Rice University

Role of Glucose and Glutamine Metabolism in the Growth of a Human T-lymphoblastoid Line, Jurkat

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree Masters of Science

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Abstract

The interrelationship between glucose and glutamine metabolism was examined using Jurkat, a human T-lymphoblastoid line. This study utilized three experimental approaches to examine the metabolism of Jurkat: (i) an in vitro whole cell 'plate assay', (ii) continuous culture bioreactor pulse changes, (iii) a proliferative assay, using the tetrazolium salt MTT.

(i) In the plate assay, cells were incubated in Kerbs-Ringer buffer at an initial density of 2-7x10^6 cells/ml. The utilization of select nutrients, and subsequent product formation was followed over a period of 8 hours. Both intercellular and extracellular nutrient levels are measured in this technique.

(ii) Jurkat maintained at a dilution rate of 0.372 day^{-1} was subjected to a number of pulse changes(10 mM glucose, 5 mM pyruvate, 4 mM glutamine, 10 mM oxaloacetate plus 2.5 mM glutamate, and 0.5x amino acids) and the resulting shifts in metabolism were followed. The 10 mM oxaloacetate and 2.5 mM glutamate pulse resulted in a marked decrease glutamine utilization and a subsequent drop in ammonium production. This pulse also provided the only instance in which increased levels of aspartic acid were detectable.

(iii) The MTT assay was modified to replace the more commonly used 'growth assay' for the measurement of cellular proliferation/activation.

Results observed in this work indicate:(a) that both aspartate-aminotransferase and alanine-aminotranferase are involved in the breakdown of glutamine;(b) aspartate's carbon skeleton is derived from glutamine, while alanine's carbon skeleton comes from glucose;(c) while glutamine utilization does not alter glucose metabolism in Jurkat, the presence of glucose not only reduced the rate of glutamine utilization, it also altered the products formed from glutamine, (d) the inability to regenerate cytosolic NAD via mitochondrial transport may contribute to the production of high levels of lactate from glucose.
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I  INTRODUCTION:

1) Overview:

This body of work describes the metabolism of one mammalian cell line grown in culture in a little more detail than other works preceding this one have attempted. Although no one thesis can completely describe the catabolic and anabolic processes that are the essence of life, the pages that follow should provide the groundwork for a continuing systematic study of mammalian cell metabolism.

The early pages of this introduction serve as a mini-review of some of the important biochemistry involved in in-vitro mammalian cell metabolism. It has been written much as any introductory biochemistry text would be.[32,56] While it requires the reader to have some background in biochemistry, it does not go into extensive detail into the complex regulation that is involved in the metabolism of mammalian cells. Alterations of mammalian cell metabolism that are associated with a tumor cell as opposed to a 'normal' cells will be emphasized.[16]

The later pages of the introduction should serve to acquaint the reader with glutamine utilization as a means of energy production for mammalian cells.[16,34] The relative importance of key enzymes in the oxidation glutamine will be reviewed.[34,54] The possibility that other amino acids present in tissue culture media may serve as an oxidative fuel is also addressed.

2) Glucose Metabolism:

A brief description of glucose utilization serves as an excellent starting point for any review on mammalian cell metabolism since its use is so vital for the survival of most, if not all, mammalian cell lines. Glucose not only serves as a major source of ATP via its partial or complete oxidation, but also has a major role in anabolic processes. NADPH and
ribose-5-phosphate, both important intermediates for biosynthetic pathways, are produced from glucose processed through the pentose phosphate pathway.[57]

Figure 1.2.1 is an overview of the reactions involved in the breakdown of glucose to pyruvate. The first half of the breakdown of glucose is regarded as energy consuming since two ATPs are utilized. The introduction of two high energy phosphate bonds on the glucose molecule serves to 'prime' it for subsequent energy production in the later half of the pathway depicted in Figure 1.2.1.

The action of the first enzyme, hexokinase (HK), 'locks' the glucose molecule in the cell by the addition of phosphate. The glucose-6-phosphate (G-6-P) formed by this reaction can proceed to fructose-6-phosphate (F-6-P) as depicted, or can be converted to 6-phosphogluconate and enter into the pentose phosphate pathway for production of NADPH and ribose-5-phosphate.

The second phosphate used to prime glucose is added to F-6-P by the action of phosphofructokinase (PFK). Both enzymes involved in the addition of phosphate to glucose serve as key regulatory enzymes in the metabolism of glucose, since their reactions are considered irreversible.

The fructose 1,6-bisphosphate (F-1,6-P) formed by the action of PFK is cleaved by the action of aldolase and yields glyceraldehyde 3-phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP). The G-3-P and DHAP formed are interconverted by the action of triose phosphate isomerase. It is G-3-P, that is carried into the second half of glycolysis.

The second half of the metabolism of glucose is considered energy yielding. After being primed in the first half, the glucose molecule upon subsequent breakdown yields both ATP and NADH.
Figure I.2.1 Oxidation of Glucose to the Level of Pyruvate
The first reaction in this half of glycolysis involves the enzyme glyceraldehydephosphate dehydrogenase. This enzyme catalyses the addition of inorganic phosphate to G-3-P and results in the production of NADH. The NADH formed has a number of possible fates. The most rewarding (energy wise) to the cell is oxidation in the respiratory chain, where 3 ATPs may be produced per NADH molecule oxidized. Another fate is its oxidation back to NAD via the action of lactate dehydrogenase. Although no energy is produced NAD is regenerated and another round of glucose metabolism is possible.

The next reaction in the pathway generates ATP. The enzyme phosphoglycerate kinase, removes the phosphate added to G-3-P to produce ATP from ADP. Here the inorganic phosphate added to G-3-P yields useful, high energy ATP.

The actions of phosphoglycerate mutase and enolase serve to convert the 3-phosphoglycerate(3-P-G) formed by phosphoglycerate kinase to phosphoenolpyruvate (PEP) in preparation for the final energy yielding step in the breakdown of glucose to pyruvate.

Pyruvate kinase catalyzes the irreversible conversion of PEP to pyruvate. In the process, ATP is produced, thus completing the final energy yielding step. Pyruvate kinase(PK), like HK and PFK, appears to be a major regulatory enzyme.

The overall balance sheet for the breakdown of glucose to pyruvate is:

\[
\text{GLUCOSE } + 2\text{Pi } + 2\text{ADP } + 2\text{NAD}^+ \quad \rightarrow \quad 2\text{PYRUVATE}^- + 2\text{ATP} + 2\text{NADH} + 2\text{H}^+ + 2\text{H}_2\text{O}
\]

Thus, from 1 mole of glucose, 2 moles of pyruvate, 2 moles of ATP, and 2 moles of NADH are produced. The ultimate fate of the NADH and pyruvate formed will be discussed in greater detail in the sections that follow. However, before this is done a brief comparison between glucose catabolism in 'normal' and 'tumor' cells needs to be presented. Since the ultimate fate of pyruvate/NADH is intimately depended on whether a
cell can be considered 'normal' or 'tumor like', the following two sections will serve to introduce the distinction between these two terms.

A) Normal Cell Metabolism:

In 'normal' cells the breakdown of glucose is tightly regulated. This regulation allows the maximum amount of energy to be produced per glucose molecule, as long as energy levels are sufficiently high. This allows a normal cell to conserve its glucose stores for times when energy levels are low. Under conditions where a normal cell is energy limited (low ATP), glucose metabolism shifts to produce energy immediately. As a consequence of the cell trying to meet its immediate energy demand, glucose is utilized less efficiently.

For the normal cell, glycolysis is tightly controlled by oxygen or ATP levels since a majority of their ATP is produced by respiration.

When normal cells are grown in an environment with sufficient oxygen, the pyruvate formed from glucose is further oxidized in the TCA cycle. As acetyl-CoA formed from pyruvate via pyruvate dehydrogenase condenses with oxaloacetate (OAA) in the mitochondria, pyruvate from the cytosol enters the mitochondria to replace the acetyl-CoA used.[16,32] In this manner only enough pyruvate is formed from glucose to replace the pyruvate that enters into the TCA cycle.[16]

If the TCA cycle is functioning in a fully respiring cell, ATP levels are high. The high levels of ATP markedly inhibit PFK, thus slowing the breakdown of glucose to perfectly match the needs of the cell.

The inhibition of PFK causes a buildup of G-6-P, the substrate utilized by PFK. The buildup of G-6-P in turns inhibits hexokinase (HK), thus adding even a finer level of control on the utilization of glucose.
The end result is that ATP through the action of HK and PFK inhibits its own production, keeping ATP at proper levels for cellular functions. As a final level of control the enzyme pyruvate kinase(PK) also serves a major regulatory function. Its activity is also reduced in the presence of high levels of ATP and acetyl-CoA. This provides the cell with one final way in which glycolysis can be tuned to meet the energy demands of the cell.

As the inhibition of HK, PFK, and PK continues, ATP levels begin to fall, or under conditions of oxygen limitation (where ATP levels would be low), the inhibition of PFK ends. As F-1,6-P levels begin to rise, they in turn stimulate PFK even more, and the flow of glucose thru this pinch point rapidly increases.

With an increasing flux through PFK the levels of G-6-P soon begin to drop and the inhibition of hexokinase is relaxed. The net result, is that glucose rapidly enter the glycolytic pathway. With ATP levels still critically low the inhibition of pyruvate kinase is overcome and large amounts of pyruvate are produced.

With a 'normal' respiring cell line with sufficient oxygen, this control scheme repeats itself over and over, with the production of just the right amount of pyruvate. If the normal cell is in an environment where oxygen is limiting, ATP can be produced by respiration, the rate of glucose oxidation remains high due to the relaxation of HK, PFK and PK inhibition. Since the low oxygen levels inhibit pyruvate dehydrogenase, pyruvate can not enter the TCA cycle and pyruvate and NADH build-up. Since NADH oxidation is limited by the availability of oxygen, the levels of NAD begin to fall. Without the regeneration of NAD, the breakdown of glucose would cease. The cell overcomes this limitation by the use of lactate dehydrogenase(LDH). LDH catalyzes the reaction of pyruvate to lactate, with the conversion of NAD to NADH. By using LDH to regenerated the NAD needed for G-3-P dehydrogenase, the cell is able to maintain a high rate of glycolysis to provide ATP when oxygen is limiting.

The features described above for normal cells allow them to maximize the amount of energy derived from each molecule of glucose by channeling the pyruvate and NADH
formed from glycolysis into the mitochondria (and therefore into the respiratory chain). If the normal cell becomes oxygen limited, and hence ATP limited, it has another (inefficient) method in which glycolysis can produce energy in the form of ATP. This results in pyruvate and NADH being used to produce lactate and NAD. The cells ability to shift its metabolism in this way allows it to survive under conditions where respiration is limited.

B) Tumor Cell Metabolism:

The tumor cell on the other hand has some interesting isozymes which allow a continual high rate of glucose breakdown under most conditions, including conditions with sufficiently high oxygen and ATP levels. The resultant high rate of pyruvate formation is often greater than the cells capacity to bring the pyruvate into the TCA cycle and results in much of the glucose being converted to lactate.

It is generally believed that all tumor cell lines, and other cell lines with unusually high rates of aerobic glycolysis, possess a special hexokinase isozyme. The form of hexokinase found in most tumor cell lines is associated with the outer surface of the mitochondria, as opposed to the normal hexokinase which is found free in the cytosol. The tumor form of hexokinase is not inhibited by high levels of G-6-P, as is the case with the normal HK. This form of hexokinase pushes glucose into the glycolytic pathway, even when there is inhibition of PFK.

There is also a special isozyme form of pyruvate kinase (type M₂) in tumor cells. This enzyme has a low affinity for PEP and is strongly inhibited by alanine. These properties allow the buildup of glycolytic intermediates from PEP to F-1,6-P. It is the ultimate buildup of F-1,6-P that allows the tumor cell with these special enzymes to bypass all the regulation involved in maintaining glycolysis under strict control. The high level of F-1,6-P overcomes the inhibition of PFK caused by high levels of ATP. The net result is that tumor cells metabolize glucose to lactate in the presence of high levels of ATP.
Consequently, the tumor cell wastes energy as glucose is uncontrollably metabolized to lactate.

3) **TCA Cycle:**

   **A) Energy Production**

   The tricarboxylic acid cycle (TCA) takes pyruvate formed from glycolysis and oxidizes it completely to CO$_2$ and H$_2$O. Pyruvate oxidation in the TCA cycle results in a substantially higher energy yield from glucose than when pyruvate is oxidized to lactate. The NADH formed in the TCA cycle increases the production of ATP to 38 per glucose molecule, if the cytosolic NADH produced during glycolysis is also oxidized. Recall pyruvate oxidation to lactate results in only 2 moles of ATP being formed.

   Figure I.3.1 depicts the TCA cycle. As with glycolysis, the TCA cycle is tightly regulated in normal cells to insure its proper functioning.[32]

   The first point of regulation that occurs is the conversion of pyruvate to acetyl-CoA. High levels of NADH, ATP, or acetyl-CoA within the mitochondria all inhibit this reaction.

   In the next step, acetyl-CoA and oxaloacetate condense to form citrate. The formation of citrate is also tightly regulated, with compounds associated with a high energy state, i.e. elevated levels of succinyl-CoA, NADH, fatty acyl-CoA, all inhibiting the formation of citrate. But by far the most important factor regulating the TCA cycle is the availability of oxaloacetate. Under most conditions the levels of oxaloacetate present in the mitochondria are extremely low.[32,65]

   In subsequent reactions, NADH is produced: the conversion of isocitrate to 2-oxoglutarate; the conversion of 2-oxoglutarate to succinyl-CoA; the conversion of malate to oxaloacetate. The conversion of succinyl-CoA to succinate yields GTP and the conversion of succinate to fumarate reduces of FAD to FADH$_2$. 
Figure I.3.1  Tricarboxylic Acid (TCA) Cycle
The TCA cycle as depicted in Figure I.3.1 shows the importance of this cycle in production of energy for mammalian cells. Not shown in this figure is that TCA intermediates often exit the cycle to serve in biosynthetic pathways. Intermediates like 2-oxoglutarate, succinate, and oxaloacetate can be removed from the cycle to serve as amino acid precursors. Citrate, and hence acetyl-CoA, can leave the cycle for the synthesis of fatty acids.[47]

When TCA intermediates are channeled into other pathways, they must be replenished if the cycle is to continue to function. One important anaplerotic reaction involves the carboxylation of pyruvate to form oxaloacetate. The enzyme pyruvate carboxylate utilizes ATP to catalyze the incorporation of CO$_2$ onto pyruvate to form oxaloacetate. This reaction allows the TCA cycle to proceed even if important intermediates have been drained off.

**B) Possible Points of Entry into the TCA Cycle for Amino Acids**

If important intermediates of the TCA cycle are continuously drained off, for nucleic acid and fatty acid synthesis, the addition of filler reactions to feed the cycle may be required for it to maintain its energy producing function.

Standard mammalian cell culture media attempts to provide the cells with all the essential amino acids they need. In addition, a full complement of non-essential amino acids are added. (See Table II.2.1) The addition of this full complement of amino acids, while undoubtedly serving the biosynthetic needs of the cell, may also supply cell with an unsuspected source of energy.

Figure I.3.2 depicts the possible entry points for amino acids into the TCA cycle. From this figure one can see that many points of entry exist in which excess amino acids can enter the TCA cycle. Depending on where the amino acid enters and leaves the TCA cycle, its contribution to the energy needs of the cell can be significant.
Figure 1.3.2 Possible Entry Points into the TCA cycle for Amino Acids

Most mammalian cell culture applications (studies) only consider glucose or glutamine in the realm of energy production. If some or all of the remaining 19 amino acid are catabolized in the TCA cycle (to some degree), the energy calculation based solely on glucose/glutamine could potentially be in error.

4) Glutamine Metabolism:

With mammalian cells, the most important energy source may not be glucose, but glutamine. The partial and/or complete oxidation of glutamine occurs in most mammalian
cell lines. For some cell lines the energy derived from glutamine has been calculated to equal that derived from glucose.[34,73]

Figure 1.4.1 depicts the steps of glutaminolysis (glutamine oxidation) and their potential relationship to other important pathways.

The initial step in the breakdown of glutamine is believed to occur by one of two different mechanisms. The first one involves the action of glutamine amidotransferase. This reaction is believed to be only a minor contributing factor to the breakdown of glutamine, since it contributes less than 10% of the total activity involved in the initial conversion of glutamine to glutamate.[15] The enzyme that is generally recognized as the main reaction involved in the conversion of glutamine to glutamate is glutaminase.

Glutaminase catalyzes the conversion of glutamine to glutamate with the release of $\text{NH}_4^+$ and is believed to be the first regulatory point in the oxidation of glutamine.[3,14,34] The reaction as depicted in Figure 1.4.1, therefore is generally considered to be irreversible. Two classes of glutaminase are known to exist in mammalian cells. The two classes differ in their affinity for glutamine and in their response to inorganic phosphate. The form of glutaminase that is believed to be important for mammalian cell lines (tumor lines) is the one that has a relatively high affinity for glutamine, and is activated by Pi.[34]

The glutaminase reaction is believed to occur in the mitochondria. The conversion of the glutamate to 2-oxoglutarate can occur via the action of three different enzymes. The three enzymes include two aminotransferases, alanine aminotransferase (AlaAT) and aspartate aminotransferase (AspAT), and glutamate dehydrogenase (GIDH). A considerable amount of research has focused on determining which enzyme predominates in the conversion of glutamate.
Figure 1.4.1  
Potential steps of Glutaminolysis and their Relationship to Other Important Pathways
Newsholme[43,44,45], and Brand[9,10,11,12] show AspAT as being the most important enzyme in the formation of 2-oxoglutarate from glutamate when their incubation buffers contained only glutamine. The action of AlaAT, on the other hand was not believed to be important in the breakdown of glutamine under the condition employed in the experiments of Newsholme, and Brand.

Once glutamate is converted to 2-oxoglutarate, it enters the TCA cycle where partial oxidation takes place. The longer the carbon skeleton of glutamine remains in the TCA cycle the larger the energy yield is.

For complete oxidation to occur, without the entrance of 'outside' intermediates, each molecule of glutamine that enters the TCA cycle must be converted to acetyl-CoA via pyruvate dehydrogenase and be reintroduced to the cycle. Several feeder reactions which convert glutamine derived malate or oxaloacetate into pyruvate are required for the complete oxidation of glutamine.

One of this reactions is catalyzed by the malic enzyme and converts malate to pyruvate.[34,40,59] Three isozymes of the malic enzyme are known to exist. They differ in their requirement for NAD and NADP. Malic enzymes 1 and 3 require NADP and catalyze both the production of pyruvate from malate, and the production of malate from pyruvate. Malic enzyme 2 on the other hand, catalyzes the irreversible conversion of pyruvate from malate with either NAD or NADP providing the reducing power. Malic enzyme 2 is found either in the cytosol or mitochondria of many mammalian cell lines.[34,54] The activities of the 3 isozymes of malic of the enzyme could supply the pyruvate required for the complete oxidation of glutamine.

Another way in which pyruvate could be formed is by the actions of oxaloacetate decarboxylase, or by the actions of phosphoenolpyruvate carboxykinase(PEPCK) and pyruvate kinase. The first enzyme would produce the needed pyruvate directly from oxaloacetate. The action of PEPCK would result in the formation of PEP first, the PEP formed then could be converted to pyruvate by the action of pyruvate kinase.
The ultimate role of each enzyme system in the final steps of glutaminolysis remain to be determined. However, if glutamine is to be completely oxidized, at least one set of reactions that produce pyruvate must be active.

5) Interaction of Glucose and Glutamine Oxidation:

For many mammalian cell lines growing in a media that contain high levels of both glutamine and glucose, glucose is usually completely converted to lactate. The glutamine utilized by cells growing under these conditions provides much of the cell's energy by its partial or complete oxidation in the TCA cycle. The question arises as to why is this the case. Why is much of the glucose utilized converted into lactate as opposed to being oxidized in the TCA cycle? While a number of possibilities exist, only two of the most interesting are highlighted below.

One possibility relates to the problem of oxygen radical formation. The final event in the respiration chain, the addition of electrons to oxygen, is critical. If the four electrons are not accepted by oxygen, toxic oxygen radicals are formed. The addition of two electrons results in the production of hydrogen peroxide, while the addition of only one electron would produce superoxide.

Superoxide formation appears to inhibit important steps in pyruvate oxidation. The production of acetyl-CoA from pyruvate is inhibited as well as the production of 2-oxoglutarate from isocitrate.[16] The other dehydrogenase enzymes in the TCA cycle appear to remain relatively active in the presence of superoxide. The net result is that pyruvate entry into the TCA cycle is inhibited. The enzymes involved in the partial oxidation of glutamine on the other hand remain active and glutamine readily enters the TCA cycle.

The second possibility that may explain why the conversion of pyruvate to lactate dominates over the oxidation of pyruvate in the TCA cycle deals with the transfer of NADH
into the mitochondria. Recall that during the conversion of glucose to pyruvate two molecules of NADH are generated. The NAD converted in the reaction involving G-3-P needs to be regenerated if additional glucose is to be broken down. The regeneration of NAD can occur by two mechanisms, one being the formation of lactate, the other being the shuttling of NADH into the mitochondria in return for NAD.[21,47] The most prominent shuttle system for transferring NADH into the mitochondria is the malate-aspartate shuttle. (Figure I.5.1)

Figure I.5.1 Malate-Aspartate Shuttle

If the malate-aspartate shuttle, or some other shuttle system[49], for some reason was inactive, the only way in the cell line could regenerate the needed NAD is via the LDH reaction. While the role of defective shuttles are often dismissed as being the reason behind excessive lactate formation, this possibility does provide an excellent explanation of the energy metabolism observed for many 'tumor' lines.
II MATERIALS AND METHODS:

1) Cell Lines and Their Maintenance

All cell lines used in this study were obtained from American Type Culture Collection (ATCC). The cell lines used include: FO, ATCC CRL 1647, a non-secreting mouse myeloma; Sp2/0-Ag14, ATCC CRL 1581, a non-secreting mouse myeloma; αIR-1, ATCC HB 175, a mouse-mouse hybridoma with FO as the fusion parent, that produces a IgG1κ that reacts to the human insulin receptor; FD441.8, ATCC TIB213, a mouse-rat hybridoma with Sp2/0-Ag14 as the fusion parent, that produces an IgG2b to LFA-1; 116NS-3d, ATCC CRL 8019, a mouse-mouse hybridoma with P3X63Ag8 as the fusion parent, that produces an IgM reactive to high molecular weight CEA; Jurkat, clone E6, ATCC TIB 152, a human T-cell leukemia line that produces interleukin 2 (IL-2) upon stimulation.

All lines were received frozen, packed in dry ice, from ATCC at an unknown passage number. Upon receipt the cell lines were rapidly thawed in a 37°C water bath, or under stream of hot water. The various cell lines were diluted 1:10 with RPMI-1640 containing 10% Fetal Bovine Serum (FBS), 100 mg/L streptomycin, 10^5 U/L penicillin, and 26.2 mM sodium bicarbonate (base-RPMI) that had been previously equilibrated in a 37°C, 5% CO₂, 100% RH incubator. Depending on whether the cells were anchorage dependent in base-RPMI or not they were handled one of two ways three hour after thawing. For the adherent cell lines (FO, Sp2/0-Ag14) the media containing residue DMSO was aspirated off and the cells were washed once with base-RPMI. The cell lines that grow in suspension (α-IR1, 116NS-3d, FD441.8, and Jurkat, clone E6) in base-RPMI were spun-down (x200g, 10 min). The media containing residual DMSO was aspirated off and the cell lines were resuspended in base-RPMI.

All cell lines were maintained in base-RPMI until a freezer stock of each line could be prepared. The maintenance of each cell line was contingent on their anchorage dependency and their ability to grow at limiting dilutions. Anchorage dependent cell lines were passed at
developing a completely defined media can not be overemphasized. From Table II.2.1, which list the components of RPMI, one can begin to understand the complexity of the nutritional requirements of mammalian cells grown in culture. For comparison, one can often grow bacteria such as *E. coli* in a simple chemically defined media that provides a carbon source (usually glucose), a nitrogen source (ammonium nitrate) and a phosphorus source (sodium phosphate).

Table: II.2.1 Components of RPMI-1640 Tissue Culture Media

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/L</td>
<td></td>
<td>g/L</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>0.2</td>
<td>Biotin</td>
<td>0.0002</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>0.05</td>
<td>D-Panthenic Acid,Ca</td>
<td>0.0025</td>
</tr>
<tr>
<td>L-Aspartic Acid</td>
<td>0.02</td>
<td>Choline Chloride</td>
<td>0.003</td>
</tr>
<tr>
<td>L-Cystine-2HCL</td>
<td>0.0652</td>
<td>Folic Acid</td>
<td>0.001</td>
</tr>
<tr>
<td>L-Glutamic Acid</td>
<td>0.02</td>
<td>Myo-Inositol</td>
<td>0.035</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>0.3</td>
<td>Niacinamide</td>
<td>0.001</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.01</td>
<td>PABA</td>
<td>0.001</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.015</td>
<td>Pyridoxine HCl</td>
<td>0.001</td>
</tr>
<tr>
<td>L-Hydropoline</td>
<td>0.02</td>
<td>Riboflavin</td>
<td>0.0002</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0.05</td>
<td>Thiamine HCl</td>
<td>0.001</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0.05</td>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;</td>
<td>0.000005</td>
</tr>
<tr>
<td>L-Lysine-HCL</td>
<td>0.04</td>
<td>Glutathione, Reduced</td>
<td>0.001</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0.015</td>
<td>Calcium Nitrate</td>
<td>0.1</td>
</tr>
<tr>
<td>L-Proline</td>
<td>0.02</td>
<td>Potassium Chloride</td>
<td>0.4</td>
</tr>
<tr>
<td>L-Serine</td>
<td>0.03</td>
<td>Magnesium Sulfate</td>
<td>0.04884</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0.02</td>
<td>Sodium Chloride</td>
<td>6.0</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0.005</td>
<td>Sodium Phosphate</td>
<td>0.8</td>
</tr>
<tr>
<td>L-Tyrosine-2Na</td>
<td>0.0283</td>
<td>Phenol Red, Na</td>
<td>0.0053</td>
</tr>
<tr>
<td>L-Valine</td>
<td>0.02</td>
<td>D-Glucose</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Mammalian cell culture media, on the other hand contains a wide assortment of amino acids, vitamins and other important nutrients. However, this still is not enough. The undefined/ill-defined component serum must be added for successful growth of most mammalian cells. Table II.2.2 list some of the typical substances found in fetal bovine serum (FBS). The lot to lot variation of FBS along with its undefined nature makes development of a serum-free media important for the study of mammalian cell metabolism.
Table: II.2.2 Partial List of Components Commonly Found in Fetal Bovine Serum

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin</td>
<td>8.0 mg/dl</td>
<td>Endotoxin</td>
<td>0.025 ng/ml</td>
</tr>
<tr>
<td>Sodium</td>
<td>141 meq/L</td>
<td>Potassium</td>
<td>11.2 meq/ml</td>
</tr>
<tr>
<td>Chloride</td>
<td>104 meq/L</td>
<td>Creatinine</td>
<td>3.2 mg%</td>
</tr>
<tr>
<td>Albumin</td>
<td>2.5 gm%</td>
<td>Uric Acid</td>
<td>3.5 mg%</td>
</tr>
<tr>
<td>Alkaline Phosphatase</td>
<td>358 mU/ml</td>
<td>Lactate Dehydrogenase</td>
<td>372 mU/ml</td>
</tr>
<tr>
<td>Progesterone</td>
<td>&lt;20 ng/dl</td>
<td>Testosterone</td>
<td>9 ng/dl</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&lt;1 mg/dl</td>
<td>Corticosterone</td>
<td>48 ng/dl</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>179 ng/dl</td>
<td>Thyroxine</td>
<td>13 μg/dl</td>
</tr>
<tr>
<td>Luteinizing hormone</td>
<td>1.3 ng/ml</td>
<td>Parathyroid hormone</td>
<td>1669 pg/ml</td>
</tr>
<tr>
<td>Insulin</td>
<td>6.2 μU/ml</td>
<td>Calcitonin</td>
<td>&lt;25 pg/ml</td>
</tr>
</tbody>
</table>

A considerable amount of effort has gone into the development of serum-free media that would be capable of sustaining the growth of a wide variety of different mammalian cell lines.[18,23-27,59,66] The serum-free media developed usually are based on RPMI-1640 or a mixture of DMEM and Ham's F-12 nutrient media. Though any common tissue culture media could conceivably serve as a starting point.

The use of ultra pure water in tissue culture applications is the primary concern that underlies the addition of a trace mineral supplement with many serum-free media formulations. Table II.2.3 lists some of the trace metals that Kovar[23] included in his serum-free media formulation, and represents the final concentrations of trace minerals utilized in the sfRPMI media developed in this study.

Table: II.2.3 Components of Trace Mineral Solution used in sfRPMI

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Form</th>
<th>Final Concentration [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cadmium</td>
<td>CdSO₄·8H₂O</td>
<td>50</td>
</tr>
<tr>
<td>Cobalt</td>
<td>CoCl₂·6H₂O</td>
<td>10</td>
</tr>
<tr>
<td>Copper</td>
<td>CuSO₄·5H₂O</td>
<td>10</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>(NH₄)₂Mo₇O₂₄·4H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>Manganese</td>
<td>MnCl₂·4H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>Nickle</td>
<td>NiSO₄·6H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>Selenium</td>
<td>Na₂Se·6H₂O</td>
<td>40</td>
</tr>
<tr>
<td>Tin</td>
<td>SnCl₂·2H₂O</td>
<td>0.25</td>
</tr>
<tr>
<td>Vanadium</td>
<td>NH₄VO₃</td>
<td>2.5</td>
</tr>
<tr>
<td>Zinc</td>
<td>ZnSO₄·7H₂O</td>
<td>1000</td>
</tr>
</tbody>
</table>
Trace metal often serve as cofactors, or are located in the active site of some enzymes. Listed below are some possible sites of action for some of the mineral used in sfRPMI.

<table>
<thead>
<tr>
<th>Mineral</th>
<th>Possible Site of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co</td>
<td>Component of vitamin B₁₂</td>
</tr>
<tr>
<td>Cu</td>
<td>Prothetic group of cytochrome oxidase</td>
</tr>
<tr>
<td>Mn</td>
<td>Enzyme cofactor, arginase</td>
</tr>
<tr>
<td>Fe</td>
<td>Heme enzymes; catalase, cytochrome oxidase</td>
</tr>
<tr>
<td>Zn</td>
<td>Enzyme cofactor; dehydrogenases, DNA polymerase</td>
</tr>
<tr>
<td>Ni</td>
<td>Enzyme cofactor, urease</td>
</tr>
<tr>
<td>Se</td>
<td>Enzyme cofactor; glutathione peroxidase, others</td>
</tr>
</tbody>
</table>

By far one of the most important trace minerals, other than iron, for mammalian cells is selenium. Selenium is an important trace mineral in sfRPMI because of its role in oxygen radical detoxification reactions.[16,18] The absence of serum proteins, some of which may be involved in oxygen radical detoxification, make the inclusion of selenium in sfRPMI very important. Selenium and glutathione are used by the glutathione peroxide enzyme in the breakdown of toxic hydrogen peroxide. This reaction and some other methods that mammalian cell have of dealing with oxygen radicals are shown below.

\[
O_2^- + O_2^- + 2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2 + O_2
\]

Superoxide dismutase removes the highly toxic \( O_2^- \) radical, however it generates \( H_2O_2 \) which is also toxic. The enzyme catalase can remove the \( H_2O_2 \) formed or glutathione peroxidase can be utilized.

\[
2H_2O_2 + 2GSH \xrightarrow{\text{Glutathione peroxidase}} 2H_2O + GSSG
\]

Most serum free media formulations also attempt to add other important growth factors that maybe required for successful growth of mammalian cells without serum. The two most commonly included growth factors are insulin and transferrin. Concentration ranging from 5 to 50 \( \mu g/ml \) are often employed.[23] Both insulin and transferrin are included in sfRPMI at concentrations of 5 \( \mu g/ml \). The effect that these proteins exert vary from cell line
to cell line, with some lines showing a marked stimulation, while other show none at all. Some other growth factors commonly added in serum-free media formulation include: EGF, PDGF, T₃, and IL-2.

Other ingredients often added in serum-free medias, but not included in sfRPMI include: ethanolamine; reducing agents, β-mercaptoethanol or dithiotrietol; hydrocortisone; linoleic acid; ascorbic acid. All attempt to fill a particular deficiency or requirement a cell line might have. Reducing agents are often added to protect cell and media proteins from the oxidizing environment that often exist in tissue culture applications.[18] However, the reducing agents listed here appear to do more harm than good for some of the cells lines used in this study.

In summary the serum-free media developed here (sfRPMI) consists of the following:

- RPMI-1640 media
- Trace minerals at the concentration listed in Table II.2.3
- 5 μg/ml Bovine Insulin and 5 μg/ml Iron Saturated Human Transferrin

B) Adaption of Mammalian Cell Lines to a Serum-Free Media.

Adaption of all lines studied was attempted with varying degrees of success. All lines did adapt to growth in a serum-free environment. However while some lines were able to reach a level of growth comparable, but slightly depressed from that observed in base-RPMI, other lines ability to grow in sfRPMI were erratic and unstable.

The procedure commonly used in adapting cells to a new environment is a gradual change in conditions until the desired new state is reached. In the case of adaption to a serum-free media, the serum content in the media is decreased in successive small steps, with the culture stabilizing at each step until the serum level is zero. The approach undertaken here can be described as the "cold turkey" approach. In one step, base-RPMI was replaced with sfRPMI. While the former approach presents more technical difficulties
than the latter, the final results achieved were the same. The benefit of the latter approach is a considerable savings in the length of time the adaption process takes.

With the one step approach, the best result was achieved if the cell density always remained high. Generally, culture densities should not be allowed to drop below 750,000 cells/ml. The cultures were by passed by a 1:2 dilution into a 1:1 mix of "conditioned" media: fresh sfRPMI. Over a period of 4-6 weeks the cultures reached a maximum level of growth (adaption). As a note: All cell lines used in this study were anchorage independent when grown in sfRPMI. Table II.2.4 list a qualitative representation of the ability of the six lines studied to adapt to, and to grow stably in sfRPMI. Also a representation of the maximum cell density achievable with each line is shown.

Table:II.2.4 Growth Characteristics of Six Mammalian Cell in sfRPMI.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Potential for stable growth in sfRPMI</th>
<th>Maximum attainable cell density (cell/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sp2/0-Ag14</td>
<td>+++</td>
<td>750,000</td>
</tr>
<tr>
<td>FO</td>
<td>+++</td>
<td>750,000</td>
</tr>
<tr>
<td>aIR-1</td>
<td>+</td>
<td>500,000</td>
</tr>
<tr>
<td>TIB 213</td>
<td>++</td>
<td>500,000</td>
</tr>
<tr>
<td>116NS-3d</td>
<td>+++++</td>
<td>1,000,000</td>
</tr>
<tr>
<td>Jurkat, clone E6</td>
<td>+++++</td>
<td>2,500,000</td>
</tr>
</tbody>
</table>

Figure II.2.1 shows the adaptation process for 116NS-3d. Here the cells were passed in early-late exponential phase to give an initial concentration of ~125,000 cells/ml. Over a period of 3-4 weeks, after a long lag phase, 116NS-3d reached its maximum ability grow in sfRPMI. The maximum growth rate in sfRPMI was 75% of that obtainable in base-RPMI and the maximum obtainable cell density was reduced by 40%.
Jurkat, clone E6 was chosen from the six lines screened for all future experiments based on its growth and the ability to attain high cell densities (2-2.5 million cells/ml) in sfRPMI. In addition, Jurkat is a widely used cell line for the study of T-cell function, in particular, the events associated with T-cell proliferation[1,19,61-64]. Jurkat has also been used as a fusion partner for the production of stable human hybridomas.[46]
3) Detection and Elimination(?) of Mycoplasma and Other Forms of Microbial Contamination

In the study of mammalian cell metabolism the maintenance of a 'pure', contamination free culture is an absolute must. Any form of contamination can greatly affect all of the results observed by either competing for important nutrients, or by the production of uncharacteristic wastes.

The media used in this study includes the antibiotics penicillin and streptomycin. Their addition greatly reduces the risk of contamination by many species of gram negative and gram positive bacteria. However these antibiotics are ineffective against some species of bacteria, yeast, fungus, and mycoplasma. Before the possibilities of contamination elimination are discussed, the methods for the detection of contamination will be reviewed.

By far the most commonly employed method to detect a contaminated culture is by direct visual and microscopic viewing. Breakthrough bacterial contamination will result in a explosive increase in the number of bacteria present in a culture, in a very short period of time. Often this explosive growth is fueled by aerobic and anaerobic glycolysis. The end result is a rapid drop in the media pH(visually a yellowing of standard tissue culture media).

Often contamination caused by yeast and fungus, which grow considerably slower than most bacteria, will not produce a significant drop in the media pH. However, yeast characteristically reproduce by budding and fungus often grow as a mycellium formation, both of which are visible upon direct microscopic viewing.

Contamination caused by moderately susceptible bacteria or mycoplasma would not be detectable by the means discussed above, and other methods of detection must be employed if these forms of contamination are to be detected.

Moderately susceptible bacteria can be detected by a simple plating of the suspect culture onto a blood agar plate(bap). Most bacterial species will produce visible colonies within 24
to 48 hours. The procedure employed in this work for periodically screening Jurkat cultures for the presence of moderately susceptible bacterial was as follows:

1) A sample of suspect culture media is centrifuged at x500g for 15 minutes
2) The supernatent containing the antimicrobial agents is discarded
3) The pellet is resuspended in a small amount of PBS or antibiotic free RPMI
4) The resuspended sample is struck out on a blood agar plate
5) The bap is incubated for 24 -48 hours at 37°C, 5% CO₂
(Colony formation is indicative of a contamination problem)

Blood agar plates used in this method can be purchased ready made, or can be prepared as follows:

1) 40 g/L of trypticase soy agar(TSA) is heated to a boil for 1 minute to completely dissolve the powdered TSA
2) The TSA solution is autoclaved at 121°C for 15 minutes
3) Upon cooling to 45 - 50°C, 5% sterile defibrinated sheep's blood is added to the TSA
4) The blood agar is then quickly poured into petri dishes before it sets

Screening cultures with blood agar plates, while acceptable for detecting moderately susceptible bacteria, some yeast, and some fungi, is not useful for the detection of mycoplasma. However, there are a number of other means available for the detection of mycoplasma, including direct culture on special mycoplasma enrichment media, direct fluorescent antibody(DFA) microscopic viewing, or ribosomal RNA hybridization with tritium-labelled DNA.

The method of choice for this work was the GEN-PROBE rapid detection system for mycoplasma T.C. II, which is a DNA probe based system. The procedure, description of method, and limitations of the procedure can all be found in the package insert of the GEN-PROBE kit.
The cultures to be screened for mycoplasma contamination with the GEN-PROBE kit were passed three times in an antibiotic free media before being tested. The GEN-PROBE procedure, when followed as outlined in the package insert, takes about 3 hours to complete.

Now that the means for detecting contamination are well in hand, the next question is, what can be done to combat a contamination once it has been detected? The most common methods employed to eliminate existing contamination are based on increased additions of antimicrobial agents. Since penicillin and streptomycin are routinely used in the media employed for most of this work, the addition of other antibiotics may help eliminate a contamination. A recent paper by Karl Schmitt[55] outlines an antibiotic treatment (ciprofloxacin) for mycoplasma eradication.

However, since many antimicrobial agents are only bacteriostatic, the return of the contamination problem is inevitable. Even antimicrobial agent that are considered bactericidal often do not completely eradicate a contamination problem.

Since the emphasis of this work is study of mammalian cell metabolism (not bacterial metabolism) contamination on any level is unacceptable. Therefore the method employed in this work to eradicate a contamination problem is the addition of a equal amount of bleach and the prompt disposal of all suspected cultures. This is truly the only means in which a culture can be decontaminated with 100% certainty.
4) Enzymatic Assays

A) Glucose

Glucose concentrations were measured using Sigma procedure No. 16-UV which is similar to the method described by Bondar [8]. The reactions utilized by the procedure are:

\[
\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{G-6-P} + \text{ADP}
\]

\[
\text{G-6-P} + \text{NAD} \xrightarrow{\text{6-Phosphate Dehydrogenase}} \text{6-PG} + \text{NADH}
\]

The oxidation of glucose to 6-PG results in the production of one mole of NADH from NAD. The increase in absorbance at 340nm that follows when NAD is reduced to NADH is directly proportional to the initial concentration of glucose.

The reconstituted reagent consists of:

- NAD: 1.5 mmol/L
- ATP: 1.0 mmol/L
- Hexokinase: 1000 U/L
- G-6-PDH: 1000 U/L
- Mg: 2.1 mmol/L

and buffered to a pH of 7.5 ± 0.1

The assay was modified from the Sigma procedure as follows.

1) 1 ml of assay reagent was employed instead of 1.5 ml
2) The sample size was increased from 10 µl to 50 µl

The resultant 1:20 sample/reagent ratio should provide a 5.5 mM upper limit of linearity.

With each set of samples a standard curve was preformed. The results obtained from the standard curve were used to determine the initial sample glucose concentration instead of Beer-Lambert's law and the millimolar absorptivity of NADH at 340 nm.

Briefly,

1) The absorbance of the assay mixture in disposable acrylic cuvets was measured at 340 nm -vs- water as a reference, Initial [A]

2) The sample or standard is then added to the cuvet and mixed. After an incubation period of 5 - 60 minutes the Final [A] is recorded, again using water as a reference

3) The change in absorbance is calculated \(\Delta A = \text{Final}[A] - \text{Initial}[A]\), and compared with the standard curve
B) Lactate

Lactate concentration were measured using Sigma procedure No. 826-UV. The reaction employed in the procedure is:

\[
\text{Lactate} \quad + \quad \text{NAD} \xleftarrow{\text{Lactate Dehydrogenase}} \quad \text{Pyruvate} \quad + \quad \text{NADH}
\]

For the determination of lactate, excess NAD is utilized. Hydrazine is added to the reaction mixture to trap the pyruvate formed. This prevents the reverse reaction from occurring. As with the glucose assay, an increase in absorbance at 340 nm is followed as NAD is reduced to NADH.

The reaction mixture consists of:

- 32.8 V% Glycine Buffer Soln. (0.6M glycine, 0.5M hydrazine, 7.53mM NAD; pH 9.2)
- 65.6 V% Water
- 1.6 V% Lactate dehydrogenase (1000U/ml)

The assay was modified from the Sigma procedure as follows:

1) 1.4 ml of assay reagent was employed instead of 2.8 ml
2) 0.1 ml of sample was used instead of 0.2 ml

As with the glucose assay, a standard curve was prepared with each set of samples, and the concentration of lactate present was determined by comparing the results with the standard curve. Generally, the samples had to be diluted 1:4 with water to fall within the linear range of the standard curve.

Briefly,

1) 1.4 ml of the assay solution is added to each cuvet.
2) 0.1 ml of sample or standard is added to each cuvet.
3) A cuvet containing 1.4 ml of the assay solution and 0.1 ml of neutralized 8% (W/V) perchloric acid is used as a blank.
4) The cuvets are mixed well and the reaction is allowed to proceed for 45-60 minutes at room temperature.
5) The absorbance of the samples/standards at 340 nm are recorded, with the blank prepared as in #3 as the reference.
C) Oxaloacetate

Oxaloacetate concentrations were measured using a UV-method developed by Rej[7]. The reaction employed in this procedure is:

\[
\text{Oxaloacetate} + \text{NADH} \overset{\text{Malate Dehydrogenase}}{\longrightarrow} \text{L-malate} + \text{NAD} 
\]

The assay is performed at a pH of 7.8 using a Tris buffer. This pH allows for a quantitative reduction of oxaloacetate to L-malate. Tris is the buffer of choice because of the enhanced stability of NADH in this buffer. Tris also increases the rate of tautomerization of enolic oxaloacetate to the ketonic forms which appear to serve as the substrates for malate dehydrogenase.[7]

The reaction mixture consist of:

1) 33.33 V% Tris, 300mM, pH 7.8; EDTA, 20 mM; NADH, 0.81 mM
2) 66.67 V% Distilled water
3) Malate Dehydrogenase/glycerol (10 ml 1:1 water:glycerol; 0.2 ml MDH, 1200 U/mg 10 mg/ml in glycerol)

As with the other enzymatic assays, a standard curve was prepared with each set of samples, and the concentration of oxaloacetate present in each sample was determined using the results of obtained from the standard curve. Fresh standards of oxaloacetate were prepared with each set of samples due to instability of oxaloacetate in solution. See Rej[7] for further detailed on oxaloacetate's instability in solution.

Briefly, 1) 1 ml of component 1 and 2 mls of component 2 were added to each cuvet
2) 0.1 ml of sample or standard is added to each cuvet
3) A cuvet containing 3 ml of components 1 and 2 and 0.1 ml of neutralized 8% (W/V) perchloric acid is used as a reaction blank
4) An initial absorbance at 340 nm for each cuvet is recorded using 3) as a reference
5) 10 µl of the malate dehydrogenase solution is added to each cuvet
6) After a 5 minute incubation the absorbance at 340 nm is recorded
D) Pyruvate

The concentration of pyruvate was measured using Sigma procedure No. 826-UV.

The reaction employed in this procedure is:

\[
\text{Pyruvate} + \text{NADH} \xrightleftharpoons{} \text{Lactate} + \text{NAD}
\]

This assay procedure employs the reverse of the reaction used for the determination of Lactate. Excess NADH is utilized and a decrease in absorbance at 340nm is observed as NADH is oxidized to NAD.

The reaction mixture consists of:

- 35.7 V% Tris base solution (1.5 mM Tris, 0.6 mM NADH, 0.05% Sodium azide)
- 64.3 V% Distilled water
- Lactate Dehydrogenase (1000U/ml)

The assay was modified from the Sigma procedure in the following ways.

1) A dilute assay mixture was prepared, and 2.8 ml was used instead of 1 ml
2) The sample size was decreased from 2 ml to 200 μl

These modifications allowed undiluted sample to be analyzed. As with the other enzymatic assays a standard curve was prepared for each set of samples.

Briefly,

1) 2.8 ml of assay solution is added to each cuvet
2) 200 μl of sample/standard is added to each cuvet, with neutralized 8% perchloric acid used as the reaction blank
3) The initial absobance at 340 nm is recorded
4) 50 μl of lactate dehydrogenase is added to each cuvet
5) After an incubation of 2-5 minutes the final absorbance is recorded
6) The change in absorbance [ΔA] = Final[A] - Initial[A] is compared with the standard
E) **Ammonium**

Ammonia concentration were measured using Sigma procedure No. 1780-UV. The reaction utilized is:

$$2\text{-Oxoglutarate} + \text{NH}_3 + \text{NADH} \rightarrow \text{Glutamate} + \text{NAD}$$

The assay is preformed at a pH of 8 using a Tris buffer. The decrease in absorbance is directly proportional to the level of ammonia present in the sample.

The reaction mixture consists of:

- **Ammonia assay soln.** (2 mM 2-Oxoglutarate, 0.12 mM NADH, 120 mM Tris, pH 8)
- **Glutamate dehydrogenase** (1200U/ml)

The assay as described in the Sigma procedure was modified by decreasing the amount of assay solution used from 3 ml to 1 ml. As with the other assay a standard curve was prepared with each set of samples, and the concentration of ammonia present in the sample was determined using the standard curve. Generally sample had to be diluted 1:4 to fall within the linear range of the standard curve.

Briefly,
1) 1 ml of the assay solution is added to each cuvet
2) 200μl of sample/standard is added to each cuvet
3) 200μl of neutralized 8%(W/V) perchloric acid is used as a reaction blank
4) Initial absorbance at 340nm is recorded
5) 20μl of glutamate dehydrogenase is added to each cuvet
6) After an incubation period of 5 minutes the absorbance at 340nm is recorded
7) The change in absorbance ($\Delta A = \text{Final}[A] - \text{Initial}[A]$) is compared with the standard curve
F) Alanine Aminotransferase

The activity of alanine aminotransferase (AlaAT), EC 2.6.12, was determined using the method of Horder and Rej[7]. The reaction utilized in this method are:

\[
\text{Alanine Aminotransferase} \\
\text{L-alanine + 2-Oxoglutarate} \rightleftharpoons \text{L-glutamate + Pyruvate} \\
\text{Pyruvate + NADH} \rightleftharpoons \text{Lactate + NAD}
\]

The reaction between L-alanine and 2-Oxoglutarate is catalyzed by AlaAT. The resultant pyruvate formed is reduced by NADH in a reaction utilizing LDH. The decrease in absorbance observed at 340nm associated with the oxidation of NADH is proportional to the amount of AlaAT present in the sample.

The reagent utilized in this method are:

1) L-alanine/Tris (L-alanine, 615 mM; Tris, 110 mM; pH 7.3)
2) 2-Oxoglutarate/Tris (2-Oxoglutarate, 180 mM; Tris, 110 mM; pH 7.3)
3) Tris buffer (Tris, 110 mM)
4) Pyridoxal 5-Phosphate (Pyridoxal 5-Phosphate, 500 mM)
5) Lactate dehydrogenase (1 ml LDH (10 mg/ml in glycerol; 500 U/mg) + 0.8 ml 1:1 water:glycerol)

The working reagent mixture, made fresh for each set of sample, includes:

20 ml L-alanine/Tris
0.5 ml Pyridoxal 5-Phosphate
0.1 ml Lactate dehydrogenase
3.3 mg NADH

The assay procedure is as follows:

1) Pipet 2 ml of the working reagent mixture in each cuvet
2) Add 0.2 ml of cell sample (frozen/thawed, sonicated 2 times for 60s at 4°C)
3) Allow reaction mix to reach equilibrium ~10 mins or until a stable absorbance is observed at 340 nm
4) Add 0.2 ml of reagent (2) and record \( \Delta A/\Delta t \) at 340nm for ~ 3 min, or until \( \Delta A > 0.2 \) Note: \( \Delta A/\Delta t \) should not exceed 0.4/min
Method for calculating the concentration of AlaAT present in the sample can be found at the end of the procedure for aspartate aminotransferase determination. Also, the controls needed for this method can be found there.

G) **Aspartate Aminotransferase**

The activity of aspartate aminotransferase (AspAT), EC 2.6.1.1, was determined using the method of Horder and Rej[7]. The reaction employed in this method are:

\[
\text{L-aspartate} + 2-\text{Oxoglutarate} \xrightarrow{\text{Aspartate Aminotransferase}} \text{L-glutamate} + \text{Oxaloacetate}
\]

\[
\text{Oxaloacetate} + \text{NADH} \xrightarrow{\text{Malate Dehydrogenase}} \text{L-malate} + \text{NAD}
\]

The reaction between L-aspartate and 2-Oxoglutarate is catalyzed by AspAT. The resultant oxaloacetate formed is reduced by NADH in a reaction utilizing MDH. The decrease in absorbance observed at 340 nm associated with the oxidation of NADH is proportional to the amount of AspAT present in the sample.

The reagent utilized in this method are:

1) L-aspartate/Tris
2) 2-Oxoglutarate/Tris
3) Tris buffer
4) Pyridoxal 5-Phosphate
5) Malate/Lactate dehydrogenase

The working reagent mixture, made fresh for each set of sample, includes:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-aspartate/Tris</td>
<td>20 ml</td>
</tr>
<tr>
<td>Pyridoxal 5-Phosphate</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Malate/Lactate dehydrogenase</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>NADH</td>
<td>3.0 mg</td>
</tr>
</tbody>
</table>
The assay procedure is as follows:

1) Pipet 2 ml of the working reagent mixture in each cuvet
2) Add 0.2 ml of cell sample (frozen/thawed, sonicated 2 times 60s at 40°C)
3) Allow reaction mix to reach equilibrium ~10 mins or until a stable absorbance is observed at 340 nm
4) Add 0.2 ml of reagent (2) and record \( \Delta A/\Delta t \) at 340 nm for ~3 min, or until \( \Delta A > 0.2 \)  Note: \( \Delta A/\Delta t \) should not exceed 0.4/min

The possibility of interference by a number of other reactions that requires a number of blanks be used in this assay system. The following reactions were run to obtain a corrected \( \Delta A/\Delta t \).

A) The overall AlaAT or AspAT reaction described as above
B) Reagent blank: sample is replaced by 154 mM NaCl solution
C) Overall sample blank: Same as (A) except reagent (1) is replaced by reagent (3) during the preparation of the working reagent mixture
D) Reagent blank for the sample blank: Same as (C) except the sample is replaced by 154 mM NaCl solution

The concentration of AlaAT or AspAT present in the sample can be determined using the following expressions:

\[
(\Delta A/\Delta t)_{\text{corrected}} = (\Delta A/\Delta t)_A - (\Delta A/\Delta t)_B - (\Delta A/\Delta t)_C - (\Delta A/\Delta t)_D 
\]

Then:

[Unit/L] AlaAT or AspAT = 1905 * (\( \Delta A/\Delta t \))_{\text{corrected}} \text{ at } 30°C
5) Equipment Setup and Method Development

A) Water HPLC

a) Equipment

A major portion of this work involves the analysis of micromolar levels of amino acids. For this analysis a Waters HPLC system was setup and a method was developed to quantitatively measure amino acid in tissue culture media. The components of this HPLC system include the following:

1) Two Model 510 solvent delivery systems
2) Model 420AC fluorescence detector
3) WISP Model 710B, with a constant temperature refrigeration unit
4) Model 721 programmable controller
5) Model 745 data module
6) Resolve 5µ C18 Column

The model 510 high performance pumps have an adjustable high pressure limit of 0-6000 psig and are capable of delivering flow rates between 0.1 and 9.9 ml/min in increments of 0.1 ml/min. The flow rates and the high pressure limits can be set manually or can be controlled via a connection to the 721 programmable controller.

The method developed for amino acids separations involves the derivatization of primary amino acids with orthophthaldehyde (OPA).[60] The isoindole product formed is highly fluorescent and is monitored using a Waters model 420AC fluorescence detector. The model 420AC fluorescence detector is divided into two self-contained units. One houses the electronics, the other houses the the flow cell, photomultiplier tube, and the optical filters. The electronic unit provides both 10 mV and 1 V recorder outputs, and provides a means to manually adjust the Gain and Span. The flow unit has a four watt fluorescent lamp with a 325 - 410 nm spectral range. The flow cell itself is of Suprasil
quartz construction with a liquid holdup volume of 8 μl. The filters employed in this method include a 338 nm bandpass excitation filter and a 425 nm longpass emission filter.

The Waters intelligent sample processor (WISP) Model 710B is a versatile stand alone unit that may also be controlled via Interlink by the Model 721 programmable controller. The WISP is an automatic sample injector, capable of handling up to 48 samples can accurately deliver sample volumes ranging from 1 - 2000 μl while maintaining a steady flowing mobile phase. The WISP, operating as a stand alone unit, can be programmed for single and multiple injections, which allows for unattended operation. The unit's software provides excellent diagnostic features which facilitates the identification of problems that may effect sample analysis. A refrigeration unit coupled to the WISP provides a constant temperature environment, controllable from -40°C to 40°C.

While the WISP software will allow for unattended single solvent operation the model 721 controller must be used if the analysis requires gradient analysis. The 721 controller can act as a stand alone pump controller or when configured with the model 710B WISP can provide for unattended operation. When the 721 is connected to the WISP via INERLINK communication, it is capable of performing single, binary, ternary or gradient analysis. The 721 can control the flow rate from 10 μl to 67.5 ml/min. The system software also provides the ability to set high and low pressure limits and is capable of controlling up to eight external accessories.

The 721 software provides a means of writing and saving system operating conditions like: pump flow rates; high and low pressure limits; solvent compressibility compensations for pumps A, B and C; runtime; number of injection; injection volumes; gradient conditions. Some important commands include the TAG command and the gradient:CURVE command. The TAG command provides a means for the precolumn derivatization of a sample, which is central to the analysis used in this work. The CURVE command within the gradient table allows one to use preset gradient curves to change flow/percent composition in a variety of ways. See Figure II.5.1
While the WISP and the 721 systems controller can be said to be the heart of the sample processing system, it is the model 745 data module that provides all important numbers. The data module receives an analog signal from the electronic unit of the detector and an initiation signal from the WISP to signal the start of a run. The signal received from the detector is processed by the data module and is reported as a chart strip recording and can be processed to give a series of peak areas, if the integration inhibit command is not selected in the dialog mode.

Curves 2 - 5 = Convex  
Curve 6 = Linear  
Curves 7 - 10 = Concave  
Curve 1 = Go to end condition immediately  
Curve 11 = Maintain start conditions

Figure: II.5.1 Gradient Curves Available Using Waters Model 721 System Controller Software.

The dialog mode allows the user to personalize the data module's recording parameters, in a time-based fashion. The dialog command and the ability to activate and inactivate functions in a time-based manner is very important. While the data module is automatically started when an initiation signal is received from the WISP when a sample is injected, it must be told when a run has ended in the dialog command.

The data module software also contains a variety of automatic correction methods that allows one to obtain excellent information from the not so perfect chromatograms that one is often faced to analyze. Corrections include base line compensation and ways of dealing with fused and tailing peaks, front and back shoulders, and a number of other problems that often occur.
Lastly, the most important piece of equipment needed for this separation is the column. The reaction of primary amino acids with OPA yields derivatives with excellent hydrophobic characteristics, making separation via reverse phase chromatography ideal. The column utilized for this separation is a Waters Resolve 5μ C18 analytical column. The Resolve C18 packing is a spherical silica based packing with a nominal pore size of 90Å and a typical efficiency of 100,000 plates/meter. This packing has the highest carbon load of all Waters C18 packings and is stable in acidic-neutral pH's.

b) Method Modification

The Waters method for the analysis of amino acids using automated precolumn derivatization with OPA is performed using the conditioned outline lined below.

<table>
<thead>
<tr>
<th>Solvents and Reagents:</th>
<th>Solvent A</th>
<th>MeOH:THF:Water,2:2:96 containing 0.05M Na2HPO4 + 0.05 M NaOAc, pH to 7.5 with acetic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent B</td>
<td>MeOH:Water, 65:35</td>
<td></td>
</tr>
<tr>
<td>Derivatizing Soln.</td>
<td>5 mg OPA/ml in 0.5 M potassium borate buffer, pH 10 + β-mercaptoethanol</td>
<td></td>
</tr>
</tbody>
</table>

The Gradient table employed:

<table>
<thead>
<tr>
<th>Time</th>
<th>Flow</th>
<th>%A</th>
<th>%B</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>2.0</td>
<td>0.1</td>
<td>100</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>2.5</td>
<td>1.5</td>
<td>100</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>14.0</td>
<td>1.5</td>
<td>50</td>
<td>50</td>
<td>7</td>
</tr>
<tr>
<td>23.0</td>
<td>1.5</td>
<td>0</td>
<td>100</td>
<td>7</td>
</tr>
<tr>
<td>28.0</td>
<td>1.5</td>
<td>100</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>35.0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>

5 μl of derivatizing solution is automatically mixed with 4 μl of sample and the above gradient table conditions are used for the Waters separation. The chemistry utilized in the derivatization reaction to provide a fluorescent hydrophobic product is as follows:
The Waters method as outlined above was modified in a number of ways, each with the intention of improving the separation. The solvent system and the gradient table were changed with this goal in mind.

The pH of solvent A was lowered to 6.5 from 7.5. The lowering of the pH has a profound effect on the separation of the early amino acids. However, the lowering of the pH was not done to improve the separation per say. The pH was lowered to prolong column life. It is rather interesting that Waters method operates in a pH range that facilitates the solvation of the silica matrix that makes up the Resolve 5μ C18 packing. There are other methods that can be used to protect the column from the effects of an unfavorable pH, but lowering the pH to 6.5 is far easier to implement.

The gradient table was also modified from the Waters method in a number of ways. The first change was to increase the time of the 0.1 ml/min flow rate from 2 to 3 minutes. The increase in time at this low flow rate gives the derivatization reaction a little additional time to go to completion. The longer reaction time provide greater sensitivity for the method since it allows more fluorescent product to be formed.

The gradient table was also modified to improve the overall resolution of amino acids of interest for this work. This includes the resolution of histidine and glutamine in tissue culture media. While Waters method is capable of resolving equal molar amounts of glutamine and histidine, it is incapable of resolving them when their levels are in a ratio that is typically observed in mammalian cell culture.
c) Final Operating Conditions

The final operating condition used for the amino acid separation are listed below. Since OPA derivatizing solution is very unstable, a calibration curve for all amino acids tested was prepared routinely with each set of sample analyzed.

Solvents and Reagents:
Solvent A: MeOH:THF:Water,2:2:96 containing 0.05 M Na$_2$HPO$_4$
0.05 M Na OAc, pH to 6.5 with acetic acid

Solvent B: MeOH:Water, 65:35

Both solvents are degassed under vacuum.

Derivatizing Soln: 5 mg OPA/ml in (1:5 saturated potassium borate buffer, pH 10:MeOH) + 10 µl/ml β-mercaptoethanol

All reagents were fresh with each set of samples

Data module settings:
PW = 6
PT = 1500
ATTN = 256

Detector settings:
Gain = 2
Span = Min
Output = 1 volt

The Gradient table employed:

<table>
<thead>
<tr>
<th>Time</th>
<th>Flow</th>
<th>%A</th>
<th>%B</th>
<th>Curve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>*</td>
</tr>
<tr>
<td>3.0</td>
<td>0.1</td>
<td>100</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3.5</td>
<td>1.5</td>
<td>100</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>29.0</td>
<td>1.5</td>
<td>0</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>31.0</td>
<td>1.5</td>
<td>100</td>
<td>0</td>
<td>11</td>
</tr>
<tr>
<td>38.0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>11</td>
</tr>
</tbody>
</table>

Injection volume 5 µl, Derivatizing (Tag) volume 5 µl

Figure II.5.2 is a typical trace obtainable using the conditions outlined in this section. It
is left to the reader's imagination what the intermediate traces looked like during the modification process.

Figure II.5.2  HPLC Separation of OPA Pre-column Column Derivatized RPMI
Tissue Culture Media
Eluent A: 0.05 M sodium acetate, 0.05 M sodium phosphate, pH 6.5: Methanol: THF; 96:2:2
Eluent B: Methanol: Water; 65:35
Detection: Fluorescence, Ex. 334 nm, Em. 425 nm
B) **Applikon Fermenter System:**

a) **Equipment**

A major portion of this work involved the setup of an Applikon fermenter system for the cultivation of mammalian cell lines. The system was configured so it would possible to perform both batch and continuous fermentations. The cell line used in all system development test runs was Jurkat, clone E6. The major components of the Applikon system include:

1) ADI 1020 Bio-processor
2) Apple Macintosh II personal computer
3) Two 3 liter unjacketed round bottom culture vessels

Minor pieces of equipment also essential for the functioning of the Applikon fermenter system include the following:

1) Gas flow meters, and associated single solenoid valves (normally closed)
2) Variable speed and fixed speed peristaltic pumps, and associated controllers
3) Steam sterilizable polarographic oxygen probes
4) Steam sterilizable pH probe, or combination pH/ORP probe
5) Magnetic stirrer assembly, and drive/controller

The ADI 1020 Bio-processor consists of several components and is depicted in Figure II.5.3. The ADI 1020 Bio-processor builds around an outside cabinet that has been fitted with three extension racks. The cabinet and extension racks as assembled is capable of holding up to seven parameter modules (with I/O processors and power cards). The parameter modules are capable of one way communication via a standard RS 232 connector. This connection allows the transmission of data from the ADI 1020 to a personal computer, or a printer. Four parameter modules are currently in place, each with capability of controlling two separate parameters when two sensor cards are in place. Within each parameter module a maximum of four actuator cards can be installed for providing signals to actuators, or recorders.
The cabinet also houses two service modules that connect the pumps, heating blanket, and solenoid valves to the ADI 1020 Bio-processor.

The cabinet also contains a supervisor module, that hold a P.L.C. (programmable logic controller) card which can be programmed by one of two different means. One being by direct keyboard assay commands, the other being by direct interfacing with a personal computer. (Documentation on supervisor functions in Applikon's 1987 operation manual are nonexistent, and although documentation in the June 1988 operation manual has
improved, it still is rather sketchy.) The supervisor module, in theory, allows for bidirectional communication that is required for "high levels" of control, or dynamic modeling.

The personal computer used with the Applikon fermenter system was a Apple Macintosh II. No attempt was made to access the capabilities of the supervisor module in this work. The MAC II was connected to a parameter module and collected data on temperature, DO, and pH within the reactor. For the one way communication between the ADI 1020 and the MAC II it was necessary to write a brief basic program. The program is listed below.

```
CLOSE
N$="":N$=FILE$S(0,"FILENAME:" )
OPEN N$ FOR OUTPUT AS #2
OPEN "CLIP:" FOR OUTPUT AS #3
OPEN"com1:1200,n,7,1" AS#1 LEN=2000
INTERVAL= 600
MULT=0
STARTI>TIMER
G1$= DATES$ 
10 START = TIMER
C$="":D$="":E$="":Z$="":Y$="":X1$="":Y1$="":X1$=""
20 PAST = TIMER - START
IF PAST < -1 THEN STARTI=0: START=0: MULT=MULT+1 :GOTO 20
IF INTERVAL > PAST THEN GOTO 20
PAST1%=(MULT*86400!+(TIMER-START1))/60
C$=CHR$(2)+"M3,11": D$= CHR$(2)+"M3,12": E$=CHR$(2)+"M4,11"
Z$=CHR$(2)+"M1,11": Y$=CHR$(2)+"M1,12": X$=CHR$(2)+"M2,11"
FI$= TIMES$
PRINT #1,C$: INPUT #1,A$,B$,C1$ 
PRINT#1,D$: INPUT#1, A$,B$,D1$
PRINT #1,E$: INPUT #1, A$,B$,E1$
PRINT #1,Z$: INPUT #1, A$,B$,Z1$
PRINT#1,Y$INPUT#1, A$,B$,Y1$
PRINT #1,X$INPUT #1, A$,B$,X1$
PRINT #2,C1$:"":D1$:"":E1$:"":Z1$:"":Y1$:"":X1$:"":F1$:"":G1$
PRINT #3,C1$:"":D1$:"":E1$:"":Z1$:"":Y1$:"":X1$:"":F1$:"":G1$
PRINT PAST1%;"","TEMP IS",D1$,"TEMP2 IS",Y1$
PRINT PAST1%;"","PH IS",C1$,"PH2 IS",Z1$
PRINT PAST1%;"","DO IS",E1$,"DO2 IS",X1$
GOTO 10
CLOSE
END
```
This program records values for pH, temperature, and DO in a basic file and in a Clipboard file, as well as displaying the values on the screen. Data stored in the Clipboard file can be directly accessed by any number of spread sheet application programs that are commercially available for the MAC II. (ie Cricket Graph) The data once transferred from the Clipboard into an application can be graphically displayed.

The final major piece of equipment making up the Applikon fermenter system is the reactor vessel itself. The units employed in this work were 3-liter unjacketed, round bottom glass vessels with a working volume of 600 to 2500 mls. The nonwelded headplate provides, three 6-mm ports, six 10-mm ports, six 18-mm ports, and one threaded center port for a magnetic stirrer assembly.

Minor pieces of equipment rounding off the Applikon fermenter system include three Cole Parmer 65 mm air flow meters, each with a maximum flow rate of 50 ml/min. Single solenoid valves(normally closed), also used with gas addition system, were supplied directly by Applikon.

Cole Parmer fixed speed peristaltic( 1 rpm motor) pumps were used for base addition. These pumps provided flow rates of 0.06, 0.21, and 0.8 ml/min when fitted with size 13, 14, or 16 masterflex pump heads.

Cole Parmer variable speed peristaltic pumps were used for media addition and media withdraw when the bioreactor was operated in a continuous fashion. For use in this work 1 - 100 rpm motor/controllers were used. With a size 13 master flex pump head flow rates from 0.06 to 6 ml/min were obtainable.

The stirrer assembly and controller were also supplied directly by Applikon. For use in this work, and all mammalian cell culture applications the use of a magnetically coupled stirrer assembly is preferred over a lipseal stirrer assembly because of its enhanced ability for contamination free operation. The stirrer motor/controller operated as a stand alone unit in this work, though the possibility for remote control operation does exist. The DC
stirrer/motor is capable of speeds ranging from 0 to 1250 rpm. The speed used throughout this work was 80 rpm.

b) Final System Configuration and Operating Parameters

1) pH Control

pH was controlled by the addition of CO₂ to the headspace via a solenoid valve placed after a Cole Parmer flowmeter (Acid Control), or by the addition of 320 mM sterile NaOH (Base Control).

For base addition, a size 13 pump head was used with C-Flex tubing. C-Flex is a biocompatible tubing with an improved resistance to NaOH. An attempt to use Norprene–food grade, a tubing also with supposedly resistant to NaOH, resulted in color leaching and therefore its use was discontinued.

Due to the different response times of CO₂ and base addition, an attempt should be made to operate under a "single control" mode, if at all possible. If simultaneous acid and base control is needed, the rate of base addition(size 13 tubing) and the rate of CO₂ gassing should be to as low as possible. For our applications the maximum CO₂ flowrate was between 10 and 15 ml/min. If the rates of base and acid addition are maintained as low as possible, the 'bouncing' observed because of the differences in response times associated with each kind of addition can be minimized.

pH probe calibration is performed at room temperature prior to vessel being sterilized. A two point calibration is performed. With the F2X6 = 2 command set, the parameter module will use the temperature input from the same module to perform an automatic temperature compensation of the pH reading.

The values of the user installable PID constants for pH control were determined by the instructions given in the Applikon users manual. The values used for all experiments were
F111 = 100, F112 = 0, and F113 = 0. These values, and the considerations for CO₂ and NaOH addition previously mentioned, provided excellent pH control.

2) Dissolved Oxygen Control

Dissolved Oxygen (DO) was controlled by head space addition of pure oxygen and nitrogen to the fermenter. Gas addition to the reactors was via the actuation of solenoid valves placed after Cole Parmer gas flow meters.

One flow meter was used for each gas. After passing through the flowmeter, the gasses path was split and flowed into two solenoid valves (one for each reactor) placed in line to regulate the addition of each gas to the reactor. After passing through the solenoid valves the gasses were humidified at 37°C before addition to the reactor. All tubing used before the point of containment (a glass wool filter) was Tygon. After the filter, Norprene food grade tubing was used to carry the gasses into the reactor.

The best DO control was obtained when the flow rates of oxygen and nitrogen were adjusted to maintain a rate of flow that approximately equaled the composition of air.

The DO probes were calibrated after the vessels were autoclaved, and the medium was stabilized at 37°C at pH 6.95 via the control units. A one point calibration was performed using air. The PID controller was set at: F111 = 55, F112 = 2000, and F113 = 500.

DO, as controlled with the above conditions, oscillated by about 15% of air saturation, which was deemed acceptable for the experiments undertaken in this work.

3) Temperature Control

Temperature was controlled by an electric heating blanket that fits snugly around the glass reactor vessel. No method for cooling the reactor contents was available with the current reactor configuration.
Temperature probes were calibrated at the reactor conditions using a reference thermometer. A single point calibration was performed. The PID controller was set at: F121=27.7, F122=130, and F113=32.5.

The system as described above provided excellent temperature control, with temperatures varying by less than 0.1 °C.

4) Continuous Culture Operation

For continuous culture operation, a medium addition and withdrawal system was installed. Medium addition and withdrawal was accomplished using a variable speed Cole Parmer pump. Media was added to the reactor using a size 13 pump head, while medium was withdrawn from the top of the liquid surface using a size 14 pump operating in the opposite direction, on the same drive, as the addition pump.

Norprene food grade tubing was used for both media addition and removal. This tubing has a longer service life, and is less permeable to oxygen, than both silicone and C-flex tubing. Feed medium was stored in the dark and cold(40°C) in 2 liter vessels. Maintaining feed media under these conditions prior to reactor addition minimizes the breakdown of important media components.

Medium addition into the reactor was measured by an inline flow measuring device, consisting of a 1 ml graduated glass pipette. With this system, the flowrate of the medium into the reactor could be periodically checked to verify that a constant flow was maintained.
6) MTT Assay Development for the Measurement of Cellular Proliferation

The most common method for measuring the effects of a substance has on mammalian cell growth is a physical or visual counting of cells after a period(s) of exposure to the substance being tested. This 'growth assay' method of measuring biological response to a given compound is undesirable. The growth assay is time consuming, labor intensive, and prone to a high degree of scattering. Clearly, if the effect exerted by a large number of substances is of interest, an alternate method for measuring cellular response is warranted.

The use of tetrazolium salts for measuring the response of bacterial species to various substances has been widely used.[58,70] Recently, the use of tetrazolium salts to measure cellular proliferation of mammalian cells has been suggested as an alternative to 'growth assays', and to [³H]thymidine incorporation assays.[42]

The reaction described by Mosmann involving the tetrazolium salt 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide) MTT has been modified slightly for use in this study. Mosmann found that MTT has several desirable features. The formazan product formed is directly proportional to the number of cells active or viable and enables the assay to be used as a measure of cellular activation in the absence of cellular proliferation.

The assay described by Mosmann involves the following reaction:

MTT light yellow color

Tetrazolium salt

MTT formazan purple color

Formazan
The disappearance of MTT or the appearance of the MTT formazan product can be followed using a multiwell spectrophotometer (Elisa reader). The use of an Elisa reader greatly enhances the user's ability to perform a large number of tests in a short period of time.

The reagents employed in the method are:

1) MTT solution
   (MTT 5 mg/ml in PBS, 7.4; 0.2 μm sterile filtered)

2) Developing solution
   (Acid-isopropanol; 0.04 N HCl in isopropanol)

The method as described by Mosmann is as follows:

1) Using 96 well plates, test wells are seeded with an appropriate concentration of the cell line of interest.

2) The test compound(s) are incubated at 37°C with the cell line to be tested for an appropriate length of time, with a total liquid volume per well of 100μl

3) After the desired incubation time has elapsed, 10μl of MTT solution is added to each well, and the reaction of MTT is allowed to proceed for four hours.

4) After the four hour incubation period, 100μl of developing solution is added to each well and is thoroughly mixed.

5) The absorbance of the test wells are read as soon as possible using an Elisa plate reader.

A) Modification of Existing Method

a) Selection of New Wavelength(s) to Analyze the MTT Reaction

Mosmann utilized a reading wavelength of 570 nm and a blanking wavelength of 630 nm for analyzing his MTT reaction. The reading at 630 nm (cell blank) is subtracted from the reading at 570 nm (MTT formazan peak) to give a corrected absorbance. The Elisa plate reader available for use in this work contains filters necessary for reading at the following wavelengths; 405, 455, 490, and 540 nm.
An absorbance versus wavelength plot for MTT, reaction products, and reaction controls was performed to determine if the Elisa reader as equipped would be adequate for the needs of this assay. Figure II.6.1 depicts the results of this test. A Gilford spectrophotometer was used to scan wavelengths from 475 to 675 nm. From this figure one can see that reacted MTT / Cells / Acid alcohol have an absorbance maximum extending from 470 nm to 615 nm. Cell / Acid alcohol minus MTT have a relatively constant absorbance spectrum from 470 - 675 nm. From this figure the use of wavelengths 490 or 540 nm would provide acceptable values for the complete MTT reaction. A cell blank if it was to be subtracted as in the Mosmann method should be read at a wavelength from 655- 675 nm, not the wavelength Mosmann used (630 nm).

For the method described in this work, the reading absorbance used was 540 nm. No blanking absorbance was used, but a reaction blank (without MTT) at 540 nm was used.

Figure II.6.1 Spectral Characteristics of MTT
b) Initial Cell Density Selection and Its Importance for the MTT Assay

One of the most critical aspects of this assay outside of the selection of the wavelength is the initial cell concentration in each well and the length of exposure to the substance(s) being tested. Figure II.6.2 Shows the effect of initial cell concentration on the absorbance as a function of incubation time. Cells were diluted in sfRPMI and no test compounds were added. From this figure, one can see that increasing cell density improves the sensitivity of the assay (larger absorbance window), but shortens the length of time the cells can be exposed to a particular test compound.

For further use in this study, an initial cell density of 500,000 cells/ml was used. This initial cell concentration provided a good window for short exposure times. This cell density also allows the cells to have an exposure time as long as 60 hours before a natural decrease in absorbance would occur.

![Graph showing the effect of initial cell concentration on absorbance as a function of incubation time.][1]

**Figure II.6.2 MTT Assay - Selection of Optimum Initial Cell Concentration and Incubation Time**
Development of a Plate Assay for Measuring the Utilization and Production of Select Metabolites.

A) Modification of Existing Procedures Used in Radiolabeling Studies

A simple "plate assay" was developed in this work to measure the utilization of important nutrients and the subsequent products produced. This assay requires very small sample volumes (0.5ml) and cell densities ranging from $2 \times 10^6$ to $7 \times 10^6$ cells/ml. The assay can be performed as a kinetic assay for times ranging from 30 minutes to 8 hours, or can be run as a fixed time assay at select times within a 8 hour period. The assay as described in the pages that follow allows one to isolate the utilization of a single nutrient, or can be used to look at the interplay that exists between a number of nutrients and their products.

The plate assay was developed follows from the procedures outlined by Lanks[28-31], Newsholme[43], and Brand[9,10,11,12] in their studies on mammalian cell metabolism. The procedure developed here is a modification of their methods with the primary difference being that radiolabeled substrates were not used. However, the use of radioisotopes is required to derive any definitive conclusions from the results observed here.

The methods described by Lanks, Newsholme, and Brand assay whole cell metabolism. A sample of cells, usually at a very high cell density (~$10^8$ or 130 mgs wet wt./ml), is incubated with a radiolabeled substrate for a fixed length of time (usually less than 2 hours). After the allotted incubation period, the cells are lysed and the protein precipitated. The processed sample generated are analyzed for nutrient utilization and product formation. Newsholme[4,43] and Kovacevic[22] also have described procedures to look at the kinetics of nutrient utilization, though their methods usually involve an incubation time of less than 2 hours.

As mentioned, the incubation times used in previous works are often less than 2 hours. Over this short time period, $^{14}$CO$_2$ is evolved at a constant rate. For longer incubation
times, the rate is nonliner. For the purpose of this work, lower cell densities were utilized and linear rates of nutrient utilization and intermediate product formation were observed for incubation times up to 8 hours. (See Figure II.7.1 and Figure II.7.2.) CO2 evolution was not evaluated

The procedure for the plate assay used in this work is as follows:

-1  A sample of cells to be used for the plate assay is centrifuged (x200g, 4°C) for 10 mins.
-2  The supernatent is discarded, the cells are resuspended in a volume of assay-buffer (Kerb-Ringers[20,21] buffer; 25 mM sodium bicarbonate; pH 7.4).
-3  The resuspended cells are spun for 10 minutes (x200g, 4°C).
-4  The wash procedure (steps 2 & 3) is repeated.
-5  The final cell concentrate is resuspended in assay-buffer at 2X the final concentration to be employed in the plate assay.
-6  The cells(2X) are added to preequilibrated (37°C, 5% CO2, 100% RH) test-solution (assay solution to which the test substances have been added).
-7  The plate(s) are then incubated for the specified amount of time at 37°C

After the allotted amount of time has passed, the samples(plates) are processed in the following way:

-1  The reaction is stopped by the addition of an equal amount of cold 8% w/v perchloric acid
-2  The acid-treated cells, after standing for 10 minutes(cold), are spun for 5 minutes in a microfuge (x16,000g)
-3  The resultant supernatant is neutralized with the addition of cold 1M potassium carbonate
-4  After standing (10 mins, cold) the potassium chlorate precipitation is removed by an additional spin (5 mins., x16,000g)
The supernatants recovered after the final spin are stored at -20°C until they could be analyzed for nutrient utilization/product formation.

B) Cell Density Selection and the Benefits of a Longer Incubation Time

By far one the most crucial elements for the performance of the plate assay is the final cell density utilized in the incubations. Previous authors have used very high cell densities (~10^8 cells/ml) and short incubation times. The use of high cell densities has the advantages of providing a strong signal, reproducibility, and shortening the incubation time needed for signal detection. However the use of high cell densities has the disadvantages of increased background noise and changing assay conditions. The media composition can change drastically if very high cell densities are used. Nutrient concentrations can be significantly reduced within the time of the assay and waste products can build up to high levels. Another disadvantage of high cell densities is the availability of cells for the assay. If cells are limiting using high cell densities may limit the number and the kinds of experiments that can be preformed.

Based on these criteria, preliminary studies were initiated to determine the optimal cell density. Also, it was deemed important to keep the cells in an environment that is as close as possible to the environment that they were removed from. In others words if a chemostat was to provide the cells for the assay, an attempt was made to use an assay cell density in a range that would be typically found in the chemostat (5x10^5 to 2.5x10^6 cells/ml). Also, in removing cells from a chemostat, one would like to cause as little a disturbance as possible.

Initially three possible cell densities were examined high(7x10^6), intermediate(3.5x10^6), and low(7x10^5). These three densities were chosen because at the high end they approach the densities used by others and should provide a detectable signal, while the low end would prove be useful when coupled with chemostats if a signal could be detected. From Figure II.7.1 which represent glutamate formation from an incubation buffer containing 4
mM glutamine, one can see that incubation times to 8 hours provide a linear signal. Times ranging from 30 minutes to 8 hours were used in this preliminary experiment to determine if the use of a non-radioisotope method was feasible.

![Graph](https://via.placeholder.com/150)

**Figure II.7.1** Production of Glutamic Acid from an Incubation Buffer Containing 4 mM Glutamine. Assay procedure as described in text. High cell density \( \sim 7 \times 10^6 \), Intermediate cell density \( \sim 3.5 \times 10^6 \), Low cell density \( \sim 7 \times 10^5 \)

Both the high cell density and intermediate cell densities provide strong measurable signals. However the signal from the low cell density assay was very weak.

As an additional test, the production of lactate from an incubation buffer consisting of 10 mM glucose was also followed for a period of 8 hours. The result of this test for both high
cell density and intermediate cell density inoculation is depicted in Figure II.7.2. Here again, a high degree of linearity of the signal produced (lactate formation) is observed. Lactate levels from the low cell density inoculation were not determined.

![Graph showing lactate concentration over time for high and intermediate cell densities.](image)

**Figure II.7.2**  
Production of Lactate from an Incubation Buffer Containing 10 mM Glucose  
Assay procedure as described in text. High cell density $\sim 7 \times 10^6$.  
Intermediate cell density $\sim 3.5 \times 10^6$

From Figures II.7.1 and II.7.2, the ability to use incubation times up to 8 hours was confirmed. Also, while the signal of the low cell density inoculation was difficult to detect, the use of cell densities substantially lower than the $10^8$ cells/ml used by others was indeed possible.
The importance (benefits) of longer incubation times, up to 8 hours, can be seen from the data presented in Figures II.7.3a, II.7.3b and Figure II.7.4. Figures II.7.3a and II.7.3b depict alanine production (accumulation) from incubation buffers containing 4 mM glutamine, 10 mM glucose, or 4 mM glutamine plus 10 mM glucose for both high and intermediate cell densities.

**Figure II.7.3a** Production of Alanine from Incubation Buffers Containing 4 mM Glutamine, 10 mM Glucose, or 4 mM Glutamine plus 10 mM Glucose for High Cell Densities.
Figure III.1.2b Effect of Increasing Concentrations of Pyruvate on Jurkat, clone E6

Figure III.1.2c Effect of Increasing Concentrations of Malate on Jurkat, clone E6
From these Figures, safe levels of pyruvate and oxaloacetate were selected for addition to cultures of Jurkat grown continuously in the Applikon fermenters. Concentrations of each of these four compounds employed in the plate assay (section II.7 and III.2) should also result in no inhibition of Jurkat during the assay.

From Figure III.1.2a and Figure II.1.2b it is interesting to note that pyruvate shows a greater degree of inhibition of Jurkat than similar concentrations of lactate. Jurkat shows only a slight degree of inhibition at 50 mM lactate at both 24 and 48 hours. Pyruvate on the other hand shows greater than 50% inhibition at concentrations approaching 50 mM.
2) Plate Assay:

A) Preliminary Results:

The plate assay describe in section II.7 was used to probe the metabolic response of Jurkat cells to various environmental conditions. Recall that this assay measures both the intra- and extracellular metabolite concentrations of cells incubated in a buffer dosed with test compounds.

Before looking into select areas of the metabolism of Jurkat in more detail, some additional results obtained during the development of the plate assay need to be addressed. In the course of the development of the plate assay, observations were made that affected the direction of the experiments to follow.

First, lactate accumulation and/or glucose utilization in incubations buffers containing either 10 mM glucose or 4 mM glutamine plus 10 mM glucose are approximately the same. (Figure III.2.1) This suggests that glutamine does not affect the lactate production (from glucose), or the rate of glucose utilization.

This is in contrast to the results obtained by Adawi[2], and Zeilke[72] but in agreement with those of Brand[12]. Adawi observed that for human peripheral lymphocytes the addition of glutamine increased glucose utilization and lactate formation. Zielke observed for fibroblasts that glutamine inhibited glucose utilization and that glucose inhibited glutamine utilization. On the other hand, Brand observed no change in the rate of glucose utilization or lactate formation for rat thymocytes when glutamine was added to an incubation buffer containing glucose.

Secondly, while the presence of glutamine does not appear to have any effect on the disappearance of glucose or the accumulation of lactate, the presence of glucose appears to reduce the rate of glutamine utilization by ~40% in Jurkat. This is in agreement with the results observed by Brand[12] for rat thymocytes, and Zielke for fibroblasts.
Figure III.2.1 Glucose Utilization and Lactate Accumulation for Incubation Buffers Containing either 10 mM Glucose or 10mM Glucose plus 4 mM Glutamine. (Cell inoculation density – 7*10^6)

Figure III.2.2 Glutamine Utilization for Incubation Buffers Containing either 4 mM Glutamine or 10mM Glucose plus 4 mM Glutamine
Cell inoculation density – 3.5*10^6
The sensitivity of the HPLC to detect small changes in glutamine concentration in the presence of high background levels (4mM) is questionable, but the results appear to be in agreement with current literature. The effect that glucose has on glutamine utilization/glutamate accumulation can also be observed in the higher levels of glutamate that accumulate when glucose is added to an incubation buffer that contains glutamine. (Figure III.2.3a and Figure III.2.3b).

Here though there appears to be a contradiction. If less glutamine is utilized when glucose is present one would suspect that the glutamate accumulation/formation would also be depressed. A possible solution to this problem could be that while glucose does indeed depress glutamine utilization it also depresses, to a greater extent, the subsequent conversion of glutamate to other substances. In fact, this theme will be shown time and time again: glutamate production is tightly controlled and insensitive to the cellular environment as long as glutamine is present.

Figure III.2.3a  Glutamate Accumulation for Incubation Buffers Containing 10 mM Glucose, 4 mM Glutamine, or 10mM Glucose plus 4 mM Glutamine
Cell inoculation density – 3.5*10^6
Finally, the presence of glucose (10mM) greatly reduces the amount of aspartic acid accumulation observed when the incubation buffer contains 4 mM Glutamine. Figure III.2.4 shows an almost complete loss in aspartate accumulation when glucose is added to the incubation buffer.

These results may indicate that glucose: (i) inhibits aminotransferase activity, or (ii) increases the utilization of aspartate for nucleic acid synthesis. AspAT inhibition may explain why glucose stimulates glutamate accumulation while simultaneously inhibiting glutamine utilization.
From these figures and the ones presented in Section II.7, the complexity of the interaction between glucose and glutamine begins to come to light. Many of the results compare favorably to recent literature, confirming the ability of this assay to replace radioisotope studies in performing preliminary experiments.

It is very important to realize that the data presented to this point and results to be discussed in the pages that follow in this section are for Jurkat, clone E6. This cell and other mammalian cells are not the same and therefore care must exercised in comparing their metabolism because they may be completely different. In other words, results are cell specific.

It is also important to realize that the measurements of nutrients levels made here can not be assigned quantitatively to one product/substrate as may possible with radiolabeling studies. In looking at nutrient utilization and product formation an attempt is made to
identify trends. The determination of rates and/or carbon balances are not attempted because of the limited nature of the data gathered.

B) The Effects of Glucose and Glycolytic Intermediates on Product Formation from Incubation Buffers Containing Glutamine

The preliminary results obtained during the development of the plate assay suggest a complex interaction between glucose and glutamine utilization and the resultant distribution of products that accumulate. The interaction of glucose and glycolytic intermediates on product distribution from an incubation buffer containing glutamine were examined in a little more detail. The results presented in this section all utilize a cell density of ~2,000,000 cells/ml. This cell density was chosen because provides a strong enough signal and it doesn't require an enormous number of cells. This should allow the assay to be coupled to reactor steady states if one so desires.

First of all, the relationship between glucose levels and product accumulation was examined. From the figures previously presented one can see that the addition of glucose has a strong effect on both alanine and aspartate accumulation. Figure III.2.5 and III.2.6 both show that this effect is dependent on the initial glucose concentration in the buffer.

The addition of 10 mM glucose greatly increases the amount of alanine that accumulates suggesting that the carbon skeleton of alanine is primarily derived from glucose. As previously observed, the addition of 10 mM glucose causes a marked increase in the rate of alanine accumulation between 6 and 8 hours. This effect is not observed with 0.1 mM glucose additions. The addition of 10 mM glucose greatly reduces the amount of aspartate that is detected, while the addition of 0.1 mM glucose only slightly inhibits aspartic acid accumulation.[72]
Figure III.2.5 Alanine Accumulation as a Function of the Initial Glucose Concentration

Figure III.2.6 Aspartate Accumulation as a Function of the Initial Glucose Concentration
To further examine the effect that glucose and glycolytic products have on the accumulation of alanine and aspartate, test wells were established that replaced glucose with pyruvate or lactate. Lactate is known to be inhibitory to the growth of mammalian cells and its production from glucose may alter the metabolism of the cells. Pyruvate is an important glucose breakdown intermediate capable of entry into the TCA cycle, for regeneration of cytosolic NADH via lactate dehydrogenase, and for direct transamination with glutamate to alanine via alanine aminotransferase. Figures III.2.7 and Figure III.2.8 show the effect that lactate and pyruvate have on alanine and aspartate accumulation in an incubation buffer that also contains 4 mM glutamine.

Figure III.2.7 Effect of 2.5 mM Lactate or 2.5 mM Pyruvate on Aspartate Accumulation in an Incubation that also Contains 4 mM Glutamine
As could be predicted, the addition of pyruvate resulted in a pattern of aspartate "inhibition" and alanine "stimulation" similar to that observed with glucose.

The results obtained from the addition of lactate also show a similar pattern, however, its presence appears to cause a greater degree of inhibition of aspartate accumulation. Whether the inhibition is directly caused by the presence of lactate or the conversion of lactate to pyruvate with the production of NAD from NADH may provide important information about the interregulation of glucose and glutamine metabolism for Jurkat, clone E6.
In an attempt to gain a better understanding of glucose and glutamine metabolism for Jurkat, clone E6 the effects that some other important glutaminolysis intermediates exert were examined with the plate assay. This time, the plate assay was performed at two selected time points. The first time point, 4 hours, should provide peak (or near peak) levels of aspartic acid accumulation as evident from previous experiments. The second time point, 8 hours, should provide peak levels of alanine accumulation. The exclusion of the three other time points previously used greatly reduces the amount of work involved in the procedure, while hopefully maintaining the level of information gathered about the accumulation of alanine and aspartic acid.

Table III.2.1 represents the results obtained in the first half of this experiment. Cell densities utilized for this data set are ~2,000,000 cells/ml. The results from control test substances at 4 and 8 hours compare well with the values obtained in previous assays.

Incubations with 4 mM glutamine (taken as the control or base case) produced high levels of aspartate at the 4 hour time point, with levels dropping off at 8 hours. As observed before, alanine levels were highest at the 8 hour time point. Cells incubated with only 4 mM glutamine do not produce any measurable lactic acid, suggesting that the complete glutaminolysis pathway may not be active in Jurkat, clone E6. These results need to be confirmed with a more sensitive assay (like a radioisotope experiment).

Incubations containing 4 mM glutamine and β-chloroalanine or aminooxyacetic acid (AOA) confirm the involvement of both aspartate-aminotransferase (AspAT) and alanine-aminotransferase (AlaAT) in the accumulation of alanine and aspartate. β-chloroalanine is a suicide inhibitor specific for alanine aminotransferase[13], and its inclusion in the assay buffer resulted in a marked decrease in the amount of alanine detected. With 4 mM glutamine as the primary carbon source a 2 fold decrease in the amount of alanine that
accumulated was observed. The specificity of β-chloroalanine for AlaAT was confirmed in that aspartate levels were not inhibited in its presence.

Table III.2.1  

<table>
<thead>
<tr>
<th>Incubation Component</th>
<th>Aspartate Level [μM]</th>
<th>Alanine Level [μM]</th>
<th>Glucose Level [mM]</th>
<th>Lactate Level [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 hrs</td>
<td>8 hrs</td>
<td>4 hrs</td>
<td>8 hrs</td>
</tr>
<tr>
<td>2mM GLN (Base)</td>
<td>65.4 ± 0.3</td>
<td>46.7 ± 5.4</td>
<td>12.55 ± 0.8</td>
<td>23.4 ± 1.4</td>
</tr>
<tr>
<td>10 mM Glucose</td>
<td>3.15 ± 1.6</td>
<td>1.7 ± 0.6</td>
<td>18.9 ± 1.7</td>
<td>25.7 ± 0.7</td>
</tr>
<tr>
<td>Base + 2.5 mM βChloroalanine</td>
<td>95.9 ± 15</td>
<td>85.2 ± 1.9</td>
<td>8.25 ± 1.2</td>
<td>10.8 ± 0.2</td>
</tr>
<tr>
<td>Base + 2.5 mM AOA</td>
<td>3.5 ± 1.3</td>
<td>4.6 ± 0.7</td>
<td>6.40 ± 0.9</td>
<td>9.1 ± 1.5</td>
</tr>
<tr>
<td>10 mM Glucose</td>
<td>12.7 ± 0.2</td>
<td>11.75 ± 0.4</td>
<td>26.2 ± 0.3</td>
<td>78.4 ± 14.1</td>
</tr>
<tr>
<td>Base + 5 mM Glucose</td>
<td>15.85 ± 2.3</td>
<td>15.35 ± 0.2</td>
<td>32.7 ± 3.8</td>
<td>80.7 ± 1.7</td>
</tr>
<tr>
<td>Base + 0.1 mM Glucose</td>
<td>43.1 ± 8.2</td>
<td>36.5 ± 5.4</td>
<td>15.35 ± 3</td>
<td>31.6 ± 5.9</td>
</tr>
<tr>
<td>Base + 10 mM PYR</td>
<td>19.7 ± 3.1</td>
<td>23.1 ± 1.1</td>
<td>66.25 ± 6.3</td>
<td>144.9 ± 10.3</td>
</tr>
<tr>
<td>Base + 5 mM PYR</td>
<td>23.5 ± 3.8</td>
<td>27.5 ± 1.1</td>
<td>55.4 ± 4.4</td>
<td>127.8 ± 6.6</td>
</tr>
<tr>
<td>Base + 1 mM PYR</td>
<td>35 ± 9.6</td>
<td>35.6 ± 0.5</td>
<td>50.8 ± 17</td>
<td>95.9 ± 2.4</td>
</tr>
<tr>
<td>Base + 5 mM Glucose + 2.5 mM Oxamate</td>
<td>7.75 ± 0.8</td>
<td>13.3 ± 1.4</td>
<td>15.8 ± 1.4</td>
<td>65.9 ± 7.21</td>
</tr>
<tr>
<td>Base + 5 mM PYR + 2.5 mM βChloroalanine</td>
<td>13.85 ± 6.7</td>
<td>16.45 ± 3</td>
<td>16.15 ± 4.7</td>
<td>30.8 ± 4.1</td>
</tr>
</tbody>
</table>
When β-chloroalanine was added to the 4 mM glutamine and 5mM pyruvate incubation, alanine production was inhibited by 4-fold. This supports the contention that AlaAT is involved in the breakdown of glutamine, and the conversion of excess pyruvate to alanine. In addition, with 4 mM glutamine as the sole substrate, the presence of β-chloroalanine resulted in an increase in aspartic acid accumulation, suggesting a partial transfer of the activity lost (AlaAT) to aspartate aminotransferase. The inclusion of AOA, a nonspecific aminotransferase inhibitor, results in a decrease in both aminotransferase activities (only background levels of alanine and aspartate were detected).[9,48]

The addition of glucose or pyruvate showed the same product patterns as previously seen, with the level of 'stimulation' and/or 'inhibition' being dose dependent. Higher levels of aspartic acid accumulation are observed for decreasing levels of glucose or pyruvate, while alanine levels increase as pyruvate concentrations climb.

The inclusion of oxamate, a lactate dehydrogenase (LDH) inhibitor, with an incubation buffer containing 4 mM glutamine and 5 mM glucose resulted in a decrease in the amount of alanine and aspartate that accumulated at 4 hours. The amount of lactate that was produced was also depressed. Oxamate, while being a potent inhibitor of LDH, has been reported to be fairly impermeable to the cell membrane. This may explain why the observed inhibition of lactate production was not complete.

Table III.2.2 presents the data from the second half of this experiment. In this set some additional compounds were screened for their effect on glutamine utilization/alanine and aspartate accumulation.

The addition of glutamate to an incubation buffer containing 10 mM glucose resulted in an increase in the amount of alanine and aspartate detected. This may imply that glutamate availability limits the activity of aminotransferases involved in the production of aspartic acid and alanine.
Table III.2.2 Results from the Second Half of the 24 Plate Assay Looking at the Effect of Various Metabolites on the Production of Alanine and Aspartate from Glutamine and Other Compounds

<table>
<thead>
<tr>
<th>Incubation Component</th>
<th>Aspartate Level [(\mu\text{M})]</th>
<th>Alanine Level [(\mu\text{M})]</th>
<th>Glucose Level [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4 hrs 8 hrs</td>
<td>4 hrs 8 hrs</td>
<td>4 hrs 8 hrs</td>
</tr>
<tr>
<td>2mM GLN (Base)</td>
<td>51.6 34.1 (\pm 4.7)</td>
<td>10.8 21.9 (\pm 1.5)</td>
<td>ND ND</td>
</tr>
<tr>
<td>Base + 2.5 mM GLU</td>
<td>50.4 36.6 (\pm 5.9)</td>
<td>10.5 23.4 (\pm 1.3)</td>
<td>ND ND</td>
</tr>
<tr>
<td>10mM Glucose</td>
<td>5.4 4.6 (\pm 0.4)</td>
<td>17 26.3 (\pm 2)</td>
<td>8.75 8.71 (\pm 0.2) ±0.12</td>
</tr>
<tr>
<td>10mM Glucose + 2.5 mM GLU</td>
<td>9.3 15.2 (\pm 0.1)</td>
<td>21.8 43.5 (\pm 0.3)</td>
<td>9.04 8.74 (\pm 0.9) ±0.09</td>
</tr>
<tr>
<td>Base + 10 mM Malate</td>
<td>59.9 48.4 (\pm 0.6)</td>
<td>12.1 29.3 (\pm 0.8)</td>
<td>ND ND</td>
</tr>
<tr>
<td>10 mM OAA</td>
<td>40.7 53.4 (\pm 2.5)</td>
<td>65.5 136.7 (\pm 5)</td>
<td>ND ND</td>
</tr>
<tr>
<td>Base + 5 mM OAA</td>
<td>38.8 39.4 (\pm 4)</td>
<td>58.9 114.2 (\pm 4.5)</td>
<td>ND ND</td>
</tr>
<tr>
<td>Base + 1 mM OAA</td>
<td>39.7 39.9 (\pm 2.1)</td>
<td>47.6 91.4 (\pm 2.7)</td>
<td>ND ND</td>
</tr>
<tr>
<td>Base + 10 mM Lactate</td>
<td>8.1 8.7 (\pm 0.5)</td>
<td>27.8 65 (\pm 2.8)</td>
<td>ND ND</td>
</tr>
<tr>
<td>Base + 5 mM Lactate</td>
<td>10.1 9.5 (\pm 0.9)</td>
<td>29 64.3 (\pm 2.4)</td>
<td>ND ND</td>
</tr>
<tr>
<td>Base + 5 mM Lactate + 2.5 mM Oxamate</td>
<td>9.8 9.1 (\pm 0.6)</td>
<td>22.3 45 (\pm 1.9)</td>
<td>ND ND</td>
</tr>
<tr>
<td>Base + 5 mM OAA + 2.5 mM GLU</td>
<td>135.8 108.4 (\pm 1)</td>
<td>64.2 119.4 (\pm 0.9)</td>
<td>ND ND</td>
</tr>
</tbody>
</table>

Malate and its conversion to pyruvate is central to functioning of the glutaminolytic pathway. The addition of 10 mM malate to 4 mM glutamine did not significantly affect the
results. Brand[11], on the other hand, observed for proliferating thymocytes that the addition of 2 mM malate resulted in a decrease in glutamine utilization with a higher percentage of aspartate being formed.

The increased activity of the malic enzyme or malate dehydrogenase, key enzymes in the glutaminolytic pathway, would result in the production of pyruvate. Pyruvate, as previously observed, is readily transaminated to alanine. An increase in alanine accumulation would therefore represent a potentially active glutaminolysis pathway. This does not appear to be the case for Jurkat, clone E6. (However, it is not at all certain if malate can enter the Jurkat cell.)

Oxaloacetate would represent another point in which the glutaminolytic pathway could generate the pyruvate needed for its functioning. Here oxaloacetate is decarboxylated to phosphoenol pyruvate (PEP) via the action of PEP carboxykinase. The PEP generated is then converted to pyruvate via the action of pyruvate kinase. The data presented in Table III.2.2 shows a marked increase in alanine accumulation with increasing initial incubation levels of oxaloacetate. While this was encouraging at first it was later discovered that oxaloacetate is spontaneously converted to pyruvate in the incubation media as described. At 8 hours, ~65% of the initial OAA had spontaneously decomposed to pyruvate in cell-free control wells. Therefore, the action/activity of PEPCK cannot be proven to be responsible for the increase in alanine detected. Radiolabeling studies with mercaptopicolinate, an inhibitor of PEPCK have been performed by Ardawi[4], and have shown that PEPCK is active in lymphocytes of the rat. He observed a 50% reduction in lactate production when 0.5mM of mercapopicolinate was included in the incubation buffer. Similar experiments need to be undertaken in the future if the involvement of PEPCK in the glutaminolysis pathway is to be confirmed for Jurkat, clone E6.

With addition of oxaloacetate in an incubation buffer containing 4 mM glutamine one would also expect an increase in aspartic acid accumulation since oxaloacetate is converted to aspartate via the action of aspartate-aminotransferase. However this was not the case.
Aspartate levels were slightly less than the base case, possibly indicating that some, but not all, of the oxaloacetate did indeed breakdown to pyruvate.

Several possibilities exist that could explain the lack of aspartate accumulation observed under these conditions. First, all the oxaloacetate in the incubation buffer could have been decarboxylated to pyruvate before it was able to enter the cell. However, the differences between the pyruvate and oxalacetate results indicate that this decarboxylation was not complete. Secondly, the possibility exists that oxaloacetate is unable to enter the cell. It is well established that oxaloacetate produced in the TCA cycle is unable to directly exit the mitochondria. Thirdly, an important component(intermediate) involved in the transamination reaction may be in short supply, resulting in an apparent inactivity of AspAT. While we can not completely rule out the first two hypotheses, we have independent data to support the later hypothesis.

The possibility that glutamate availability limits the activity of AspAT was examined. If glutamate availability is indeed a problem, two possibilities exist. First, the Km for glutamate for AlaAT may be considerably lower than that for AspAT. This would result in an active AlaAT at lower concentrations of glutamate than what would be required for AspAT. Secondly, glutamate production from glutamine via glutaminase occurs in the mitochondria.[34] Therefore, the glutamate generated may be 'trapped' in the mitochondria; thus, if AspAT activity is primarily located in the cytosol and if oxaloacetate that enters the cell is 'trapped' in the cytosol, unable to enter the mitochondria, AspAT will be starved for glutamate. The addition of 2.5 mM glutamate to the incubation buffer containing both oxaloacetate and glutamine resulted in a marked increase in aspartate production, indicating that glutamate was indeed limiting. However, whether the glutamate produced from glutamine is more readily utilized by AlaAT, or whether compartmentalization of glutamate/oxaloacetate is the cause, needs to be addressed in future work.
As previously observed, the addition of lactate in an incubation buffer containing 4 mM glutamine resulted in a strong inhibition of aspartate accumulation and an increase in the amount of alanine detected. The addition of oxamate in an incubation buffer containing glutamine and lactate resulted in a decrease in the amount of alanine observed, confirming that the conversion of lactate to pyruvate via lactate dehydrogenase is involved in the increase in alanine levels. However, whether lactate itself, or the increased levels of NAD that result from its conversion to pyruvate, is behind the depressed aspartic acid production observed for lactate incubations could not be determined.

This final set of plate assay data again shows the utility of the procedure. The assay as outlined can provide valuable information when performed as a fixed time or kinetic assay. It allows one to screen a number of compounds quickly, safely, and inexpensively. Important observations can be confirmed using the same procedure outlined here with the addition of radioisotopes to the incubation buffer.
3) Continuous Culture Bioreactor Results:

In order to gain some additional insight into the metabolism of Jurkat, clone E6 the transient responses of this cell line to various metabolite pulses were examined in some detail. The use of continuous culture and its ability to maintain mammalian cells in an apparent metabolic steady state allows one to systematically examine how the cells react to changes in the prevailing steady state. The chemostat was operated as follows:

The dilution rate for the results in the following section was 0.37 day\(^{-1}\) and was periodically checked and remained relatively constant throughout all experiments. This suggests that during the 45 - 50 days of operation both the pumps and the tubing used performed flawlessly.

The working volume of the chemostat was maintained at 1250 ml via a crude level control. (See section IV.3 for problem associated with this method of media removal.)

The DO was controlled at a set point of 30% air saturation via headspace gassing with pure oxygen and nitrogen. The gasses were humidified at reactor conditions to minimize evaporative losses. Maximum gas throughput was ~ 1 - 2 VVH.

Agitation was maintained at 80 RPM throughout all experiments. A 2 inch marine (vortexing) impeller provided the mixing.

pH was controlled via the addition of 320 mM NaOH (base control) or head space CO\(_2\) addition (acid control). A relatively constant pH of 6.95 was maintained throughout most of the experiments.

Temperature was maintained at 37\(^\circ\)C via an electric heating blanket. No method for negative temperature control was available with the current reactor configuration.

The level of control provided by the Applikon reactor sytem is shown in figure II.3.1. While the levels of control, as evident in this figure is adequate, many improvements can be implemented to improve future runs. (See section IV.3)

Cells were grown in the dark to eliminate any negative effects that exposure to the light might have on their growth.

Preliminary reactor experiments indicated that the sfRPMI developed in this work was not capable of supporting the growth of Jurkat, clone E6 under reactor conditions. The addition of 125 mg/L of crystalline serum albumin to sfRPMI allowed Jurkat to thrive under the reactor conditions outlined above.

The nutrient feed consisted of sfRPMI + 125 mg/ml crystalline serum albumin.(reactor-sfRPMI)
Pulse additions of select metabolites were prepared in 20 ml of a 1:1 mix of reactor-sfRPMI and Kerbs-Ringer buffer. The concentrated pulse addition was pH to ~ 7.4 prior to addition to the reactor. Upon the addition of the pulse( time = 0\(^{-}\)) both the feed and the waste pumps were disabled. The pulse was
the addition of the pulse (time = 0+ ) both the feed and the waste pumps were disabled. The pulse was allowed to thoroughly mix in the reactor for 10 minute under zero flow conditions. After the 10 minutes passed the pumps were restarted. After an additional 5 minutes the time = 0+ sample was withdrawn.

![Graph](image)

Figure III.3.1   Sample Reactor Conditions for a Three and a Half Day Period

Table III.3.1 presents the steady-state concentration and specific consumption rates for select compounds for Jurkat, clone E6 maintained at a dilution rate of 0.372 day⁻¹. Prior to the introduction of metabolic pulses the cell density obtained at this dilution rate was
~2,500,000 cells/ml with an apparent viability in excess of 98%. The viability remained at essentially this level throughout the course of all pulses performed.

The activities of AlaT and AspAT in cell suspensions taken from the steady state bioreactor were determined to be 8,000 and 16,480 μmol/min per mg protein for Jurkat maintained at the conditions described above. Newsholme[5] found the activity of AlaAT and AspAT to be 10.3 and 129 nmol/min per mg of protein, respectively, in rat lymphocytes. Brand[11] found the activity of AspAT to be 22,140 μmol/10^{10} cells in proliferating thymocytes and the activity to be 3,900 μmol/10^{10} cells in resting thymocytes.

<table>
<thead>
<tr>
<th>Table III.3.1</th>
<th>Steady State Metabolite Concentrations and Utilization Rates, Prior to the 10 mM Glucose Pulse for a 0.372 Day⁻¹ Dilution Rate.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient / Waste</td>
<td>Input (μM)</td>
</tr>
<tr>
<td>Glucose</td>
<td>11125</td>
</tr>
<tr>
<td>Lactate</td>
<td>0</td>
</tr>
<tr>
<td>Ammonium</td>
<td>0</td>
</tr>
<tr>
<td>Alanine</td>
<td>0</td>
</tr>
<tr>
<td>Arginine</td>
<td>1226</td>
</tr>
<tr>
<td>Aspartate</td>
<td>157</td>
</tr>
<tr>
<td>Asparaginate</td>
<td>384</td>
</tr>
<tr>
<td>Glutamate</td>
<td>143</td>
</tr>
<tr>
<td>Glutamine</td>
<td>2052</td>
</tr>
<tr>
<td>Glycine</td>
<td>127</td>
</tr>
<tr>
<td>Histidine</td>
<td>104</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>424</td>
</tr>
<tr>
<td>Leucine</td>
<td>402</td>
</tr>
<tr>
<td>Lysine</td>
<td>206</td>
</tr>
<tr>
<td>Serine</td>
<td>304</td>
</tr>
<tr>
<td>Threonine</td>
<td>189</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>133</td>
</tr>
</tbody>
</table>
A) 10 mM Glucose Pulse:

The results obtained upon the addition of a 10 mM glucose pulse are presented in Figures III.3.2a-d. From Figure III.3.2a one can see that upon the addition of glucose the cell concentration decreased dramatically. This was result puzzling at first, but was later determined to be a direct consequence of the addition of NaOH to the reactor to combat the increased lactic acid production. (Figure III.3.2b) See section IV.3 for additional comments.

![Graph](image)

**Figure III.3.2a** Transient Response of Cell Concentration During a 10 mM Glucose Pulse.

Figure III.3.2b shows that the addition of the 10 mM glucose resulted in a marked increase in lactic acid production. Apparently, most of the additional glucose provide was converted into lactic acid.
Figure III.3.2b Transient Responses of Lactate, Glucose, and Ammonium Levels During a 10 mM Glucose Pulse.

Figure III.3.2c Transient Responses of Alanine, Aspartate, Glutamate, and Glutamine Levels During a 10 mM Glucose Pulse.
In Figure III.3.2c the response of glutamate, glutamine, aspartate, and alanine levels to the 10 mM glucose pulse are presented. Here alanine and glutamine levels climb upon the addition of glucose, while the level of glutamate detected drops slightly. Aspartate levels remain unchanged (essential the same as feed conditions). The response to the glucose pulse observed here for these nutrients closely parallel those observed by Miller[36,38].

![Graph showing transient responses of serine, glycine, leucine, and isoleucine levels during a 10 mM glucose pulse.]

Figure III.3.2d Transient Responses of Serine, Glycine, Leucine, and Isoleucine Levels During a 10 mM Glucose Pulse.

Figure III.3.2d shows the response of four other amino acid to the glucose pulse. Here the levels of leucine, isoleucine, and glycine remained unchanged throughout the duration of the experiment. On the other hand serine levels rise sharply upon the addition of glucose. This probably represents the increased flux of glycolytic intermediates, one being 3-Phosphoglycerate(3-P-G). 3-P-G is the starting point for serine synthesis.
B) 4 mM Glutamine Pulse

Eight days after the glucose pulse, a 4 mM pulse of glutamine was added to the bioreactor. (Figures III.3.3a - Figure III.3.3d) Figure III.3.3a shows that the cell concentration remained approximately constant throughout the 4 mM glutamine pulse, signifying that glutamine was not the sole limiting nutrient.

From Figure III.3.3b one can see that glucose, lactate, and ammonia levels remained constant throughout the pulse. These results compare well with plate assay data, where the addition of glutamine in the assay buffer had no effect on the rate of glucose utilization or lactate formation. These results cast doubt on to whether or not the complete glutaminolysis pathway is an important component of the metabolism of Jurkat.

Figure III.3.3a Transient Response of Cell Concentration During a 4 mM Glutamine Pulse.
Figure III.3.3b Transient Responses of Lactate, Glucose, Ammonium, and Glutamine Levels During a 4 mM Glutamine Pulse.

Figure III.3.3c Transient Responses of Alanine, Aspartate, Glutamate, and Glutamine Levels During a 4 mM Glutamine Pulse.
Figure III.3.3c shows that there is an increase in both alanine and glutamate levels upon the addition of 4 mM glutamine. Aspartate levels remain unchanged throughout the course of the experiment.

**Figure III.3.3d Transient Responses of Serine, and Glycine Levels During a 4 mM Glutamine Pulse.**

Figure III.3.3d shows an increase in both serine and glycine levels at about 90 hours after the glutamine pulse. Leucine and isoleucine levels, while not shown in Figure III.3.3d, remained essentially unchanged.

C) 5 mM Pyruvate Pulse:

Nine days after the glutamine pulse, a pulse of 5mM pyruvate was added.(Figures III.3.4a - Figure III.3.4d)
Figure III.3.4a show the change in cell concentration during the 5 mM pyruvate pulse. The cell density remains essentially constant after the addition of pyruvate; however, a slight decrease in cell concentration occurs after the resumption of base addition.

During the glucose pulse base control initially was required to maintain a constant pH, but was later discontinued when it was no longer needed. During the glutamine pulse, base control was not required. For the pyruvate pulse, the rate of NaOH addition initially emptied the based reservoir, causing the pH to temporarily fall (lowest point 6.82). (Figure III.3.1) This problem was remedied at ~77 hours, at which point NaOH addition resumed. The drop in cell density observed occurred at a point when base control resumed. From preliminary (unsuccessful) reactor experiments, the toxicity of NaOH addition was well established. With the current reactor configuration nothing could be done to alleviate the need for NaOH addition. (See discussion for additional details/recommendation.)

![Figure III.3.4a](Image of Graph)

Figure III.3.4a Transient Response of Cell Concentration During a 5 mM Pyruvate Pulse.
Figure III.3.4b shows the response of glucose, lactate, pyruvate and ammonium levels to the addition of pyruvate. Upon addition of pyruvate, there is a rapid increase in the level of lactate produced, suggesting that much of the pyruvate added goes to lactate.

![Graph showing transient responses of lactate, glucose, ammonium, and pyruvate levels during a 5 mM pyruvate pulse.](image)

**Figure III.3.4b Transient Responses of Lactate, Glucose, Ammonium, and Pyruvate Levels During a 5 mM Pyruvate Pulse.**

In the plate assay, it was observed that upon the addition of pyruvate to an incubation buffer also containing glutamine (but no glucose) resulted in no appreciable lactate accumulation. With the conditions employed in the plate assay the level of cytosolic NADH required for the conversion of pyruvate to lactate is probably in short supply since no glucose was available to produce NADH. Consequently, much of the pyruvate was converted to alanine instead of lactate. Within the reactor, the need to remove excess NADH
and regenerate NAD probably results in much of the pyruvate added being driven to lactate formation. The apparent differences between reactor results and plate assay results, not only points out that care must be exercised in applying the results obtained from one system to another (different) system, but also demonstrates the increased insight that may be gained from comparing the results obtained by each method.

![Figure III.3.4c Transient Responses of Alanine, Aspartate, Glutamate, and Glutamine Levels During a 5 mM Pyruvate Pulse.](image)

Figure III.3.4b and Figure III.3.4c show unchanging levels of glucose, ammonium, and aspartic acid during the pulse. Levels of alanine and glutamate on the other hand, both increase during the pyruvate pulse. Glutamine levels also show a slight increase during the
pulse indicating a slight inhibition in glutamine utilization in the presence of elevated pyruvate levels. Radiolabeling experiments are required to determine if the increase in glutamine levels is indicative of a reduced role for glutamine in energy production due to possible pyruvate oxidation.

![Graph showing transient responses of serine, glycine, leucine, and isoleucine levels during a 5 mM pyruvate pulse.]

**Figure III.3.4d** Transient Responses of Serine, Glycine, Leucine, and Isoleucine Levels During a 5 mM Pyruvate Pulse.

Serine and glycine levels remain approximately unchanged during the pyruvate pulse (Figure III.3.4d). Isoleucine and leucine both show a dramatic decrease when pyruvate is added. The levels of other essential amino acids remain constant during the pulse.
D) 10 mM Oxaloacetate and 2.5 mM Glutamate Pulse Changes:

Figures III.3.5a through Figure III.3.5e summarize the results obtained from a 10 mM oxaloacetate pulse followed (~40 hours) by a 2.5 mM glutamate pulse. Within this experimental set, an attempt was made to induce a measurable change in aspartic acid levels. From plate assay data, it was determined that the addition of glutamate enhanced the levels of aspartate produced from an incubation buffer containing oxaloacetate. While the residual levels of glutamate in the reactor might have been sufficient, additional glutamate was added to be certain that the stimulation of aspartate production was not limited by glutamate availability. Glutamate was added after the oxaloacetate pulse so that the effects of the oxaloacetate pulse alone could be evaluated.

Cell concentration remained approximately constant after the addition of both the glutamate pulse and the oxaloacetate pulse (Figure III.3.5a).

![Graph](image-url)
Figure III.3.5b shows the change in oxaloacetate, lactate and pyruvate levels during the course of this experiment. The possible problem of spontaneous decarboxylation of oxaloacetate mentioned in Section III.2.C is quite evident in this figure. Only 20% of the initial amount of oxaloacetate remained upon its addition to the bioreactor. Oxaloacetate was readily decarboxylated to pyruvate during its preparation. As observed with the 5 mM pyruvate pulse, there is a sharp increase in the amount of lactate produced when oxaloacetate(pyruvate) is added to the reactor. The subsequent glutamate pulse appears to have increased both the levels of pyruvate and lactate detected.
From Figure III.3.5c one can see that the goal of producing a measurable change in aspartic acid levels was accomplished. While the addition of oxaloacetate turned out to be considerably lower than desired, an increase in aspartate levels is evident immediately following the pulse.

The addition of oxaloacetate also results in a marked decrease in glutamine utilization, and therefore a decrease in glutamate levels. Alanine levels initially rise, but fall off sharply after the addition of glutamate. This is in contrast to results obtained in the plate assay, where the addition of glutamate had no effect on the level of alanine detected.
As can be expected, the fall in glutamine utilization results in a decrease in the ammonium levels detected. (Figure III.3.5d) The addition of glutamate results in a sharp increase in ammonium levels. Does the addition of glutamate change the way glutamine is metabolized, or is the resultant increase in ammonium levels detected produced from the direct catabolism of glutamate? As the concentration of glutamine begins its descent to prepulse levels, ammonium levels begin its climb back up. As with the pyruvate pulse, glucose levels remained unchanged throughout this experiment.

The oxaloacetate pulse also produced a decrease in isoleucine and leucine levels previously observed for the pyruvate pulse. (Figure III.3.5e) However, since the addition of 10 mM oxaloacetate actually resulted in the addition of ~ 8 mM pyruvate and ~ 2 mM...
oxaloacetate, the decrease in leucine and isoleucine levels may not be a result of oxaloacetate. The sharp decrease in the levels of these two essential amino acid contrasts with the constant levels maintained by the other amino acids and warrants future investigation into their role in the metabolism of Jurkat.

Figure III.3.5e  Transient Responses of Serine, Glycine, Leucine, and Isoleucine Levels During a 10 mM Oxaloacetate and 2.5 mM Glutamate Pulse

Glycine levels markedly increased after the addition of oxaloacetate and dropped off after about 75 hours. Serine levels drop off sharply immediately after the glutamate pulse.
In summary, the use of continuous culture allows one to examine changes in metabolism in response to changes in the steady state of the reactor. The reactor data obtained, while providing some insight as to how metabolic pathways respond to environmental changes, fails to provide conclusive evidence as to what changes are occurring. Carefully controlled radiolabelling experiments are a necessity if one desires to know what actually is happening, and why.

In this work, an Applikon reactor system was setup, and the conditions were determined that would allow for long term culture of Jurkat, clone E6. However, it should be noted that the data collected represents the first test of the system as described in this work and a number of problems were encountered that affect the results obtained. The problems encountered and possible solutions are discussed in greater detail in section IV.3.

Upon correction of these problems, the Applikon reactor will be an indispensable tool for studying mammalian cell metabolism.
IV DISCUSSION AND RECOMMENDATIONS:

1) MTT Assay

The MTT Assay provides an excellent alternative to the standard procedures commonly employed to measure cellular proliferation/activation. The MTT assay has many advantages over the more commonly employed "growth assay", and [14C]thymidine incorporation assay.

The MTT assay is quick. Assay development takes only four hours. Incubations of test compounds can be as short as a few hours or as long as a few days. The cost of the MTT assay make it considerably more economical than the thymidine incorporation assay, and the potential hazards of working with radioisotopes are avoided. The MTT assay requires considerably fewer cells than a growth assay would require. This allows one to perform more tests or replicates with the same numbers of cells. Also, since cells are not counted, the MTT assay greatly reduces the labor involved in screening a cell line for reactivity to a number of test compounds.

Within this body of work, the MTT assay was used primarily to screen cultures of Jurkat, clone E6 for sensitivity to a number of substances. In performing the MTT assay side by side with the growth assay to determine the sensitivity of Jurkat to increasing levels of ammonium, the results from each method compare fairly well with each other. The MTT assay however, was more sensitive for identifying initial effects of the test compound. MTT assays were also performed on a number of other compounds (lactate, pyruvate, oxaloacetate, and malate). The results obtained from the MTT assay were used to select safe levels of these compounds to be administered to the reactor. Additionally, the results of the MTT assay suggest that the levels of ammonium and lactate present in the reactor at steady state conditions should not be inhibitory to the growth of Jurkat. The sensitivity of Jurkat, clone E6 to both lactate and ammonium appear to closely parallel that observed for the cell line used in Miller's work.[38]
The ability of the MTT to measure cellular activation in the absence of proliferation allows one to examine what effect limiting nutrient levels have on cellular stimulation. Figures IV.1.1a and Figure IV.1.1b show the ability of the MTT assay to detect cellular response to limiting levels of glucose and glutamine. While the figures present data for a 12 hour incubation time, significant differences are also observable at a 6 hour incubation time, making the MTT assay useful for the determination of 'initial' effects.

Figure IV.1.1a Ability of Varying Glucose Concentrations to Stimulate Jurkat at Three "Limiting" Glutamine Concentrations
The ability of the MTT assay to detect stimulation at limiting nutrient levels, its ability to examine the toxicity (both long and short term) of metabolites, and its relative ease of use should make MTT analysis an excellent choice for statistically designed experiments that involve very large data sets. The MTT assay would allow a large sets of experiments to be performed in considerably shorter period of time than a growth assay would require. This should allow the selection of optimal growth conditions for any cell line to be determined with the least amount of work, and in the shortest period of time. This would greatly shorten the time involved in developing specialized serum-free media formulation that perfectly match the individual needs of a cell.

Figure IV.1.1b Ability of Varying Glutamine Concentrations to Stimulate Jurkat at Three "Limiting Glucose Concentrations."
MTT and other tetrazolium salts also present another potential labor reducing feature. The enzymatic determinations used in this work all utilize the UV detection of the NAD/NADH pair. The enzymatic assays as performed by the procedures outlined in Section II.4A-E using a narrow band Gilford are labor intensive. If the procedures as outlined in section II could be modified in such a way that a standard Elisa plate reader could be used, much effort could be saved. The use of Elisa reader and 96 well plates would allow smaller samples to be used (very important with limited sample volumes). Additionally, performing multiple replicates would be possible and would greatly improve the accuracy of the results.

With the use of an electron carrier such as phenazine methosulfate (PMS), the enzymatic procedures that utilize the NAD/NADH pair could be coupled with a tetrazolium salt (MTT, INT), making the use of a standard Elisa reader possible. Recently Farmillette et. al. [17] described an assay for lactic acid determination that utilizes p-iodonitrotetrazolium (INT) and the electron carrier PMS.

![Lactate Dehydrogenase Reaction](image)

**Figure IV.1.2** Determination of Lactic Acid Concentrations Using a Coupled LDH/PMS System with the Tetrazolium Salt INT

The procedure as outlined in Farmillette's et. al. paper could conceivably be modified for any enzymatic procedure that utilizes NADH/NAD. The modified assay procedures, using a tetrazolium salt, would have all advantages listed above.
2) **Plate Assay:**

The plate assay as described in this work, when used either as a fixed time or a kinetic assay, can provide valuable information about cellular metabolism. In the initial experiments the feasibility of using a non-radioisotopic method to measure *in vivo* whole cell metabolism was verified.

Cell densities ranging from 2-7x10^6 cells/ml provided strong detectable signals. However, reliable (reproducible) measurements of substrate disappearance are only possible using higher cell densities >5x10^6 cells/ml. If cell numbers are limiting the plate assay can be performed with a low cell inoculum and product formation can be followed. If cell numbers are not limiting, a higher cell density could also provide additional information about substrate utilization.

The assay is by no means a complete substitute for radioisotopic procedures, but is offered as an alternative procedure for preliminary experiments.

Results obtained from preliminary experiments compared fairly well with the radiolabeling experiments by Brand[10] using a different clone of Jurkat and rat thymocytes. The results Brand obtained for rate of glucose utilization and lactate production are about 20% lower than those observed in this work.

From preliminary experiments using Jurkat, clone E6 some, important insight was gained concerning the metabolism of glucose and glutamine by this cell line.

First, the presence of glutamine(4mM) does not effect glucose utilization or lactic acid formation of Jurkat incubated with 10 mM glucose. The effect that glutamine has on TCA cycle oxidation of glucose needs to be verified using [6-14C] glucose. The inability of glutamine to disturb glucose utilization and lactate formation observed in this work has also been observed by others: Ashby[6] for human enterocytes, Brand[12] for rat thymocytes. However, other authors have observed changes in glucose metabolism upon the addition of glutamine. Newsholme[43] observed a decrease in glucose utilization and an increase in
lactate production for mouse macrophages, while Ardawi[2] observed an increase in glucose utilization and lactic acid production for human peripheral lymphocytes.

All this serves to emphasize that the results obtained in any experiment performed are very cell specific, and the results observed for one cell line should not be used to generalize about the metabolism of other cell types.

Second, the presence of glucose in any incubation buffer that contains glutamine inhibits glutamine utilization and aspartate formation while an increase in glutamic acid and alanine accumulation is observed. These results closely parallel Brand's[13], who observed that glucose inhibited aspartate formation(13% as opposed to 25%), while stimulating glutamate production(72% as opposed to 61%). Conversely, Newsholme[4] observed an increase in glutamine utilization(1.4 fold), with more glutamate and aspartate being produced. Brand's observation were made with immature rat thymocytes, whose metabolism is probably closer to that of Jurkat's( a T-lymphosblastoid) than the 'mature' lymphocytes Newsholme used in his work. The possibility that aspartate accumulation is low in the presence of glucose because of increased nucleic acid synthesis needs to be addressed in future work.

Third, while the inhibition of aspartate accumulation in the presence of glucose has been observed by others little(or nothing) has been said about the kinetics of aspartic acid accumulation over long incubation times.

Kinetic experiments with an incubation buffer containing only glutamine shows that there is a rapid increase in aspartic acid accumulation, followed by a sharp decline in the levels detected. With only glutamine present in the incubation buffer, high(unusually high) levels of oxaloacetate(OAA) apparently would build up in the mitochondria as glutamine enters the TCA cycle. Without the presence of acetyl-CoA(derived from glucose, pyruvate, or lactate) the OAA formed from glutamine has no where to go other than to aspartate if the key enzymes in the later stages of glutaminolysis are inactive. If the key enzymes, malic enzyme or malate dehydrogenase:PEPCK:PK of the glutaminolysis are inactive high levels of OAA would result, and consequently drive aspartate formation.
From the kinetic data for aspartate accumulation, a number of questions arise as to what causes the drop in aspartate accumulation observed at later incubation times. All other products/substrates, with the exception of alanine in an incubation buffer containing high levels of glucose in addition to glutamine, maintained a linear increase or decrease throughout the entire incubation time. The possibility that aspartate enters in nucleotide synthesis or protein synthesis seem unlikely given the incubation conditions utilized.

One possibility that may exist is that aspartate plays an important role in the adenine nucleotide cycle.[20,21](Figure IV.2.)

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**Figure IV.2.1** Metabolic Pathways Involved in the Degradation and Resynthesis of Adenine Nucleotides
At early incubation times, the possibility exist that ATP levels are critically low. The cell responds by breaking down AMP to IMP with the action of adenine deaminase. Adenylate kinase, then converts ADP to ATP and AMP. This causes a rise in the ATP/ADP levels in the cell. High ATP/ADP level are critical for the functioning of many cellular operations.

As incubation time increases, ATP levels rise as a result of the partial oxidation of glutamine in the TCA cycle. With levels of ATP production sufficiently restored, the cell respond by rebuilding its adenine nucleotide pool. Here aspartate donates its nitrogen group to IMP. The use of hadacidin, a adenylatosuccinate synthase inhibitor, may shed some light onto the importance of the nucleotide cycle in Jurkat. And the role, if any, it has in the kinetic behavior observed for aspartate.

While this is one possibility, the complete resolution of this problem will require a kinetic assay using [U-14C]glutamine. The disappearance of the 14C-aspartate formed from glutamine could be followed to determine what compounds aspartate is converted to at long incubation times.

The other plate experiments preformed in this work shed some light on the relative importance of AspAT in the metabolism of glutamine. Newsholme and Brand both conclude that AspAT plays a major role in glutamine metabolism of mammalian cells. Within this work, the importance of AspAT in the breakdown of glutamine is uncontested, if the conditions are such that OAA levels are high in the mitochondria.(i.e. no acetyl-CoA enters the TCA cycle)

This would be the case if glutamine was only the incubation nutrient and the activity of the malic enzyme, which produces pyruvate from malate, and oxaloacetate decarboxylation or PEPCK:PK, which produce pyruvate from OAA, were low or nonexistent. With glutamine as the sole nutrient, it appears that its conversion to pyruvate does not take place in Jurkat, for if it did, we would see lactate formed in the plate assay(which we do not). Therefore OAA levels in the mitochondria should be high and AspAT would be active.
However, with most tissue culture applications, glutamine is not alone. Since glucose and lactate were both shown to inhibit aspartate accumulation and are present at high levels, it may be assumed that the levels of OAA are very low. Therefore under normal tissue culture conditions the OAA levels may be limiting the activity of AspAT. However, it must be noted that just because glucose inhibited aspartate accumulation, this does not necessarily mean it inhibits aspartate formation, since aspartate is an important precursor for many biological compounds.

Additional experiments are required to determine the relative importance of GIDH and/or AlaAT in the breakdown of glutamine. Newsholme and Brand believe that GIDH is active. However, both authors believe that AlaAT plays no role in the breakdown of glutamine. Again, the information in this work tends to contradict the belief that AlaAT is not important in the breakdown of glutamine.

First, when β-chloroalanine was included in an incubation buffer that contained glutamine a 50% reduction in alanine levels was observed. If β-chloroalanine was added to an incubation buffer containing glutamine and pyruvate, a 4 fold reduction in alanine was observed. These results show that β-chloroalanine is a strong inhibitor of AlaAT. The incubation buffer that contained only glutamine and β-chloroalanine also experienced a sharp increase in aspartate levels. The fact that aspartate levels increase while alanine levels decreased suggest that the activity of AlaAT lost was replaced by AspAT activity. This seems to verify the role of AlaAT in the breakdown of glutamine for Jurkat.

Some confusion lies in the literature here. Other authors often measure AlaAT activity by the production of labeled alanine from radiolabeled glutamine. However, the carbon chain of alanine may come from glucose. In this work, the production of pyruvate from glutamine is questioned. So if the above logic was applied, no AlaAT activity would be detected. Clearly this is not the case. The problem lies in the fact that while glutamine is the only nutrient added to the incubation buffer, it not the sole nutrient available to cells. Most cell lines have high intracellular lactate levels[28,29], and this lactate(pyruvate) could be
used in the AlaAT reaction. The end result would be a active AlaAT with no labeled alanine detected.

In a sense this hypothesis has been shown to be the case. Brand[11] observed that for an incubation buffer containing [U-14C] glutamine plus glucose that labeled glutamate and a little labeled aspartate was formed, while large amounts of unlabeled alanine was produced. This suggests the carbon chain of alanine is derived from pyruvate while the nitrogen group is derived from glutamine.

Experiments with 15N glutamine would be required to confirm that glutamate used in the AlaAT reaction is derived from glutamine. If 15N alanine was produced, the importance of AlaAT in glutamine metabolism would be confirmed.[68,69]

Experiments with OAA resulted not only in the addition of OAA to the incubation media but also pyruvate. Pyruvate is formed from OAA by the spontaneous decarboxylation of OAA in solution. This experiment attempted to look at the importance of PEPCK and oxaloacetate decarboxylase in the metabolism of Jurkat; however, because of the decarboxylation, this was not possible.

With the OAA addition, high levels of alanine were produced as expected, since pyruvate was also added. The level of aspartate 'inhibition' was not as large as previously observed for incubation buffers containing pyruvate. Hence, some OAA survived and resulted in additional aspartate production. Also, the trend for changing aspartate levels with the OAA addition was different than that previously observed. With the OAA addition aspartate levels did not drop off at the 8 hour incubation time.

With the addition of glutamate a large increases in aspartic acid levels were observed. During this incubation alanine levels did not respond to the glutamate addition. The fact that alanine production does not change and the inability of OAA to readily cross into the mitochondria suggests that glutamate is limiting in the cytosol for the AspAT reaction.
The addition of oxaloacetate and the addition of OAA+glutamate both point out the importance of OAA in the metabolism of Jurkat. Future kinetic experiments where OAA is added, and prepared under alkaline condition to reduce its spontaneous decarboxylation, with and without mercaptopicolinate may answer some question regarding the importance of PEPCK and oxaloacetate decarboxylase in the breakdown of glutamine. Additional kinetic experiments with glutamate and OAA may shed some light onto the importance of compartmentalization of important intermediates in Jurkat.
3) Reactor Experiments:

The use of continuous culture affords the user a wonderful opportunity to examine mammalian cell metabolism. With a properly functioning continuous culture bioreactor setup (chemostat), once the cell line within the reactor reaches a steady state, time is essentially frozen. This is in contrast to the use of a batch culture system where time passes in a blink of the eye, and what is observed is often gone before one even has a chance to see it. With a chemostat the operator conceivably has all the time in the world to perform his experiments so long as he can maintain a steady state.

At a steady state endless possibilities exist for the scope of experiments contemplated. In this work the way in which a human T-lymphoblastoid line, Jurkat, metabolizes important nutrients was the focus of the experiments. However, experiments looking at gene induction in Jurkat, in particular the IL-2 gene, would also be possible with the use of continuous culture.

Within the study of cellular nutrition the use of a chemostat is a valuable tool. Looking at different steady states (degrees of cellular proliferation) allows one to examine how cells are able to survive in a wide range of growth conditions. At low dilution rates, cells are crowded, important nutrients are in short supply, toxic product of metabolism (ammonium, lactate) reach their highest levels, and metabolic pathways have to adapt to the harsh conditions if the cell is to survive. On the other hand, at high dilutions, cell-cell contact is not as great, nutrients are abundant, the medium is devoid of high levels of harmful wastes, and the cells thrive. Their metabolism shifts from that of survival to unbounded proliferation. The use of a chemostat allows the operator to examine a number of steady states and determine what changes are occurring in the metabolism at each operating condition. This information gives one the opportunity to see how cells respond to the their changing environment.
Within this body of work pulse changes in important nutrients were administered to Jurkat maintained at a 'steady state' environment and the changes in metabolism that resulted were followed in some detail.

The dilution rate used in this work was 0.372 day\(^{-1}\). At this dilution rate Jurkat's environment was a harsh one. Important nutrients were in short supply (glucose ~0.3 mM, glutamine ~0.1 mM), and harmful waste were reaching high levels (lactate ~21 mM, ammonium ~2 mM). Jurkat's response to a number of nutrient pulses (glucose, glutamine, pyruvate, oxaloacetate, or 0.5X amino acids) showed that a state of multiple substrate limitations existed within the reactor. The overall response to each pulse was the essentially the same. The pulse administered was rapidly utilized and no net increase in cell mass (numbers) occurred.

The response to the 10 mM glucose pulse was a rapid utilization of the added energy source. The utilization of the added glucose by Jurkat was relatively inefficient, with a large increase in lactic acid production resulting. High rates of aerobic glycolysis is often the norm for tumor cell lines grown in culture.

The increased flux through the glycolytic pathway resulted in an increase in both serine and alanine accumulation within the reactor. The increase in serine production started immediately upon the addition of the glucose pulse, and ended abruptly when all the added glucose was consumed. The response of alanine (and hence glutamate since it is intimately involved in alanine production) was quite different. Immediate upon the addition of glucose alanine levels increased slightly. Alanine levels remained slightly elevated for approximately 30 hours at which time another increase in alanine levels occurred. The second increase was gradual, reaching its maximum well after all the added glucose was utilized. The resultant increase in alanine persisted for greater than 200 hours after the glucose pulse was initiated.
One possibility (one of many) for the behavior of alanine production observed is that the increase in the level of glucose (or resultant pyruvate) brought about a delayed increase in AlaAT protein synthesis. As the ability of the steady state levels of AlaAT were taxed the cells responded by increasing the rate of AlaAT synthesis, and a new (increased) level of AlaAT was reached within the cells. If the half life of AlaAT was relatively long and the $K_m$s for pyruvate and glutamate relatively low, the result would be an increase in alanine production even as the levels of glucose declined.

This hypothesis and the data from this pulse bring to light some very fundamental question regarding reactor operation.

What is a steady state?

If the level of cell mass (numbers), concentration of lactate, concentration of serine, concentration of glucose, concentration of ammonium, and concentration of aspartate all returned to the values observed before the 10 mM glucose pulse, have the cells returned to the steady state? What criteria should be used to define a 'steady state'? Has a new steady state been reached with increased levels of alanine and decreased levels of glutamate?

As the degree of completeness in studying transients responses of mammalian cells improves in the future the question mentioned above will need to be addressed. In this work the conclusion drawn is that the cells have not returned to a steady state. If given sufficient time, it is believed that the levels of glutamate and alanine would return to their steady state values. (However, the possibility of multiple steady states should be considered in future work.)

After the glucose pulse a 4 mM glutamine pulse was given to Jurkat. The response of select nutrients observed is essentially as predicted given the information gathered in the plate assay.

Upon addition of glutamine there was a rapid increase in both alanine and glutamate production. The addition of glutamine resulted in no change in the levels of glucose and
lactate detected. The stability of lactate production observed cast doubt on the whether the **complete** glutaminolytic pathway is functioning in Jurkat, but can not be used as definitive proof that this pathway is not important. Future work with [U-14C] glutamine is required for determining what role glutaminolysis plays in the metabolism of glutamine in Jurkat.

Aspartate levels do not change during the course of the glutamine pulse. From the plate assay, it was observed that lactate greatly reduces the level of aspartate that accumulates. With steady state lactate levels at ~20 mM this may explain why no net increase in aspartate levels were observed when 4 mM glutamine was added to the reactor. This also cast doubt as to the relative importance of AspAT in the breakdown of glutamine under reactor conditions. (But again it must be emphasized, that aspartate is also a precursor for many important biosynthetic products. Therefore, the lack of aspartate accumulation should not be misinterpreted as a lack of aspartate formation.)

The disturbance in amino acid levels at 75 hours, just as glutamine levels return to normal, will require further investigation to determine if the increases observed in serine, glycine, alanine, aspartate, and glutamate levels are real or just an experimental artifact.

The pyruvate pulse produced a large increase in lactic acid production as expected. Also, a slight decrease in glutamine utilization was observed. Pyruvate utilization while apparently not causing any noticeable changes in glucose metabolism, appears to affect glutamine metabolism to some extent.

Almost all the pyruvate formed from glucose appears to be required to regenerate NAD via LDH (Table III.3.1), this results in little, if any, glucose derived pyruvate being available for complete oxidation in the TCA cycle. (The high rate of lactate production also implies that the cells ability to transport NADH into the mitochondria, via the malate-aspartate shuttle, to regenerate NAD may be limited.) The pulsed pyruvate, on the other hand, is not required for the regeneration of NAD. The decrease in glutamine utilization (oxidation) therefore may be the result of increased pyruvate (pulse) oxidation.
While most of the amino acid data for this run is considerably more scattered than usual one important observation can be seen in regard to changing amino acid levels. The pyruvate pulse causes a very rapid drop in the levels of both isoleucine and leucine detected. Other essential amino acids remain relatively constant throughout the pulse, suggesting that an increase in protein synthesis is not responsible for the drop in isoleucine and leucine levels.

Very little information is available on branched chain amino acid and their role in mammalian cell metabolism. But from the dramatic decreases observed in this pulse and the potential use of branched chain amino acids as an oxidative fuel, future consideration as to their effect on mammalian cell metabolism and energy production is warranted.

The pyruvate pulse also solved the mystery behind the decrease in cell concentration observed after the glucose pulse was initiated. Throughout the early part of the pyruvate pulse, the addition of base to the reactor was not required since the pH in the reactor remained very close to the setpoint value. However, in the later half of the experiment as lactic acid concentrations began to rise the pH began to drop and NaOH addition was once again required. While the resumption of base addition restored the pH to its set point value, it also resulted in a sharp decrease in cell concentration.

Figure IV.3.1 is the cumulative cell concentration data for all pulse experiments performed. From this figure the problem of NaOH additions is apparent. After the glucose addition a very dramatic decrease in cell concentration is observed, as the demand for NaOH addition reached its peak with the maximal rates of lactate production. After about 5 days the cell levels returned to approximately its prepulse value. During this time, the base reservoir became empty and NaOH addition ceased (lowest pH ~6.82). Over the course of the next 12 days the cell concentrations obtained during the glutamine and pyruvate pulses appear to reach a new steady state level that was significantly higher than those previously observed. As mentioned above with the pyruvate pulse came the resumption of base
addition, the steady state cell concentration returned to the levels previously observed when NaOH was added to the reactor.

From figure IV.3.1 it is evident that the base addition system as currently configured has serve limitations, and corrective measures need to be taken before future experiments begin. Below are listed some possible solutions to this problem:
1) The addition of NaOH for base control should be discontinued, and other means of base control should be found. Other possible methods include the direct addition of a NaOH substitute (Perhaps sodium bicarbonate would work). Or, if the rate of CO₂ stripping can be increased the pH of the media would rise and base addition per say would not be required(Perhaps increased gas flow rates could be used to raise the pH of the media).

2) Part of NaOH toxicity is derived from the way initial contact is made with the reactor media(cells). Base is pumped directly into the reactor, and the point where initial contact takes place is marked by strands of dead cells and cell debris. If a cell free area could be isolated in the reactor where base can be diluted with media before coming into contact with cells, the toxicity of NaOH observed may be greatly reduced.

Listed above are a few ways in which it may be possible to eliminate the problem associated with NaOH addition. With the problem of NaOH toxicity eliminated, the results obtained in future experiment will be a better reflection of the changes in mammalian cell metabolism associated with a change in nutrient level.

The pulse of 10 mM oxaloacetate and 2.5 mm glutamate was one of the more interesting reactor experiments preformed. In this experiment the intention was to pulse with oxaloacetate and glutamate. Instead the pulse consisted of oxaloacetate, glutamate, and PYRUVATE. The spontaneous decarboxylation of oxaloacetate to pyruvate proved to be a bit of a surprise. But nevertheless interesting results were observed.

The addition of oxaloacetate(pyruvate) resulted in a dramatic decrease in glutamine utilization. While this may have resulted, in part, from the pyruvate addition, the decrease in glutamine utilization and ammonium production was far greater than those observed with the pyruvate pulse alone. Additionally, this was the only pulse that was able to affect
aspartate production (accumulation), albeit only very transiently. The pulse also caused a rapid rise in the levels of alanine and glycine, while the levels of glutamate, leucine, and isoleucine decreased.

The lack of available oxaloacetate and glutamate appear to be the reason behind the relative inactivity observed for AspAT in reactor experiments. The conclusions reached by Newsholme \[44\] in his radiolabelling studies as to the importance of aspartate aminotransferase in the breakdown of glutamine appear to be in question. Under his assay conditions, the levels of oxaloacetate and glutamate may be abnormally high. Under 'normal' condition of cell growth, the level of oxaloacetate (and possibly glutamate) in the mitochondria is probably limiting, hence the apparent activity of AspAT would be low. Thus under 'normal' conditions the relative importance of glutamate dehydrogenase or AlaAT in the breakdown of glutamine need to be examined more closely in the future works.

Future experiments would also benefit from pulses of important TCA cycle intermediates like citrate, oxaloacetate, 2-oxoglutarate, and malate. The addition of this data should make addressing the question as to how glutamine and glucose metabolism effect each other more manageable.

A final pulse of 0.5X RPMI amino acids (excluding glutamine) was performed to determine if any of the essential amino acids were in limited supply. As seen in Figure IV.3.1 the addition of this pulse did not bring about any significant increase in cell numbers. Since no one pulse of a given nutrient (each of which are essential for growth) increased cell levels, the reactor as described at a dilution rate of 0.372 day\(^{-1}\) was operating in region of multiple nutrient limitation.

All the reactor data presented to this point was for a dilution rate of 0.372 day\(^{-1}\). As mentioned at the beginning of this section it would of interest to compare the results
obtained at this dilution rate to those from a higher dilution rate where nutrients would be abundant. The comparison of these two states could provide some valuable insights as to how Jurkat shifts its overall metabolic rate.

With the two reactor system described in Section II.5 the question arises: why wasn't this done in this work? This answer to question serves to introduce the most serious problem encountered with the current reactor configuration.

Figure IV.3.2 represent the failed attempt to observe transient responses of Jurkat to metabolite changes at a dilution rate of 0.49 day⁻¹.

![Graph showing the inability of Jurkat to reach a steady state after a 10 mM pulse change in glucose concentration.](image)

**Figure IV.3.2 Inability of Jurkat, Maintained at a Dilution Rate of 0.49 day⁻¹, to Reach a Steady State after a 10 mM Pulse Change in Glucose Concentration.**
At time=0, a 10 mM glucose pulse was added to the reactor. From this figure it is evident that for a time span of 300 hours the culture never reached a steady state. This figure clearly shows a gradually increase in cell concentration with time. It appears that the current reactor setup is a perfusion system in disguise at a dilution rate of 0.49 day\(^{-1}\)

The seriousness of this problem makes it an absolute must that some corrective measure be taken before any additional experiments are performed. The basis of the problem lies in both the mixing capabilities of the current marine impeller (at 80 RPM), and the way in which spent media is removed from the reactor.

1) The marine impeller operating at 80 RPM results in incomplete mixing of the reactor contents. With critical, poorly mixed regions confined an area around the impeller shaft, and a thin film that covers the surface of the reactor fluid.

2) Media is withdrawn from the reactor surface as described in Section II.5. The media withdrawn from the surface of the reactor unfortunately has a lower concentration of cells than that of the bulk of the reactor medium has. Hence, the reactor as describe performs as a perfusion reactor when operated continuously.

Some possible corrective measures that could be implemented include:

1) Keeping the media removal system the same and making correction that end the mixing problem, is one possible alternative. Mixing can be improved in a number of ways. A faster agitation speed can be used or a new impeller with improved mixing capabilities can be selected. With Jurkat being relatively sensitive to shear either corrections mentioned above must be approached with caution, or the results could be the improved mixing of dead cells.

2) Keeping the agitation system as it is and improving the media removal system may be a more attractive method of solving this problem. The easiest solution here would be to place the draw off tube well below the surface of the liquid where
mixing is improved, and drawing liquid at the same rate as it is added. One problem that exist here is that the flow rates used are very low. Perfectly matching the flow rate in and the flow rate out would be next to impossible. Using the same pump with two heads, one for inflow and one for outflow, may solve this problem. However, the tubing used for media addition has an inside diameter of 0.8 mm and a very real possibility exists that the outflow tubing would become clogged with cells.

One method that should work would be the use of some sort of level sensing devise (like an antifoam probe). When the liquid reaches the probe the waste pump would be turned on. When the liquid is below the probe the waste pump would be turned off. With this system the draw off tube can be placed anywhere in the reactor, and any tubing can be used. This solution would require an additional parameter module with two level control sensor cards.

The final problem with the current reactor system to be addressed can be seen in Figure II.3.1. Here for some unknown reason the temperature in the reactor spikes. Two such instances are clearly visible in this figure. The temperature control system outside of these spikes appears to being functioning well. The temperature spikes observed here can be harmful to the cells if the temperature rises high enough. Also, the temperature spikes may result in (un)known changes in the metabolism of Jurkat. Changes in metabolic functions associated with temperature increases are well documented. Lanks[31] observed both an increase in glutamine oxidation and lactate production after cells were exposed to elevated temperatures.

The reactor system as currently configured has no means of cooling, therefore the temperature spikes cannot be controlled by cooling. The only method available for eliminating the spikes would be to shut down the heating system after a high alarm has been breached. While the present system software does provide for alarms (high and low), the alarms turn accessories 'on' not 'off'. Therefore the only way to prevent this problem
would be to use an external device (PC) to receive the alarm warning and send a message to the reactor system (supervisor module) to disable the heating blanket. Once the temperature has returned to a normal level the computer can send another signal telling the supervisor to enable the heating blanket.

In this section the results obtained from the reactor studies were examined in some detail. The limitation of the current Applikon configuration were addressed, and possible course of action were suggested.

The use of continuous culture provides many opportunities for studying mammalian cell metabolism. With the problems mention in this section resolved, a great deal can be learned about the metabolism of Jurkat through the use of continuous culture.
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