640 (Dutch Modification).

Component	mg/L	Component	mg/L
Arginine	200	Thiamin HCl	1
Asparagine	56.82	Vit B_{12}	0.005
Aspartic acid	20	$Ca(NO_3)_2$	69.49
Cystine	59.15	KCl	400
Glutamic acid	20	$MgSO_4 \cdot 7H_2O$	100
Glutathione	1	NaCl	6400
Glycine	10	$NaHCO_3$	1000
Histidine	15	Na_2HPO_4	800.7
Hydroxyproline	20	Glucose	2000
Isoleucine	50	HEPES	4770
Leucine	50	Na Phenol Red	5
Lysine	40		
Methionine	15		
Phenylalanine	15		
Proline	20		
Serine	30		
Threonine	20		
Tryptophan	5		
Tyrosine	20		
Valine	20		
Biotin	0.2		
Ca Pantothenate	0.25		
Choline Cl	3		
Folic acid	1		
Inositol	35		
Nicotinamide	1		
pABA	1		
Pyridoxine HCl	1		
Riboflavin	0.2		

5.2. The composition of αMEM (1)

Component	mg/L	Component	mg/L
Alanine	25	KCl	400
Arginine	105	$MgSO_4 \cdot 7H_2O$	200
Asparagine	50	NaCl	6800
Aspartic acid	30	Lipoic acid	0.2
Cysteine	100.24	$NaHCO_3$	2200
Glutamic acid	75	$NaH_2PO_4 \cdot 2H_2O$	140
Glycine	50	Glucose	1000
Histidine	31	Na Pyruvate	110
Isoleucine	52.5	Na Phenol Red	10
Leucine	52.4		
Lysine	58		
Methionine	15		
Phenylalanine	32		
Proline	40		
Serine	25		
Threonine	48		
Tryptophan	10		
Tyrosine	36		
Valine	46		
Ascorbic acid	50		
Biotin	0.1		
Ca Pantothenate	1		
Choline Cl	1		
Folic acid	1		
Inositol	2		
Riboflavin	0.1		
Thiamin HCl	1		
Vit B_{12}	1.36		
$CaCl_2$	200		
Nicotinamide	1		

αMEM (2) as above except that $NaHCO_3$ 458 mg/L and 20mM HEPES is added.

5.3. Statistical analysis by the Student t-test.

$$\bar{x}_2 - \bar{x}_1 \gg t\sqrt{\mathcal{E}_1^2 + \mathcal{E}_2^2}$$

$$t = \frac{\bar{x}_2 - \bar{x}_1}{\sqrt{\mathcal{E}_1^2 + \mathcal{E}_2^2}}$$

where $\bar{x}_1$ = arithmetic mean of controls

$\bar{x}_2$ = arithmetic mean of tests

$\mathcal{E}_1$ = standard error of the mean (SD/ N)controls

$\mathcal{E}_2$ = standard error of the mean tests.

Rates of substrate consumption and/or production in Tables were produced by subtraction of assayed substrate concentrations at the time given and recalculation of the denominator. Thus, on obtaining triplicate measurements at two time points the rate mean +/- SEM was obtained by the evaluation of mean +/- SEM of: (A-D) (A-E) (A-F) (B-D) (B-E) (B-F) (C-D) (C-E) (C-F) where A B and C are measurements at the first time point and D E and F at the second, n=9.

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