MEDIA FOR SEMIBATCH CULTURING OF MAMMALIAN CELLS

A THESIS

Presented to

The Faculty of the Division of Graduate Studies

By

Roberto Rafael Rivera Castillo

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Chemical Engineering

Georgia Institute of Technology

May, 1978

MEDIA FOR SEMIBATCH

CULTURING OF MAMMALIAN CELLS

Approved:

Ronnie S. Roberts, Chairman

James Diez

William Ernst

Date approved by Chairman May 19, 1978

ACKNOWLEDGMENTS

I was very fortunate to have as advisor, Dr. Ronnie S. Roberts, who besides being the author of the original idea, taught me, with Weyman Donaway, most of what I know of tissue culture and provided multiple suggestions, encouragement and friendship that made this work possible. I am also very grateful to Dr. James Diez whose invaluable help led this work to conclusion and introduced to me the use of gas-liquid chromatography to analyze amino acids. I would like to thank Dr. G. L. Bridger who provided economic support and to Dr. William Ernst who acceded, at the last minute, to revise my thesis draft.

I received many valuable suggestions and help from Dr. Sheldon May, Dr. W. T. Ziegler, Larry Stratchan, Maria de los Angeles Contreras, Dr. William Bale, Dr. Jerry Hubbard, Dr. Mark White, Dr. Roy Wiggins and Michael Soule-Hinds. I was fortunate to receive the assistance of the staff of Chemical Engineering and in particular the aid of Nancy Staton, Mary Dupriest, Cassie Harmon, Janice Cox and Charlie Blackwood.

I am grateful to the staff of the School of Biology, to Don Lillie and Gerry Doninger in the glass blowing shop and to the staff at Price Gilbert Library. I received greatest collaboration from my laboratory coworkers, David Moore, who saved my cells several times, and lately, David Johnson.

My parents, my brother, Xavier and my sister, Ana Elena provided me with all the support and love that made possible the conclusion of this work that is dedicated to them.

Finally, I would like to thank the typists, Susan Fuller and, in particular, Hazel Gaines for the outstanding job in writing and more than occasionally correcting my work.

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c	Concentration (Moles or cells/unit of time)
k	Specific growth rate (Moles or cells/time · cell)
K	Concentration of substrate S when $k_{max}/_{k} = 2$ (Moles/unit of volume)
Q	Cell and Molar flow rate (Moles or cells/unit of time)
s	substrate (moles)
t	time
T	temperature (°C)
V	volume

Subscripts for a variable "L"

i	variable "L" at time i
j	variable corresponding to species "L"
max	maximum of species "L"
o	initial

SUMMARY

The design of media suitable for the culture of mammalian cells in vitro in semibatch operations is considered with regard to its application in improving significantly the production of such cells. The major limitation in efficiency of past semibatch operations has been recognized as being nutrient depletion. The present study concentrates in feeding the culture a supplement of glucose and several amino acids without upsetting the cell's environment significantly. The concepts involved in the design of semibatch media were tested in the mouse leukemia L 5178 Y. Osmotic pressure, glucose consumption and amino acid metabolism data are reported for both batch and semibatch operations. Amino acid analysis was performed through gas-liquid chromatography. Cell yield of L 5178 Y cells was increased from 1.6×10^6 viable cells/ml to 4.3×10^6 viable cells/ml.

Abstract

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Roberto Rafael Rivera Castillo

69 Pages

Dicected by Dr. Ronnie S. Roberts

The design of media suitable for the culture of mammalian cells in vitro in semibatch operations is considered with regard to its application in improving significantly the production of such cells. The major limitation in efficiency of past semibatch operations has been recognized as being nutrient depletion. The present study concentrates in feeding the culture a supplement of glucose and several amino acids without upsetting the cell's environment significantly. The concepts involved in the design of semibatch madia were tested in the mouse leukemia L 5178 Y. Osmotic pressure, glucose consumption and amino acid metabolism data are reported for both batch and semibatch operations. Amino acid analysis was performed through gas-liquid chromatography. Cell yield of L 5178 Y cells was increased from 1.6×10^6 viable cells/ml to 4.3×10^6 viable cells/ml.

CHAPTER I

INTRODUCTION

The present work is concerned with large scale production of suspended mammalian cell lines. Several authors have pointed out the value of mammalian tissue culture as a biochemical research tool and a means to manufacture important chemicals such as enzymes, hormones, and vaccines (1, 2, 3). Past efforts have been directed toward scale up of conventional tissue culture techniques into semibatch fermentations and chemostats (4, 5, 6, 7).

Mammalian suspension cultures are grown in media which originally were designed for batch culturing *in vitro* (8, 9). The media are such that cells under favorable environmental conditions are able to survive and propagate in it. The media used in the scale up of traditional culture methods for mammalian cells can be modified so as to be suitable for continuous and semicontinuous culturing of mammalian cell lines. The purpose of this work is to introduce the concept of media which are specifically designed for maximum cell yield in the semibatch culturing of mammalian suspensions and apply it to the culturing of the mouse leukemia L 5178 Y.

Problems Encountered in Culturing Mammalian Cell Lines

Mammalian cell culturing introduces new challenges to the treatment of biological systems as encountered in traditional bioengineering technology (7). Mammals are generally known to require at least eight

essential amino acids and mammalian cells require several more when cultured *in vitro*. Furthermore, there are specific requirements for vitamins and growth promoting substances. Tissue culture cells also require an exogenous supply of carbohydrates and a balanced salt solution (8, 9, 10).

There is an absolute necessity to maintain sterile conditions in culturing cells. The use of antibiotics and rigorous application of the aseptic technique makes the process difficult and expensive, particularly when large volume equipment is used (11).

Optimal growth of suspension cells requires very narrow environmental limits. The most important factors, other than nutrition, effecting suspension culturing of cells are (12, 13):

(1) Temperature

(2) Hydrogen Ion Potential (pH) and Carbon Dioxide Tension

(3) Osmotic Pressure

(4) Oxygen Tension and Oxidation Reduction Potential (ORP)

(5) Agitation

(6) Cell Concentration and Density Inhibition

(7) Innocula Sizes

(8) Serum and Growth Promoting Factors

(9) Hormones

When cells are cultured under batch conditions, the population dynamics as described by Hahn (14) apply in three main phases as shown in Figure 1.

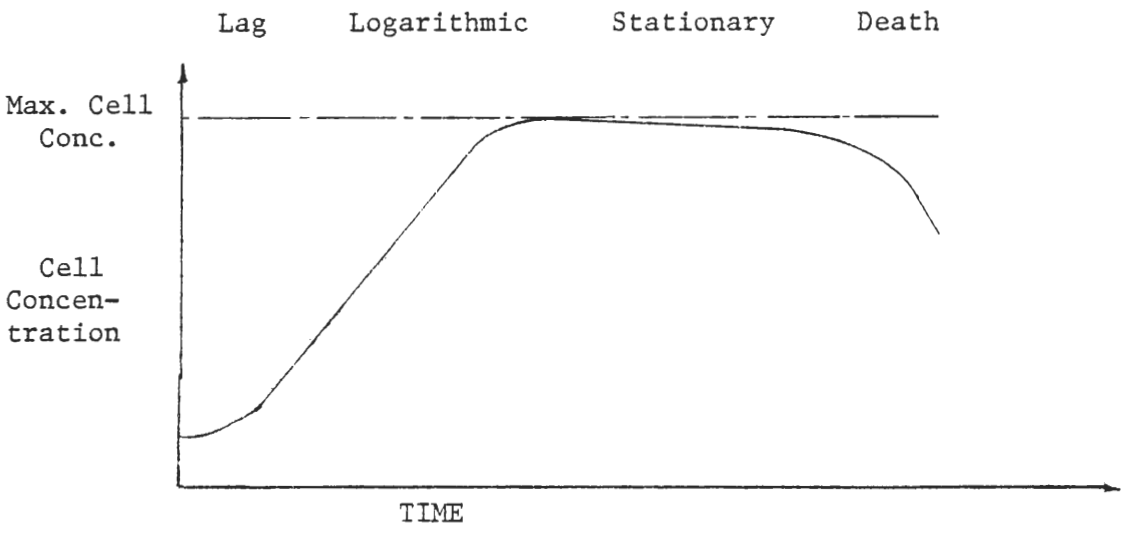

Figure 1. Mammalian cell Population Dynamics

First a lag phase may appear as a result of initial conditions or the cultures previous history (15, 16). Second the cells reproduce themselves exponentially according to the following reaction (17):

$$\text{Cells} + \text{Substrate} \rightarrow \text{More Cells} + \text{Energy} + \text{Byproducts} \qquad (1)$$

Exponential growth corresponds to the logarithmic phase. The growth of the cells follows the form of an autocatalytic reaction in batch culturing. This is represented by Equation 2.

$$\frac{dN}{dt} = kN \qquad (2)$$

Where k is defined as the specific growth. Integration from time t = 0 and initial cell number N_0 to time t and cell number N yields:

$$N = N_0 e^{kt} \qquad (3)$$

The doubling time t_d occurs when $N/N_0 = 2$. This leads to Equation 4.

$$t_d = \frac{\ln 2}{k} \tag{4}$$

It follows immediately that

$$N = N_0 2^{t/td} \tag{5}$$

After the logarithmic phase the cells enter a plateau called the stationary phase. Cell size distribution measurements show that protein synthesis decreases as cells find difficulty in producing the necessary metabolites for mitosis. This is possibly due to nutrient depletion. The cells subsist for a while at an essentially constant concentration but after some time, they die (15, 18).

All phases vary from cell line to cell line but in general in agitated suspension cultures after the culture begins dying it will continue to do so, regardless of changes made to the medium. Hence, once the cells reach the stationary phase it becomes very difficult or impossible to save the culture.

As a consequence, laboratories that cultivate mammalian suspensions follow an endless cycle of logarithmic growth and successive dilution. Figure 2. (19).

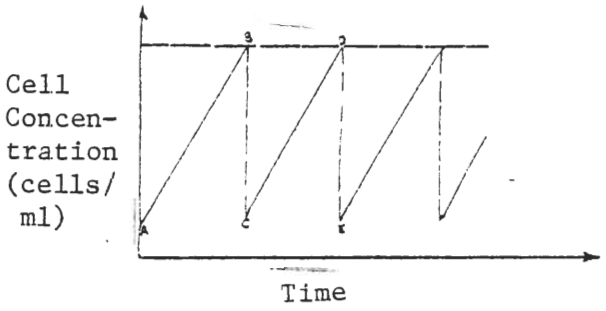

Figure 2. Pattern of Cell Concentration in Traditional Semibatch Growth
Line segment AB - represents a period of batch growth.
BC - is a dilution point. The cycle is repeated in CD and DE.

CHAPTER II

REVIEW OF PERTINENT LITERATURE

Suspension cultures were first reported by Owens, Gey and Gey in 1954 (20). One year later Earle et al. (21) published a paper that aroused widespread interest in suspension cultures. By 1957 McLimans and coworkers reported the use of a five liter fermentor and a year later, a twenty liter unit (22, 23). In the decades that followed, a number of massive suspension culture methods were reported (24, 25, 26, 27).

The most common method in massive cultivation consists of innoculating a small suspension culture vessel and allowing the concentration of cells to grow to a point just before maximum population density. The next step is to sequentially transfer the culture to larger vessels containing fresh medium. Finally, when the full capacity of the combined vessels is reached, the culture is harvested, perhaps leaving a small portion to start the process again (28). This is shown in Chapter III. Another procedure is to innoculate the cells in a large vessel containing a small amount of fresh medium. As the population doubles the suspension is rediluted with fresh media. The cell population is not allowed to achieve maximum concentration until the vessel is filled (27).

Another approach to massive suspension cultures is to employ continuous cultivation of cells. Tempest (1965) described a chemostat for culturing microorganisms and pointed out the value of obtaining steady state with constant influx of media and outflow of a microorganism suspension. However, Tempest's chemostat was used for bacteria with doubling

times of the order of 40 minutes as opposed mammalian cell lines generally with doubling times in excess of ten hours. Mammalian cells also have much more rigorous environmental and nutritional requirements than bacteria (29).

Pirt and Callow (30), in reporting a chemostat culture, encountered poor reproducibility in maintaining cell concentration constant. L. K. Nyri (31) has considered the problems and applications of control of environmental factors in semicontinuous cultures using computers. Nyri stresses the need for research in cell-environment interaction and development of new sensor systems.

Himmelfarb (32) described in 1964, a device that could yield suspension concentrations above 10^7 cells/ml, that is five to ten times the concentration usually achieved in semibatch cultivation. The device, known as spin filter, allows fresh medium to be pumped into the culture vessel and removes cell-free medium by means of a rotating filter. Himmelfarb acknowledged that the medium utilized was similar to conventional batch cultivation. Graham and Siminovitch (33) working with monkey kidney cells had previously devised a method to obtain high population densities. The rather cumbersome method consisted of centrifuging and resuspending in fresh medium a cell population without disturbing the logarithmic phase.

In 1976, Nundel et al. (34) described an apparatus that allowed continuous cultures, but had the option of concentrating the suspension by using a filter that permitted detritus to pass but stopped the whole cells. Further clogging was prevented by placing a four-bladed stirrer at the top of the filter. Without the filter, the apparatus would work as an ordinary chemostat, very much like the apparatus of Pirt and Callow.

CHAPTER III

PROPOSED ALTERNATIVE TO MASS CULTURING OF MAMMALIAN CELLS AND IN PARTICULAR TO THE L 5178 Y MOUSE LEUKEMIA LYMPHOBLAST

The present work proposes an alternative approach to mass culturing of mammalian cell lines. The proposed method introduces the concept of a specific medium suitable for semibatch culturing. All the systems described in the previous section make use of conventional batch culturing media. The scope of this work is to introduce and justify the use of medium specifically designed for semibatch culturing and to test the validity of the concept, using an established cell line. In this case, the murine lymphoblast cell line, L 5178 Y, will be used.

Ham (9) gives an interesting account of how conventional media was first designed and Charity Waymouth (11) points out the current thrust in cell nutrition and media design research.

It appears that cell nutrition research peaked in the early sixties. A typical area was the design of a medium for a then recently established cell line. The medium design started with a balanced salt solution containing small amounts of dialyzed serum, amino acids, vitamins, and a source of carbohydrates. A growth curve was determined by minimum doubling times and highest cell population densities. The nutrient concentration was usually selected to be the middle point between minimum and maximum concentrations for optimal growth (9, 10).

Media were designed for a limited number of cell strains and cell nutrition studies appear to be tilted toward Hela and L cells (9).

The question that arises is what causes the cell population to peak after a period of logarithmic growth. One possibility is that a control substance or chalon is excreted by the cells. The chalon reaches a concentration at which, by feedback mechanism, growth is regulated in eucaryotic cells (35). Contact inhibition is possibly more prevalent in monolayer cultures where there is a constant membrane interaction (36) as opposed to a suspended culture where the only cell to cell contact is by collision.

Growth could also be impaired by deficiencies of nutrient diffusion in cell populations of elevated density (37). Cooper, Burt, and Wilson (38) found that cell growth rates were strongly dependent on oxygen levels. Oxygen tension has been related to culturing of Hela cells by Barton (39) who uses a Michaelis-Menten expression for the oxygen uptake by the Hela cells.

The use of Michaelis-Menten expressions (Equation 6) was first suggested by Monod for bacterial systems (40). Equation 6.

$$k_j = \frac{k_{maxj} s_j}{K_j + s_j} \tag{6}$$

Where k_j is the specific growth rate (Equation 2) and k_{maxj} is the maximum specific growth rate. s_j is the concentration of a given nutrient and K_j is the concentration of s_j at $k_{maxj}/k_j = 2$. If K_j is much smaller than s_j or s_j is in large supply (and provided large amounts of S are not toxic or produce a feedback inhibition) then:

$$k_j \simeq k_{maxj} \tag{7}$$

The fact that nutrient material is incorporated into the cellular material has led to the assumption that Equation 2 applies to the depletion of nutrients. The right hand side of Equation 2 would be a change in concentration of a particular substrate and "k" is called the rate of consumption of the given nutrient. If K_j is small as compared to s, then "k" is essentially constant.

This means that the consumption of a given nutrient is proportional to the number of cells in culture. Nundel et al. (34) have used precisely this assumption in the computation of medium requirements for their continuous system with a concentrating device. The assumption of proportionality between cell numbers and nutrient concentration does not always hold. Munyon and Merchant (41) found that lactate was being consumed by the cells even when the concentration of glucose was 50% of the initial level. Consequently, Munyon and Merchant (41) found no correlation between cell number and glucose depletion (LS fibroblasts).

An explanation came in 1957 by Leslie, Fulton, and Sinclair (42) who found that there was a linear relationship between cell number and glucose which was utilized but not converted into lactic and keto-acids.

Zuartouw and Westwood (43) studied the effect of pH, serum, and oxygen concentrations. They concluded that maximum cellular growth occured when pH, oxygen-tension, and serum concentration were such that cell glycolysis rate was lowest. Pirt and Griffiths (44) found that the uptake of most amino acids was greatest during lag and initial logarithmic growth. Since cells have a net production of some nonessential amino acids such as alanine and glycine, it appeared to Pirt and Griffiths that the excreted amino acids would have a sparing action on the uptake of essential amino

acids.

In conclusion, while it may be possible to find a linear relation between cell concentration and nutrient uptake, the presence of other compounds such as intermediary metabolites must be taken into account. The problem of analyzing "rates of consumption" of nutrients is vital to the design of media for batch, semibatch and chemostats cultures. Pirt and Griffiths (44) show that the uptake of amino acids is different from batch to chemostat cultures. Most important of all considerations is that there is good evidence that the first cause of formation of a stationary phase is the depletion of glucose (43) and amino acids (44, 45, 46). In 1967, Griffiths and Pirt (47) determined that maximum population was limited by the supply of amino acids (particularly glutamine) at the end of the logarithmic phase. Griffiths and Pirt added to their cultures initial supplements of amino acids. They found an increase in cell yield of 33% for one supplement and an increase of 14% for another supplement. However, when they combined the supplements they found an increase in cell yield of only 16%. Furthermore, Griffiths and Pirt found that glutamine would limit growth if glucose were present in excess. In 1971 Baker, Birch and Pirt (45) found that increasing amino acid supply by 50% in Hela cell cultures, produced an increased maximum density of 40% with no effect on growth rate and did not introduce a lag phase. Roberts (12) was able to obtain over 100% increase in the yield of mouse plasmacytoma cells by analysis of amino acid requirements of the cells in culture and supplementing the recommended medium by the American Type Culture Collection.

Supplementation of media with nutrients has certain limitations. Roberts (46) et al. found that further attempts to increase yields by

addition of nutrients and vitamins did not improve results. Griffiths and Pirt (44) found also that there was a concentration of glutamine beyond which the maximum yield of LS cells decayed. Furthermore, high concentrations of glucose appear to be toxic (43). Ham (9) investigated the role of six amino acids in reversing toxicity of excess tryptophan.

Devices such as the spin filter of Himmelfarb et al. (32) and elaborate methods such as the ones employed by Graham and Siminovitch (33) show that populations of the order of 10^7 cells/ml or more can be achieved at the expense of large amounts of medium. In such systems the balance in nutrient, vitamins, and growth factors does not change drastically enough to induce departure from logarithmic growth.

The question is whether there is an optimal way to provide the nutrients in order to increase cell yields while keeping maximum growth rates. This should be done without resorting to expensive and complicated devices as the spin filter (32) or very elaborate methods such as the one described by Graham and Siminovitch (33). Furthermore, the spin filter and Graham's and Siminovitch's method do not improve the efficiency of media utilization.

The cell populations even at concentrations of 10^7 occupy relatively a small volume in the medium itself. For example the spherical volume of an L 5178 Y cell was reported by Irimayiri, Hamai, and Inouye (47) to be 1.05×10^{-12} liter which means 10^7 cells per ml would occupy 1% of the solution. Therefore, there is considerable advantage in increasing maximum cell population per unit volume of media used. The use of conventional media usually leads to maximum densities of the order of $1\text{-}2 \times 10^6$ cells/ml. Since the ratio cell-environment is so small, it should be possible to

introduce small changes in the medium so as to optimize the growth of cells.

If cells were allowed to grow under batch conditions and just before they depart from logarithmic growth were supplied with a small concentrated solution of depleted nutrients, the cells would continue to grow beyond the limits encountered by batch growth. (See Figure 3). Furthermore, by appropriate addition of nutrient concentrates, the cells would conceivably attain the high concentrations of Himmelfarb's device or Graham's and Siminovitch's procedure. A constraint is that the addition of nutrients should not affect environmental conditions that would inhibit normal growth or induce cell death.

Under a semibatch regime, the culture of cells could follow one of the following patterns:

(1) A vessel almost filled to capacity allows cells to reach a concentration slightly below the maximum density allowed by batch culturing. By subsequent additions of nutrients the suspension is permitted almost to reach maximum concentrations, then a portion of the system is removed and fresh medium is added. The cycle can be repeated ad infinitum. (See Figure 4).

(2) There is possibility of continuous or semicontinuous withdrawal of suspension. The effect of continuous withdrawal in semibatch reactors is considered by Smith (48). By addition of small substrate supplements the overall volume could be approximated as constant. Furthermore, replacement of solution is needed since there is evaporation of water due to gassing the system with a stream containing nitrogen and small amounts

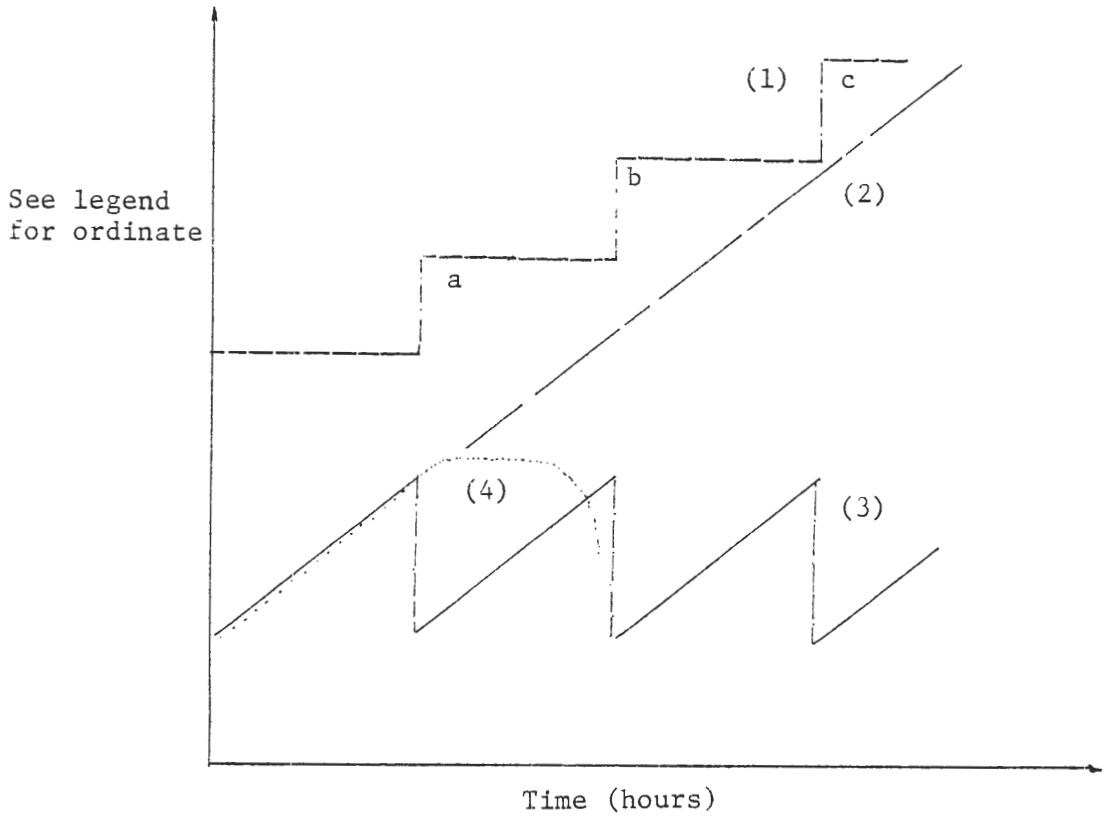

Figure 3. Pattern of Semibatch Culturing Using Sequentially Larger Vessels

(1) Ordinate is culture vessel volume. Starting with a small part of the vessel occupied by the innoculum, the culture size increases as the system is regularly supplied with fresh medium. The "step" increase "a" is half the size of "b" and one fourth the size pf "c".

(2) Ordinate is logarithm of total cell concentration. By taking as a unit volume the space occupied by the innoculum, this line indicates how the overall cell number increases through conventional semibatch culturing.

(3) Ordinate is logarithm of cell concentration (cell/unit volume). This plot is the same as the one shown in Figure 2. The dilution corresponds to an increase in the occupied volume of the vessel. The change in volume is proportional to the overall cell population in the culture. By supplying the culture with fresh medium the depletion of nutrients essential to keep the culture in logarithmic growth is offset. However, the concentration of amino acids that are not preferentially used increases. The amino acids whose concentration levels were formulated in the initial medium to offset the toxic effects of other medium components such as triptophane are wasted.

(4) Ordinate is logarithm of cell concentration (cells/unit volume). The dotted line represents growth of a batch culture and is included to show the effect of not supplying the innoculum with fresh medium.

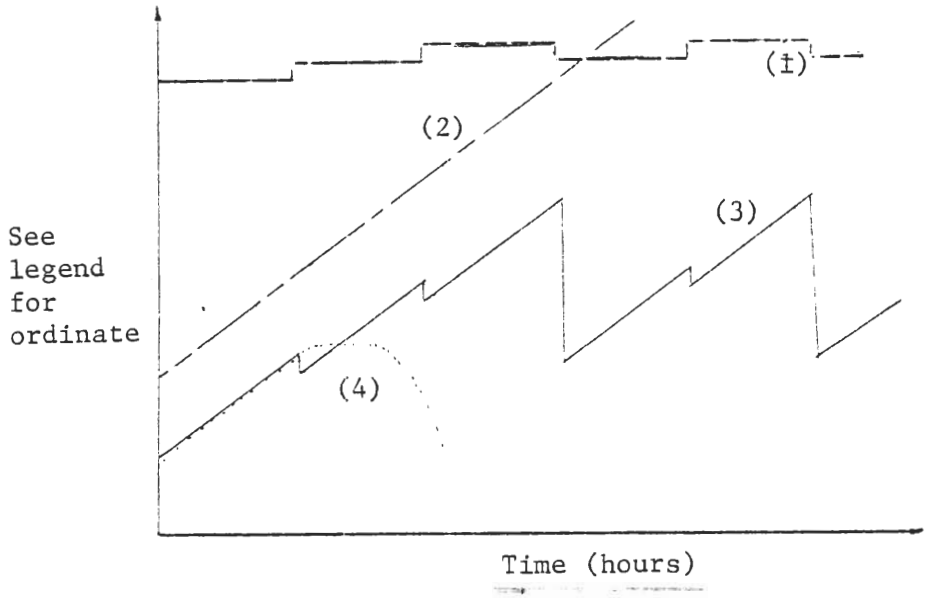

Figure 4. Pattern of Semibatch Mammalian Cell Culture with Medium designed for Semibatch Culturing

(1) Ordinate is culture vessel volume. The plot represents small increases of the vessel which is used for this mode of culture almost at full capacity. The addition of supplements in the experiments carried in the foregoing supplements caused an increase in the vessel volume of the order of 0.5% each time the culture was fed.

(2) Ordinate is logarithm of total cell number. The increase in total cell yield is similar in form as the total cell plot of Figure 3 except that it is expected to be larger as discussed in this chapter. Because the initial vessel is almost filled, the innoculum is larger for a same culture vessel than in Figure 3. Since the cell doubling time is the same in both cases (Figures 3 and 4) the total cell number for Figure 4 is always greater than in Figure 3.

(3) Ordinate is logarithm of cell concentration (cells/unit volume). If the cause of departure from logarithmic growth in a culture is nutrient(s) depletion, then after the culture is fed, the cell concentration should continue to increase as the suspension maintains logarithmic growth. The feeding points are slightly exaggerated to indicate the dilution effect caused by adding a concentrated supplement. The culture is also resuspended in fresh medium or diluted to a large extent to replace "spent" serum and other nutrients (such as vitamins) that may not be present in the supplement.

(4) Ordinate is logarithm of cell concentration (cells/unit volume). The dotted line represents batch culturing as in Figure 1 and is shown for reference purposes.

of oxygen and carbon dioxide. (See Experimental Methods).

Equation 2 could be expressed in terms of concentration dividing both sides of equation 2 by the volume of the vessel.

$$\frac{dc}{dt} = kc \geq k \max c \tag{8}$$

A cell balance along the culture vessel (semibatch reactor) is:

rate of output of cells	+	rate of generation of cells	−	total accumulation or depletion of cells

or:

$$-Qc + kcV = V \frac{dc}{dt} \tag{9}$$

where Q is the exit rate in volume per unit of time. kcV is the rate of generation of cells of $\frac{dc}{dt}$ for batch culturing. Rearrangement of Equation (5) leads to:

$$\frac{dc}{dt} = -\frac{Q}{V} + k\,c \tag{10}$$

finally, integrating from c_0, initial concentration to c and from time zero to t:

$$c = c_0 e^{(-Q/V + k)t} \tag{11}$$

Figure 5 shows the effect of continuous withdrawal at increasingly larger -Q/V (inverse of the residence time). There is a point at which -Q/V = k where the concentration is constant. If the concentration in which -Q/V = k is the highest possible concentration, then the semibatch system operates at highest efficiency.

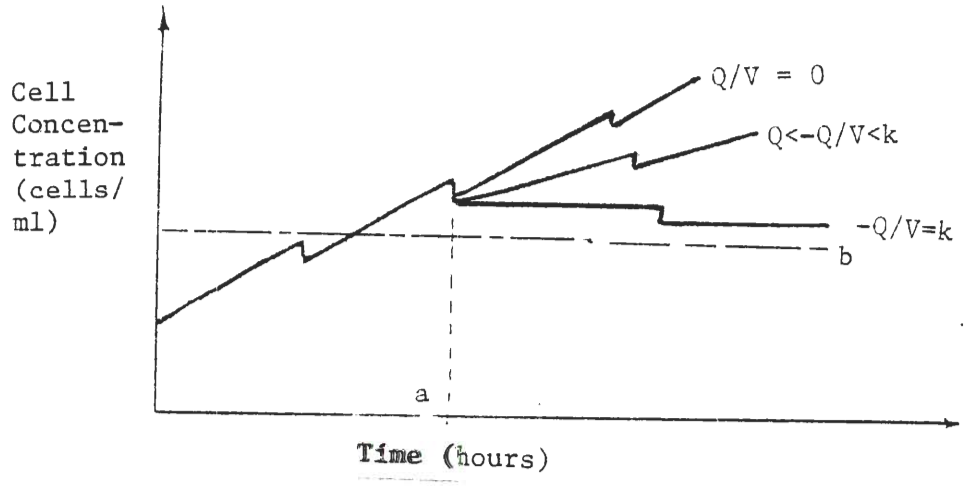

Figure 5. Effect of Continuous Cell Withdrawal in Proposed Semibatch Culturing

a - Point at which withdrawal starts (arbitrary, in this figure chosen as a dilution point). Dilution points shown as a small decrease of cell concentration are also feeding points where the dilution is caused by the addition of the nutrient supplement.
b - Maximum cell population in conventional cell culturing.

The semibatch patterns described in the literature survey could be benefitted by:

(1) Employing the total volume of the culture vessel:

(2) Achieving cell concentrations in excess of those obtained by using batch media;

(3) Maintaining higher cell concentrations than in batch culturing. (This would result in higher production rates per unit volume);

(4) Choosing media so as to minimize costs of nutrients and serum by taking advantage of substrates that are not consumed in batch media but are kept to maintain a balanced solution (10). (That is a solution in which the particular combination of nutrient does not allow one nutrient to become toxic).

CHAPTER IV

MATERIALS AND METHODS

Cell Strain

L 5178 Y mouse lymphoblast cells were obtained from Dr. T. J. Yang, University of Connecticut, Storrs, Connecticut, U.S.A.

The cells have been grown in a 10% heat inactivated horse serum solution of Fisher's media and each liter was supplemented with 10 mls of 100 mMolar solution of glutamine. The L 5178 Y strain has been cultured in our laboratory since March 1976, always under logarithmic phase. The cells were passaged on the average of every two days.

The L 5178 Y have an average diameter of 12.6 microns as determined by David Moore in our laboratory, using a Model F. Coulter Counter and reported elsewhere in the literature (48). The media contained 100 units/ml of penicillin and 100 mg/ml of streptomycin. The media was sterilized by filtration using glass filters.

Culture Methods

Cell counts were performed with an haemacytometer and occasionally verified with the Coulter Counter. All counts in this work are reported as viable cells. Viability was 95% or above unless otherwise stated. Viability was determined by exclusion of 0.5 percent trypan blue solution.

The cells were cultivated in three modes. First, they were kept in small bottles in a constant temperature incubator under conditions in Table 1. Second, the cells were cultured in sealed small bottles,

Table 1. Stock Solution Culturing Conditions

pH	7.3- 6.8
Temperature	37.8 $\pm$ 0.2°C
Agitation	60-70 rpm for bottles with stirrer
Gas stream	sealed
Surface to depth ratio	variable
Average culture size	50 ml
Average innoculum [a]	1.5×10^5 cells/ml

a - viable cells

Stock solution refers to a culture of L 5178 Y cells in sealed non-agitated flasks and in sealed flasks provided of a magnetic stirrer. The cells from non-agitated flasks were transferred to agitated flasks so that all cells involved in spinner flasks experiments had already been grown in agitated suspension for at least one generation time period.

each provided with a teflon covered magnetic bar stirrer in a full view incubator. These two modes were considered stock and all experiments innocula were taken from closed sealed suspensions that had been under agitation for at least 20 hours, which is larger than the reported generation time for this strain (49).

Third, the experiments were performed with Bellco 250 ml spinner flasks. The apparatus is depicted in Figure 6 and with cultivation conditions given in Table 2. The main components of the apparatus are as follows:

(1) Bellco Spinner Flask 250 ml with magnetic stirrer

(2) Sampling needle

(3) Cotton filled glass vent

(4) Cotton filled gas inlet (Clinical Blood Gas)

(5) Full view constant temperature (6) incubator with inlet gas flow meter

(6) Thermometer

The cell innocula were 250,000 cells/ml that were previously centrifuged at 120g and resuspended in fresh media.

There was no attempt to measure and control oxygen tension (PO_2) and oxidation-reduction potential (ORP). The relationship between ORP and PO_2 is discussed by Fish et al (13). The importance of analyzing PO_2 has been established by Daniels, et al. (50) and Cooper, Burt, and Wilson (30). It also has been shown that oxygen tension is related to enzymatic activity and respiration (51) and consequently glucose uptake (43). Kilburn and coworkers (51, 52) have studied oxygen tension in uncontrolled environments. They determined a sharp drop in oxygen tension during

Table 2. Growth Conditions in Spinner Flask Cultures (L 5178 Y Cells)

pH	7.2 - 6.9
Osmotic pressure	300-340[a] mosm
Temperature	37.8 $\pm$ 0.1°C
Agitation	60 - 70 rpm
Open surface to depth ratio	8 cm (average)
Gas stream	10 ml/min [b]
Innoculum	2.5×10^5 viable cells/ml
Average culture size	70 ml

[a] - The suspension register 350 mosm at late stationary phase.

[b] - Gas stream is 3.5% CO_2, 6% O_2, balance in nitrogen.

Spinner flask cultures of L 5178 Y cells were innoculated with cells resuspended in fresh medium by means of a 50 ml syringe screwed into the feeding needle. The collection of the newly resuspended cells was performed with a hypodermic syringe whose needle was unscrewed and then attached into the feeding needle under flame.

logarithmic growth which falls short of the 40 mmHg to 100 mmHg of PO_2 (5% to 13% PO_2) range estimated to be optimum (43, 53, 54, 55). The maximum cell growth yields and rates are generally decreased by lack of control of PO_2 and ORP. Taylor et al. (53) found that for L cells (with optimum PO_2 at 9%), there was a 20% decrease in cell yield oxygen tensions as low as 5% or as high as 20%.

There is, however, an advantage in testing a medium for semibatch culturing in an uncontrolled ORP. A predicted increase of cell yield in this case would have to be attributed to depleted nutrient replacement rather than improvement in control of oxygen tension and oxygen reduction potential.

Serum replacement and the addition of hormones has been considered by Jainchill and Todaro (56). The loss of effectiveness in the growing factors after several days could only be solved by replacing the serum or by resolving analytically the serum composition. Since the growing factors in the serum have not yet been fully identified, the continuous supply of serum is necessary in semibatch operations (57).

Physical Parameter and Nutrient Consumption Measurements

Osmotic pressure was estimated using an osmometer Osmete 2000 pH measurements were evaluated using a pH meter, and by phenyl red. Glucose concentration was measured using a YSI analyzer. Amino acid levels were analyzed by gas-liquid chromatography using the method of Gehrke et al. (58) with some modifications (see Appendix I). A Varian two column 7240 gas-liquid chromatograph with differential flame ionization detectors was employed in the analysis. A Houston recorder and an Elmer-Perkins integrator were attached for data acquisition. Temperature

programming was set at $4^{\circ}C$/min. from five minutes preheating at 98°C to 228°C. The amino acid analysis was performed in glass columns 1.5 meters long and 4 mm in internal diameter. All reagents were of analytical grade. A typical chromatogram of Fisher's medium is shown in Appendix I.

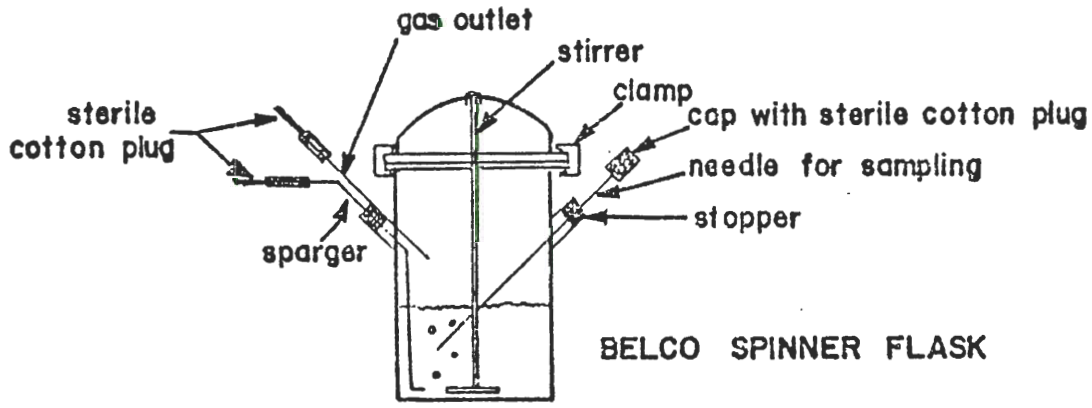

Figure 6. Schematic Equipment for Batch and Semibatch Culturing of Mammalian Cells

CHAPTER V

PRELIMINARY EXPERIMENTS AND DESIGN CONSIDERATIONS FOR A MEDIUM SUITABLE FOR SEMIBATCH CULTURING OF L 5178 Y CELLS

The first step toward designing an improved method was to consider the traditional culture conditions of the L 5178 Y. After gaining experience in batch cultivation, some possible environmental effects were studied. The last step was to determine glucose and amino acid consumption to design a new medium based on the depletion of a few selected nutrients.

The methods and conditions of culturing L 5178 Y cells as described in Chapter IV have been used by a large number of researchers (49, 59, 60). There are other media and serum that could be used to culture L 5178 Y cells, Yang and Vas (61) were able to adapt growth of L 5178 Y cells from horse to rabbit serum and Brennan (62) et al. have used McCoy's (double strength) medium.

Results

The growth dynamics of a spinner culture with conditions as in Table 2 are depicted in Figure 7 and the results summarized in Table 3. When the cell population entered the stationary phase, viability decreased approximately 10% after several hours. Then the cells started clumping into groups of four or more cells. The cells also showed membrane damage (apparition of multiple blebs). After a long period (about 24 hours), the culture showed a large number of dead cells. When

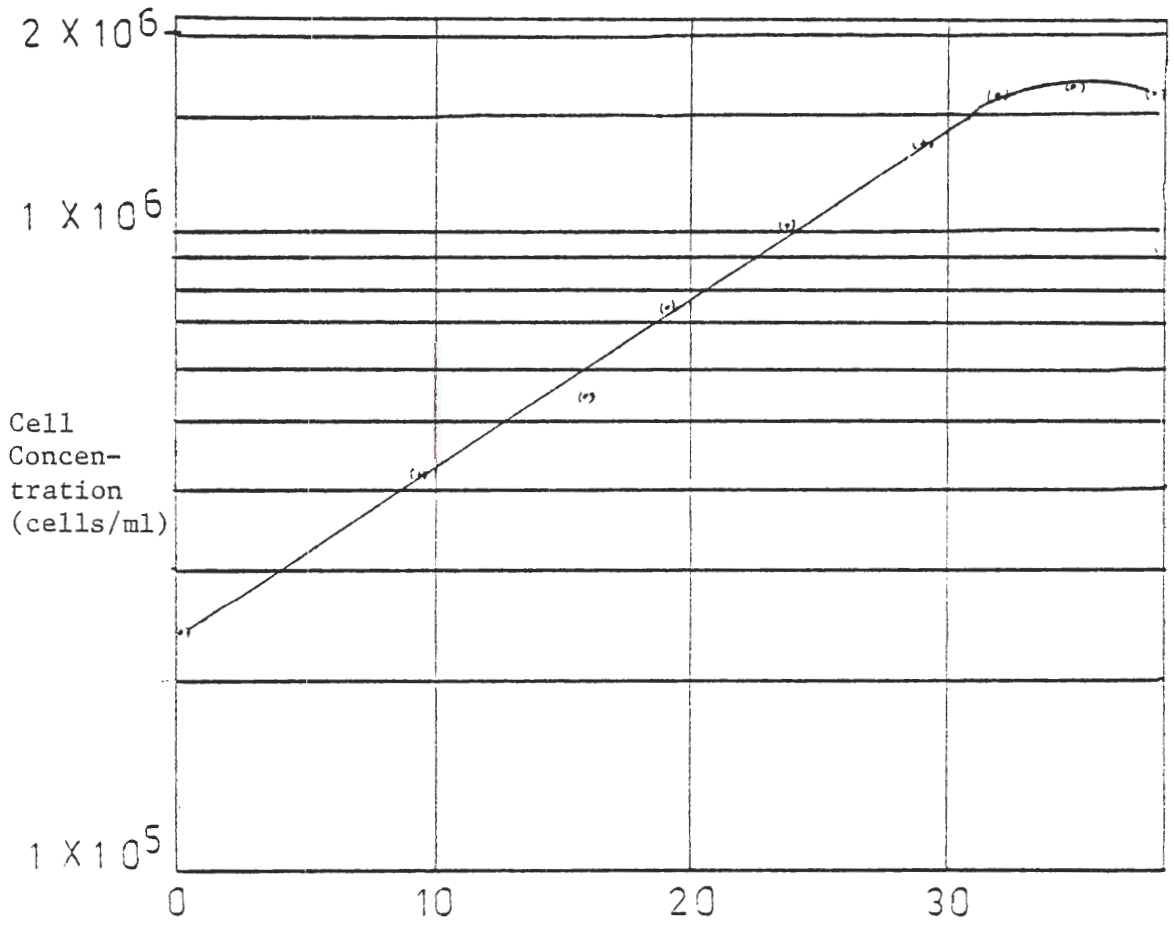

Figure 7. Population Dynamics of Batch Culture of L 5178 Y Cells

The plot was obtained by plotting data from four experiments. The population dynamics of L 5178 Y cells follows the traditional growth curve described in Figure 1. The lag phase for the L 5178 Y cells with culture conditions as in Table 2 appears to be very small to the point of not being noticeable at all. Haemacytometer counts have an error of $\pm$ 5% and each count is the average of three to six observations.

Table 3. Population Dynamics of L 5178 Y Cells in Batch Culture

Time (hours)	Viable Cell Concentration (cells/ml) ± 5%
0	2.5×10^5
36	1.65×10^6*
48	1.35×10^6

*Maximum cell concentration.

Results were the arithmetic average at 36 hours of four experiments. The minimum was 1.6×10^6 cells/ml and the maximum 1.7×10^6 cells/ml. STD deviation 4×10^4.

Table 4. Glucose Metabolism of L 5178 Y Cells

Time	Concentration of glucose in the medium (mg/liter)	Uptake rate from zero hours (mMoles/cell x hour) x 10^{11}
0	9.9×10^2	18.9
36	1.8×10^2	18.9
48	0.05×10^2	

Results for 0 and 36 hours were obtained by arithmetic average of three determinations. The average rate of uptake from equation 13 had a std. deviation of .353. The result at 48 hours was taken from a single determination.

innoculum was of the order of 1.5×10^5 cell/ml, the maximum population achieved was about 1.2×10^6 cell/ml. The pH started sometimes at about 7.5 but after two hours it would invariably turn to 7.2. Probably a pH of 7.2 represents the clinical blood gas reaching equilibrium with the bicarbonate buffered solution. The pH changed in the culture media from 7.2 to about 6.8 because the cells produced carbon dioxide and other metabolic products. Osmotic pressure varied between 323-338 mosm. It was found that under batch culturing osmotic pressure would remain at a constant value until after the cells had reached stationary phase. After the cell suspension had peaked an increase in the osmotic pressure was recorded to approximately 340 mosm or about an 8% increase.

The rate of consumption of nutrients is obtained from Equation 5. Equation 5 assumes that the uptake of a nutrient is proportional to the number of cells. The limitations of this assumption were briefly discussed in Chapter II. If s_j is the amount of substrate

$$\frac{ds_j}{dt} = k_j N \qquad \text{Equation (5)}$$

dividing s_j by the volume V. The concentration cs_j is obtained by integration if the volume is constant.

$$\int_{C_{s_{ji}}}^{C_{s_{ji+1}}} dCs_i = \int_{t_i}^{t_{i+1}} k_{i/V} \, N dt = \int_{t_i}^{t_{i+1}} ks_i N_i 2^{t/td} dt \qquad (12)$$

or if:

$$k_{j/_V} = k_{s_j} \tag{13}$$

$$k_{s_j} = \frac{\left[C_{s_{j_{i+1}}} - C_{x_{j_i}} \right]}{\left[t_{i+1} - t_i \right]} \quad \frac{\left[\ln N_{i+1} - \ln N_i \right]}{\left[n_{i+1} - N_i \right]}$$

Glucose consumption under conditions of Table 3 is reported on Table 4 and consumption of certain amino acids is depicted on Table 5.

Discussion of Results

The results were in good agreement with those obtained by Watanabe and Okada (49) who studied the effect of temperature on batch culturing of L 5178 Y cells. At 37 Okada and Watanabe report a doubling time of 11.5 hours which is somewhat lower than 14 hours as recorded in Table 3. A discrepancy between results of this work and those of Okada and Watanabe is the absence of a lag phase in the cultures of this work. The doubling times of the present work are in better agreement with those obtained by Yang and Vas (61). As far as the maximum population is concerned, Okada and Watanabe arrived at concentrations of the order of 1.2×10^6 cells/ml (18, 49). This is consistent with the results of this work when innocula were of the order of 10^5 cells/ml. Okada and Watanabe started their cultures at 5×10^4 cells/ml.

The absence of a lag phase may be related to the culture conditions. Merchant et al. (15) found no lag when cells in early logarithmic period

Table 5. Amino Acid Metabolism in Batch Culturing of L 5178 Y Cells

Amino acid	Concentration initial (mg/l)	Concentration final (mg/l)	Rate (Uptake) (mMoles/hr x cell x 10^{-11})	% Consumed
L-alanine	5.8	32	.67	produced
L-valine	62	35	-0.98	-43%
L-glycine	5.4	1.8	-0.21	-66%
L-isoleucine	54	34	-1.01	-38%
L-leucine	28	7.3	-0.68	-74%
L-proline	6.1	2.6	-0.13	-57%
L-threonine	26	14	-0.42	-45%
L-serine	19	7.0	-0.49	-63%
L-methionine	158 a	93	-1.88	-41%
L-phenylalanine	59	37	-0.60	-38%
L-aspartic acid [b]	8.2	4.6	-0.12	-43%
L-glutamic acid [b]	252	133	-3.48	-47%
L-tyrosine	61	58	-0.07	-4.8%
L-lysine	29	17	-0.34	-40%

a - Approximately 30% more methionine than expected appeared in the initial medium by gc analysis.

b - See notes on Tables 8 and 9.

The results of this table correspond to a single experiment (one determination). Uptake rates were computed using Equation 13. Initial culture conditions correspond to those of Table 2. Data from Figure 7 was used to estimate results on third column by means of Equation 13. Results on the first two columns were obtained by gas-liquid chromatographic analysis as outlined in Chapter IV and Appendix I.

were placed in fresh media. Lag phase may be related to the ability of cells to "condition" their media. The use of healthy population even in the late logarithmic period at high initial concentrations (2.5×10^5 cells/ml) could explain the unexpected lack of lag phase. The term "to condition the medium" is an alteration to the medium by cells (54).

Okada and Watanabe (49) had determined the optimal temperature for the growth of L 5178 Y cells to between 37°C and 38°C. Eagle (63) determined that for mouse cell lines, variations of pH from 7.4 to 6.8 did not greatly affect growth dynamics. Pirt and Thackeray (64) found that their chemostat system (ERK cells) had a pH variation from 7.2 to 6.8.

Agitation (approximately 70 rpm) in 250 Bellco spinner flasks had no apparent effect on the viability of cells. Surface to volume ratio was slightly decreased by evaporation (calculated to be a maximum of 0.6 ml/day) of water and by 1-2 ml sample withdrawals. Changes in surface to volume ratio did not have a significant effect on population doubling times. The centrifugation of cells (120 g) and resuspension also had no apparent effect on the growth of cells.

Osmotic pressure has usually only been considered in the design of new media (13). Several authors (46, 64) have tried to supplement their medium to a point where subsequent increase of substrates carried an inhibitory effect due to high osmotic pressure.

The cells shrink at elevated osmotic pressures and explode when the osmotic pressure is very low. Waymouth (65) has estimated osmotic pressure of plasma of animals from which numerous cell lines have been derived. It has been observed that optimal osmotic pressures for mouse cell lines lie

between 300 mosm and 340 mosm.*

Sodium chloride accounts for nearly 90% of the osmotic pressure and glucose for about 3% in the modified Fisher's medium employed in this work. The addition of a high osmotic pressure supplement will proportionately modify the osmotic pressure of the suspension culture. Hence, it is of critical importance to maintain osmotic pressure within optimal range. It has been found in this work that L 5178 Y cell suspensions showed a constant osmotic pressure when grown under conditions of Table 2.

Trowell (66), while working with lymphocytes, concluded that optimal growth and lactate production were related to osmolarity.

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*One mosm is the quantity of solute molecules in solution that produces a freezing point depression of 1.86×10^{-3}°C. Osmolarity of a solution is related to tonicity of a solution and these terms have been used interchangeably. Waymouth (65) explains the difference between isosmotic and isotonic solution.

CHAPTER VI

RESULTS AND DISCUSSION

The final testing of designing a medium suitable for semibatch culturing was done by one set of duplicate experiments. The experiments involved the same conditions as in batch culturing except that the medium was supplemented with a solution of glucose plus amino acids as shown in Table 6.

The supplement was originally prepared so that all amino acids and glucose were in ratio to Fisher's medium formula. Not all amino acids were included in the supplement but only those which were suspected of being the cause of inhibiting normal growth by their depletion. The amino acids that were suspected of being such cause were selected from the literature (46).

The results of Table 5 indicated, however, that the amino acids in batch culture were not consumed in the same proportion as in the Fisher's medium formulation. Therefore, a second supplement was formed such that the amino acids present in this supplement were in the same proportion as they were consumed in batch culturing. Lysine was omitted from this formulation which is shown on Table 6. Finally since in semibatch culturing it was found that consumption rates changed, a final modification to the supplement was used in the final experiments as shown in Figure 8. All supplements were successful in the sense that all increased considerably the maximum cell yield as shown in Table 7.

Table 6. Glucose and Amino acid Supplements (50 ml)

Amino acid	Final Supplement (mg)	First Supplement (mg)	Intermediary Supplement (mg)
Glucose	2000	2500	4000
L-Glutamine	625	510	610
L-Isoleucine	100	188	160
DL-Phenylalanine	60	150	120
L-Leucine	60	75	120
L-Lysine	60	125	-
DL-Valine	50	175	140

The first supplement was obtained by comparing uptake rates in MOPC cells (see reference 12) and the ratio of the amino acids chosen in the supplement is the same as in Fisher's formulation. The intermediary supplement was formulated by analysis of batch data obtained from gas-liquid chromatography in this laboratory for L 5178 Y cells. (See Table 8). The final supplement was achieved by analysis of semibatch data. (See Table 9). The key factor in formulating a supplement is to keep the same relative ratio among the nutrients whose concentration levels are being changed by the culture. The actual amount of supplement fed to the culture is estimated from Equation 13 which according to the discussion in Chapter III does not necessarily hold. The maximum cell population with the first supplement was 7.4×10^6 cells/ml but the experiment was not duplicated. The intermediary supplement achieved a maximum population of 2.8×10^6 cells (duplicate experiment). The final supplement yielded a maximum

cell concentration of 4.3×10^6 cells/ml in a duplicate experiment. The osmotic pressure of the first, intermediary and final supplements were approximately 900,700 and 340 mosm respectively. The final supplement has the advantage of nutrients economy and ease of osmotic shock.

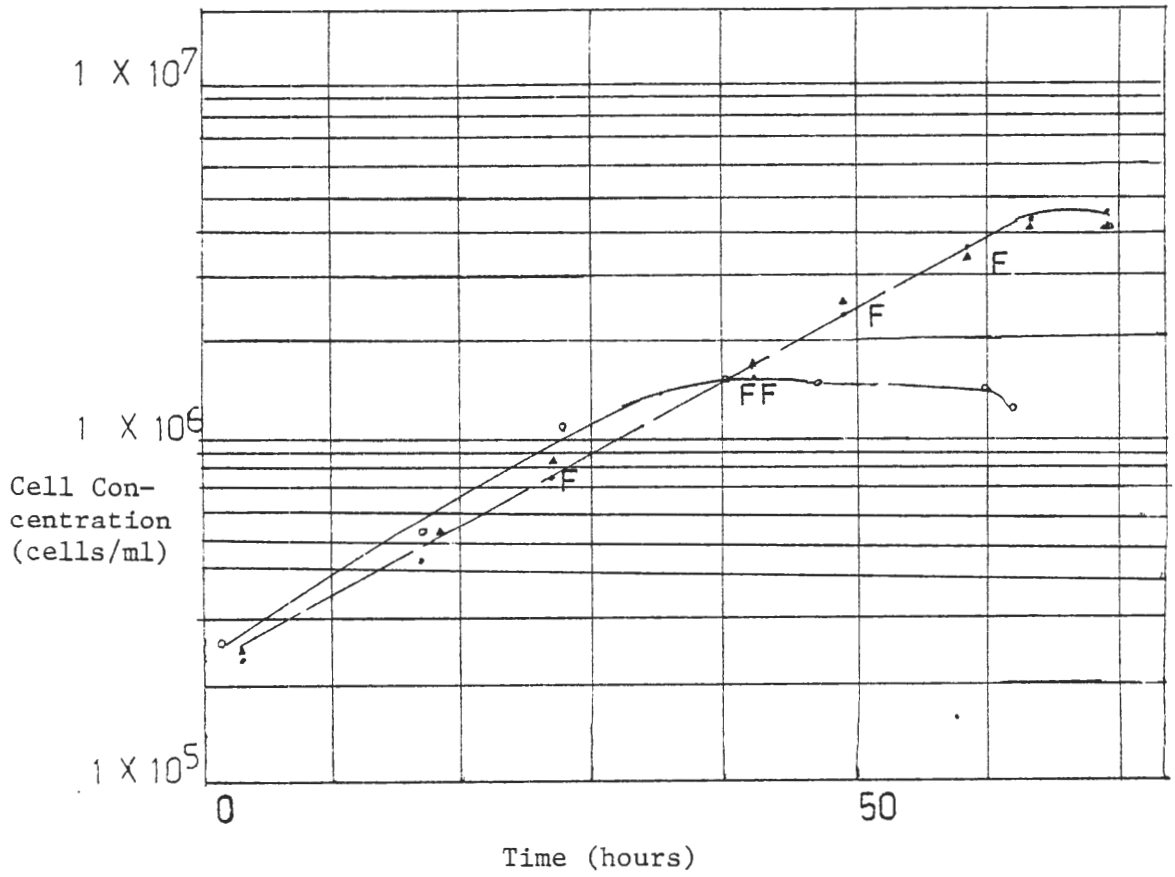


Figure 8. Population Dynamics of Semibatch Culture of L 5178 Y Cells

The graph presents results of the final (duplicated) experiment with one control culture. The ability of the suspension culture to maintain logarithmic growth beyond the maximum concentration attained by conventional batch culturing (control) shows the potentialities of designing media for semibatch culturing. The increase in cell concentration is approximately 2.7×10^6 cells/ml beyond the 1.6×10^6 cells/ml achieved by the control. It is believed that although the supplement is far from "perfect" it carries the implication that the cause of departure from logarithmic growth is not the accumulation of toxic substances. Another implication results from the fact that enriching the initial media as has been done in the past with a certain lack of complete success can be substituted by this procedure. This is because it eliminates the problem stated by R. G. Ham (9) that "too much is as bad as too little" in reference to the amino acid levels in the formulation of initial medium. The feeding points are marked with F at a CLA of about 7×10^{-11} cells^{-1} hour^{-11} except where it is marked with a double F (FF) where it was fed twice and the culture was allowed to grow undisturbed to a CLA of 4×10^{-11} hour^{-11} cell^{-1}.

Table 7. Population Dynamics of Final Experimental Runs

Time (hours)	Control Concentration (cells/ml)	Semibatch fed Concentration (cells/ml)
0	2.5×10^5	2.5×10^5
38	1.6×10^6	1.5×10^6
60	1.32×10^6	4.3×10^6

The tabulated results were compiled from one experimental run for the control (or batch) culture and the arithmetic average of the final heamacytometer duplicate experiment. Each entry is the arithmetic average of 3 to 6 haemacytometer counts. Both control and duplicate experiments had same stock culture innoculum and started at the same time. The doubling time for the control culture of 13.3 hours is slightly inferior to the average of the final runs (14.3 hours). These doubling times were estimated using only date at 0 and 38 hours. Haemacytometer counts carry an error of at least $\pm$ 5% and it was not felt necessary to make a more elaborate curve fitting since the cell number counts were in close prediction with exponential growth behavior. This can be seen in Figure 8. Since the difference is of the order of 7% in doubling times, this difference has not been considered significant. The average doubling time in the semibatch region is approximately 15 hours, still within 5% of the doubling time for the first 38 hours.

The experiments consisted of initially allowing the culture to function as batch culture up to a point before the maximum cell density was reached. Thereafter, a supplement was periodically added to offset the depletion of amino acids by the suspension. A detailed discussion of the choice of a time period to add a supplement and the amount of supplement that was consequently added follows.

The supplement was added with a syringe. The interruption of culture conditions for less than 10 minutes was found not to change the maximum cell yield or doubling time.

The results in terms of cell yield and doubling time are shown in Figure 8 and Table 7. Table 7 shows an average maximum population of 4.3 x 10^6 cells/ml which is 2.6 times greater than 1.65 x 10^6 cells/ml achieved in batch culturing of L 5178 Y cells.

Amino Acid Consumption

Equation 13, with the limitations discussed in Chapter II can be written from a time period t_i to t_{i+1} as:

$$c_{j_i} - c_{j_{i+1}} = \frac{k_j}{\frac{\ln N_{i+1} - \ln N_i}{(t_{i+1} - t_i)\,(N_{i+1} - N_i)}} \qquad (14)$$

The factor $\frac{\ln N_{i+1} - \ln N_i}{(t_{i+1} - t_i)\,(N_{i+1} - N_i)}$ will be called henceforth cells logarithmic average (CLA). It has units of $\text{cell}^{-1}\ \text{hr}^{-1}$ and it refers to a specific volume (in this work 1ml). The CLA is inversely proportional to

a change in nutrient concentration provided k, the substrate uptake rate is constant.

In batch culturing, starting with an innocula of 250,000 cells/ml mammalian cells (Table 3) the cells peaked at 1.65×10^{6} cells/ml after approximately 36 hours. The CLA in 36 hours is 3.74×10^{-11} cells hr^{-1} at which the amino acid concentrations changed as depicted in Table 5. The rate k under this condition is calculated by Equation 13. Assuming k is constant $c_{j_i} - c_{j_{i+1}}$, or the amount of a given depleted nutrient, can be supplied if CLA is known. It is also assumed that if CLA decreases to a value below 3.74×10^{-11}, the cells peak since in that case they become deficient in one or more amino acids. Table 5 shows that leucine had been depleted to a large extent in batch culturing L 5178 Y cells.

Since the doubling time is fairly constant (straight line of cell concentration as time in semilogarithmic plots) a point is selected usually at twice or less the ratio:

$$2 > \frac{CLAi}{CLA\ batch} > 1 \qquad (15)$$

where CLAi is the cell logarithmic average from a point i to i+1 and CLA batch is 3.74×10^{-11} cell hr^{-1}. (See Figure 9).

When $\frac{CLA_i}{CLA\ batch}$ is equal to two, half of the amino acids are assumed to be depleted. When $\frac{CLA_i}{CLA\ batch}$ is equal to one, a limiting growth nutrient(s) has been depleted and the culture departs from logarithmic phase.

Table 8 suggests that the consumption rates of some amino acids is essentially the same after 24, 27, or 36 hours. The cell concentration peaked after 36 hours. The consumption rates of the supplied amino acids

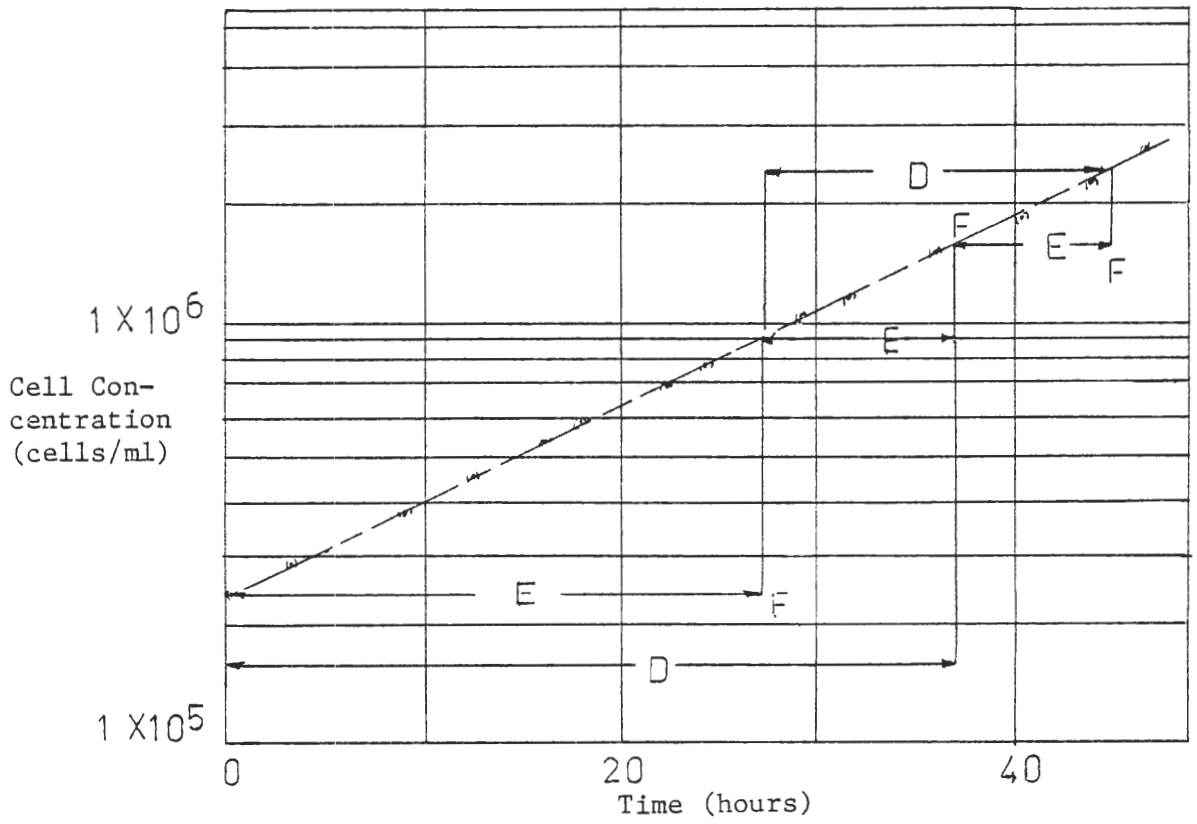


Figure 9. Criteria for Selection of Feeding Periods

The diagonal line is the representation of an hypothetical growth curve of the L 5178 Y cell culture. The segments marked "E" represent a CLA of approximately 8 x 10^{-11} $cell^{-1}$ $hour^{-1}$ or approximately 50% of the nutrients that are consumed before the culture attains maximum population. The segments marked "D" indicate a CLA of 4 x 10^{-11} $cell^{-1}$ $hour^{-1}$. If the uptake rates for all the nutrients k_{ij} that are essential to maintain logarithmic growth are constant "D" segments also indicate the maximum change in nutrient's medium concentration before the culture passes to stationary phase. Therefore, if the intent is to replace nutrients to a concentration similar to the one in the initial medium each time the culture is fed with the supplement, the feeding interval should always be greater than CLA equal to 4 x 10^{-11} $cell^{-1}$ $hour^{-1}$. The problem of "overfeeding" the culture exists because high concentrations of certain nutrients may have toxic effects as discussed in Chapter III.

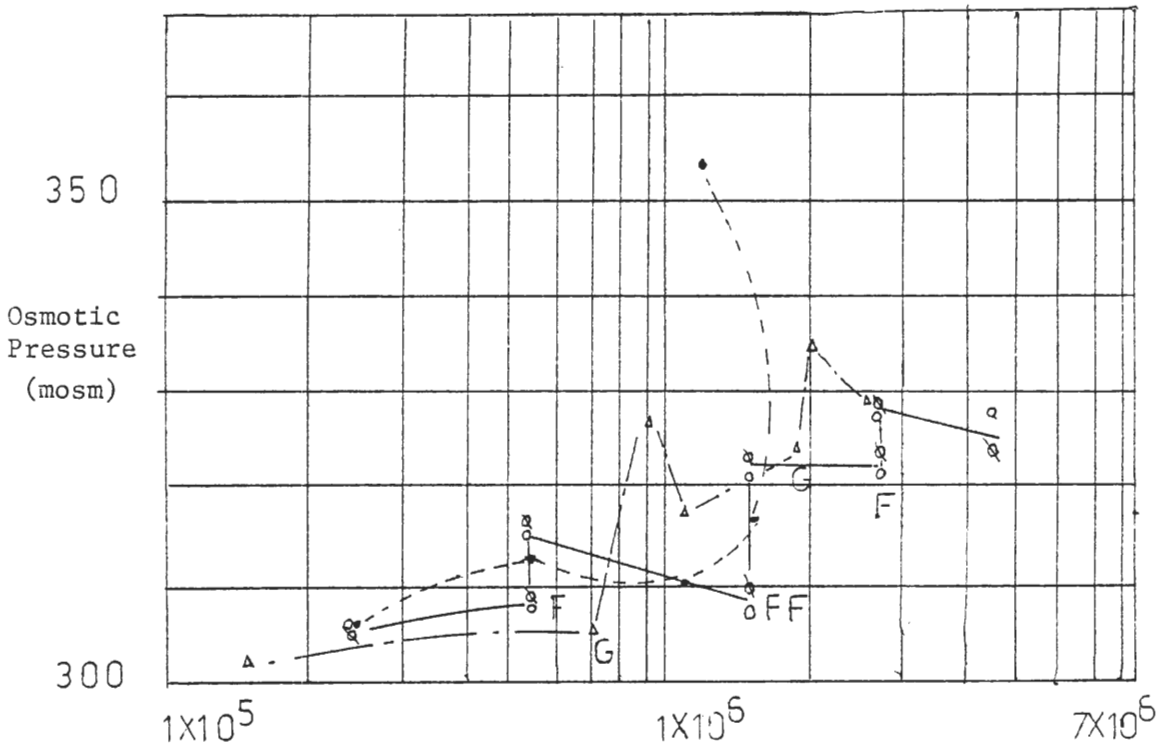


Figure 10. Osmotic Pressure versus Cell/Concentration

• ..... Batch culture (control of final duplicated experiments)
o ..... Final duplicate experiments (semibatch fed)
Δ ..... Semibatch fed experiment using intermediary medium (see Table 6)

The plot representa the change in osmotic pressure of the L 5178 Y cell cultures throughout the experiments. There is an advantage in plotting the change in osmotic pressure versus cell concentration rather than versus time in that changes of osmotic pressure due to supplementation and possibly nutrient depletion are a function of both population and culture age. With approximate doubling times fairly established it is possible to relate time to cell number since the initial cell concentration can be read from Figure 10 (See Equation 3). Figure 10 shows how the osmotic pressure changes in stationary phase for the control culture. The figure also appears to indicate an improvement by the final supplement over the intermediary supplement both in maximum cell number and osmotic "shock". Both experiments (with final and intermediary supplements were performed in duplicate).

Table 8. Amino Acid Metabolism in Batch and Semibatch Growth

Amino Acid	0 to 24 hours	0 to 27 hours	0 to 36 hours	49 to 58 hours
	(Amino Acid level differences by Equation 13 in mMoles/ cell x hour x 10^{-11})			
L-alanine	.66	.64	.67	1.62
L-valine *	-1.70	-1.88	-0.98	-0.80
L-glycine	-0.3	N.C.[a]	-0.21	N.C.
L-isoleucine	-0.89	-1.05	-1.01	-1.40
L-leucine	-0.70	-0.60	-0.68	-0.90
L-proline	.07	N.C.	-0.13	.17
L-threonine	-0.38	-0.49	-0.42	-0.14
L-serine	-0.58	-0.28	-0.49	-0.10
L-methionine	-1.71	-1.78	-1.88	b
L-phenylalanine *	-0.60	-0.76	-0.69	-0.78
L-aspartic acid c	-0.12	N.D.[d]	-0.08	N.C.
L-glutamic acid e	-3.48	-3.49	-3.50	-3.71
L-lysine	-0.34	-0.36	-0.32	-0.87
L-tyrosine	-0.07	N.D.	-0.21	-0.08

* - During semibatch medium regime a racemic mixture of the amino acid was supplied.

a - Negligible change.

b - An unexplained increase in amino acid level (see Table 9).

c - Asparagine is converted to aspartic acid during derivatization.

d - Not determined due to poor resolution in the chromatograph.

e - Glutamine is converted to glutamic acid during derivatization. The tabulated results indicate a decrease in glutamine levels plus any amount of glutamic acid released by the culture. Other cell cultures (McCarthy, K. "Selective Utilization of Amino acids by Mammalian Cell Cultures") release from 10 to 25% of the glutamine consumed as glutamic acid into the medium.

Table 8. Amino Acid Metabolism in Batch and Semibatch Growth

(Continued)

The results are taken from a single determination and each column represents a different experiment except for the first and fourth columns. The fourth column results belong to semibatch medium. The results were arrived at by use of Equation 13. The initial experimental conditions were those of Table 2.

increased slightly, possibly as the essential amino acids are used to synthesize non-essential amino acids. The difference in valine may be attributed to a higher uptake at first (0.89×10^{-11}/cell hours) followed by a decline in consumption as the amino acid concentration is lowered. The detection of amino acids that were not present in Fisher's medium formulation is explained by the presence of serum. Glycine, a non-essential amino acid, supplied by the serum was depleted.

Table 8 shows that after 36 hours in the experimentally fed culture, the consumption of leucine, isoleucine, and lysine show a marked increase. One possible reason is the fact that the medium is more concentrated and the cells would tend to increment their intracellular amino acid pool (67). Another possible reason is that the depletion of other amino acids forces the cells to use supplied amino acids to synthesize them (68).

Table 8 shows the depletion of glutamine plus the production of glutamic acid. Since glutamine and asparagine are converted to glutamic and aspartic acids respectively, in the derivatization, there is a slight increase in glutamine depletion.

Since L-glutamine is converted to L-glutamic acid in the gc sample preparation, the analysis is for both L-glutamine and L-glutamic acid. This consumption then represented the net L-glutamine converted into something other than L-glutamic acid. Since cells cannot normally use L-glutamic acid in place of L-glutamine, this represents a problem in the data. Of the supplied amino acid consumption rates, valine seems to decrease and phenyalanine remains approximately the same.

The slight increase of the rate of utilization of glutamine offers an interesting occurrence since there is also a net production of proline

of about the same magnitude as the glutamine depletion. Proline and glutamine have both as precursors glutamate (60) in metabolic pathways of E. Coli.

The origin of alanine is unknown though it could be obtained by the cells from the serum protein or even by transamination from glutamic acid.

Glycine, supplied by the serum, is depleted and this seems in agreement with Mohberg (70).

Essential amino acids that were not in the supplement formulation seem to be consumed and then reach a plateau at a low concentration. Table 9 shows a decrease in tyrosine after 24 hours, but a higher than initial concentration after 49 hours. There is, however, a possibility that tyrosine was partially destroyed in the derivatization and the reported results are incorrect.

The period between 45 and 58 hours has a CLA of 3.94×10^{-11} hr^{-1} $cell^{-1}$ and it corresponds to twice the amount of amino acids in the initial medium.

$$\frac{CLA\ batch}{CLA_{49\text{-}58\ hours}} = \frac{3.74 \times 10^{-11}\ cell^{-1}\ hr^{-1}}{3.94 \times 10^{-11}\ cell^{-1}\ hr^{-1}} = 95\%$$

There is usually a 10% increase each time the suspension is fed with supplement. It is expected that the final concentration exceeds the initial concentration by 32%. Table 10 shows the relative percent difference between initial and the final amino acid cencentration, the latter being decreased by 32%.

Table 11 shows a comparison between some of the cell's metabolism described by McCarthy (71) and the myeloma cells studied by Roberts (46).

Table 9. Concentration of Amino Acids in Semibatch Growth

Amino Acid	Batch Region 0 hours (mg/l)	Batch Region 24 hours (mg/l)	Semibatch Region 49 hours (mg/l)	Semibatch Region 58 hours (mg/l)
L-alanine	N.[a]	7	48	85
L-valine	53	24	38	13
DL-valine			83	58
L-glycine	1	N.	3	2
L-isoleucine	48	31	127	73
L-leucine	21	11	13	29
L-proline	N.	N.	9	14
L-threonine	21	14	13	16
L-serine	13	9	11	14
l-methionine	67	33	61	85
L-phenylalanine	41	26	40	7.5
DL-phenylalanine			92	60
L-aspartic Acid [b]	4	3	3	3
L-glutamic Acid [c]	185 [d]	120	374	215
L-tyrosine	25	15	31	35
L-lysine	16	10	58	21

a - Negligible amount of amino acid detected by the chromatograph or not detected at all.

b - Asparagine was converted to aspartic acid during derivatization.

c - Glutamine was converted to glutamic acid during derivatization. (See notes on Table 8.)

d - Chromatographic analysis registered slight variations in amino acid composition regarding the formula published by the supplier. (See Appendix II).

This table summarizes one experiment and each number is product of a single determination. There is an unexplained appearance of methionine

Table 9. Concentration of Amino Acids in Semibatch Growth (Continued)

in the semibatch region since this particular amino acid was not supplied to the culture throughout the experiment. Chromatographic determinations were performed using methods described in Chapter IV and Appendix I. A typical chromatograph is presented on Appendix II. Data on this table and cell numbers and time periods of Figure 8 were utilized by means of Equation 13 to calculate the results of the first and fourth columns of Table 8.

Table 10. Difference Between Estimated Consumption and Actual Consumption of Amino Acids

Amino acid supplied to medium in semi-batch regime	% Change[a]
L-valine *	-18%
L-isoleucine	39%
L-leucine	32%
L-phenyalanine *	13%
L-Glutamine [b]	6%
L-Lysine	170%

* A racemic mixture was supplied to the culture

a calculated by:

$$\frac{\left[\text{rate of uptake from 0 to 36 hr.} - \text{rate of uptake from 49 to 58 hr.}\right]}{\text{rate of uptake from 0 to 36 hr.}} \times 100$$

b - see note on Tables 8 and 9

This chart was prepared with data from Table 8.

Because of the size of the injecting syringe (1 ml) and other difficulties in the feeding process an excess of 10% was included in the supplement, the compounded error $(1.1)^3 - 1$ amounts to 33%. The excess was added so that depletion of an amino acid due to the difficulties outlined above would not provoke departure from the culture's logarithmic growth. According to Eagle (8), the size of the cell's internal pool depends upon the exogenous concentration of a given amino acid. Furthermore, according to Lemonnier (79) not only a given amino acid exogenous concentration has an effect upon the cells internal concentration of a given essential amino acid but also the outside concentration of the non-essential amino

acids. This could be explained by the cell's use of essential amino acids to synthesize non-essential ones and a corresponding increase in uptake of essential amino acids. The increase of the essential amino acids exogenous concentration may also be a factor in the increased uptake rates under semibatch fed cultures. The large increase in lysine uptake is unexplained but in a previous experiment in which lysine was omitted (see Table 6) the culture only attained 2.8×10^6 cells/ml as opposed to the final supplement containing lysine (see Table 6) with 4.3×10^6 cell/ml. The exception would be glutamine which is always supplied in excess both in batch and semibatch culturing (see Table 9 and Appendix II. In support of this hypothesis the increase of glutamine is only 6%.

Table 11. Comparison of Amino Acid Metabolism

Amino Acid	Cell Lines					
	L 5178 Y (batch)	L 5178 Y (semi-batch)	Hela[a]	KB[a]	MOPC[b]	HEP-2[a]
	metabolism rates in (mMol/cell x hour) x 10^{11}					
L-alanine	.67	1.62	.14	1.58	-	.3
L-valine	-1.7	-0.8*	-0.88	-0.5	-0.16	-0.97
L-glycine	-0.3	negligible	.6	.15	.19	.03
L-isoleucine	-0.89	-1.4	-0.99	-0.90	-1.51	-1.21
L-leucine	-0.70	-0.9	-1.17	-1.02	-1.01	-1.36
L-proline	.07	.17	.15	.26	.15	.14
L-threonine	-0.38	-0.14	-0.40	-0.13	-0.31	-0.35
L-serine	-0.49	-0.1	.4	--	-0.26	-
L-methionine	-1.71	c	-0.3	-0.3	-0.19	-0.31
L-phenylalanine	-0.76	-0.78*	-0.51	-0.2	-0.13	-0.15
L-aspartic acid[c]	-0.12	negligible	.31	.11	-	-
L-glutamic Acid[c]	-3.48	-3.71	-5.12	-5.8	-2.62	-7.17
L-lysine	-0.34	-0.87	-0.00	-1.02	-0.87	-0.98
L-tyrosine	-0.07	-0.08	-0.58	-0.24	-0.07	-0.46

* - Supplement contained a racemic mixture of valine and phenylalanine.

a - Source: McCarth, K., "Selective Utilization of Amino Acids by Mammalian Cell Culture."

b - Source: Roberts, R. S., "Growth Dynamics in Vitro and in Vivo." 10^{11}

c - See notes on Tables 8 and 9.

Table 11. Comparison of Amino Acid Metabolism
(Continued)

The batch period is the first column of Table 8 and the semibatch period the fourth column of Table 8. Both batch and semibatch periods belong to the same experiment. One determination was achieved for each result. Both experimental and literature data are calculated from Equation 13. The population dynamics data necessary to estimate amino acid metabolism rates are contained in Figure 8 and Table 7.

Table 11 shows, that like the other cells, there is a net production of alanine (except for the myeloma cells) and of proline.

As with other cells, glutamine requirement seems to be highest. McCarthy (71) observed the constancy of the rate of consumption of lysine for all different cell lines. In the L 5178 Y cells this observation applies in the semibatch region. The results show that the amino acid metabolism of L 5178 Y cells is well within range of the cell lines studied by McCarthy (71) and Roberts (46).

Glucose Consumption

Glucose consumption requirements are also estimated using Equation 14. The results for the supplemented semibatch experiments are depicted in Table 12. The increase in glucose consumption at elevated densities is not in agreement with batch culture results. There are several possible reasons for the increase in glucose consumption. One reason is the depletion of oxygen and an increase in glycolysis (72). Another possibility is carbon dioxide tension and pH (53, 54).

The glucose consumption is compared with results obtained from several authors in Table 12. Table 12 shows that glucose consumption for L 5178 Y is of the order of magnitude of most tissue culture cells encountered in the literature survey (41, 71, 73).

Supplementation of glucose or other nutrients have been reported to yield "diminishing returns" (74). Diminishing returns means that the amount of nutrients depleted by the suspension is not incorporated into cellular material as effectively as in early batch culturing.

The results in Table 8 and 12 indicate that both glucose and amino acid consumption increased after the suspension has been supplemented.

Table 12. Comparison of Glucose Consumption

Cell Lines	Batch designed (mMoles/cell x hour)x10^{11}	Semibatch designed (mMoles/cell x hour)x10^{11}
L 5178 Y	18.9	29.1
Hela*	22.7	
H. Ep. 2*	27.1	
Monkey Kidney*	9.25	
L 929 Fibroblasts*	21.6	
KB*	15.5	

* - Sources: McCarthy, K. "Selective Utilization of Amino Acids by Mammalian Cell Cultures" and Bryant, J. C., "Glucose and Lactic Acid Trends in Suspension Cultures of Two Established Cell Strains in Chemically Defined Media."

The result in Batch medium represent the average of experiments. Semibatch data corresponds to one experiment using intermediary points (49 to 58 hours) while batch data comprises the period from 0 to 36 hours. Both numbers were obtained through Equation 13.

There is no evidence that this consumption results in "diminishing returns. However, the increase in consumption of nutrients appears to contradict an earlier report by Fish et al. (13). Fish and coworkers had suggested that L (strain Earle) cells would only consume amino acids within the first day in culture, leaving the rest of the amino acids almost untouched for the rest of the growth period.

If the cells consume a large amount of amino acids and glucose within the first hours of growth and continue to proliferate subsequently, then they are able to accumulate a pool of amino acids and other substrates within the cell. Studies (10) have shown, for example, that although mammalian cells are able to synthesize all their lipids, they will capture fatty acids from the medium up to a point at which they literally burst (75).

Osmotic Pressure

One of the most important parameters in designing semibatch media is osmotic pressure. Figure 10 represents a plot of osmotic pressure versus cell number for the final duplicate experiments, the control batch run of the final experiment and one experiment where an intermediate supplement was used (Table 6). In the control experiment there is a marked increase in osmotic pressure during the stationary phase when cell viability decreases.

The increase in osmotic pressure with cell death has been reported in the literature (76) to be the effect of large molecules breaking during lysing. A small increase in osmotic pressure was caused by adding the final supplement at 340 mosm. This increase was offset by the culture,

possibly by increase in glucose and amino acid consumption.

The supplement was added so that it would cause an increase of the total suspension volume of either 0.5% of 1%. The 0.5% increase (340 microliters in 70 ml) was used when CLA / CLA_{batch} was approximately two, or 50% of the nutrients that are depleted in barch culture before growth is inhibited were consumed. The 1% increase resulted when CLA_i/CLA_{batch} was approximately one.

At 1.6×10^6 cells/ml a 1% increase in the suspension volume was caused by adding twice the supplement that was used before (700 microliters). The increase in osmotic pressure was not offset by the suspension completely to the point where it was before. It is interesting that in this case the osmotic pressure does not appear to be additive. The reasons for this observation are unknown.

The fact that the suspension was not allowed to return to it's original state suggests that all supplement additions should be such that the suspension remains at a constant osmotic pressure. That is, only additions of the final supplement, where CLA_i is around 8×10^{-11} $cell^{-1}$ $hour^{-1}$ should be used. This is the same as 0.5% increases in the suspension volume or CLA_i/CLA_{batch} equal to two. The osmotic pressure contribution of consumables amounts to less than 5% which makes changes in osmotic pressure difficult to establish.

Serum and Limiting Factors

Griffiths (74), Meisler (77) and Pirt and Lambert (78) have concluded that provided oxygen, amino acids and glucose are not in short supply, growth inhibition is serum dependent. Serum main function has been recognized to be in growth promotion although it has also been

reported to be involved in amino acid transport (77). Sera that have been "conditioned" loose the bulk part of their growth promoting capabilities (54). Conditioned serum is that which has already been involved in cell culture and is employed again after removal of the first culture cells.

Results from batch culture indicate that practically all glucose was depleted within 48 hours (Table 4). This may be an indication that cells are able to break down lactic acid. Such ability may explain the rather long stationary phase encountered in the control cultures (Figure 6). There is at the end of the stationary phase a dramatic fall of cell viability (at least 10%) after 60 hours. At the end of the final experiment a much shorter stationary phase was encountered. These results suggest the possibility of serum being the growth limiting factor if, after 60 hours or so, the serum looses the bulk of its growth promoting capabilities. In the final experimentation DL mixture was used for valine and phenylalanine. Since the cells only consume L isomers it is also possible that growth was limited by exhaustion of all L isomers supplied to the culture.

Several researchers have attempted to isolate and identify the components of serum that allow the cells to grow. Although some cell lines have been able to grow on chemically defined medium (78), the fast proliferating lymphoblasts definitely require serum.

Dulbecco (79) has identified at least four different functions of the serum, one of which is to prevent irreversible changes that lead to cell death. Results by Clarke et al. (80) show that suspension cultures of BHK (Baby Hamster Kidney) cells are up to 50% less dependent upon

serum concentration to generate DNA synthesis than monolayer cultures.

Serum has also been found to reduce its effectiveness in crowded populations (63).

The experiments of Todaro et al. (81) and Schutz and Mora (82) provide good evidence that accumulation of toxic substances does not limit growth.

Griffiths (78) supplied mixtures of isoleuine, arginine, and leucine, vitamins, glucose, and cystine to Wl-38, Human diploid monolayer cells, with 20% increase from control. Glucose addition by Westwood and Zuartouw (43) also seemed to increase the cell maximum concentration but none of these authors attempted to formulate a system and medium to be used as a generalized technique for the culturing of mammalian cell lines.

Figure 6 shows the fact that a brief and incomplete analysis of the cell environment can be greatly rewarding in designing a millieu for semi-batch culturing.

CHAPTER VII

CONCLUSIONS AND RECOMMENDATIONS

It is believed that the goals of the project have been accomplished. The results in the previous chapter indicate that for L 5178 Y cells, the first cause of departure from logarithmic phase is nutrient depletion. Furthermore, an increase in cell yield predicted by the use of a medium for semibatch culturing has been encountered. Simple changes in nutrient content of the medium resulted in 2.65×10^6 cells/ml in excess to 1.65×10^6 cells/ml obtained by conventional batch culturing of the L 5178 Y cells. This suggests that some basic research on nutritional requirements of a particular cell line and the application of the concept of media for semibatch culturing of mammalian cells could lead to an increased production of mammalian cells several fold of that obtained by traditional batch culturing. The application of the methodology described and documented in this work could conceivably cause enormous savings in the large scale operations, such as the ones described by Girard (25) for production of BHK cells. BHK cells application is in the production of livestock vaccine doses of the Foot and Mouth Disease virus. Increased cell yields are not the only advantage since a more effective use of serum and ability to operate at almost maximum volume in bioreactors is also accomplished (Chapter III).

The equipment used in this work is possibly the simplest and most inexpensive. Experimental conditions are also easy to reproduce.

The amino acid analysis was not complete since histidine, arginine, triptophan and cystine were not analyzed. Also, a method such as the one devised by Hediger et al. (83) should be employed to differentiate glutamine from glutamic acid. Nevertheless, a very simple analysis of amino acid and glucose consumption and osmotic pressure considerations has led to an enormous increase in the maximum cell concentration. There is also need for further research on osmotic pressure changes produced by adding concentrated supplements.

Control of environmental parameters such as oxygen tension is likely to enhance the results even more. A more extensive research of nutrient and oxygen carbon dioxide requirements could be very profitable in terms of further enhancement of cell productivity. However, it is believed that the second limiting factor in growth is the loss of effectiveness of the serum. A second series of experiments with shorter periods of growth (i.e., starting at higher concentrations) or replenishment of serum is therefore recommended. However, a complete development of media for semi-batch growth of the mouse leukemia L 5178 Y and of other mammalian cell lines as well will come when a better understanding of cell biology and in particular, of the nature of serum is achieved.

APPENDIX I

Several modifications of Gehrke's procedure for amino acid analysis for sera were applied with respect to hydrolizates clean-up. An anion resin was employed (Dowex 50x, 100-200 mesh). The resin was cleaned with acetic acid, potassium hydroxide and water in a similar fashion to the clean-up of the cation resin. The amino acids were eluted to the anion exchange resin from the cation resin in Pasteur pippetes.

All evaporations were done by atmospherically gassing the solutions with an air stream. Esterification was done at 100°C for 15 min. and acylation at 150°C for 7 min. Sample chromatograph of serum containing medium is annexed.

APPENDIX II

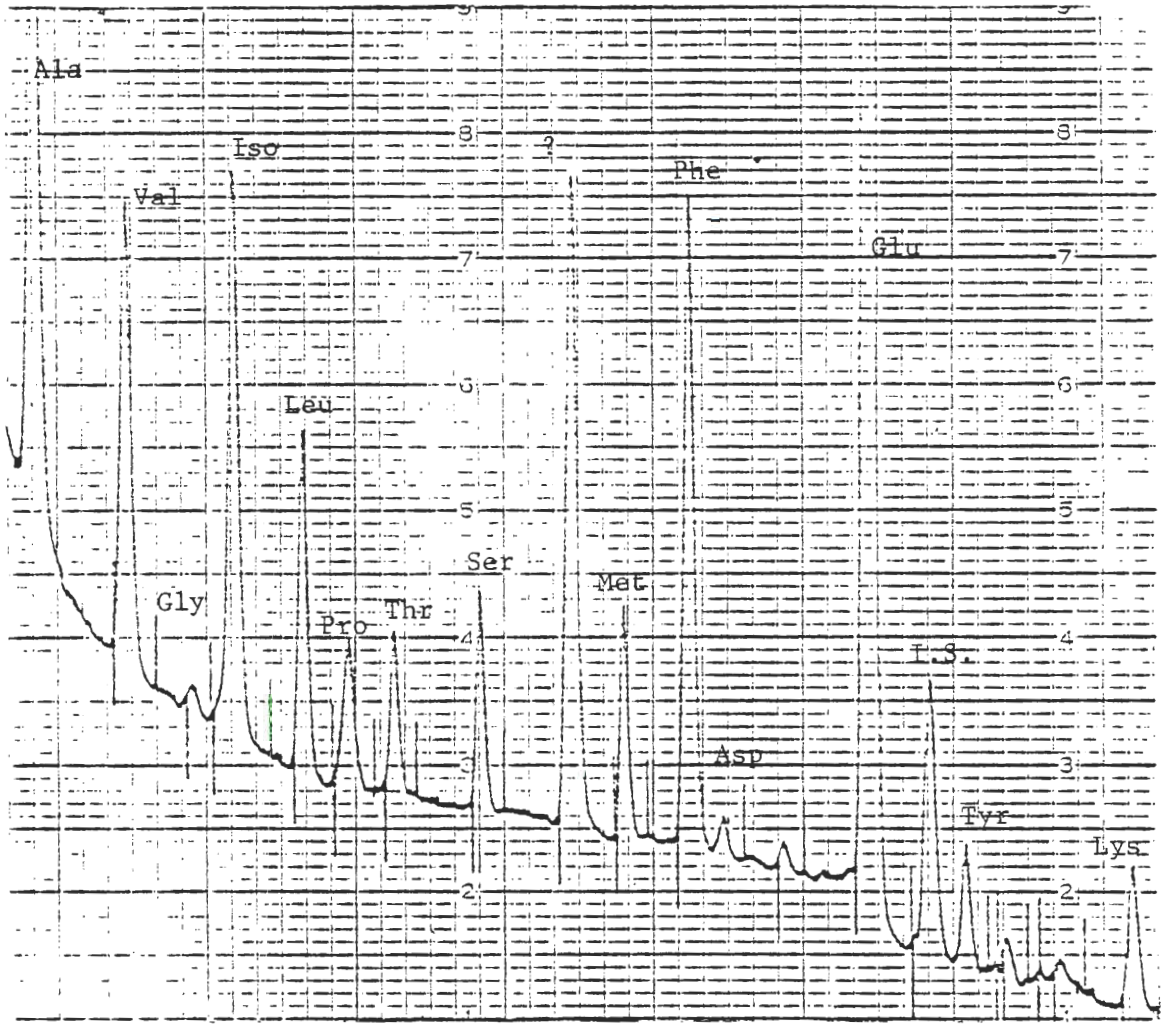
one Determination by Gas-Liquid Chromatography and a Sample Chromatogram.

Amino Acid	Suppliers Catalog	Initial Medium from Gas Chromatography (with 10% serum) (mg/L)
L-alanine	-	5.8
L-valine	70	62
L-glycine	-	5.4
L-isoleucine	75	64
L-leucine	30	28
L-lysine	50	29
L-methionine	100	158
L-phenylalanine	60	59
L-serine	15	19
L-threonine	30	26
L-triptophane	10	Not determined
L-tyrosine a	60	61.4
L-aspartic acid	10	8.2
L-glutamine	230	250 b
L-proline	-	6

a - provided as disodium sale (86.88 mg)

b - This entry includes glutamine and glutamic acid. (See note on Table 9).

Note: The presence of amino acids in the initial medium that do not appear on Fisher's formulation is explained by the addition of 10% serum to the solution fed to the cultures.



Serum Containing Culture Spent Medium

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