AN EXPERIMENTAL STUDY IN THE USE OF INSTRUMENTATION TO ANALYZE METABOLISM AND PRODUCT FORMATION IN CELL CULTURE

by

ROBERT JAMES FLEISCHAKER JR.

B. S. Harvey Mudd College

(1974)

M. S. Massachusetts Institute of Technology

(1977)

SUBMITTED TO THE DEPARTMENT OF NUTRITION AND FOOD SCIENCE IN PARTIAL FULFILLMENT OF THE REQUIREMENTS OF THE DEGREE OF DOCTORATE OF PHILOSOPHY

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 1982

Signature of Author _____

Department of Nutrition and Food Science

May 21, 1982

Certified by _____

Anthony J. Sinskey

Thesis supervisor

Accepted by _____

Anthony J. Sinskey

Chairman, Departmental Graduate Committee

This doctoral thesis has been examined by a committee of the Department of Nutrition and Food Science as follows:

Prof. Daniel I. C. Wang _____ Chairman

Prof. Anthony J. Sinskey _____ Thesis Supervisor

Prof. Henri Brunengraber _____

Prof. Phillips W. Robbins _____

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Submitted to the Department of Nutrition and Food Science
on May 21, 1982 in partial fulfillment of the
requirements of the Degree of Doctorate of Philosophy in
Biochemical Engineering

ABSTRACT

The use of instrumentation to monitor and control animal cells in culture has been investigated and developed in this thesis. Instrumentation for use in cell culture is subject to a very different set of constraints than those encountered in microbial fermentations. Most, notably the lower rates of metabolism combined with the lower densities obtained in culture require a higher degree of sensitivity and stability in the sensors used. Furthermore, because the nutritional requirements of animal cells are much more complex than those of the microbial organisms, a very different approach must be used in developing strategies to monitor and control cell growth.

The FS-4 cells, a human diploid fibroblast cell strain and interferon producer, were grown in microcarrier culture as a model system. Instrumentation was developed to monitor the energy metabolism of these cells. The ATP flux was calculated by monitoring the influx and efflux of substrates and products of the primary energy pathways, glycolysis and oxidative phosphorylation. Measuring the energy metabolism of these cells was shown to be a useful methodology for monitoring cell growth and product formation. Futhermore, it was determined that most of the energy generated by these cells is utilized for cell maintenance.

Finally, the metabolism of glucose by the FS-4 cells was controlled using a measured ATP flux, such that glycolysis was significantly reduced. The ATP flux was used to control the glucose feed rate to the culture. Thus, it is possible to minimize glycolysis by maintaining the glucose concentration at very low levels, while providing an adequate supply of glucose so that cell growth is not impaired. Additionally, we have shown that most of the glucose utilized by these cells can be accounted for by the formation of lactic acid and carbon dioxide.

Thesis Supervisor: Dr. Anthony J. Sinskey
Professor of Applied Microbiology

ACKNOWLEDGEMENTS

I want to extend my deep felt gratitude to the following people:

Dr. Sinskey, for his support, understanding, and guidance through this project.

Dr. Thilly, who is the one most responsible for my being at MIT and has on many occasions been most helpful and generous.

Don Giard, for many discussions and the use of the Cell Culture Center facilities

Michele Frabricant for her excellent technical assistnace and also to the technicians of the Cell Culture Center for their efforts that made these large runs feasible.

The National Cancer Institute, for having made available the traineeship that funded much of this research.

My wife, and family for their love, help, and constant support.

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INTRODUCTION

Our present understanding of the growth and metabolism of animal cells in culture is somewhat limited. This is particularly evident when that knowledge is compared to the current body of information accumulated for the microbial organisms. Reasons for this disparity are principally historical. Although many of the foundations of modern biochemistry were studied using animal tissue (notably pigeon liver slices), the bacteria and yeast soon proved to be a more convenient subject. They are simple to grow, multiply rapidly, and can attain relatively high densities in culture. In contrast, the growth of animal cells in culture was slow in development until after World War II, at which time antibiotics became available. Prior to this, the problem of contamination had made work very slow and troublesome. Since then, a number of advances have been made and currently cell culture is utilized in many basic biological studies (cancer research, endocrinology, virology, immunology, and embryogenesis) and commercially for the production of certain cell products, the principle ones being the vaccines.

Although animal cell culture technology has made great strides, notably in the formulation of defined media compositions, its development has been hindered by fundamental problems in the growth of normal diploid cells on a large-scale. Animal cells, in stark contrast to microbial organisms, require a surface for growth. Thus, traditionally animal cells have been cultivated on the surface of petri plates, or using relatively minor variations of the same theme. This highly inefficient use of volume for suitable growth surface and requisite manual labor has created a serious bottleneck in the production of cells and cell-derived products on a large scale. The situation, however, is slowly changing as a number of groups are working on methods to increase both the scale and density of cell culture. Our laboratories, in particular, have been working with the microcarrier method of cell culture. In short, previous work in our labs was responsible for removing many of the original shortcomings with microcarriers and generating a renewed interest in the subject.

Figure 1 Electron Micrograph of FS-4 Cells on Microcarriers

0690 005 X952 0X02

Microcarrier culture consists of using positively charged microspheres as a surface for cell growth (see Figure 1 on page 16 for an electron micrograph of human fibroblasts on microcarriers). These microspheres are kept in suspension by gentle agitation. The benefits of such a method are those of

- Greatly increasing the scale of cell culture because of the very favorable surface to volume (S/V) ratio. This also greatly increases the number of cells per unit volume.

- Making it possible to obtain a representative culture sample for observation and enumeration.

- Maintaining uniform environmental conditions throughout the culture. Thus a measurement in one region is representative of the entire vessel.

Not only are these beneficial in growing cells and in the production of cell derived products, but they make it feasible to monitor and control the environment surrounding the cells. This is essential if instrumentation is to be used in cell culture, and in fact it is this aspect that has made this study possible.

Those who have worked with animal cell culture are very much aware that many of the day-to-day decisions are made on a subjective basis, particularly upon how the cells look under the microscope. The microscope is used to judge if the cells are growing well, if they are confluent, or if the cells are otherwise generally 'healthy'. The point is not that the microscope lacks objectivity, but rather that we are faced with a serious lack of quantitative data describing pertinent variables upon which decisions can be rationally formed. For example, in the optimization of virus production, one would want to first correlate cell metabolism, growth rate, and the phase of growth with virus yield. Second, one would want to be assured that these conditions could be reproduced. If animal cell culture is ever to be brought into parity with the culture of the bacteria and

yeasts, then much more will have to be done other than simply developing the technology to grow large quantities of animal cells. It is essential that one first identify those biochemical processes of importance and their corresponding observable parameters. Second, one must analyze and develop suitable methods to measure these observables[1]. Third, one must develop methodologies to interpret this data and use the information to understand and control cell growth as well as product formation.

The object of this study will be to examine mammalian cell growth and metabolism, and the subsequent formation of an induced product. The latter is of interest because many of the important cell products (viruses, interferons, etc..) are in fact induced products. The direction that I will follow is to concentrate upon monitoring the energy metabolism of these cells by quantitating the flux of substrates into these pathways (ie. sugar and oxygen) and the flux of products from them (ie. CO_2 and lactic acid). The rate of energy formation, and thus its general biochemical availability, is of importance to cell maintenance and cell growth as well as to product formation. This will begin, hopefully, to introduce some objectivity other than simply 'how do they look?'.

The particular system used in this study is the FS-4/Interferon one. FS-4 cells are normal human fibroblasts which are noted for being relatively good producers of interferon.

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[1] For example, oxidative phosphorylation (the biochemical process) can be observed by quantitating the uptake of oxygen (the observable). Additionally, the availability of oxygen (ie. its concentration) influences this process. Thus, one is faced with developing both the means to measure the oxygen uptake by the cells and the means to control the oxygen concentration.

INSTRUMENTATION FOR PROCESS CONTROL IN CELL CULTURE

The increasing use of instrumentation in the fermentation industry has led to significant advances in the understanding of microbial processes and their optimization (Wang *et al.*, 1979, Ryu and Humphrey, 1973). In contrast, the use of similar instrumentation for the study and control of cell culture had been rare, even though cell culture is used increasingly for the production of vaccines and cell products. The adaptation and development of better instrumentation for environmental control and monitoring of cell function can contribute to the improvement of cell yields and productivity. The intent of this survey is to review the instruments and measurement techniques used by the fermentation industry, and assess the potential of these and emerging techniques for use in cell culture.

An important first step in the development of an instrument system is the selection of the quantities to be measured. The choice will generally depend upon (1) the overall aim of the project and (2) the nature of the biological system that is utilized. For the purposes of this review I will concentrate primarily upon determining cell mass and secondly upon cellular metabolism. The specific system to be considered is the growth of anchorage-dependent animal cells in microcarrier culture. [e.g. WI-38 cells, a normal human diploid fibroblast cell strain derived from fetal lung tissue (Slonim, 1974). These cells are of importance because of the many studies that have been conducted using them and their use in the commercial production of certain vaccines.] Instrumental methods can be classified into two general categories: off-line and on-line. Off-line methods typically involve removal of discrete samples for subsequent treatment and analysis. Typically, off-line methods, such as wet chemical or immunological methods, involve more processing than on-line methods. Although off-line methods are becoming more automated and rapid, they are still relatively slow and cumbersome by comparison to on-line methods. In contrast, on-line methods involve measurements performed directly on or within the biological system. These measurements are made either without any sample removal or utilizing a continuous (generally small)

sample withdrawal. Owing to the real time nature of on-line methods, direct interfacing to a computer is usually possible. The emphasis here will be primarily with on-line methods of analysis.

COMPARISON BETWEEN ANIMAL AND MICROBIAL CELLS

Although animal and microbial cells are subject to the same fundamental laws, there are a number of essential differences between them, as summarized in Table 1 on page 21. From the viewpoint of measurement, the most significant differences are the lower metabolic activities per cell mass of animal cells and their lower cell densities (seldom more than 1 gm/liter of dry cell weight). As a consequence, during a given period of time, the effects that animal cells have upon their environment or the demands they make on it are considerably less than those of microbial cells, so that there is comparably less to measure.

In addition, growth of animal cells is restricted to a narrow range of environmental conditions, and strict control of these conditions greatly enhances growth. For example, Ceccarini and Eagle (1971) showed that diploid fibroblasts in a controlled pH environment showed significantly better growth than under variable pH conditions (CO_2-bicarbonate buffer system). Similarly, Kilburn and Webb (1968) showed that if dissolved oxygen was maintained within the range of 40-100 mm Hg, cell growth and peak cell densities were maximal.

In the case of microbial cells, there is considerable flexibility in the composition of the medium. Many researchers have taken advantage of this to simplify subsequent instrumental methods. For example, Wang et al. (1977) used NH_4OH as the sole nitrogen source in the growth of baker's yeast, the addition of which was regulated by pH control. Consequently, measurements of base addition were used to the estimate of cell mass (or more specifically total cellular nitrogen). Such flexibility does not exist with cell culture, where the medium consists of a complex mixture of vitamins, minerals, and amino acids supplemented with animal sera. These nutritional requirements are complex and difficult to determine and furthermore, many of these nutrients are tolerated only within a relatively

TABLE 1
COMPARISON OF MICROBIAL AND ANIMAL CELLS

	Microbial	Animal
Size (diameter)	1-10 μm	10-100 μm
Metabolic regulation	Internal	Internal and hormonal
Nutritional spectrum	Wide range of substrates	Very fastidious nature
Growth rate (doubling time, Hr)	Typically 0.5-2.0	Typically 12-60
Environment	Wide range of tolerance	Narrow range of tolerance
Others		Limited population age of normal cells general
		Lack of protective cell wall

narrow concentration range. In general, it is very difficult to make any changes in medium composition without adversely affecting cell performance. As case in point, several researchers have noted the instability of glutamine in culture giving rise to the formation of ammonia (Higuchi, 1970; Tritsch and Moore, 1962; Griffiths and Pirt, 1967), thus requiring the addition of supplemental glutamine. In this case, both the loss of glutamine and the formation of ammonia are undesirable, yet efforts towards replacement of glutamine with glutamic acid have had only mixed results. Apparently glutamine is also a primary source of usable ammmonia by animal cells.

MICROCARRIERS

The cultivation of anchorage-dependant mammalian cells is one of the main problems in the large-scale production of cellular products and virus vaccines. A primary difficulty is one of providing large, accessible surfaces for cell growth. A number of techniques have been proposed as means of solving this problem, including the roller bottle (Ubertini *et al.*, 1960), multi-plate propagators (Schleicher and Weiss, 1968), spiral plastic film bottles (House *et al.*, 1972), artificial capillary propagators (Knazek *et al.*, 1972), and membrane tubing reels (Jensen, 1977). In general, however, these systems suffer from the following shortcomings.

1. limited potential for scale up,
2. difficulty of taking cell samples,
3. limited potential for monitoring the system, and
4. difficulty of maintaining homogeneous environmental conditions throughout the culture.

In an effort to overcome these limitations, van Wezel (1967, 1973, 1977) and van Wezel *et al.*(1978) examined the use of the commercial ion exchange resin, DEAE-Sephadex A50 for large scale cultivation of anchorage-dependent cells. When suspended in culture medium by gentle stirring, the Sephadex beads provided a large surface for attachment growth by normal diploid cells. However, a toxicity was observed at bead concentrations exceeding about 1 gm of DEAE-Sephadex per liter, as indicated by increased inoculum losses and a diminished capacity for cell

growth. Complete death of the inoculum was observed at bead concentrations exceeding 5 gm/liter.

Our laboratories found that this toxicity could be largely eliminated by reducing the exchange capacity of Sephadex beads (Levine *et al.*, 1977a,b; Levine 1977). Using beads prepared with an exchange capacity of 2.0 meq/gm dextran (approximately 40% of the exchange capacity of the commercial preparation), normal human embryonic lung fibroblasts (HEL 299) showed good growth and proliferation at Sephadex concentrations up to 5gm/liter dextran. The potential of microcarriers for scale-up is illustrated by the fact that 1 liter of culture with a DEAE-Sephadex concentration of 5 gm/liter has enough available surface area (27,000 cm^2) to replace approximately 55 roller bottles (490 cm^2 each).

Experiments by Giard et al. (1977,1979) indicate that cells grown on beads are normal with respect to product formation. Their work shows that microcarrier-grown cells can produce viruses and peptide products (interferon) at per cell yields typical of cells grown by more conventional techniques.

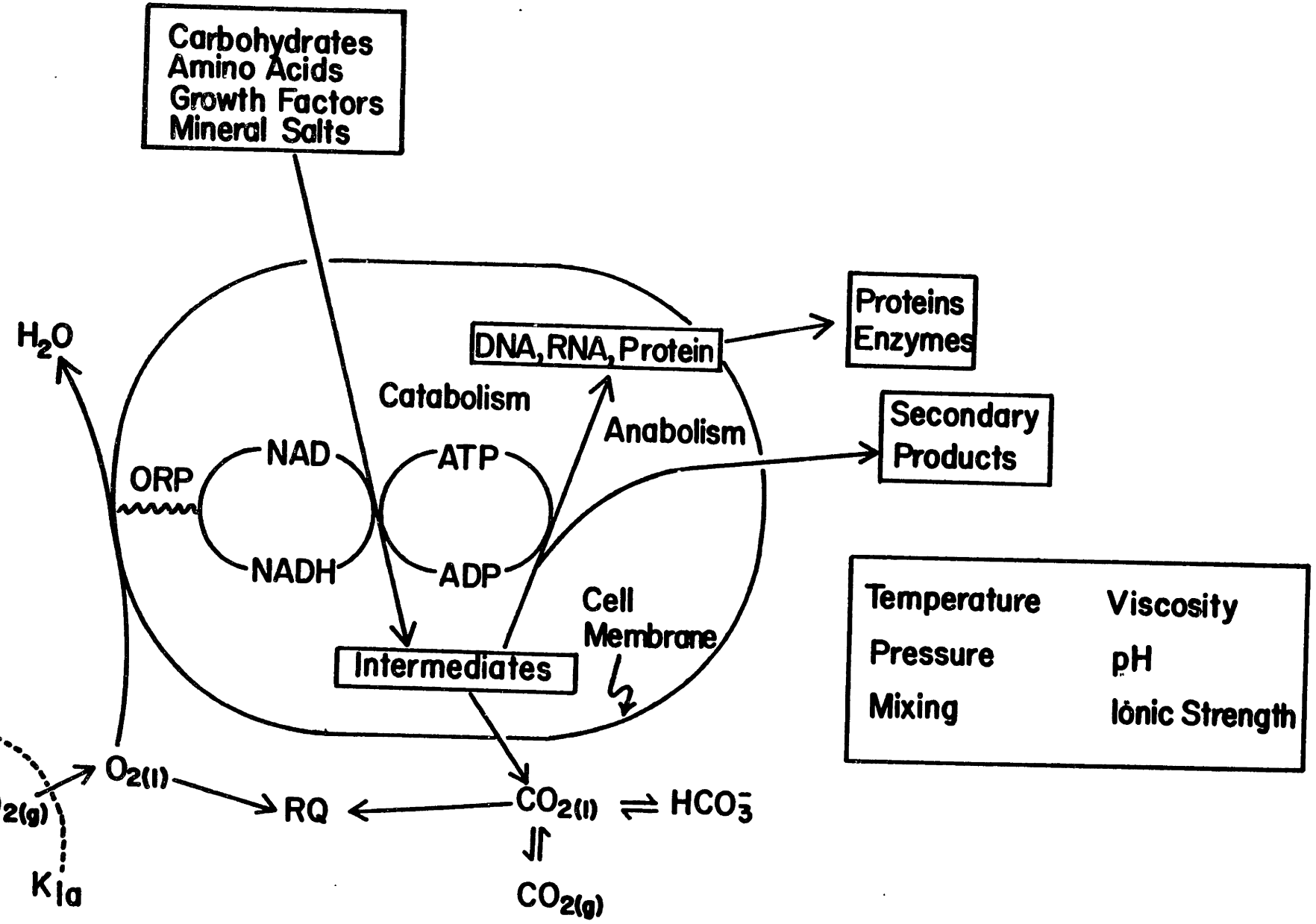
GENERALIZED MODEL OF CELL GROWTH

Most of the measurements made using on-line sensors provide indirect estimates of cell growth and metabolism. As cells grow and divide, they produce changes in their liquid environment, which can be measured and used to monitor cell processes. Thus, for example, measurement of the rate of oxygen utilization provides a direct indication of metabolic activity, which can be used to estimate cell mass. In order to devise a system for measuring cell growth, an understanding of the underlying processes of growth is essential.

Cell-Environment Interactions

In the course of growth and metabolism, the cells consume nutrients from the medium and secrete back into it a number of metabolic by-products,

Figure 2 Generalized Model of Animal Cell Metabolism


Carbohydrates
Amino Acids
Growth Factors
Mineral Salts
H_2O
DNA, RNA, Protein
Proteins
Enzymes
Catabolism
Anabolism
Secondary
Products
ORP
NAD
NADH
ATP
ADP
Cell
Membrane
Intermediates
Temperature
Viscosity
Pressure
pH
Mixing
Ionic Strength
$O_{2(l)}$
$O_{2(g)}$
K_{la}
RQ
$CO_{2(l)}$
HCO_3^-
$CO_{2(g)}$

as shown in Figure 2 on page 24. Animal cells are very sensitive to the concentration of these nutrients, since they have very "leaky" membranes. Hence, if the nutrients in the medium fall below a certain concentration, nutrients will diffuse outward from the cells. This is of particular importance with respect to certain growth factors produced by the cells themselves. As a direct consequence, the cloning of animal cells is typically very inefficient and, in addition, losses in cell inocula incurred during passaging increase with lower cell densities.

Cell-Cell Interactions

Different types of animal cells are capable of influencing one another by hormonal secretions. However, since cell culture usually deals with growth of a single cell type, this interaction is not often encountered. On the other hand, density-dependent interactions (which were previously termed "contact inhibition") are frequently observed; that is, for a given medium composition, cell growth stops at a given surface coverage. This phenomenon has been shown by Folkman and Moscona (1978) to reflect the fact that DNA synthesis is strongly dependent on the curvature of the cell membrane; i.e., as the cell becomes more spherical, DNA synthesis declines. This interaction has been viewed by some as an explanation for the anchorage-dependency of these cells. It should be noted that this phenomenon puts a limit on the minimum diameter acceptable for microcarriers. Marcoudas (1972) determined this limit to be about 55 μm for glass beads using baby hamster kidney cells (BHK21-C13).

PROCESS CONTROL IN CELL CULTURE

The objective of developing instrumentation for animal cell culture is two fold: one, that of further increasing our understanding of animal cells and two, that of achieving process control in cell culture. If we concentrate upon the latter, i.e. process control, we must ask ourselves, what in fact are the products, that one would want to produce using animal cells? Table 2 on page 28 is an effort to list several of these products and can be divided into two categories. In the first group, the cells are the final product, while the second group consists of cell-derived pro-

ducts. The objective, then, of process control is to maximize productivity and product yields while minimizing the variability of these yields over time. However, from an engineering viewpoint the problem of developing process control in animal cell systems is quite different from that of chemical engineering systems. In the typical chemical engineering problem we deal with only a few chemical reactions, and this is not by chance, but rather this is the fundamental design of these systems. Second, by this stage (i.e. when developing process control) these reactions are very well characterized and models have been developed to describe the rates of reactions, and the concentration profiles of the substrates, intermediates, and reactants.

By comparison animal cells are:

- more complicated systems,
- less understood, and
- influenced by a greater number of variables.

Additionally, not much of the data available has been obtained using large scale cell culture and the indiscriminate use of this data to the problems of process control in cell culture can lead to some very misleading (and wrong) conclusions.

A generalized approach toward developing process control in animal cell systems is outlined in Table 3 on page 29. The bulk of this process involves the development of a suitable system's model. If we go back and again compare the animal cell system to the 'typical' chemical engineering system, the animal cell system is both 'data poor' and 'data rich'. Unquestionably, a tremendous amount of data has been accumulated concerning animal cells, but in general the data amassed for any of these individual reactions is rather sparse. It would be neither practical nor useful to attempt to include all of this information in a system's model, but rather the first step must be the formation of a simplified system's model. In essence, one must define a very limited set of biochemical reactions and the corresponding set of variables. The objective is not to completely describe the animal cell but rather to attempt to understand the effect of the environment upon the cell and the production of the cell product while preserving both simplicity and utility. This is an important step and unfortunately its determination is largely subjective. The second step is

TABLE 2

LIST OF POTENTIAL PRODUCTS FROM CELL CULTURE

Group I: Cells as an end product

- Artificial skin
- Artificial organs
  - Beta-islet cells (pancreas)
  - Hepatocytes (liver)
- Bone marrow
- Lymphocytes

Group II: Cell-derived products

- Growth factors
  - Nerve growth factor
  - Epidermal growth factor
- Blood factors
- Monoclonal antibodies
- Interferons
- Proteases (Urokinase, etc.)
- Viruses
- Hormones
  - Human growth hormone
  - Insulin
  - Calcitonin
  - Parathyroid hormone

TABLE 3

GENERALIZED APPROACH TO PROCESS CONTROL IN CELL CULTURE

1. Define the model
2. Define the measurable variables
3. Develop suitable instrumentation
4. Data Acquistion
5. Determine if steps 1 and 2 have been properly formulated. If not repeat these steps.
6. Introduce process control

to examine this model and to determine a set of measurable parameters sufficient to define both the variables of the model and the reaction rates. Next, one needs to develop and obtain the instrumentation needs to measure these parameters and then begin to amass a data base. The purpose of this data acquisiton step is two fold: First, to insure that the measured variables do in fact define the system's model and that the model itself is adequate as a basis for process control, and second, to remove the system from a 'data poor' position and further improve upon the system's model. Once, this has been achieved (ie having gone through step 5), the rest is relatively straight foreward, because at this point the following has been attianed; a simplified, but useful, system's model, a large data base, and the instrumentation to monitor the variables and reaction rates of the model. In fact at this point from an engineering viewpoint the problem of developing process conrol is equivalent to that of the so-called typical chemical engineering problem and for which there is a moderately well developed process for achieving this goal.

EVALUATION OF INSTRUMENTAL METHODS

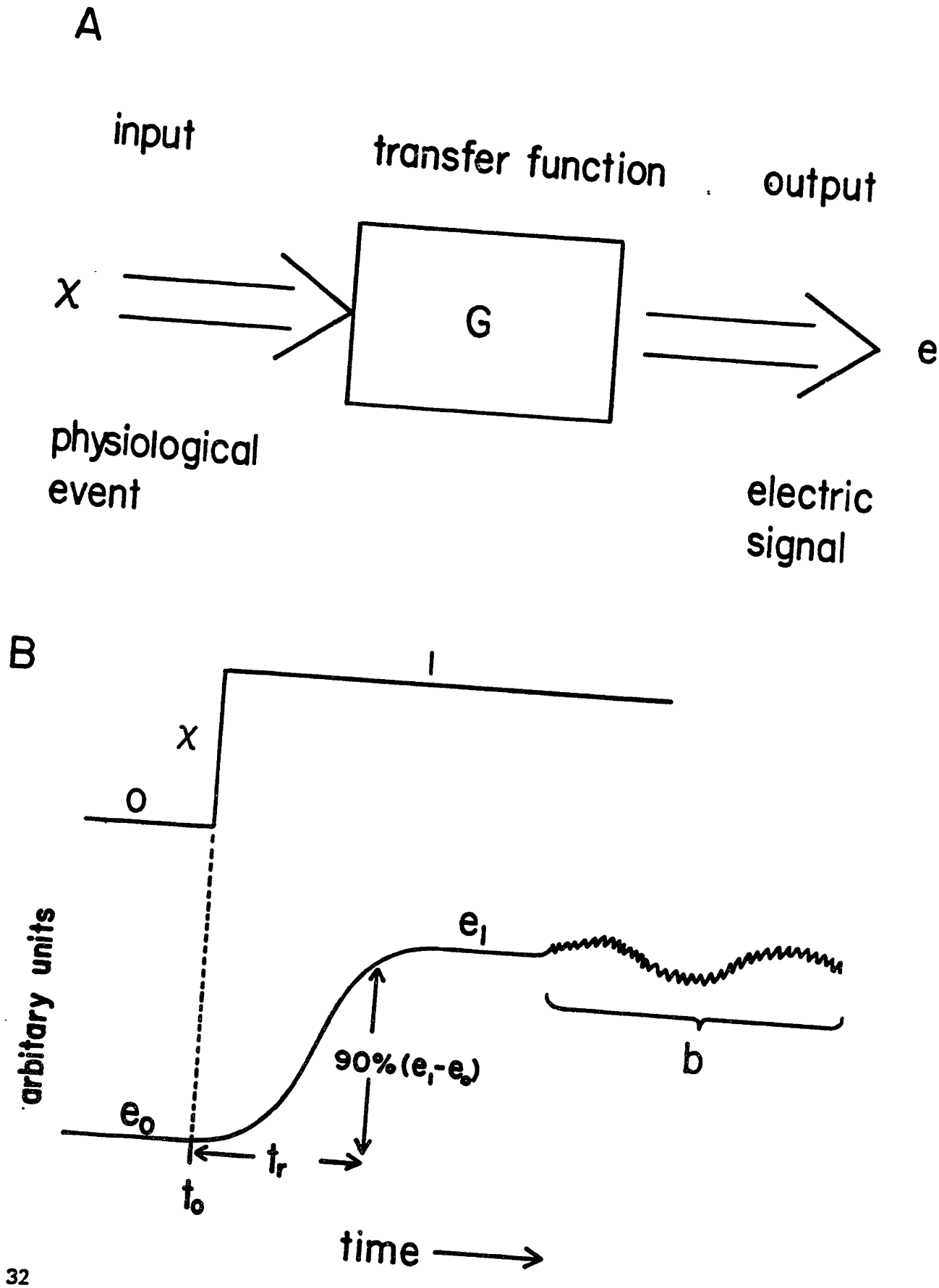
In order to establish a basis for evaluating the performance of instrumental techniques, a number of terms need to be introduced and defined. Most important are the concepts of response time, gain, specificity, signal-to-noise ratio, and drift.

Response Time and Gain

The fundamental process is the translation of some microscopic (or physiological) event into an electric signal, which is then recorded by either a computer or a stripchart recorder. Such a translation is called a transduction, and the associated devices are referred to as transducers. An example of a transducer is a pH electrode, which converts hydrogen ion activity (the microscopic event) into an electric potential that is subsequently recorded. Similarly, the Clark oxygen electrode produces an electric current proportional to the oxygen partial pressure in the surrounding fluid. The exact relationship between the microscopic activity and the electric signal can be very complicated, but for our purposes the

Figure 3 Transfer Function and Transducer Output

(A)Transducer represented in "black box" form. The relationship between the output signal of the transducer and events occurring at the physiological level are described by the transfer function G.
(B) Typical transducer output response to a step change in the input.


A
input
transfer function
output
X
G
e
physiological event
electric signal
B
1
X
0
arbitary units
e_1
90% (e_1-e_0)
b
e_0
t_r
t_0
time

transduction can be adequately described in a "black box" fashion, as shown in part A of Figure 3 on page 32. The ability to describe the transfer function (G) mathematically is very valuable, particularly from the viewpoint of process control (D'azzo and Houpis, 1975). This is because many process controls involve feedback loops, the design of which requires a knowledge of the transfer functions, in order to ensure stable control operations.

Transfer functions can be characterized by the concepts of response time and gain, which can be evaluated by following the system response to a step change (e.g., Cobbold, 1974). Given a step change at the microscopic level (as shown in part B of Figure 3 on page 32), the instruments will show a change (typically slower than the input function) from some value e_0 to a value e_1, where e_0 and e_1 are steady-state values. The response time (t_r) is defined as the time required for the output signal to change from the initial value to 90% of the final value, following the step change at the microscopic level. The reason that the response is defined in terms of the 90% value is that small errors in the steady-state value lead only to small errors in evaluating the time required to reach the 90% values, whereas these same errors can lead to significant errors in estimating the time required to reach the steady state values.

The gain is simply e (output) divided by the x (input) and may or may not be constant over the range in question. A plot of gain versus x represents the calibration curve for the transducer. The term "sensitivity", which is closely related to gain, refers to $\Delta e/\Delta x$.

The acceptable values for these measurements will depend upon the particular system in question. In the case of animal cells grown in culture, the doubling time is about 24 hours, with a typical experiment lasting 150 hours. Thus, a response time of 30 minutes or less would be adequate to resolve the doubling time to within 5%. Most sensors by themselves have response times on the order of seconds to minutes, but the overall response time of the system can be considerably longer. Delays can be caused by flow through tubing and the time required for homogeneous mixing in a large vessel. Additionally, if the information needed is a rate

of change, 10-30 minutes may be required, even though the response time of the transducer is on the order of seconds.

Signal-To-Noise Ratio

Another concept to be defined in relation to the transducer is the signal-to-noise (S/N) ratio. In the example shown (see part B of Figure 3 on page 32), the signal is measured as the difference (e_1-e_0) and noise is measured as the root mean square (rms) of the fluctuation about the time average of signal e_1. Signal noise can generally be segregated into low- and high-frequency components. The high frequency component consists of those fluctuation with frequencies lower than $1/t_r$, and the low-frequency component consists of fluctuations with frequencies lower than $1/t_r$. The high-frequency signal noise often causes the most serious limitation to the overall accuracy of measurement and is the dominant component of the noise in the S/N ratio. The detection limit of the instrument is the smallest quantity that can be measured above the system noise at the physiological level. Typically, one wants to operate with an S/N ratio of 10 or greater.

DRIFT

The low-frequency portion of signal noise contains components referred to as signal drift, the magnitude of which determines how frequently an instrument must be recalibrated. For many instruments, such as an external gas analyzer, recalibration does not represent a serious problem. However, for a number of instruments such as dissolved oxygen probes, recalibration may be difficult or impractical, and it is important to determine beforehand whether such drift is going to cause serious problems over the course of the experiment. In some instances, the use of an internal standard, differential measurement, or a pulsed addition will satisfactorily recalibrate a transducer *in situ*.

In summary, a transducer that converts a biological activity into an electric signal can be characterized by its response time, specificity, gain or sensitivity, S/N ratio, ease of recalibration, and the frequency with

which it must be recalibrated. Two other important considerations are sterility (that is, whether the instrument can be sterilized if it is to come in contact with the culture broth) and cost.

TEMPERATURE

Monitoring and control of temperature are important for a number of reasons. First, animal cells can grow only within a narrow temperature range, and second, since the transfer functions of many sensors are strongly dependent upon temperature, errors can result if the temperature is neither controlled nor corrected. Hence, the ability to maintain the temperature of the culture within a narrow range is essential for cell growth and for reducing measurement errors.

In general, biological systems are not efficient in converting their substrates into cell mass. It is estimated that *Escherichia coli* converts only about 50% of the glucose consumed into cell mass, the other 50% is utilized to generate energy (for both the formation of new cell mass and cell maintenance) with the corresponding liberation of heat (Wang *et al.*, 1976). By comparison, animal cells, because of their proportionately higher maintenace requirements, produce less cell mass in relation to the heat generated.

There are essentially three methods for measuring heat produced: measurement of cooling requirements, flow calorimetry (Eriksson and Holme, 1973),and dynamic calorimetry (Cooney *et al.*, 1969; Mou and Cooney, 1976).

Measuring the heat of fermentation by monitoring the cooling requiremnts is easily adapted to the very large industrial fermentors. Laboratory fermentors, however, are much smaller and tend to lose significant quantities of heat to the surrounding environment by means (such as radiation) for which accounting is difficult. Consequently, other methods for measuring the heat of fermentation are generally used with these smaller vessels (Swartz and Cooney, 1978).

Flow calorimetry involves the continual removal into an insulated chamber of a stream of culture from the fermentation vessel and the measurement of the temperature rise in a small flow cell (see Figure 4 on page 38). The temperature rise, ΔT, depends upon the cell mass concentration and the residence time of the flow cell. The dynamic technique involves periodic disconnection of the temperature control and measurement with a sensitive thermistor of the heat generated in the fermentor. The heat accumulated in the fermentor is calculated as

$$Q_{acc} = kT,$$

where Q_{acc} is the rate of accumulation, K is a lumped parameter representing the heat capacity of the system, and T is the rate of change of temperature. The heat released during growth (Q_H) can then be determined by evaluating the heat of agitation (Q_{ag}) and the heat lost to the surroundings (Q_{surr}), so that

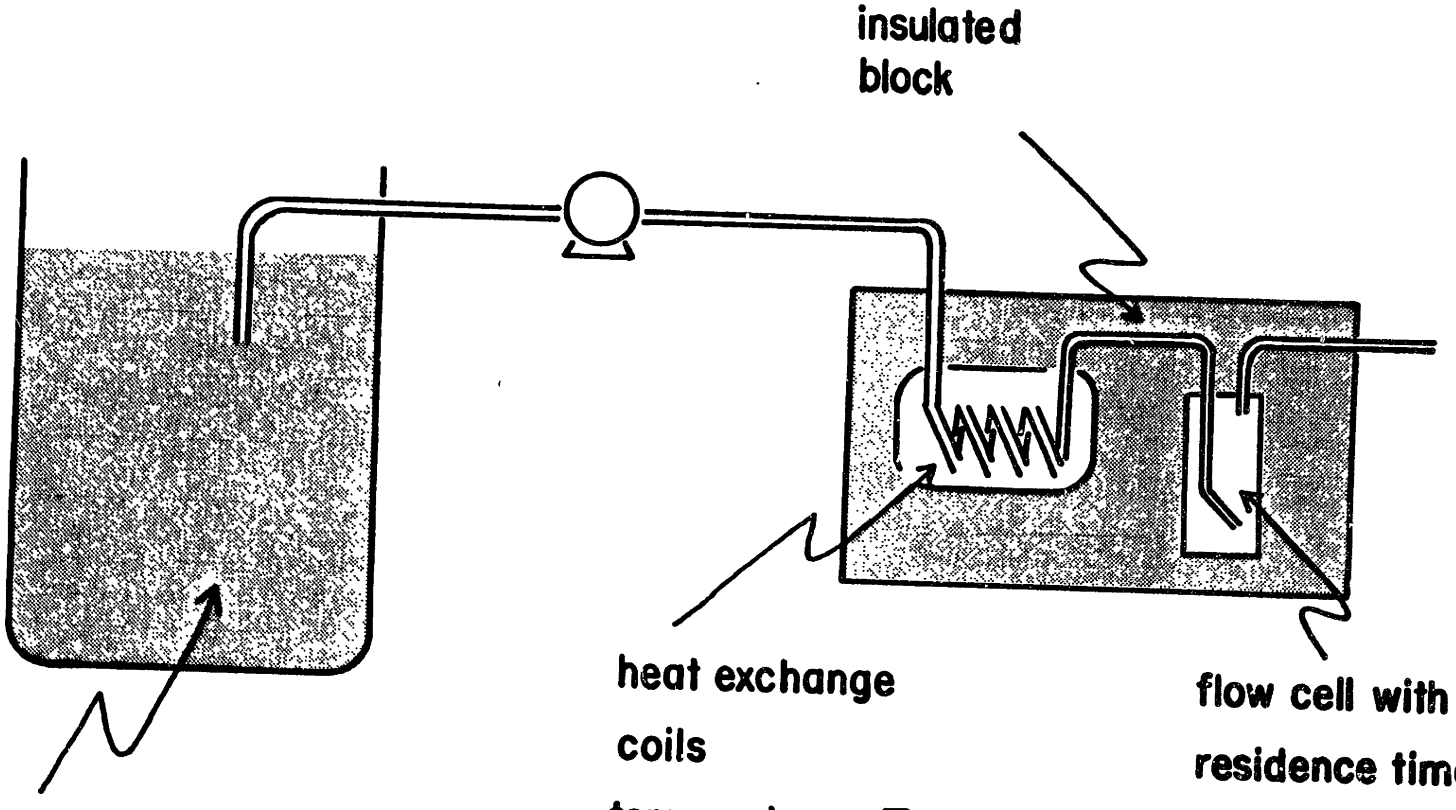
$$Q_H = Q_{acc} - Q_{ag} + Q_{surr}.$$

The heat losses due to the evaporation and the differences in humidity of the air blown into the fermentor can be neglected if the air is saturated with water at the operating temperature of the fermentor.

Both flow calorimetry and dynamic calorimetry have distinct advantages and disadvantages. A flow calorimeter can be used to measure heat evolution independent of culture volume, contributions due to stirring, the addition of gas, alkali, or nutrients, and inadequate insulation of the fermentor, whereas these parameters limit the accuracy of the dynamic technique. However, flow calorimetery is believed to be inadequate for use with filamentous organisms or non-Newtonian fluids, organisms that tend to form wall growths, and cultures above a moderate density owing to the potential for depleting oxygen or other nutrients within the flow cell.

To evaluate the potential of these thermal methods, an estimation of the heat produced by animal cells in culture is required. Given a density of 10^6 WI-38 cells/ml (achievable with microcarriers), with an expected oxy-

Figure 4 Assembly for Flow Calorimetry


insulated
block
culture
vessel
heat exchange
coils
temperature, T_1
flow cell with
residence time, τ
& temperature, T_2

gen consumption of 0.15 mmoles of O_2 /liter-hour (Mclimans *et al.*, 1969), and a heat conversion of 0.11 kcal/mmole O_2 (Cooney *et al.*, 1969), then a heat production of about 0.017 kcal/liter-hour is expected. The draw back to the dynamic method of calorimetry, is that this rate of heat production by the culture is minor compared to the rate of heat lost by the vessel to the environment for most laboratory sized vessels. In our laboratory, in a well insulated 10-liter fermentor (1-in.-thick aircraft fiberglass with aluminum foil on the outside), temperature decreased at a rate of 1.1 °C/hour when it was agitated at 60 rpm at 37 °C without aeration. The rate of heat loss was five times greater without the insulation. Consequently, the dynamic means of calorimetry would involve determining the difference between two large numbers, which is inherently inaccurate.

A flow microcalorimeter while supplying adequate resolution and sensitivity has two serious problems; that of pumping a representative bead suspension to the flow cell and that of beads settling in the flow cell in the absence of stirring. Hence, it appears that a special adaptation of calorimetry is needed to measure cell growth in microcarrier culture. However, for suspension culture the flow microcalorimeter should be directly applicable, if the problem of wall growth in the flow cell can be eliminated.

MIXING AND VISCOSITY

The interactions involved in the rheology of a culture fluid can be complex (Charles, 1978). Adequate mixing is required to ensure uniform environmental conditions and to prevent beads from settling. Since the cells are attached to a surface, the diffusional layers controlling the maximal rates of nutrient uptake will be determined by the velocity of the liquid relative to the bead. In general the effect of high-velocity gradients (shear stress) upon animal cells has not been extensively characterized. Augenstein *et al.*, (1971) have shown that animal cells are relatively tolerant of momentary exposure to relatively high shear stress, but it is the long term effects of viscous shear forces upon cell growth (i.e. is this

going to reduce cell viability, attachment, or cell density) that is most in need of further understanding.

Viscosity changes in culture medium have long been advocated as a possible means of measuring cell growth. However, this approach has not yet been fully developed, as measurement of viscosity in a non-Newtonian fluid is complicated, and to date there is not enough research in the area. The technical aspects of the rheological properties of microbial culture have been extensively reviewed by Charles (1978). Perney *et al.*, (1979) attempted to measure yeast concentration by means of a continuous flow viscometer, which has the advantages of being easy to operate and inexpensive. In principle, viscosity is proportional to the pressure drop for constant flow across a capillary tube. However, in practice the measurement of viscosity was found to be of little value, except at high cell concentrations. The explanation for this is that changes in the yeast morphology also make a nontrivial contribution to the viscous changes in the fluid rheology, consequently at low cell concentrations it is difficult to distinquish between these effect and those due to increases in the cell number. The flow viscometer is probably not well suited for microcarrier culture on account of the problem of bead settling in the capillary tube. However, the method may be well suited for monitoring growth in suspension cultures.

Alternatively, viscosity can be determined from measurements of the power requirements of the impeller system in the fermentor. In the laminar flow region for an ungassed, Newtonian fluid, the power number will be inversely proportional to the Reynolds number. Hence, given a constant geometry, the power uptake is related to the viscosity (μ) and the impeller speed (n) as:

$$P \propto n^2\mu$$

True power measurements can be determined by means of a strain gauge on the impeller shaft.

The microcarrier system consists of cells growing on spherical beads with the system being nongassed (in any case, periods of nongassing pose no

real problems) and the broth viscosity is expected to be Newtonian. As the cells grow on the beads, their effective diameter increases and viscosity is expected to rise. Given an original effective bead diameter of 168 μm, as the cells grow to confluence over this surface, bead diameter can be expected to increase approximately 10 μm. Using the Einstein equation for the modelled viscosity of suspended spheres (Einstein, 1906), where μ_c is the viscosity of cell suspension, μ_r the viscosity of the supernatant, and C is the volume fraction of the suspended particles,

$$\mu_c/\mu_r = (1 + 0.5C)/(1-C).$$

The ratio μ_c/μ_r is expected to change from 1.22 to 1.27 for cell confluent at a concentration of 5 gm of beads/liter. Alternately using the correlation determined by Perley et al. (1979) to relate yeast concentration to viscosity, a change of 1.25 to 1.33 would be expected in μ_c/μ_r. In principle, cell growth on microcarriers should alter the viscosity, but only very slightly (about 4%), and sensitive measurements of that change may prove to be difficult. Additionally, two other methods might in principle be used to monitor cell growth on the basis of a changing effective bead diameter. These are settling time and scattered light. The latter technique is not particularly well suited for use with particle sizes greater than 10 um in diameter. The most serious limitation to the use of any of the three techniques, viscosity, scattered light, and settling time, is that the distribution of bead sizes (168 ± 15μm) is larger than the change expected to result from the growth of a layer of cells about the beads (approximately 10 μm). The measurement suffers from a low S/N ratio and therefore the accuracy with which one is able to predict growth is very poor.

PH

pH is an important parameter to control, since many biochemical processes are sensitive functions of the ionization state of the molecular species concerned. Such processes include the rate of enzyme catalyzed reactions, protein conformation, ion-binding properties of proteins, and others. pH is commonly measured with a combined glass-reference electrode. Steam

sterilizable electrodes are commercially available, and, with proper electronics and temperature control the pH can be measured to an accuracy of 0.01 pH units.

Work by Ceccarini and Eagle (1971) indicates that the absolute value of the pH is less critical than the ability to maintain a constant value during the growth of normal cells. By controlling pH, a cell density twice the usual value can be achieved. Although the individual pH optima vary with cell type, most of the normal fibroblast cells grow fairly well at 7.4. Work by Fodge and Rubin (1975) indicates that 90% of the change in pH is due to the production of CO_2 and lactic acid, which is interesting since if the pH is kept constant, the amount of base added will give a direct and fairly good measurement of the amount of acid formed.

Our studies found CEF's to produce up to 10 m *M* lactic acid, which means that for a total volume of 5 liters, approximately 200 ml of 0.25 *M* sodium hydroxide would be required over the course of the experiment to control the pH. Base addition could best be monitored by means of a load cell to measure the weight of the contents of the bottle from which base is added. Load cells have two basic designs: position-spring transducers and strain gauges. The position-spring transducers are less expensive but also less accurate. By means of a spring, the weight of an object is translated into a vertical displacement, which is measured by the position transducer. The nonlinearity and hystresis of the spring are the major limitations on the accuracy of the devise, approximately 2% with extensive calibration (Swartz and Cooney, 1978). Strain gauges give results that are reproducible to 0.01%, display nonlinearities of about 0.03% and are essentially insensitive to change in the ambient temperature (technical information from BLH Electronics, Inc., Waltham, Massachusetts).

In summary, maintenance of a constant pH is beneficial for cell growth. In addition, growth and growth rates can be estimated by quantitating the base addition used to control pH over the course of the experiments. The measurement of base addition is simple, direct, and quite accurate.

IONIC STRENGTH

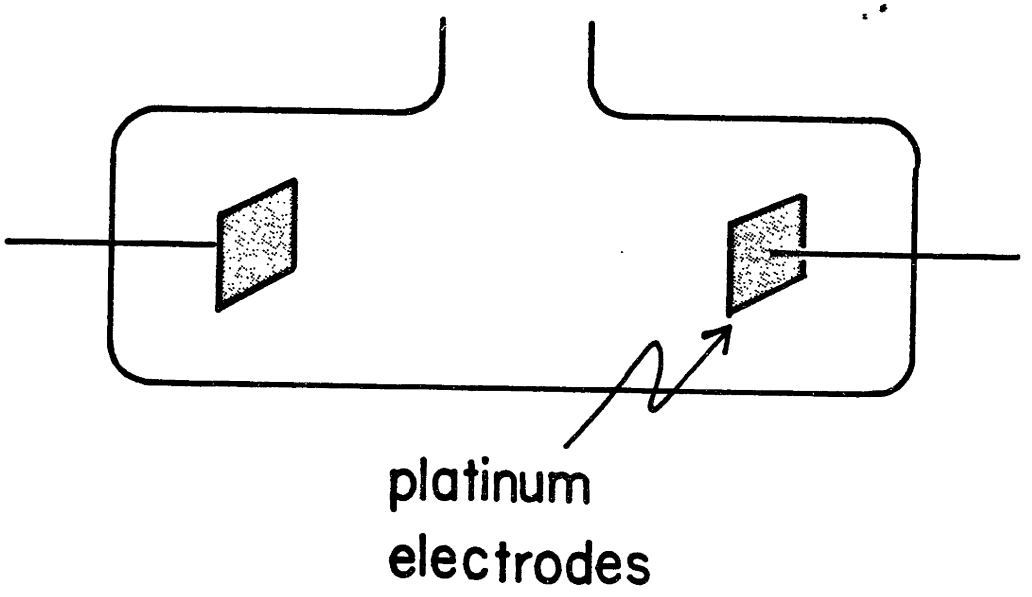
Animal cells are also sensitive to the ionic strength of their environment. However, the changes in ionic strength occurring over the normal course of growth are not large enough to be physiologically significant. Such changes are the result of the removal of charged species from the medium or the conversion by the organism of noncharged substances to charged compounds, such as glucose to lactate. These small changes can be directly measured electrically and provide a basis for a continuous measurement of the accumulation of metabolites. Hence, a number of investigators (Hardy *et al.*, 1977; Kagen *et al.*, 1977; Zafari and Martin, 1977, Richards *et al.*, 1978; Cady, 1975; Ur and Brown, 1975) have proposed using electrical conductance measurements to monitor cell metabolism. This is a relatively simple procedure, which can be rapid, sensitive, and widely applied. A conductivity measurement is usually performed using alternating current conditions of 1-10 kHz rather than direct current and utilizing two platinum electrodes in contact with the broth (see part A of Figure 5 on page 44). The impedance of a measurement cell containing bacterial culture or tissue culture medium can be crudely modeled as either a series or a parallel resistor-capacitor circuit (see part B of Figure 5 on page 44). The capacitance is believed to arise at the liquid-electrode interface (Richards *et al.*, 1978), whereas the resistance is due mainly to the ionic content of the liquid. In determinations of conductivity, certain precautions need to be observed. The conductance should be measured in an AC circuit to minimize electrode polarization (Schwan, 1968; Geddes, 1972; Cobbold, 1974). Furthermore, the voltage drop across the electrodes should be kept below one volt to minimize electrolysis of the liquid, and the temperature should be carefully controlled, since conductivity is a significant function of temperature (about 2%/°C). By way of comparison, a 1% change in conductance is expected to occur over the entire culture period due to the growth of the organisms.

Richards et al. (1978) argues that the resistive component of impedance provides a more appropriate measurement of the bacterial growth than total impedance, since this component is a measure of the ionic content of the medium. They were also able to quantitate the resistive component of

Figure 5 Diagram of Conductance Cell and Electrical Analogue

(A) Typical conductivity cell
(B) Series electrical equivalent of conductivity cell.

A



B

total impedance, using sophisticated electronics that would lock onto that portion of the output signal in phase with the input signal.

In cell culture an estimate of the expected change in conductance can be made if it is assumed that changes in the ionic content of the medium result only from production of lactic acid and ammonia (assumed to have final concentrations of 20 and 3 m *M* respectively). Conductance (L) is defined for a slab of conducting material to be:

$L = KA/l'$

where A is surface area of the slab (cm^2), l' is the thickness of the slab (cm), and K is a parameter dependent on the ion species involved and the concentration (ohm^{-1}, cm^{-1}). K is defined as:

$K = C (l^+ + l^-)$

where C is concentration (equivalents/ml), and l^+ and l^- are constants dependent on the species involved. The $l^+ + l^-$ for NH_4OH is 274 cm^2 $equiv^{-1}$ ohm^{-1} (Castellan, 1964), and about 92 cm^2 $equiv^{-1}$ ohm^{-1} for sodium lactate (assuming lactate to behave approximately as acetate). Given l' to be 7 cm and A to be 0.18 cm^2, the total conductance change would be 1×10^{-4} ohm^{-1}; therefore, a 1.3% change in conductance is expected.

In principle, conductance measurements can be used to determine inoculum size, lag time, and population-doubling times. The method is conceptually simple but requires moderately sophisticated electronics and a well-controlled temperature environment. Animal cells in culture appear to produce charged compounds in amounts sufficient to be detected by the instrumentation described for work with bacteria. A primary limitation of this method will be the stability of the medium. The decomposition of the medium (glutamine to ammonia, for example) could produce significant background drift, requiring a differential measurement. A major criticism of the technique is that one is never entirely certain exactly what chemical species is being measured, since conductance is affected by many factors.

DIALYZABLE COMPONENTS

In order to grow and/or maintain its metabolic activity, an organism must utilize a number of nutrients from the environment. Examples of these nutrients include glucose, amino acids, certain organic acids, minerals, and vitamins. Quantitating the uptake rates of these nutrients provides useful information for monitoring cell growth and metabolism. Glucose is the most commonly examined of these nutrients. A number of enzyme probes have been constructed to measure glucose concentrations (Gray *et al.*, 1977; Guilbault, 1976). The major problem with these devises is that they cannot be steam-sterilized. Alternatively, a dialysis membrane probe can be used to remove glucose from the broth to an external glucose analyzer (Zabriskie and Humphrey, 1978a) (Figure 6 on page 48). In principle, the liquid flows across one side of a dialysis membrane, and the dialyzable compounds in the bulk solution diffuses across the membrane into the liquid stream (Figure 7 on page 50). The concentration of each compound leaving the dialysis probe (S) is related to its concentration in the bulk fluid (S_b), the liquid velocity (V), the dimensions of the probe (W,D,L),and the permeability of the membrane to the dialyzable compound (U), as given by the following equation:

$$\ln(1-a) = -(UL/D)(1/V),$$

where $a = S/S_b$. The response time of the measurement will be related to the liquid velocity, but is typically about 5 minutes.The accuracy and linearity of the measurement will depend largely upon the downstream transducer and the quality of the pumps.

The dialysis probe is a promising development in the area of instrumentation for cell culture. First, it provides a means for on-line use of transducers that cannot be steam-sterilized (such as enzyme electrodes). Second, because of the transducer's external location, recalibration and other adjustments are easily made. Finally, the dialysis probe in conjunction with the appropriate transducers has the potential for measuring a wide spectrum of substances, including carbohydrates, organic acids, amino acids, vitamins, ammonia, mineral salts, and a number of growth factors.

Figure 6 Assembly for Dialysis Probe

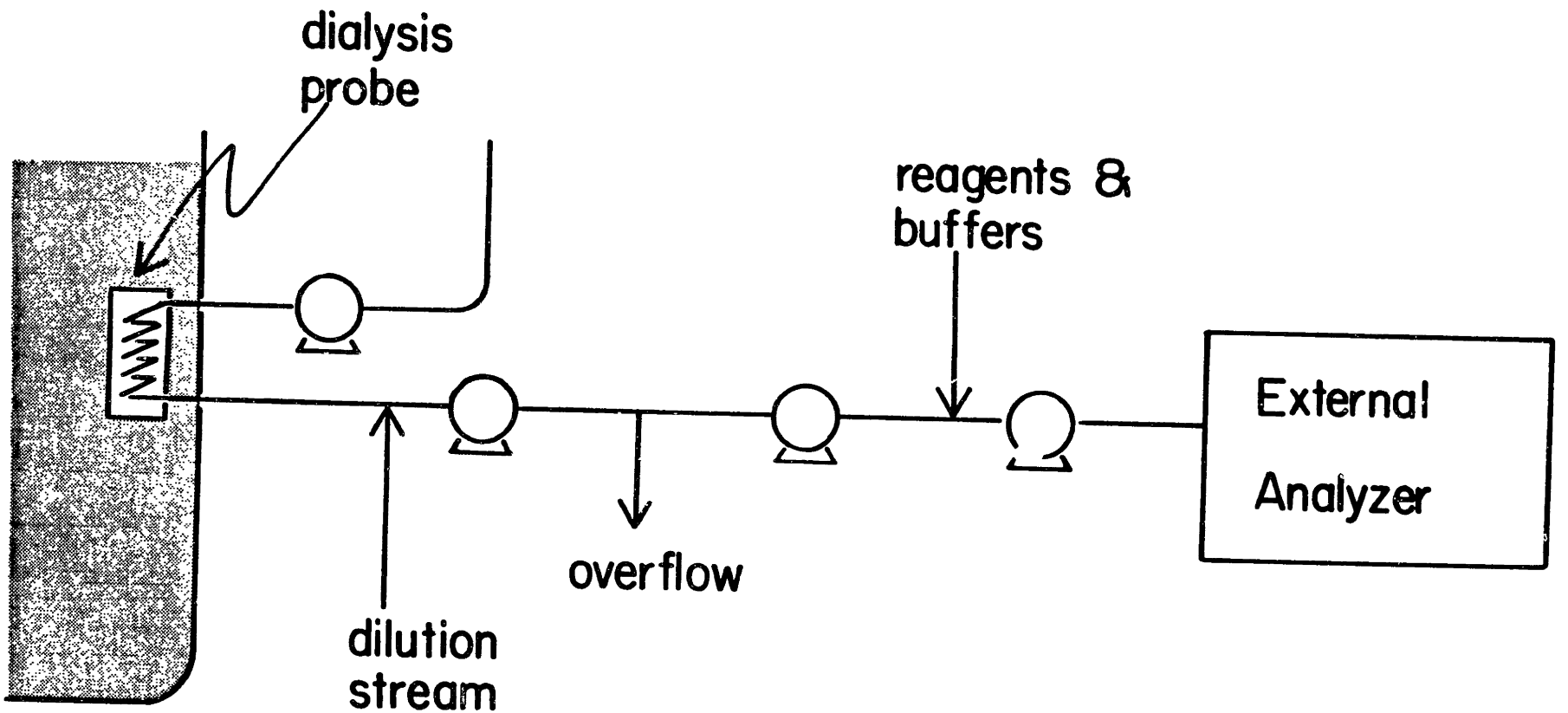
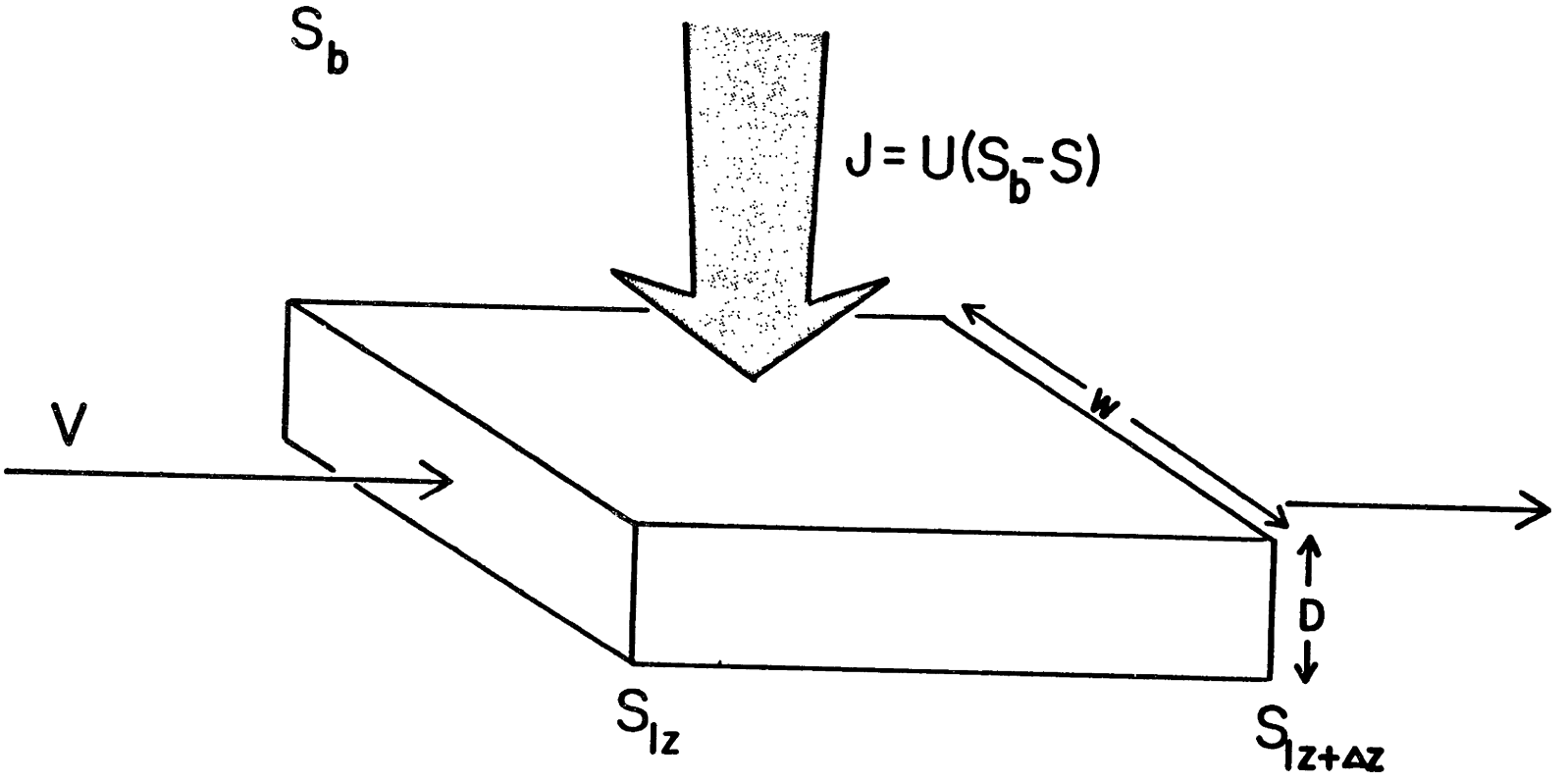

dialysis
probe
reagents &
buffers
External
Analyzer
overflow
dilution
stream

Figure 7 Diffusion of Solute Across Membrane into Channel of Dialysis Probe


S_b
$J = U(S_b - S)$
V
W
D
$S_{|z}$
$S_{|z+\Delta z}$

OXYGEN

Oxygen is another basic parameter in biological processes. Of particular importance are the effect of oxygen concentration on the growth and metabolism of the cells, and the problem of transporting oxygen from the gas phase to the cells. Kilburn and Webb (1968) have shown with mouse LS cells that optimal growth occurred when the oxygen partial pressure (PO_2) was controlled within the range of 40-100 mm Hg. At PO_2 levels below this range, metabolism became more anaerobic, as indicated by the increased production of organic acids and elevated levels of lactic dehydrogenase. Conversely, an oxygen toxicity was observed at PO_2 levels above this range, as indicated by decreasing cell growth rates, saturation densities, and with further increased concentrations (above 320 mm Hg) cell death.

Mclimans *et al.*, (1962, 1968) have reported on the importance in cell culture of adequate oxygen transfer for the establishment and growth of primary hepatic cells. In very detailed work, the depth of the liquid overlay in monolayer culture was related to overall gas exchange kinetics. In microbiological systems, the oxygen demand for cells growing in submerged culture can generally be provided by sparging the gas at the bottom of the vessel. The objective is to maximize the interfacial area between the gas and the liquid and to minimize the distance that the oxygen must diffuse from the gas to the cell. A large amount of data has been accumulated, correlating vessel geometry, impeller speed, gas flow, and broth rheology with mass transfer rates (Wang *et al.*, 1979).

However, it is not clear that sparging can provide adequate mass transfer for cell growth in microcarrier culture. In the first place, the medium contains a number of serum components that would lead to serious foaming problems. Moreover, since animal cell membranes are not covered with a rigid cell wall, many of the antifoaming agent used in microbial fermentations cannot be employed. Second, for reasons not well understood, the presence of gas bubbles causes pronounced damage to cells in cell culture (Kilburn and Webb, 1968). Similar damage (platelet losses and hemolysis) by air bubbles in blood oxygenation is well documented (Mortensen, 1977). Although there is some evidence that increasing the

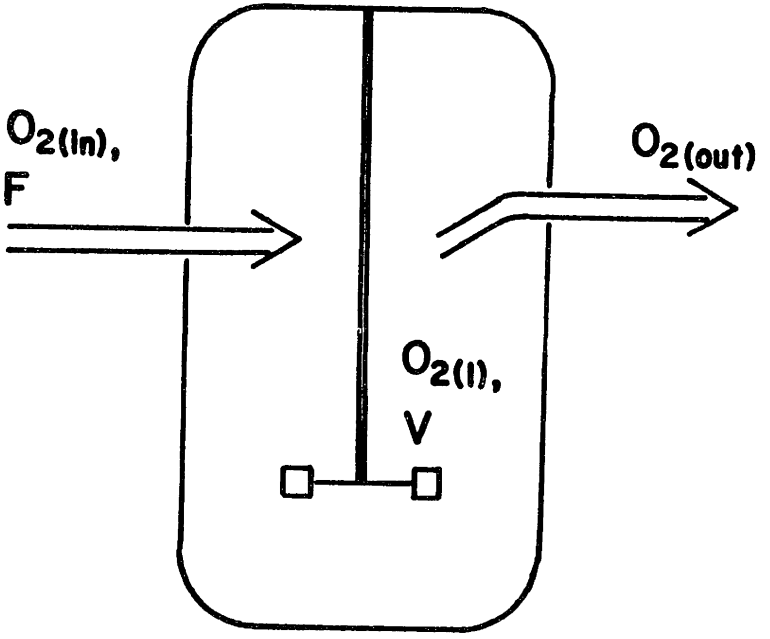
serum concentration will minimize foam damage (Kilburn and Webb, 1968), this possibility has not been well investigated nor does it promise to be realistic, since the problem of foaming will remain.

Surface oxygenation, which has been used in the laboratory, would not appear to be suitable for large-scale work, since the surface area per unit volume needed to support adequate mass transfer is not available. However, membrane oxygenators function by providing a very large surface area for gas exchange.

Quantification of oxygen demand can be used to estimate cell growth and metabolism. The oxygen uptake rate can be measured by means of off-gas analysis or dynamic assessment. The off-gas technique involves measuring the gas flow rate, the inlet oxygen concentration, and the outlet concentration. The oxygen uptake rate is equal to the product of the flow rate and the difference of gas concentrations (see Figure 8 on page 54). The usefulness of this method depends on both the sensitivity of the oxygen analyzer and the uptake rate itself. For example, the paramagnetic analyzer is capable of measuring a 2% change in the oxygen concentration with 1% accuracy (Snedecor, 1977). For a 5-liter culture of WI-38 cells growing at a density of 10^6 cells/ml, the corresponding oxygen demand would be 0.75 mmoles O_2/hour (16.8 ml O_2/hour). For a 2% change in exit gas concentration, the flow rate through the system would be 840 ml/hr. However, the major problem with the paramagnetic analyzer is that it is quite sensitive to the variation flow for low gas-flow rates. In order to maintain 1% accuracy in the oxygen uptake rate, the flow to the analyzer must be kept constant to within 0.2% (Swartz and Cooney, 1978).

Alternatively, the oxygen uptake rate can be assessed using the dynamic technique. In this procedure, the air flow to the culture is stopped and the change in dissolved oxygen is measured over time. For 10^6 WI-38 cells/ml, the dissolved oxygen concentration would be expected to change from 50% to 25% saturation in about 25 minutes. The response time for a fermentor-quality dissolved oxygen probe is about 45 seconds. The probes are accurate to 0.3% and feature a drift of less than 2%/week (technical data, Instrumentation Laboratories, Lexington, Massachusetts).

Figure 8 Mass Balance of Oxygen into and out of a Fermentor



$$OUR = F(O_{2(in)} - O_{2(out)}) - V\frac{d}{dt}O_{2(l)} \quad (\sim 0)$$

A

Finally, the mass spectrometer (MS) can be used to measure dissolved oxygen and gas phase oxygen simultaneously with other gases. Although the use of MS is not yet widespread in fermentation monitoring (Reuss *et al.*, 1975; Weaver and Abrams, 1979), it has been used successfully in many medical applications, particularly blood gas (Woldering *et al.*, 1966; Dyken, 1972).

CARBON-DIOXIDE

Measuring the production of carbon dioxide promises to be another useful means of monitoring and controlling fermentation processes. In a number of instances, cell mass has been correlated with cumulative CO_2 production (Mou 1979; Mou and Cooney, 1976), and others have used CO_2 production as a means to close carbon mass balances and estimate growth rates and cell mass (Wang *et al.*, 1976).

Carbon dioxide is the end product of a number of metabolic processes, and the total amount produced is dependent upon the activity of the individual pathways, as well as the total amount of cell mass. The respiratory quotient (R.Q.) is defined here

R.Q. = CO_2 production rate/O_2 uptake rate

and serves as a valuable indication of the cell's metabolism. A number of workers have controlled the growth of yeast by limiting the glucose addition on the basis of R.Q. values (Nagai et al., 1976; Wang, 1977). Excess glucose was observed to induce an anaerobic state in yeasts and to form ethanol instead of cell mass, the change in metabolism being concurrent with a sudden increase in R.Q. Efficient conversion of glucose to cell mass was affected by limiting the rate of glucose addition to maintain the R.Q. at a value of about 1.

Carbon dioxide plays an additional role in animal cells, since the CO_2-bicarbonate system is one of the three important buffers in the blood of vertebrates. Hence, it is of interest that in the development of basal minimal essential (BME) medium, Eagle (1971) found the CO_2-bicarbonate

system optimal for the growth of cells. Additionally, a class of nongaseous buffers has been developed for cells in mass culture (Eagle, 1971). However, colonies will grow from single cells with high efficiency only if carbon dioxide is also present. Animal cells in fact need some CO_2 for cell growth, and apparently, when growing in low density cultures CO_2 will diffuse out of the system faster than they are able to produce it, hence the need for an external source of CO_2.

There are essentially two methods to measure CO_2 production: off-gas analysis and dynamic assessment. The off-gas analysis instruments typically quantitate CO_2 concentrations by measuring the infrared absorbance of the carbonyl bond. They are considerably less sensitive to gas flow rates than the paramagnetic oxygen analyzers and have much faster response times. Whether the flow of gas through the system will give adequate resolution depends upon the concentration of CO_2 in the inlet gas stream, assuming the pH is buffered in part by a CO_2-bicarbonate system. Suppose we have 10^6 WI-38 cells/ml and the R.Q. = 1, then 5 liters of culture should produce 16.8 ml of CO_2/hour. If the concentration of CO_2 in the inlet gas stream in 2% and the desired concentration of CO_2 in the outlet stream in 2.2% then the flow would be 8.4 liters/hour. Instruments with adequate resolution are available. However, an important problem associated with CO_2 off-gas analysis is that the partial pressure of CO_2 in the off-gas is not in equilibrium with the concentration of dissolved CO_2 (Reuss *et al.*, 1975). This is owing to the fact that desorption of CO_2 from the liquid is a relatively slow process, presumably due to the slow conversion of bicarbonate to carbonic acid. The method of off-gas analysis is valuable for determining the integrated production of CO_2. With increasing flow rates, the measurements of instantaneous CO_2 production rates become more reliable.

The technique of dynamic assessment have a distinct advantage in the case of instantaneous CO_2 production rate. The methodology is essentially the same as for measurement of the oxygen uptake rate: the gas flow is momentarily interrupted and the accumulation of CO_2 in the broth is measured. There are basically two steam-sterilizable instrumental methods for measuring CO_2: the tubing method and the mass spectrometer (MS).

The tubing method (Phillip and Johnson, 1961; Kilburn and Webb, 1968; Self *et al.*, 1968; Kilburn *et al.*, 1969) involves passing a stream of inert carrier gas (e.g. nitrogen or helium) through a coil of permeable tubing (e.g. silicone rubber or polypropylene), and then measuring the dissolved gas, which enters the tubing by diffusion-permeation and is swept to a CO_2 transducer. The system has a response time of 2-10 minutes, depending on the flow rate and the permeability of the tubing. Although it has found little use with microbial systems, it is well suited for work with animal cells. Using a 3m length of silicone rubber tubing (1.0 and 2.0 mm OD), Kilburn and Webb (1968) found the CO_2 content of the carrier gas to be a linear concentration of the partial pressure of CO_2 external to the tubing. At 38 mm Hg, the CO_2 content of the carrier gas (with a flow rate of 55ml/minute) was 0.355%. The tubing can also be used to measure other dissolved gases by placing other gas transducers downstream in the carrier gas (see part A of Figure 9 on page 60).

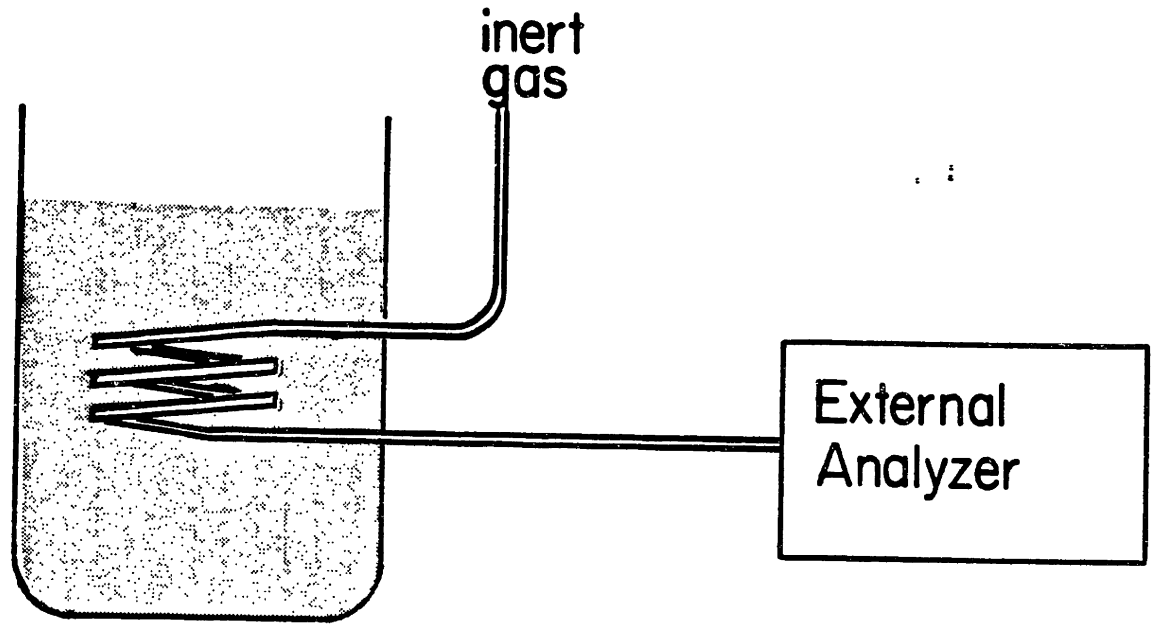
A MS can serve as a CO_2 transducer in the tubing method, or it can also be used continuously to measure dissolved CO_2. In the probe configuration (see part B of Figure 9 on page 60), using a macroscopic probe (Reuss *et al.*, 1975), the MS system consists of specially constructed membrane-covered inlet system introduced directly into the culture and coupled to the instrument by means of a stainless-steel tube. The silicone rubber membrane interfaces the culture broth to the vacuum of the MS, allowing the low molecular weight compounds to enter the MS by diffusion across the membrane.

In principle, the MS is very similar to the tubing method. Its advantages are that faster response times are obtained and that a large number of different compounds can be measured simultaneously with one instrument. The MS has been used for some time in blood gas analysis (Woldering *et al.*, 1966), and more recently in fermentation monitoring of dissolved CO_2, dissolved oxygen, and methanol concentrations (Reuss *et al.*, 1975). The technique provides good chemical concentration resolution and responds linearly to dissolved gas and dissolved volatile compounds over a wide range of concentrations (e.g., 5×10^{-6} to 1 *M* for dissolved Ethanol) (Reuss *et al.*, 1976; Weaver *et al.*, 1978).

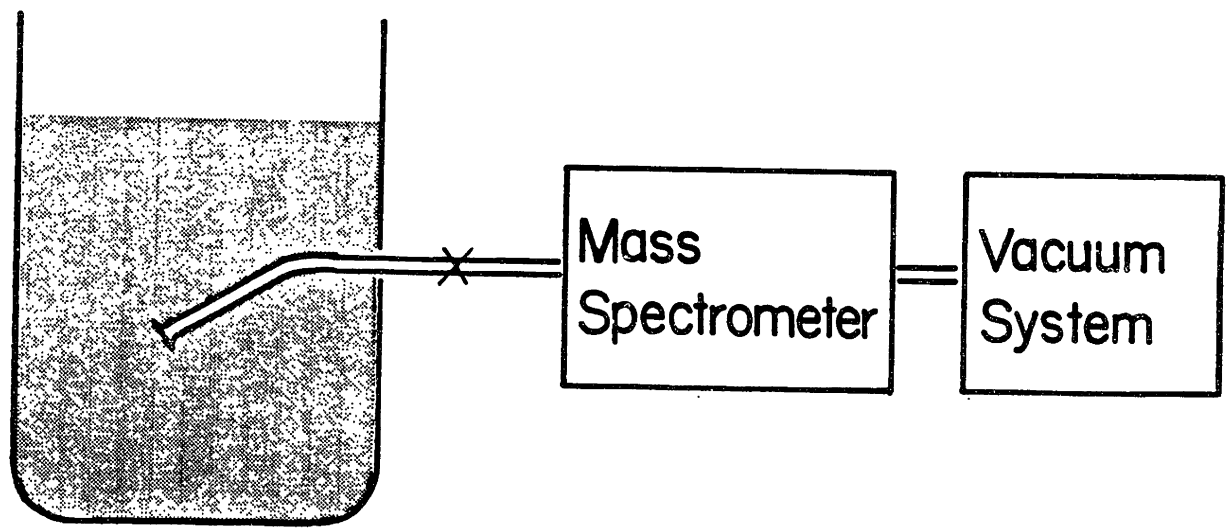
Figure 9 Assemblys for the Measurement of Carbon Dioxide

(A) The tubing method and
(B) The mass spectrometer.

A


inert
gas
External
Analyzer

B

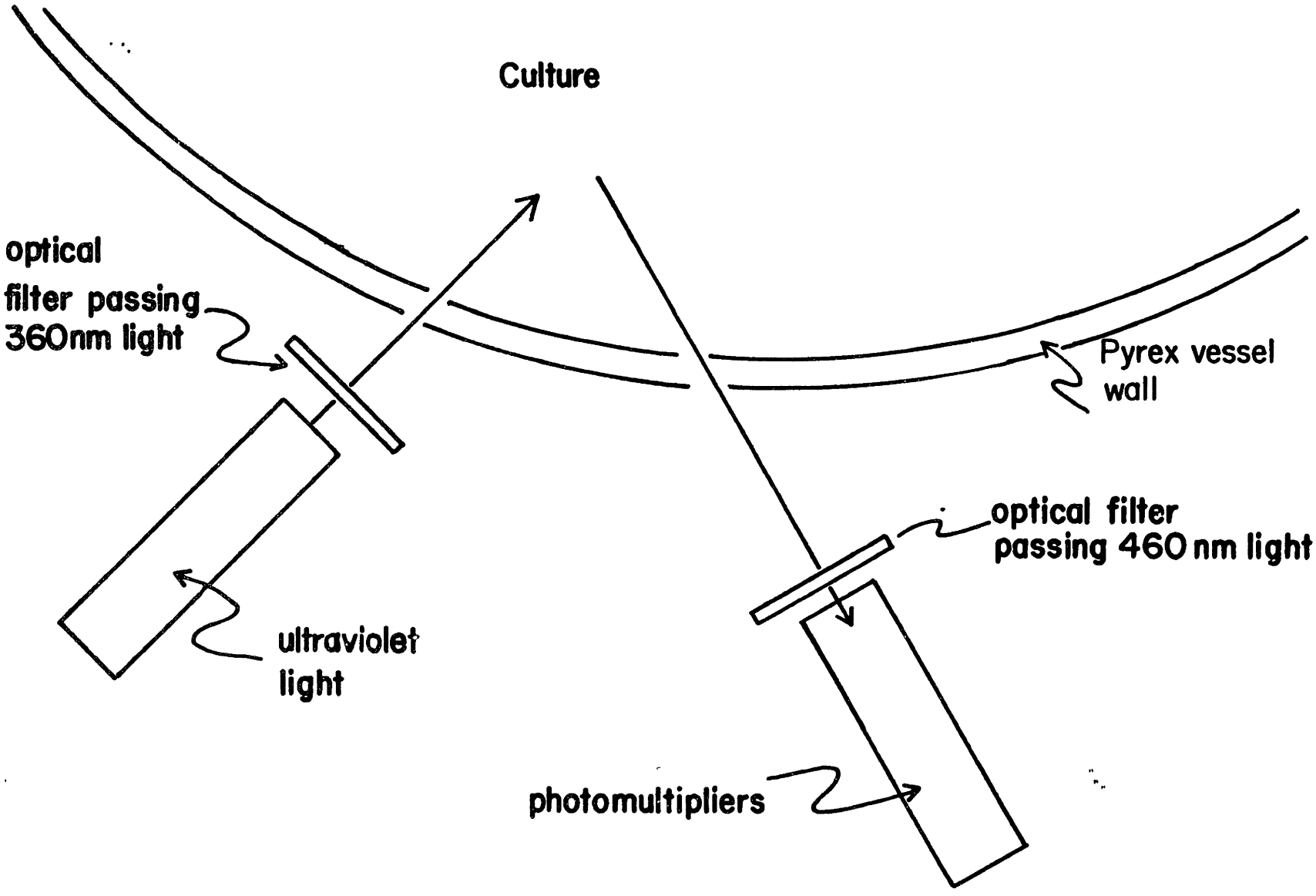

Mass
Spectrometer
Vacuum
System

Generally MS cannot be used to examine a number of substances simultaneously without additional separation techniques (such as gas chromatography). The reason for this is that mass spectra tend to be complicated and examination of several compound simultaneously can result in significant overlap, seriously compromising the quantitation of the individual species. The nightmare that would result from injecting the culture broth directly into the MS is in practice avoided by the selectivity of the semipermeable membrane. The membrane allows only the dissolved gases and highly volatile organics to diffuse into the vacuum chamber, thus greatly simplifying the interpretation of the spectrum. Recent work by Weaver and Abrams (1979) has shown that selective expression of volatile acids and bases is made possible by controlling the pH at the MS interface. Hence, overlap from organic acids and bases can, in principle, be accounted for. Nonetheless, if neutral organic compounds are not removed, the presence of several similar acids or bases may prove to be troublesome.

NAD^+-NADH

It is possible to measure the concentration of cell mass on the basis of total culture fluorescence (Figure 10 on page 62), because the intracellular reduced nicotinamide nucleotides (NADH and NADPH, although in animal cells NAD concentrations are generally much greater than NADP concentrations (Lehninger, 1980)) will fluoresce at 460 nm when cells are irradiated by 360 nm light (Zabriskie and Humphrey, 1978b; Harrison and Harmes, 1972; Harrison and Chance, 1970; Ristroph *et al.*, 1977). The fluorescence property has been used extensively by biochemists in studying the response of NAD^+-NADH levels to environmental changes. Zabriskie and Humphrey (1978b), and Harrison and Chance (1970) have taken this approach for estimation of biomass concentration in fermentor culture. They found that culture fluorescence was a function of biomass concentration, together with a number of environmental factors, such as dissolved oxygen concentration, energy substrate concentration, temperature, and pH. When these environmental conditions were carefully controlled, the log of the fluorescence was linearly related to log of the biomass. Fluorescence increased dramatically with temperature

Figure 10 Assembly for Measurement of Culture Fluorescence


Culture
optical
filter passing
360nm light
Pyrex vessel
wall
optical filter
passing 460 nm light
ultraviolet
light
photomultipliers

(2.5%/°C), which again mandates the requirement for accurate temperature control. However, the fluorescence decreased with increasing pH, an effect thought to be due to the ionic state of the fluorophores or some quenching species.

For 10^6 WI-38 cells/ml, the dry weight is about 0.6 gm/liter. If the fluorescence on a per weight basis is the same as that measured for yeast, we would expect a fluorescence signal with a S/N ratio of 50, as measured using the instrument of Zabriskie and Humphrey. Media used to support microbial growth often have a background fluorescence (Ristroph *et al.*, 1977). While it is not clear how much background signal is to be expected from the media used for cell culture, these numbers do not appear unreasonable. The use of this technique with microcarrier culture has two added advantages. One is the possibility of allowing the beads to settle and determining the background signal at any time, the other is the possibility of making the measurements without interference from air bubbles, which apparently can cause instability in the signal.

A suitable fluorescence instrument is not readily available though it would not be complicated to construct (Mayer *et al.*, (1969). However, one problem to be encountered in long term use of such an instrument are variations in the intensity of the lamp over time. In order to normalize the signal, it is essential in the construction of such an instrument to provide for measurement of the intensity of the excitation beam and to determine the ratio of output signal to the intensity of the lamp.

Matsunaga *et al.*, (1979) have reported on an electrode system for estimating cell mass directly (see part A of Figure 11 on page 66). The system consists of two electrodes, which are basically identical except that one is covered with a dialysis membrane (the reference electrode). Each electrode has a platinum anode and a silver peroxide cathode. Phosphate buffer (1.0 *M*, pH 7.0) was used as the catholyte, and an anion exchange membrane was used as a separator. The electrode is steam sterilizable, and the output is proportional to the cell number.

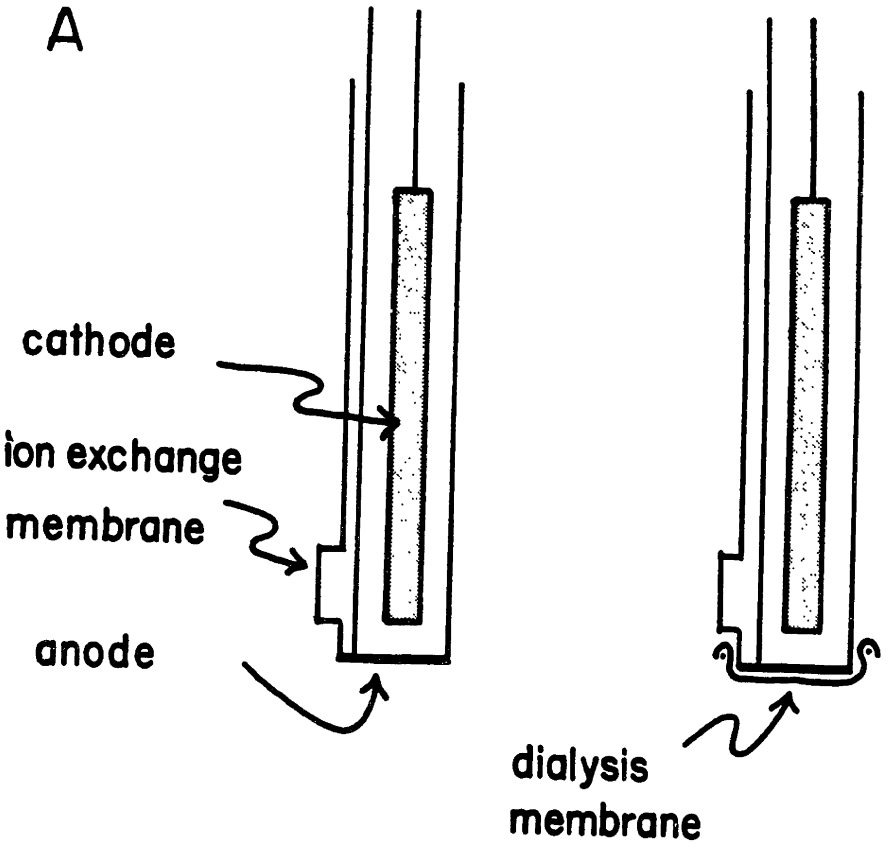
In principle, the electrode operates by accepting electrons directly from NADH-$FADH_2$ groups located on the cell surface (see part B of Figure 11

Figure 11 NADH Biomass Electrode

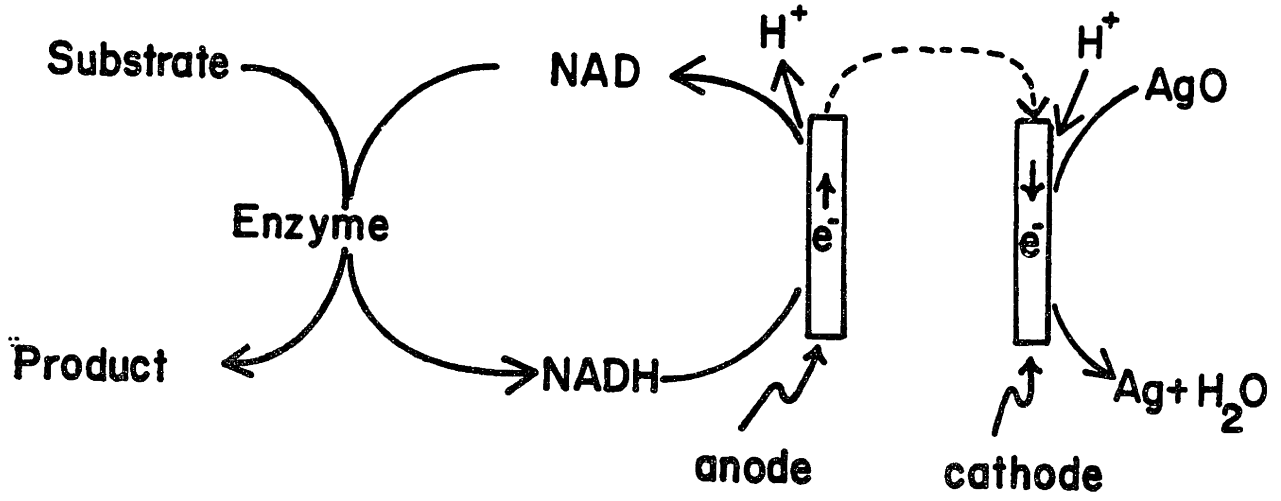
(A) Electrode for estimation of biomass

(B) Idealized process occurring at electrode surface

A


cathode
ion exchange
membrane
anode
dialysis
membrane

B


Substrate
Enzyme
Product
NAD
NADH
H+
H+
AgO
e-
e-
anode
cathode
Ag+ H2O

on page 66) (Matsunaga *et al.*, 1978). By virtue of the dialysis membrane, the reference electrode excludes the cells but not the oxidizable groups in solution, thus allowing the background signal to be determined. Only viable cells seem to contribute to the electrode signal. Since the cells themselves must be transported to the electrode surface in order to transfer electrons, the response time of this electrode should be governed by the transport rate of cells or by the diffusion of substrate materials to cells in the vicinity of the electrode surface, Hence, a slow response is expected, and in fact the electrodes have a response time of about 15 minutes. With cells bound to microcarriers, still longer response times might be anticipated.

In their work with a yeast suspension, Matsunaga *et al.*(1979) found the signal to be sensitive to temperature (about 2%/°C) and pH (about 16% per pH unit). However, with adequate control, the relative error in these signals can be reduced to less than 1%. The electrode showed a linear response with bacteria and yeast over concentration range of 10^8 to 4 x 10^9 and 10^7 to 4 x 10^8, respectively. It is not known whether this method will work for monitoring growth on microcarrier culture. If the response is assumed to be proportional to cell mass, we can estimate the bacterium culture to vary over the range of 0.1-4.0 gm/liter. Growth of WI-38 cells on microcarriers would be expected to vary over the range of 0.1-0.6 gm/liter, so that mass is of the same order of magnitude. On the other hand, surface area of the minimum number of observable bacteria or yeasts can be estimated to be about 7 cm^2/ml. The surface area of the beads is about 30 cm^2/ml. Hence, one would also hope to be able to measure the growth of cells on beads on the basis of surface area.

OXIDATION-REDUCTION POTENTIAL

The use of oxidation-reduction potential (ORP) has been advocated by a number of investigators for use in the control and monitoring of animal cell culture (Toth, 1977). The argument is that measurements of changes in the availability of electrons in the culture fluid would be valuable, owing to the importance of molecular charge conditions for the regulation of animal cell membrane activity. However, there are two major problems

involved in making these sorts of measurements with the present redox probe. The first is that under aerobic conditions, the ORP is principly (90%) a measurement of the dissolved oxygen concentration (Lengyel and Nyiri, 1965). However, a correction might be applied if an independent dissolved oxygen measurement is made. The second problem is more fundamental in that it is likely the cells are capable of adjusting their intracellular ORP independently of the ORP of their external environment. In summary, it is felt that the information gotten with the present ORP electrode can be more easily obtained with dissolved oxygen probes. Moreover, if one is interested in measuring the ORP within the cell, the fluorescence technique for measuring the NADH concentration is more effective.

ATP

Adenosine triphosphate (ATP) function as currency for the energy available in living cells. Consequently, the measurement of intracellular ATP concentrations gives a valuable indication of the metabolic state of the organism. For an actively growing culture, the total amount of ATP can be related to total cell mass. However, measuring the metabolic intermediates and coenzyme is difficult due to their rapid decay. The turnover time for the complete pool of ATP in a growing cell is ~ 1 second. Hence, measuring these compounds requires a means of sampling and quenching the culture within a small fraction of a second. Harrison and Harmes (1972) described the operation of a pneumatic ball valve rapid sampler which would allow the operator to collect a 10-ml sample of culture in a fraction of a second. Cells thus collected can be quenched with phosphoric acid and then refrigerated for several hours without loss of ATP. Measurement of the amount of ATP (ATP concentrations are on the order of 1 to 10 m *M* within the cytoplasma) can be easily accomplished by

---

[2] The energy charge of the cell is defined as:
([ATP] + [ADP]/2)/([ATP] + [ADP] + [AMP])

use of the biometer luminescence method (Trauberman, 1969). Furthermore, by measuring ATP, ADP, and AMP concentration one can calculate the "energy charge"[2] of the cell. The energy charge of the cell is a useful indicator of cell metabolism (Lehninger, 1980); higher values tend to stimulate ATP utilizing pathways, whereas ATP generating pathways are inhibited by a high energy charge.

CYTOFLUOREMETRY

In introducing this section, a general comment concerning interpretation of microbiological data seems in order. If every cell were uniform in size and in composition, the interpretation of measurements would be greatly simplified. However, cells show a great deal of variability over the course of their growth cycle with respect to composition, in particular DNA, RNA, and protein contents. Cells also show variability in relation to the environment, as changes in temperature, pH, and medium components can alter cell size, shape, composition, and metabolism. What then does one examine and how does one interpret the data? The answers will depend upon the problem being analyzed. For example, in the production of single-cell protein, one would be most interested in measurements of total cell protein. However, most situation are not so well defined and the user must determine which measured parameter can best characterize this process.

In general, the tendency (especially with microorganisms) is to treat the system as a homogenous population, uniform in composition and activity. Such treatment is especially valuable in the evaluation of bulk thermodynamic constants such as heat or grams of cells produced per mole of glucose consumed. Variations in metabolism that occur over the growth cycle of an organism are "smoothed out" by such methods of analysis.

On the other hand, analysis of population distributions are very useful for understanding those processes in which the organisms of interest undergo various forms of differentiation (e.g., the production of antibiotics by molds). Under such circumstances one is interested in

quantitating the size of a specific population and the influence of the environment on it.

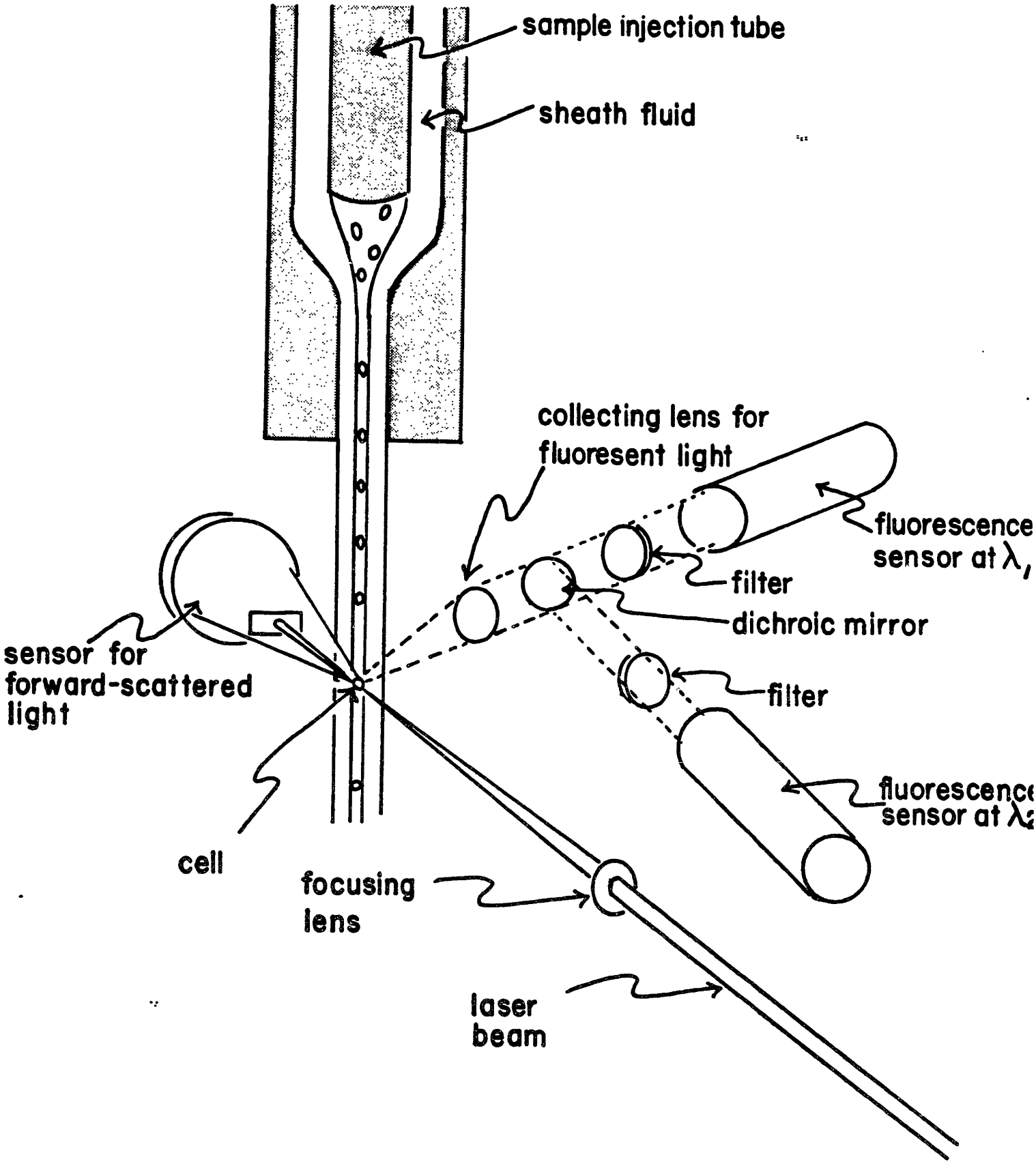
The cytofluoremeter has rapidly gained prominence as a means to determine population distributions (Horan and Wheeles, 1977; Melamed *et al.*, 1979). The instrument operates by transporting a suspension of cells past a laser beam via a laminar sheath-flow technique (Figure 12 on page 72). The sheath-flow technique confines the cells to the center of the flow stream and, by adjusting the cell concentration, the cells are illuminated one at a time. Present day electronics and mechanics allow analysis of as many as 3×10^4 cells/second. By means of various dyes, previously taken up by the cells, different cellular components can be detected by their fluorescence, Cells have been characterized by means of their size, nuclear-to-cellular diameter ratio, DNA content, RNA content, protein content, and content of certain specific enzymes. Several of these determination can be evaluated simultaneously. The use of cytofluoremetry can be quite valuable in the analysis of cells on microcarriers, provided the cells can be readily removed.

CONCLUSION

The development of microcarrier cultivation by anchorage-dependent cells promises an improvement in both the scale of operation and ease of maintaining homogenous environmental conditions in cell culture. One of the more subtle features of the microcarrier system is the potential for careful monitoring of the growth of animal cells on a surface and for quantitating the effect of the environment on the growth and production of various products.

Much of the instrumentation which is useful for obtaining kinetic data on animal cells has been used for some time in work with microbial systems. However, because of the differences in metabolism in animal and microbial cells, a number of pronounced modifications in the measurement of these biochemical events is anticipated. Many of the techniques used with microbial cells may not be sensitive enough for use with animal cells, and most will have to be somewhat modified. Nonetheless, the application of

Figure 12 Assembly for Cytofluoremetry


sample injection tube
sheath fluid
collecting lens for
fluoresent light
fluorescence
sensor at λ1
filter
dichroic mirror
sensor for
forward-scattered
light
filter
fluorescence
sensor at λ2
cell
focusing
lens
laser
beam

instrumentation to cell culture promises a rapid advance in our understanding of the physiology of animal cells and the influence of the environment upon their metabolic processes.

INTERFERON

INTRODUCTION

Interferons are a class of proteins produced by the body in response to a viral infection. An important component of the body's own natural defense system, interferons inhibit the ability of viruses to replicate in protected cells. In clinical and animal studies, interferons have shown promise in the treatment of viruses, such as hepatitis, herpes, and rabies. More recently, interferons have shown some promise to be beneficial in the treatment of cancer. Although originally interferon was thought to produce only an antiviral state, it has become increasingly clear that interferon produces a number of other cellular alterations, most notably, modulations of the immune system, inhibition of growth, and regulation of interferon synthesis. Figure 13 on page 74 is an attempt to summarize the interferon system.

HISTORICAL BACKGROUND

It had been known since the 1930's that the infection of one virus in animals and in cell cultures interfers with the subsequent infection of a second virus. It was investigations into this phenomenon that led Isaacs and Lindenmann to the discovery of interferon. In 1957 they reported that chicken egg chorioallantoic membranes infected with heat-inactivated influenza virus released a factor that transfered virus resistance to fresh, non-infected cells (see Figure 14 on page 76). This factor somehow interfered with the ability of live virus to cause a normal host infection and was given the name "interferon". It was soon established that interferon was a relatively low molecular weight protein and quite non-specific in its action, i.e. that interferon induced by one virus is

Figure 13 Host Defenses Against Virus Infection

The extracellular defenses include antibody (1 and 3) and the phagocytic macrophage (4) which may combine with and inactivate virus. Defenses against intracellularly replicating virus include, cytolysis of virus-infected cells bearing virus surface antigens by synthesized T-lymphocytes (2) acting directly or through their mediators; antibody plus macrophages (1A); antibody plus complement (1B); antibody plus T-lymphocytes (1C); and antibody plus K cells (not shown). Additional inhibition of intracellular viral replication (D and C) can be caused by the nonspecific defense mechanisms of interferon (5-5F). local acidity, local hypoxia (6), and interfering virus (not shown).

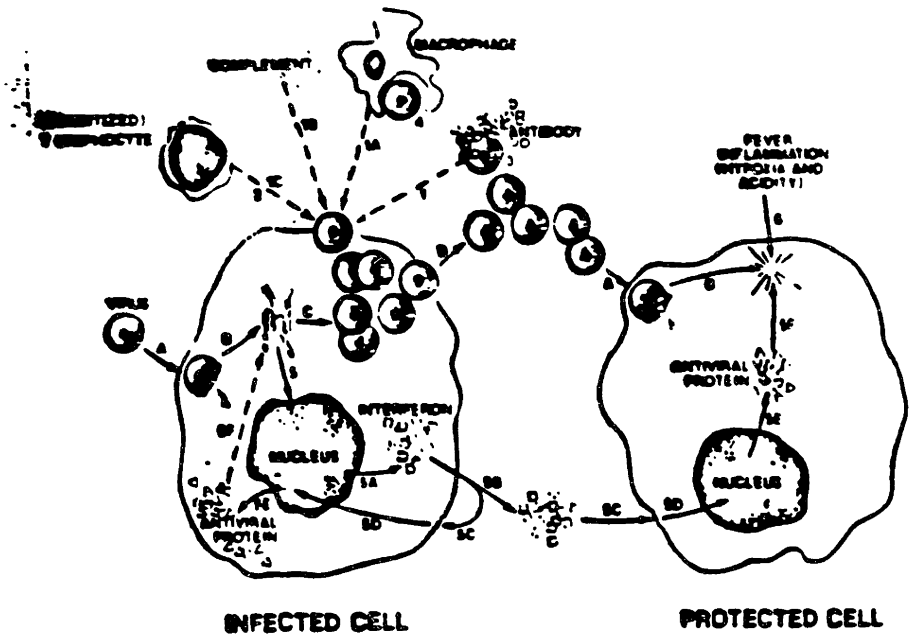
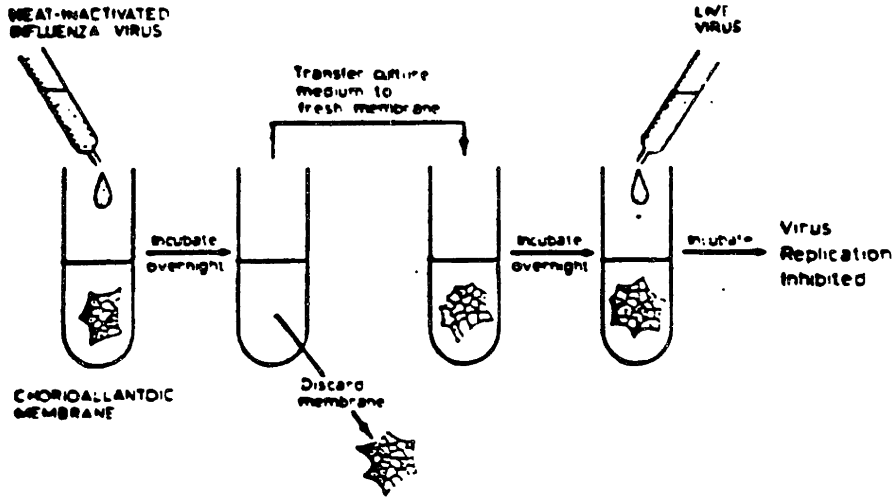

MACROPHAGE
COMPLEMENT
ANTIBODY
VIRUS
NUCLEUS
INTERFERON
NUCLEUS
INFECTED CELL
PROTECTED CELL

Figure 14 The Discovery of Interferon

A schematic representation of the original experiment of Isaacs and Lindenmann


HEAT-INACTIVATED
INFLUENZA VIRUS
LIVE
VIRUS
Transfer entire
medium to
fresh membrane
incubate
overnight
incubate
overnight
Virus
Replication
inhibited
CHORIOALLANTOIC
MEMBRANE
Discard
membrane

effective against a broad range of other viruses. However, interferons do demonstrate species-specificity in that the interferon produced by one species generally stimulates antiviral activity only in cells of that or closely related animal species. For example, human interferon protects human and monkey cells, but not mouse or chicken cells.

There is substantial evidence that interferon plays an important role in the natural recovery from viral infections. It is perhaps the earliest of the known host responses, operative within hours after infection. The most compelling evidence for this argument has come from animal studies. Gresser *et al.*, (1977) demonstrated that mice treated with antisera against mouse interferon are killed by injections of herpes simplex virus at dose levels several hundred fold less than the LD_{50} for mice not treated with antisera.

CHEMISTRY

Three distinct human interferons have been isolated and characterized (see Table 4 on page 79 for a summary). Leukocyte inteferon (Le-IF or "alpha") is the major species produced in cells of lymphoid origin; fibroblast (F-IF or "beta") is the predominant product of cells of non-lymphoid origin; and immune interferon (or "gamma")is produced in cultures of lymphocytes enriched for T cells (Berg *et al.*, 1975; Havell and Berman, 1975). Le-IF and F-IF mRNA's have been isolated, sequenced, and found to contain 836 and 865 nucleotides respectively (excluding the 3' poly A tail and a possible 5' cap structure), and there is an average homology of about 45% between the nucleotide sequences coding for amino acids (Derynck *et al.*, 1980; Mantei *et al.*, 1980; Taniguchi *et al.*, 1980). Weissenbach *et al.*, (1980) have also recently reported that two mRNA species producing biologically active interferon were isolated from human fibroblasts. The mRNA's were easily distinguished with gel chromatography and did not cross-hybridize. Yet despite the high degree of homology, it would appear that most of the protein is required to produce the antiviral activity. A study by Arnheiter *et al.*, (1981) showed that peptide fragments of the carboxyl-terminal (33 to 96 amino acids in length) were without any antiviral activity although having simi-

TABLE 4

DISTINGUISHING PROPERTIES OF HUMAN INTERFERONS

	Leukocyte	Fibroblast	Immune
Approx. Mol. Wt.	20,000 + 15,000	22,000	30,000 + 70,000
mRNA*	865 Nucleotides	836 Nucleotides	??
Stability at pH 2	stable	stable	unstable
Antiviral Activity in Human Fibroblasts	very high	very high	low
Antiviral Activity in Bovine cells	high	very low	very low

* Excluding 3' poly A tail and possible 5' cap structure

lar antigenic properties. Similarly, significantly altering the tertiary structure has also resulted in loss of activity. Shepard *et al.*, (1981) demonstrated that the changed of a single amino acid, the 141cys to a tyr resulted in complete loss of the antiviral activity, presumably due to the loss of an essential disulfide bond.

Only recently have these proteins been purified to near homogeneity (Cantell *et al.*, 1974; Friesen *et al.*, 1981; Knight 1976; Knight and Fahey, 1981) Two problems have and still continue to thwart this effort. First, there has been an overall scarcity of crude material and second, once purified these proteins are not particularly stable, particularly in dilute solutions.

Both fibroblast and leukocyte interferons are glycoproteins, yet the role of the carbohydrate moiety is poorly understood. The degree of glycosylation , and the number of terminal sialic acid residues are believed to be responsible for charge heterogeneity among interferons of given molecular weight class. In all probability the presence of the carbohydrate moiety is not essential for the antiviral action of interferon. Extensively desialated or deglycosylated interferon, as well as non-glycosylated interferon protein produced by bacteria do have antiviral activity. On the other hand, the presence of sugars in glycoproteins is generally held to be important in the pharmacokinetic behavior of these molecules (Billiau and Somer, 1980). Animal studies with human interferon have shown the desialated material to be cleared from the vascular system at a faster rate (Bocci *et al.*, 1977). Additionally, perfusion studies with rabbit liver have also shown a greater rate of uptake for the desialylated form (Bose and Hickman, 1977).

INTERFERON INDUCERS

The first step in the production of interferon involves the recognition of some chemical species leading to the derepression of genetic material coding for interferon. These chemical species are referred to as inducers. The first inducers identified were viruses. Subsequently, "foreign" nucleic acids (RNA) were also found to be capable of inducing interferon.

To date a tremendous number of different substances have been reported to be inducers. For *in vitro* studies, the two most potent and commonly used inducers are the viruses and synthetic polynucleotides.

Viruses from every major grouping have been shown to induce interferon, albeit with varying degrees of effectiveness. Newcastle Disease Virus (NDV) is considered to be an example of a "good" inducer. It is consistently capable of inducing relatively high titers of interferon, both *in vivo* and *in vitro*. Many factors affect the ability of the virus to induce interferon, a number of which are related to the metabolic changes that the virus causes in the infected cells. Many "poor" interferon inducers can be significantly improved by treatments which slightly damage the virus (such as with heat or UV light).

In 1967, double stranded synthetic polyribonucleotides were discovered to be efficient inducers of interferon (Field *et al.*, 1967). Poly I • poly C was used for this study and, despite considerable efforts to improve it, is still the most potent of the polynucleotide inducers. Recently it has been claimed that polyriboguanylate • poly C is equally as active an inducer, provided certain precautions are observed (Novakhatsky *et al.*, 1975). Despite the apparent lack of progress (ie. no new super inducers have been developed) much work has gone into characterizing the structure/activity relationships of these double stranded RNA inducers. To begin there appears to be a minimum requirement for the length of these strands (roughly corresponding to a molecular weight of 300,000 [Pitha *et al.*, 1977]). However, it is interesting to note that in poly I • poly C the length of the poly I strand appears to be more critical than the length of the poly C strand (DeClerq and Torrence, 1977). The presence of the 2'-hydroxyl group in the nucleotide is also required. Apparently any modification of this group will result in a decrease of activity and consistent with this hypothesis is the fact that the deoxyribose polymers are completely inactive (DeClerq *et al.*, 1972). Also, it is essential that these nucleotides be double stranded (Colby 1971). However, recently it has been pointed out that size and double strandedness are not the only requirements. Reovirus RNA is an inefficient interferon inducer, although its melting point and molecular weight would suggest it should be more potent (DeClerq and Torrence, 1977). Additionally, active samples

of poly I • poly C can be inactivated by incubation with S1 nuclease (Gordon and Minks, 1981). This suggests that a partly double stranded, partly single stranded structure is in fact required for activity. Finally, the packaging and stability of the inducer can influence its overall efficiency. In many *in vivo* systems complexing the polynucleotides with cationic polymers (such as polylysine or DEAE-dextran) can greatly improve their resistance to nucleolytic degradation and hence improve their apparent potency (Rice *et al.*, 1970; Dianzani *et al.*, 1968). Also the enclosure of double stranded RNA in phospholipid vesicles has increased the potency 10-fold (Magee, 1978).

It is also worthwhile to mention that in human fibroblast cells, the kind of interferon produced is dependent upon the nature of the inducer. Until quite recently leukocyte interferon production was regarded as a unique activity of lymphoid cells, and fibroblast interferon was thought to be the exclusive IF product of induced fibroblasts. Havel et al. (1978) and Hayes et al. (1979), however, have recently shed new light upon these dogmas. The authors have demonstrated that both Le-IF and F-IF are produced in human fibroblasts, and the relative proportions of each IF species in the production medium is directly related to the type of inducer. When normal and aneuploid human fibroblasts were induced with a virus (NDV), they produced up to 20% of their total interferon as Le-IF, whereas a non-viral inducer (poly I • poly C) failed to produce more than 1% of the total as Le-IF.

GENETIC CONTROL OF THE INTERFERON SYSTEM

The genes responsible for the production of human fibroblast interferon have been examined by a number of researchers. Typically, these studies have involved the construction of human-mouse or human-hamster hybrid cells. These cells initially contain a complete set of genes from both parents but are unstable and spontaneously begin to lose chromosomes. By examining the karyotype of the resultant cells and their ability to produce interferon, researchers have sought to deduce which chromosomes contain the interferon gene. Unfortunately, many differing conclusions have been reached. In 1974, Tan *et al.* reported that the

structural gene was located on chromosome 5, and sited the involvement of chromosomes 2 and 18. Other studies (Morgan and Faik, 1977; and Slate and Ruddle, 1979) also have indicated the involvement of chromosomes 2 and 5. However, more recently Burke and Meage (1980) have concluded that the gene for the fibroblast interferon protein is located on chromosome 9.

Similarly, somatic cell hybrid studies have indicated that the gene responsible for the antiviral state is located on chromosome 21. Furthermore, the genetic control of the interferon induced cell growth inhibition is located on chromosome 21 (Tan *et al.*, 1973).

KINETICS AND CONTROL OF INTERFERON PRODUCTION

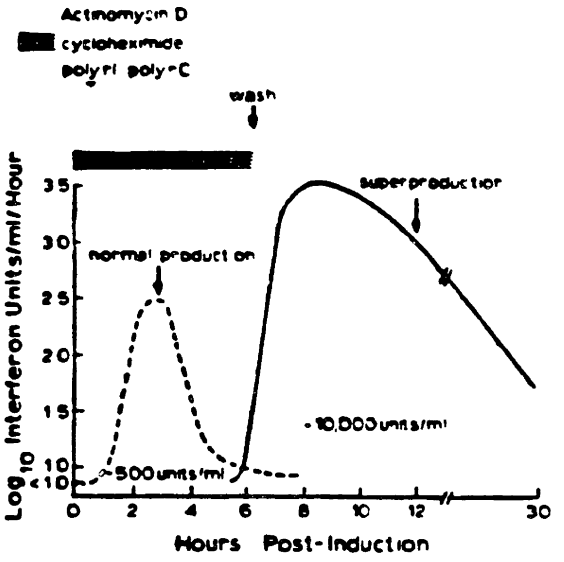
Typically, cell cultures begin to synthesize interferon approximately 2 hours after exposure to poly I • poly C. The rate of synthesis reaches a maximum in about 3 hours and then decreases at a rate as rapidly as it rises. This rapid increase and decrease is usually accompanied by a corresponding increase and decrease in biologically active mRNA in the induced cells (see Figure 15 on page 84). The generally accepted view is that the termination of synthesis is due to the irreversible inactivation of mRNA A repressor protein also induced by poly I • poly C. It is believed to be responsible for the post-transcription control of interferon synthesis. The evidence for this model is three-fold:

- Cells induced by poly I • poly C become temporarily refractive to repeated induction, perhaps due to the residual levels of the repressor protein (Kohase and Vilcek, 1977).

- Selective treatment of induced cells with various metabolic inhibitors is capable of greatly enhancing and sustaining the synthesis of interferon. This treatment is called "superinduction," and the success of this process may be due to the preferential sensitivity of the repressor protein mRNA to these antimetabolites (Vilcek *et al.*, 1976).

Figure 15 Kinetic of Inteferon Production

The Kinetics of interferon in human fibroblasts is illustrated. Additionally, the production of interferon by cells simply exposed to poly I • poly C is contrasted with cells that have been treated with antimetabolites (superinduction) according to the method described by Havell and Vilcek (1972)


Actinomycin D
cycloheximide
poly rI poly-C
wash
superproduction
normal production
~10,000 units/ml
~500 units/ml
Log10 Interferon Units/ml/Hour
35
30
25
20
15
10
<10
0
2
4
6
8
10
12
30
Hours Post-Induction

- The amount of mRNA in cells induced with poly I • poly C and those superinduced at their peak synthesis rates differs by no more than a factor of two, even though the amount of interferon produced by these cells differ by a factor of 20-fold or more, as has been demonstrated using various mRNA translation systems, such as injection into xenopus oocytes, to quantitate the amount of interferon mRNA (Raj *et al.*, 1977).

THERAPEUTIC POTENTIAL

Antiviral agent

The antiviral properties of interferon are attributed to an increase in the protected cell of three double stranded RNA enzymatic activities: an oligonucleotide polymerase, which sythesizes a series of oligonucleotides with unusual 2'5' phosphodiester bonds; an indoribonuclease;and a protein kinase (Kerr *et al.*, 1978; Lebleu *et al.*, 1976; Farrell *et al.*, 1977; Sen *et al.*, 1976). The activity of the oligonucleotide polymerase is intriguing. Apparently, the polymerase which requires double stranded RNA for activity generates a 2'5' oligoadenylate which in turn activates a latent single stranded specific nuclease. Furthermore, because the 2'5'oligoadenylate is exceedingly labile, the activity of the single stranded specific endonuclease is restricted to RNA with a partial double stranded single stranded structure. This structure is uniquely viral, eg. the RNA virus replicative intermediate (Nilsen and Baglioni, 1979).

Clinical trials using interferon (mainly leukocyte) as an antiviral agent are limited, yet the results appear to be encouraging. The drug is relatively non-toxic and has been effective in animal and human trials in the treatment of influenza, rhinoviruses, rubella, hepatitis B, cytomegalovirus, herpes, varicella-zorter, and vaccina. Interferon is more effective when administered as a prophylactic agent, i.e., given 24 hours prior to infection, but by necessity most clinical trials have dealt with it as a therapeutic agent.

Interferon may be used also as a vaccine supplement. In principle, interferon can be used to control a viral infection until the body develops antibodies against the virus. Support for this idea comes from animal studies where vaccines against rabies were more effective when supplemented with interferon (Hilfenhaus et al., 1975; Baer *et al.*, 1977).

Antitumor agent

Because some tumors are believed to be induced by viruses, interferon was examined as an antitumor agent. In animal studies when the tumor was induced by a viral agent, interferon effected a cure if the viral or tumor load could be reduced (Galasso and Dunnick, 1977). Interferon was first used to treat Hodgkin's disease and osteogenic sarcomas because of their believed viral association. In these cases, although interferon has been shown to be somewhat effective, it is not clear that the action is a result of its antiviral activity, and may in part be due to some of its other activities such as growth inhibition and enhancement of macrophage and T-cell activity.

The most extensive clinical trials to date have been with patients with osteogenic sarcoma (Strander, 1977; Strander *et al.*, 1977), a virulent form of bone cancer which, in the normal course of the disease, spreads to the lungs. On average, only 20% of the patients with no evidence of metastases at the time of the diagnosis survive an additional five years. In 1971, researchers in Stockholm began a program to determine the effectiveness of interferon against this disease. They found that of those patients treated with interferon 65% remained free of metastases 2.5 years after the diagnosis. In contrast, among patients treated in other Swedish hospitals not receiving interferon, only 30% remained disease-free over the same period of time.

Recently, Gutterman *et al.*, (1980) reported the use of leukocyte interferon in the treatment of thirty-eight patients with advanced breast cancer, multiple myeloma, and malignant lymphoma. Tumor regression was observed in approximately 50% of the patients in each diagnostic category.

Since then, clinical trials on other forms of cancer have been initiated. Those that have shown responsiveness to interferon include Hodgkins's disease, melanoma, certain forms of leukemia, and bladder cancer.

The effect of human interferons on human tumor colony formation in vitro has also been reported. Of 40 evaluable tumor specimens, 18 showed at least a 70% inhibition of colony formation in the presence of interferon (Bradley *et al.*, 1980).

Not all clinical studies, however, have been so encouraging. Interferons have been judged ineffective as single therapeutic agents in a number of human viral and tumorgenic diseases. Acute hepatitis B and rhinovirus have been reported to respond poorly to interferon (Scott *et al.*, 1980; Weimar *et al.*, 1980) and a recent human tumor study with 52 patients was not encouraging (McPherson *et al.*, 1980). It is likely that in the future interferon will be used more in combination with conventional chemotherapy and radiation treatment.

CELL CULTURE

Human diploid foreskin fibroblasts (FS-4) were obtained from Dr. Jan Vilcek, of the New York University School of Medicine, New York, and were used in all experiments. Cells were obtained at approximately the eighteenth population doubling. They were frozen in one batch for the entire series of experiments and were used between population doublings 30 and 40.

Stock cultures were maintained in Corning plastic roller bottles in a walk-in incubator at 37 °C. For experimental purposes, microcarrier cultures were seeded from these stocks. A microcarrier concentration of 5 mg/ml was used. Cultures were seeded generally in one of three basic vessel configurations: 250ml glass spinner bottles (Wilbur Scientific, Inc., Boston, MA) at a working volume of 50 to 100 ml, 2-liter spinner bottles (Wheaton Scientific) at a working volume of 500 to 1000 ml per vessel, and in a 14-liter New Brunswick fermentor with a working volume of 5 to 10 liters. Inoculum densities generally ranged from 3 to 4 x 10^5 cells/ml.

The growth medium for the stock cultures consisted of Dulbecco's Modified Eagle Medium (DME) (GIBCO Laboratories, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (Sterile Systems, Inc., Logan, Utah). For microcarrier growth the serum concentration was reduced to 5% (v/v). Antibiotics used were penicillin G (100 units/ml) and streptomycin (100 µg/ml) and were obtained from Sigma Chemical Co. (St. Louis, MO). Cultures were split using a trypsin-EDTA solution (GIBCO Laboratories, Grand Island, NY), as described by Hayflick and Morrhead (1961).

CELL COUNTS

Cells on microcarriers were counted using a modification of the method of Sanford (Sanford *et al.*, 1951, Van Wezel, 1973). A one-ml sample of the

TABLE 5
COMPOSITION OF DME

Component	mg/l	mM
Amino Acids		
Arginine HCl	84.0	0.40
Cystine	48.0	0.20
Glutamine	584.0	4.0
Glycine	30.0	0.40
Histidine	42.0	0.20
Isoleucine	104.8	0.80
Leucine	104.8	0.80
Lysine	146.2	1.0
Methionine	30.0	0.20
Phenylalanine	66.0	0.40
Serine	42.0	0.40
Threonine	95.2	0.80
Tryptophan	16.0	0.08
Tyrosine	72.0	0.40
Valine	93.6	0.80

Component	mg/l	mM
Vitamins		
Ca Panthothenate	4.0	0.01
Choline Chloride	4.0	0.03
Folic Acid	4.0	0.01
Inositol	7.0	0.04
Nicotinamide	4.0	0.03
Pyridoxal HCl	4.0	0.02
Riboflavin	0.40	0.001
Thiamine HCl	4.0	0.01
Other Components		
Dextrose	4500.	25.0
Phenol Red	15.0	0.042
Sodium Pyruvate	110.0	1.0
Minerals		
Calcium Chloride	200.0	1.8
Ferric Nitrate	0.1	0.0002
Potassium Chloride	400.0	5.37
Magnessium Sulfate	97.7	0.81
Sodium Chloride	6400.0	109.5
Sodium Bicarbonate	3700.0	44.1
Sodium Phosphate	125.0	0.9

culture is withdrawn from a well-mixed vessel and then centrifuged (2,000 rpm for 5 min.). The growth medium is removed. The pellet, consisting of cells and microcarriers, is resuspended in 1 ml of counting reagent (a solution of 0.1M citric acid (Baker) and 0.1% (w/w) crystal violet (Matheson, Coleman, and Bell)). The cells are incubated at 37 °C for 45 minutes. The suspension is then sheared with a pasteur pipet, a process which liberates and stains the nuclei. The nuclei are then counted with a hemocytometer.

Viable cell counts were obtained by diluting a freshly trypsinized cell suspension in an equal volume of trypan blue dye (0.4% in PBS and filtered, Sigma Chemical Company, St. Louis, MO.) and counting using the hemocytometer.

QUALITY CONTROL

Cultures were thoroughly screened for the presence of mycoplasma using the culture method (isolation of mycoplasma colonies on artificial medium), the uridine-uracil assay described by Schneider *et al.*, and the DNA staining method reported by Russel *et al.*. All results were negative.

INTERFERON PRODUCTION

The superinduction procedure used was a modification of the method reported by Havell and Vilcek (1972): Cells were grown to confluency on microcarriers, to a density of about 1 x 10^6 cells/ml, and induced between the sixth and eighth day of growth. Post-exponential growth cells are first primed with interferon. Cells are separated from the growth medium and washed once with DME (w/o serum) and then incubated for 16 hours in DME + 0.5% (w/w) Human Plasma Protein (HPP) + 50 units interferon/ml. Subsequently, the cells are induced by first withdrawing the priming medium, and washing the cells two times with DME, and then replacing the medium with DME + 50 µg/ml poly I • poly C + 10 µg/ml cycloheximide. The temperature during the induction period is lowered to 34 °C. After four hours of incubation, actinomycin D is added to give a

final concentration of 1 µg/ml. After two more hours of incubation, the medium is removed and the cells are washed twice with DME. The production of interferon begins after the antimetabolites are removed and DME containing 0.5% HPP is added to the culture. Initially during the production period, the temperature is at 37 °C, but is lowered to 30 °C after one hour for the remainder of the production period. After 48 hours the culture fluids are collected.

INTERFERON ASSAY

Interferon titers were determined by a microplate assay measuring inhibition of VSV-induced cytopathic effect (CPE). The interferon assay is essentially that described by Havell and Vilcek (1972): Samples were assayed in duplicate in Costar 96-well culture dishes (Microbiological Associates, Walkerville, MD). Growth medium (0.10 ml) was added to each well and two-fold dilutions of prediluted samples were made using a Titertek Multipipetter (Flow Laboratories, Rockville, MD) starting with 0.1 ml of the sample. Each well was seeded with 5×10^4 FS-4 cells in 0.10 ml of growth medium. The plates are then incubated for about 24 hours in a humidified CO_2 incubator at 37 °C. Subsequently, cells were challenged with 1000 pfu of VSV (Indiana Strain, obtained from David Baltimore, Center for Cancer Research, MIT, Cambridge MA) per well. Several wells in each plate served as cell and virus controls. The plates were incubated at 37 °C and scored microscopically after 48 to 72 hours. The highest dilution of a sample showing 50% destruction was judged to be the endpoint. An internal standard calibrated against the international standard B69/19 (obtained from the National Institute of Health, Bethesda, MD) was included with each assay.

VIRUS

Vesicular stomatitis virus (Indiana strain) was produced by the lytic infection of primary chicken embyro fibroblasts (CEF). The CEF's were grown to confluency in 490 cm^2 Corning roller bottles from minced 9-11 day old chick egg tissue and refed every 5 days with DME containing 1%

chick serum, 2% tryptic phosphate broth, and 1% calf serum. When the cells were approximately 15 days old they were infected with a multiplicity of infection (MOI) of 0.1. The supernatant was harvested 24 hours later and clarified by centrifugation. Production harvests were titrated using a plaque assay. Specifically CEF's were seeded into 60mm petri dishes (Corning) at a density of 3 x 10^4 cells/cm^2 in 5 ml of growth medium, and incubated for two days at 37 °C in an atmosphere of 10% CO_2. The cells were then challenged with 0.3 ml/dish of production virus that had been diluted to five different concentrations ranging from 1:1 x 10^{-5} to 1:1 x 10^{-9}. After a one hour adsorption (with occasional rocking) the cells were covered with 5 ml of an agar overlay[3]. The cells were then incubated for an additional 48 hours and then stained with neutral red stain (1:2500 in PBS) at which time the plaques were counted and the titers determined.

MICROCARRIERS

RECRYSTALLIZATION OF DEAE-CL•HCL

Diethylaminoethylchloride-Hydrochloride (DEAE-Cl•HCl, Sigma) is recrystallized from methylenechloride (Fisher) before it is used to prepare microcarriers. One liter of methylene chloride is added to about 300 grams of DEAE-Cl•HCl and the suspension is brought to a boil while mixing is provided by a magnetic stirbar. The beaker is then covered with aluminum foil to minimize solvent losses to evaporation. The liquid is allowed to cool at room temperature for about two hours, following which it is placed in a refrigerator (4 °C) for an additional eight hours. The crystals are collected by suction filtration and the vacuum is keep on until all traces of the solvent are removed. Typically, about 180 grams of

---

[3] The agar had the following composition; Noble agar (1.5%) in Hams F12 Medium plus 2% calf serum and 2% tryptic phosphate broth.

DEAE-Cl•HCl are recovered. The material is then stored in a desicator until used.

DERIVITIZATION OF MICROCARRIERS

The microcarriers are prepared in our laboratories by a modification of the Hartmann procedure (Hartmann, 1930), in which DEAE-Cl•HCl is bound to the carbohydrate support matrix under alkaline conditions. Uniform unreacted beads (53 - 75 μm) are obtained by sieving dry Sephadex G50, 20-80 (Sigma). One gram of dry Sephadex is hydrated with 12 ml of distilled water. The suspension is prewarmed at 50 °C in a stirred vessel. To initiate the reaction, 6 ml of prewarmed 3 *M* NaOH is added to the suspension, followed by 6 ml of prewarmed 3 *M* DEAE-Cl•HCl. The reaction is allowed to proceed for one hour. Subsequently, the carriers are separated from the reaction by filtration and washed with 0.5 liters of distilled water. Beads prepared by this procedure typically have an exchange capacity of 2.0 meq/gm dextran (however, 1.8 to 2.1 meq/gm dextran is considered an acceptable range of variation). Beads of a lower exchange capacity can be prepared by reducing the time of the reaction, as is shown in Figure 16 on page 96.

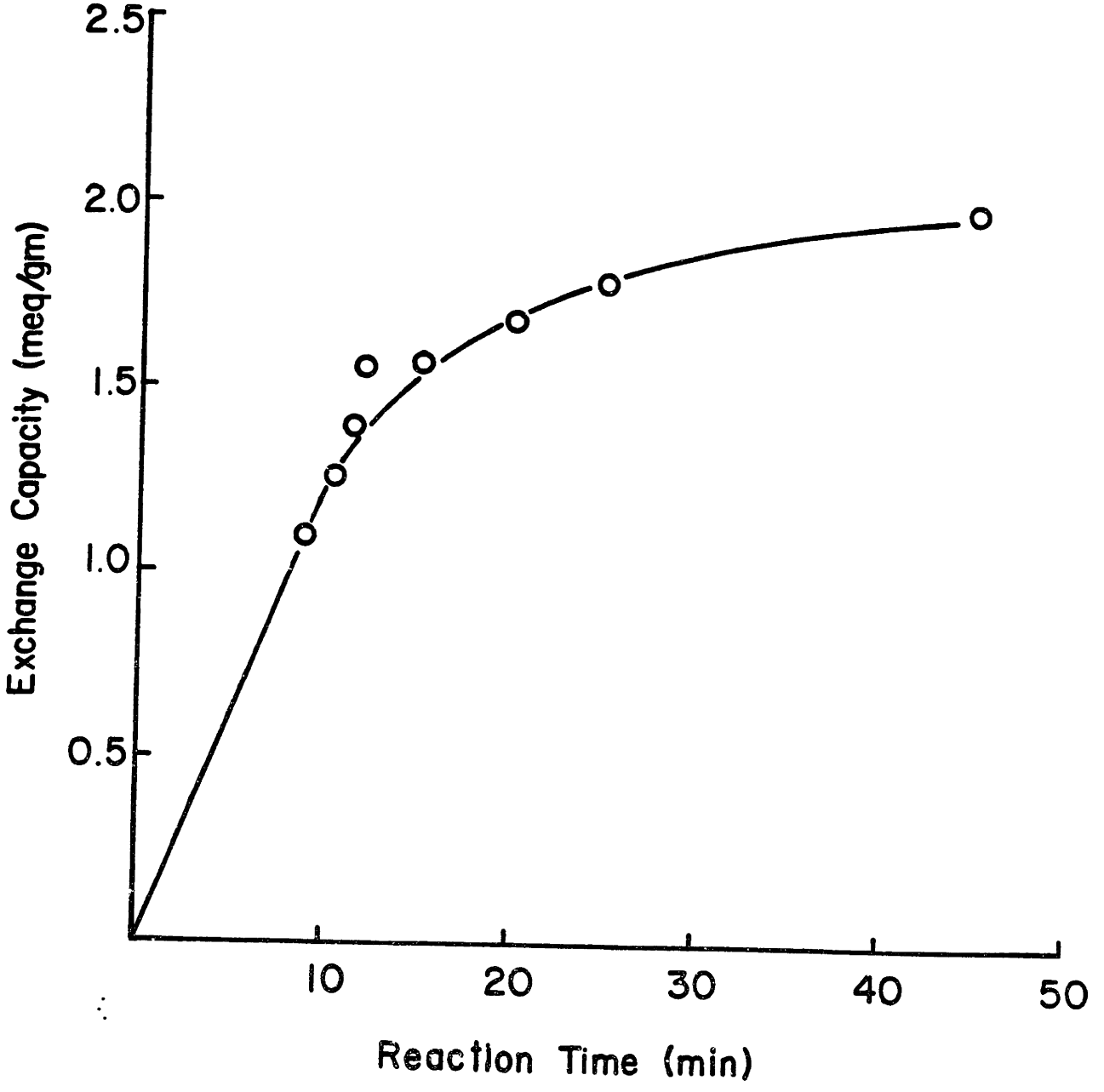
WASHING OF MICROCARRIERS AND TITRATION OF BOUND DEAE-CL•HCL

The carriers are washed thoroughly with 0.1 *N* HCl, which saturates the exchange sites with Cl^- ions (0.5 liters/gm). They are then rinsed with dilute HCl, 10^{-4}, to remove the unbound Cl^- (0.8 liters/gm). The beads are then washed with 10% (w/w) Na_2SO_4 (75 ml/gm) and the filtrate collected. This procedure displaces the bound Cl^- with $SO_4^=$.

In the titration, the filtrate volume is measured and 100 ml aliquots are titrated with 1.0 *N* Ag_2NO_3 in the presence of potassium chromate as an indicator (1 ml of 5% (w/w) solution).

Figure 16 Profile of Charge Substitute in Microcarriers vs Time

The incorporation of DEAE into the sephadex matrix at 50 °C under alkaline conditions is shown here as a function of reaction time.


2.5
2.0
1.5
1.0
0.5
Exchange Capacity (meq/gm)
10
20
30
40
50
Reaction Time (min)

Following the titration, the microcarriers are washed with distilled water (0.5 liters/gm) to remove the $SO_4^=$ and then with phosphate buffered saline without Ca^{2+} and Mg^{2+} (0.8 liters/gm) to balance the pH and the ionic strength. Completely washed beads are then resuspended in PBS at a concentration of 10 gm/l, bottled, and then autoclaved.

PHOSPHATE BUFFERED SALINE

Phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS), was prepared as a 10X stock solution. For a five liter stock solution, the following composition was used: NaCl, 400 gm, KCl, 10 gm, Na_2HPO_4, 57.5 gm, KH_2PO_4, 10 gm. All the chemicals were reagent grade quality (Baker Chemical Co.) and the water was deionized and distilled.

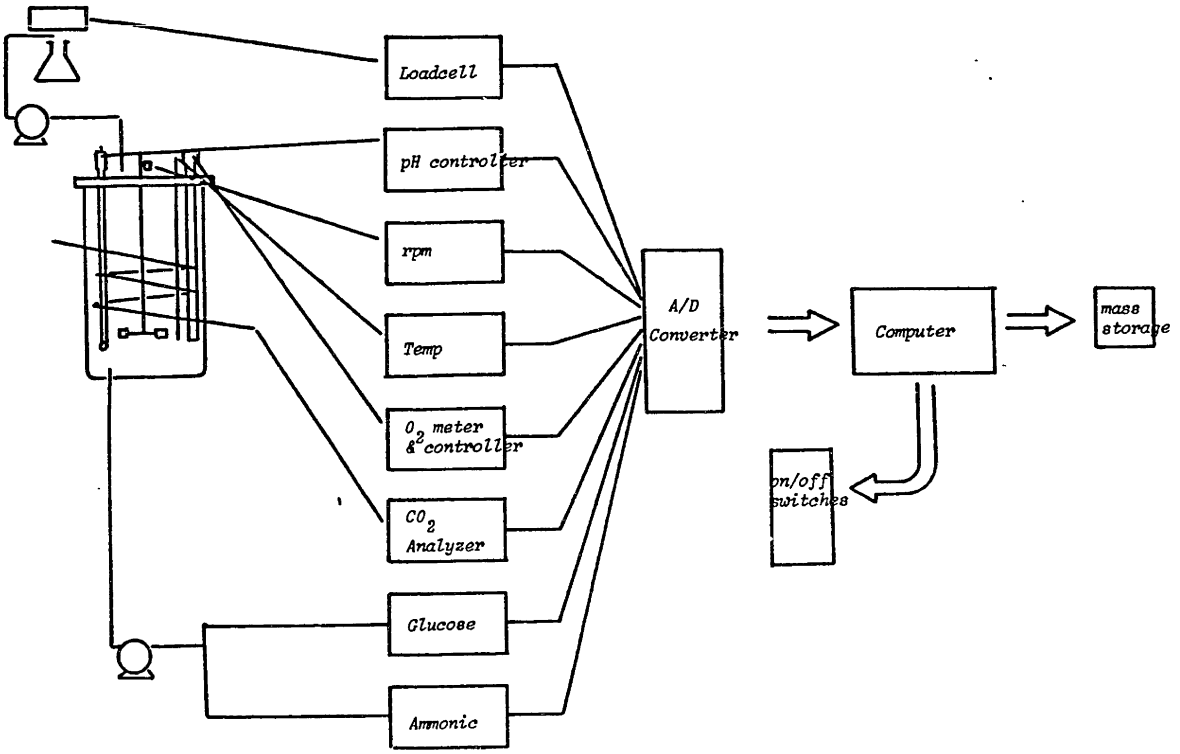
TRICINE BUFFERED MEDIA

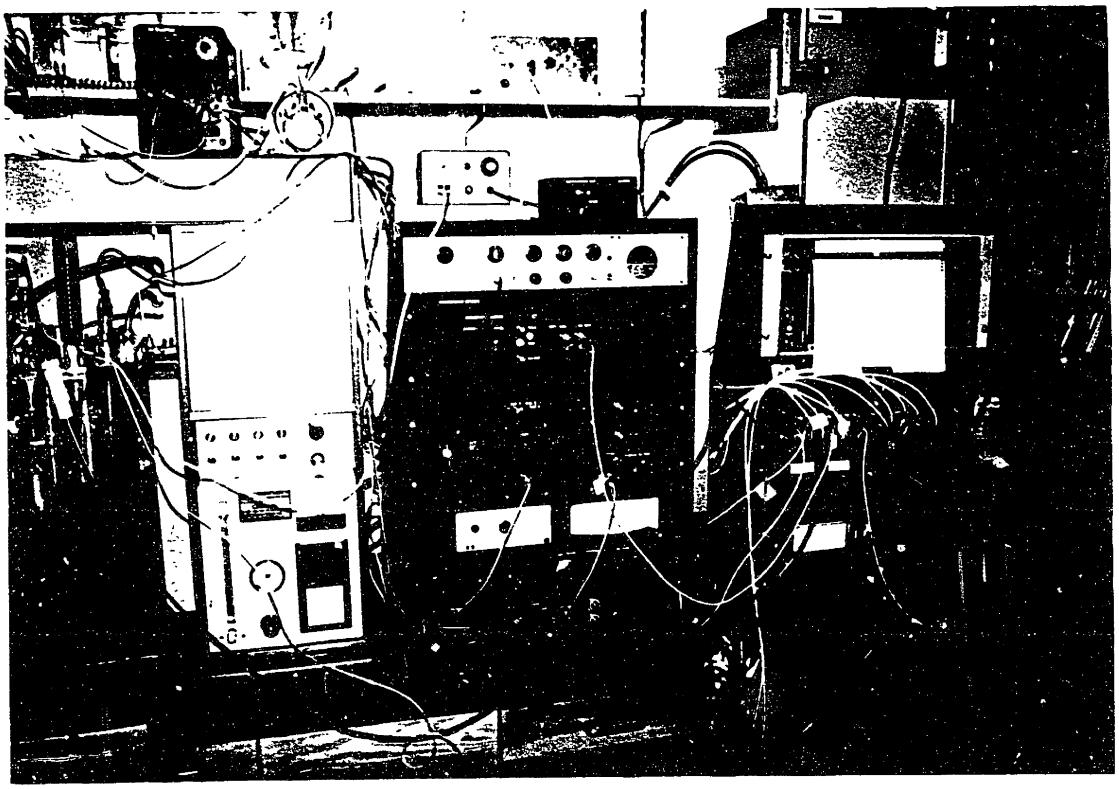
To quantitate the acid production in the large runs it was essential to replace the bicarbonate-CO_2 buffer system of DME with another buffer. Tricine was selected for this purpose, because its pKa is close to 7 and it is known to be relatively nontoxic. Media was prepared in the usual fashion except in place of sodium bicarbonate, tricine (0.864 g/l) and and NaCl (0.74 g/l) were added. The tricine (5 m *M*) provides a marginal buffering system and the NaCl is used to adjust the osmotic strength of the medium (now that the bicarbonate salt has been eliminated) to a physiological level.

INSTRUMENTAL SYSTEM

Figure 17 on page 100 shows a schematic diagram and photo of the instrumentation used in this thesis to monitor cell growth and metabolism. Basically, it consisted of a fermentor equipped with several sensors to monitor pH, temperature, dissolved oxygen, impeller speed, off-gas carbon dioxide, sugar and ammonia concentrations, and base addition used to control the pH, the individual meters and controllers, the analog to digital

Figure 17 Schematic of Instrumental System


Loadcell
pH controller
rpm
Temp
O_2 meter & controller
CO_2 Analyzer
Glucose
Ammonic
A/D Converter
Computer
mass storage
on/off switches



converter (which is the interface to the computer), the computer, a floppy disk system for mass storage, and a series of solid state relays activated by the computer to interrupt the controllers, turn on pumps, and control sensor recalibration.

MONITORING THE OXYGEN UTILIZATION IN CELL CULTURE

CALIBRATION OF OXYGEN PROBE.

The oxygen probe (Instrumentation Laboratories, Lexington, MA) is calibrated in stagnant water with air using the following correction factor:

$Cf = (Pa - Pv)/760$

where Pa is the atmospheric pressure in mm Hg, and Pv[4] is the vapor pressure of H_2O in mm Hg. (Technical data from Instrumentation Laboratories.)

DETERMINATION OF THE SOLUBILITY OF OXYGEN IN THE MEDIUM.

The solubility of oxygen in pure water at a given temperature was calculated using the empirical relationships derived by Benson and Krause (1976).[5] The difference between the solubility of oxygen in the medium and that in pure water was estimated from data by Schume et al. (1978)[6], which characterizes the solubility of oxygen in electrolyte solutions. Using these correlations, the solubility of oxygen in DME at 37 °C is estimated to be 179 µmoles/l.

---

[4] Over the temperature range of 25 to 40 °C (T), the vapor pressure of water can be approximated by the function:
$\ln(Pv) = 8.0479 - (1718.75/(T + 232.6))$.

DETERMINATION OF OXYGEN TRANSFER IN AND OUT OF THE 14-LITER VESSEL.

The mass transfer coefficient of oxygen across the surface of the 14-liter vessel was determined by exchanging the air in the head space of the fermentor with nitrogen gas and measuring the change in the dissolved oxygen concentration (C) with time. The temperature of the vessel was 37 °C for the experiments. Both the liquid volume and the agitation speed were varied. The mass transfer coefficient (Kla) is defined by the following equation:

$$dC/dt = Kla(C_{[gas]}-C)$$

where t is time in hours and $C_{[gas]}$ is the dissolved oxygen concentration of the liquid in equilibrium with the contacting atmosphere.

MEASUREMENT OF OXYGEN DEMAND

To measure the oxygen demand of the cells, a transient was periodically induced, wherein the gas in the headspace, air + CO_2 (for pH control), was replaced with a mixed gas consisting of approximately 10% O_2. The resulting decrease in the dissolved oxygen concentration is due to both mass transfer of oxygen out of the vessel and oxygen utilization by the

---

[5] The solubility of oxygen and other nonpolar gases, is strongly dependent upon the temperature of the solution. Generally, speaking the solubilty decreases with increasing temperature. For oxygen the following relationship has been determined:

$B = 1244142\rho/k$

where: B is the solubility of oxygen in cc

ρ is the density of water

k is function of temperature described as follows

$\ln(1/K) = -4.0605 - (5416.7/°K) + (1026100/°K^2)$.

cells. The oxygen uptake rate (OUR) by the cells can be evaluated using the following equation:

$$OUR = (Co-C_f)/(t_f-to) + (\int Kla\ (C_{[gas]}-C)dt)/(t_f-to)$$

where Co is the initial oxygen concentration, C_f is the final oxygen concentration, to is the initial time, t_f is the final time, and dt is the time interval between dissolved oxygen measurements.

OXYGENATION USING SILICONE RUBBER TUBING.

The oxygen transfer potential of silicone rubber tubing (Dow-Corning Silastic Tubing) was measured in a manner similar to the determination of the Kla for the 14-liter vessel, except that nitrogen was passed through a 4-meter length of tubing (0.058" x 0.077", ID x OD) spiralled around the baffles of the fermentor. The mass transfer potential was evaluated at several agitation rates. To oxygenate the culture using the silicone rubber tubing, the following control scheme was used: air was nominally passed through the tubing. However, should the oxygen tension of the culture fall below a determined set point, the air passing through the tubing would be intermittently replaced with O_2 until the oxygen tension of

---

[6] The solubility of oxygen in the medium is somewhat different than that in pure water. The data of Schume *et al.* has shown the following correlation between the actual solubility of oxygen and the ionic content of the solution:

$$\log(C_o/C) = \Sigma\ H_i \cdot I_i$$

where: C_o is the solubility of O_2 in water
C is the solubility of O_2 in the electrolyte solution
H_i is a constant of the ionic species
I_i is the ionic strength

the culture was restored to the set point. The control circuit is shown in Appendix L. Typically the oxygen tension of the culture was maintained at 60% of air saturation.

MEASUREMENT OF RPM

The agitation rate of the impeller to the vessel was determined by monitoring the magnetic coupling system of the impeller assembly. Using a hall effect sensor mounted immediately beside the magnetic coupling, the motion of the drive shaft could be translated into a digital signal and counted over a discrete time interval. The circuits for this devise are presented in Appendix D.

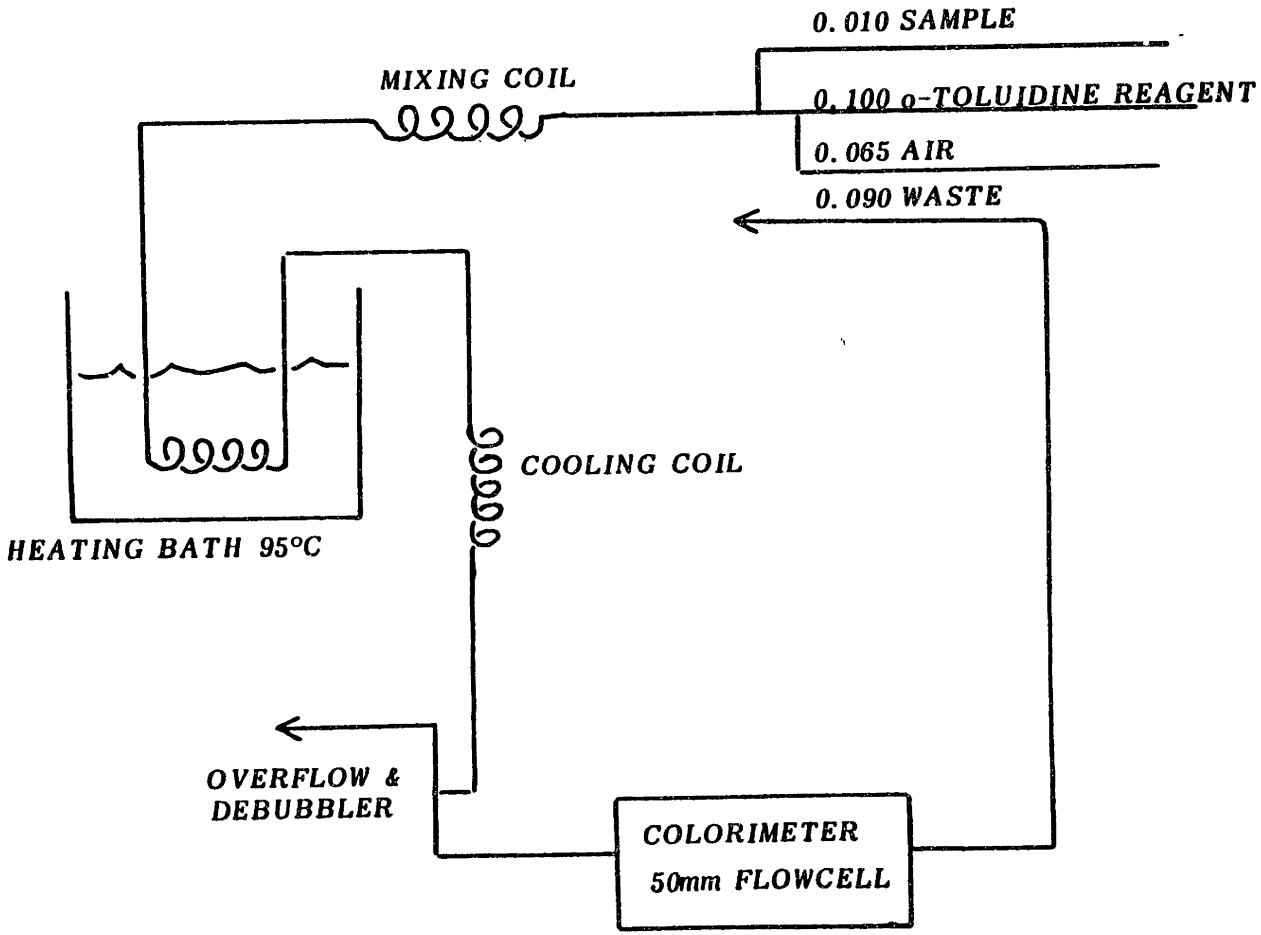
BASE ADDITION

The measurement of base used to effect pH control is a useful measurement of the total acid production (lactic acid + CO_2). The base addition was quantitated using a Gould loadcell (type L-32-2). The loadcell is a strain gauge devise and the circuit used is described in Appendix B. The pH meter and controller is described in Appendix A.

SUGAR ANALYSIS

The concentration of sugar in the fermentation broth was determined using the reaction of o-toluidine with aldohexoses (Fring, C.S., 1970). Aldohexoses react with o-toluidine in the presence of acetic acid to produce a green color, which follows Beer's law over a wide range of concentrations. Furthermore, the method can easily be automated. Figure 106 shows the flow diagram for the automated method. The equipment was largely salvaged Technicon Autoanalyzer parts. The colorimeter was modified slightly by upgrading the electronics used to monitor the output of the photodiodes (see Appendix I). To simplify matters further, the absorbance units were calculated digitally using the Apple computer. A double mixing coil is used to cool the reactant stream before the

Figure 18 Flow Diagram of Automated Method of Analysis for Sugars


0.010 SAMPLE
MIXING COIL
0.100 o-TOLUIDINE REAGENT
0.065 AIR
0.090 WASTE
COOLING COIL
HEATING BATH 95°C
OVERFLOW &
DEBUBBLER
COLORIMETER
50mm FLOWCELL

absorbance at 660 nm was determined. A heating coil with a 5 minute residence time in a hot oil bath (95 °C) is sufficient. The tubing should be replaced after each run. The o-toluidine reagent was prepared by adding to 1 liter of glacial acetic acid: 1.5 grams thiourea, 60 ml o-toluidine, and 170 ml of distilled water.

To determine glucose concentrations less than 2 m *M* the following modifications were utilized: one the water content of the o-toluidine reagent was eliminated, two the flow rate of the glucose sample stream was increased, and three the residence time of the flow stream in the hot oil bath was increased.

AMMONIA

The ammonia concentration of the culture fluid from the fermentor was measured using an automated flow method (as shown in Figure 19 on page 108). The sample is mixed with base (0.1 *N* NaOH) and air. A single mixing coil is used prior to the ammonia probe. The ammonia probe is a gas-sensing electrode manufactured by Instrumentation Laboratories (Lexington, MA). The flow-through cap was machined from a solid teflon block. The circuit interfacing the probe to the computer is shown in Appendix H.

CARBON DIOXIDE ANALYSIS

The dissolved CO_2 content of the culture fluid can be measured indirectly by analyzing the CO_2 content of the exit gas from the silicone rubber tubing for oxygenation (as shown in Figure 20 on page 110). The CO_2 in the exit gas stream is measured using an infrared CO_2 gas analyzer (Mine Safety Appliances Co., Pittsburg PA, LIRA Model #300). The concentration of CO_2 in the exit stream is a function of the pH, the dissolved CO_2 concentration, and the gas flow rate. The flow rate of the gas stream through the silicone rubber tubing is determined by measuring the pressure differential between the inlet and outlet points of the silicone

Figure 19 Flow Diagram of Automated Analysis of Ammonia

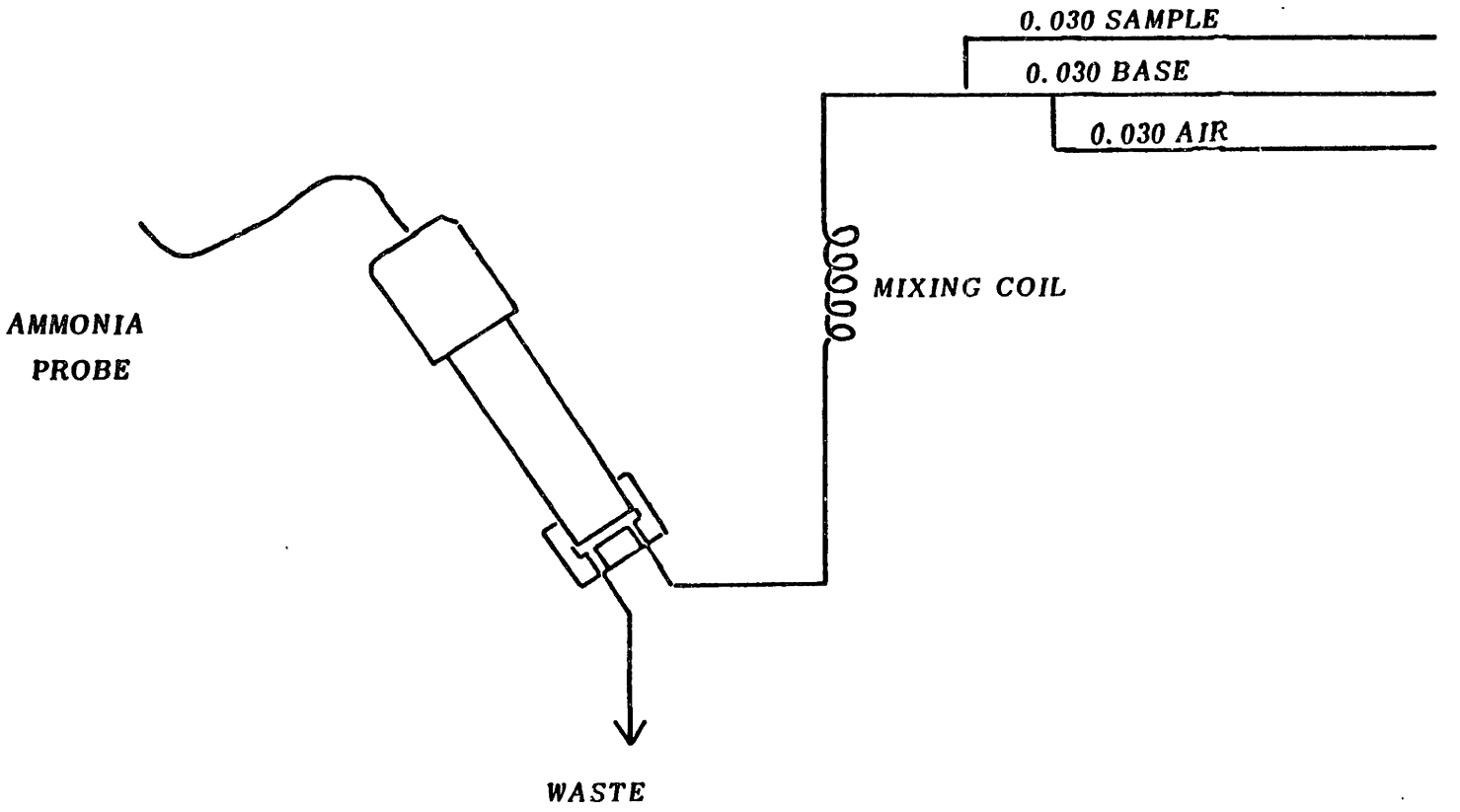
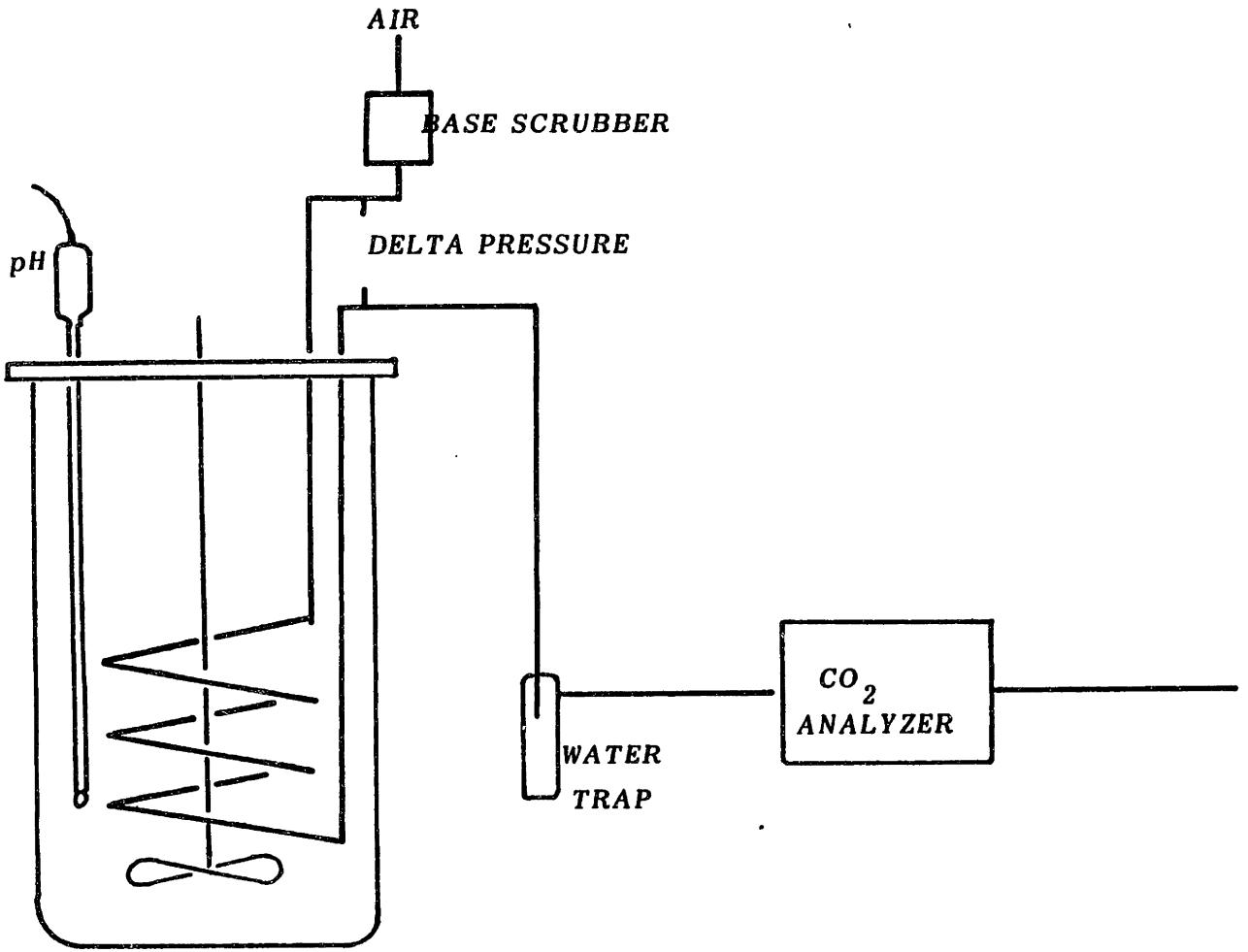

0.030 SAMPLE
0.030 BASE
0.030 AIR
MIXING COIL
AMMONIA
PROBE
WASTE

Figure 20 Diagram of Equipment Used to Measure Dissolved CO_2


AIR
BASE SCRUBBER
DELTA PRESSURE
pH
CO_2
ANALYZER
WATER
TRAP

rubber tubing with a National Semiconductor (Santa Clara, Calif.) differential pressure transducer. The circuit is shown in Appendix J.

AMINO ACID ANALYSIS

AMINO ACID CLEAN-UP

Each medium sample was prepared for amino acid analysis by a single column clean-up procedure. Norleucine (0.5 m *M*) was used as an internal standard. Each aliquot was deproteinized by perchloric acid (3%). The protein free supernatant was diluted with 0.1 *M* HCl and passed through a Dowex 50W (H^+ form) cation exchange column (8 x 1.1 cm). The resin was washed with 50 ml distilled water adjusted to pH 3. The amino acids were then eluted with 30 ml NH_4OH (3 *M*). A 3 ml sample of this eluate was dried by rotary evaporation and analysed for amino acids. The procedure was a modification of a previously reported method (Butler *et al.*, 1975).

AMINO ACID ANALYSIS

Amino acid samples (40 µl) were run on a D-500 amino acid analyzer (Dionex Corp.).The co-chromatography of peaks for serine and glutamine were resolved by running a second sample which was pretreated with 3 *M* HCl at 100 °C for 2 hrs. to hydrolyze the glutamine to glutamic acid.

CONDUCTANCE MEASUREMENTS

CONDUCTANCE CHAMBER

The conductance chambers were made "in house" by modifying the 250 ml spinner bottles (Wilbur Scientific). Platinum wire, 40 mil, was inserted

(about 0.6 cm) into the vessel (near the bottom) by a glass blower. The electrodes had a cross-sectional area of about 0.18 cm^2 and were separated by 6 cm in distance.

The chambers were sterilized by autoclaving. They were cleaned of adsorbed proteins by soaking them overnight in 1 *N* nitric acid.

MEASUREMENT OF THE CONDUCTANCE OF A SINGLE CHAMBER

The conductance of a single chamber was determined by measuring the voltage drop across the chamber in series with a fixed resistor. Three precautions were observed in making these measurements: First, the voltage drop across the conductance chamber was maintained below 1.0 volt to prevent electrolysis of the solution. Second, the current density at the Pt electrodes was limited to below 10 ma/cm^2 in order to obtain an accurate impedance measurement (Gedes, 1972). Third, the measurement was made using alternating current (10 kHz) to minimize polarization of the electrodes.

The impedance (Z) of a single chamber can be calculated as follows;

$$Z = R/(v/e - 1)$$

where R is the resistance of the fixed resistor in series with the conductance chamber, v is the voltage across both the chamber and the resistor in series, and e is the voltage drop across the conductance chamber.

DIFFERENTIAL CONDUCTANCE MEASUREMENT

The change in impedance of a single chamber can be measured as the difference between two chambers, the advantage of this method is that it can impart a certain degree of isolation against impedance changes due to changes in the temperature. In this work, a Wheatstone bridge circuit was used to make these measurements. The oscillator used to drive the circuit

was designed to be both frequency and amplitude stable. This was accomplished by using a crystal oscillator to stabilize the frequency, the amplitude was stabilized by being servo'ed against a temperature compensated diode. The construction of the circuit is described in the appendixes C.

This section is divided into essentially two parts. The first half deals with characterizing the FS-4 cells, the conditions essential for their growth, and the interferon assay. The second half presents the calibration and performance of the instrumental methods later used to monitor and control the growth of the FS-4 cells.

GROWTH AND OTHER PROPERTIE OF FS-4 CELLS

Prior to growing the FS-4 cells on a large scale it was essential to establish those variables which were critical in obtaining reproducible and successful cell growth. These turned out to be: adequate seed density, reduced serum concentration, and the use of recently subcultivated cells for the inoculum. Additionally, to aid in subsequent data analysis the dry cell weight and protein content of these cells were determined. Second, the changes in the amino acid composition of the growth medium was evaluated.

CELL GROWTH AT DIFFERENT INOCULUM DENSITIES

To determine the influence of seed density upon cell growth, a series of small spinner flasks were inoculated using varying cell densities. The resulting growth curves are shown in Figure 21 on page 116. These curves indicate than an initial cell inoculum of at least 3 x 10^5 cells per ml was required for adequate cell growth. Increasing the inoculum from 3 x 10^5 to 4 x 10^5 cells/ml showed no additional benefit with regard to cell growth or final cell density (which was slightly more that 10^6 cells/ml). At a seed density of 2 x 10^5 cells per ml, significantly less growth occurred, the final density (as judged after 7 days in culture) was considerably less than 10^6 cells/ml. Furthermore, a seed density of 10^5 cells/ml showed no significant cell growth after 7 days.

Figure 21 Inoculum Densities vs. FS-4 Cell Growth on Microcarriers

FS-4 cells from eight roller bottles were pooled and used to seed cultures, in duplicate, at the cell densities indicated. At the times indicated, samples were taken and cells were counted as described in the text.

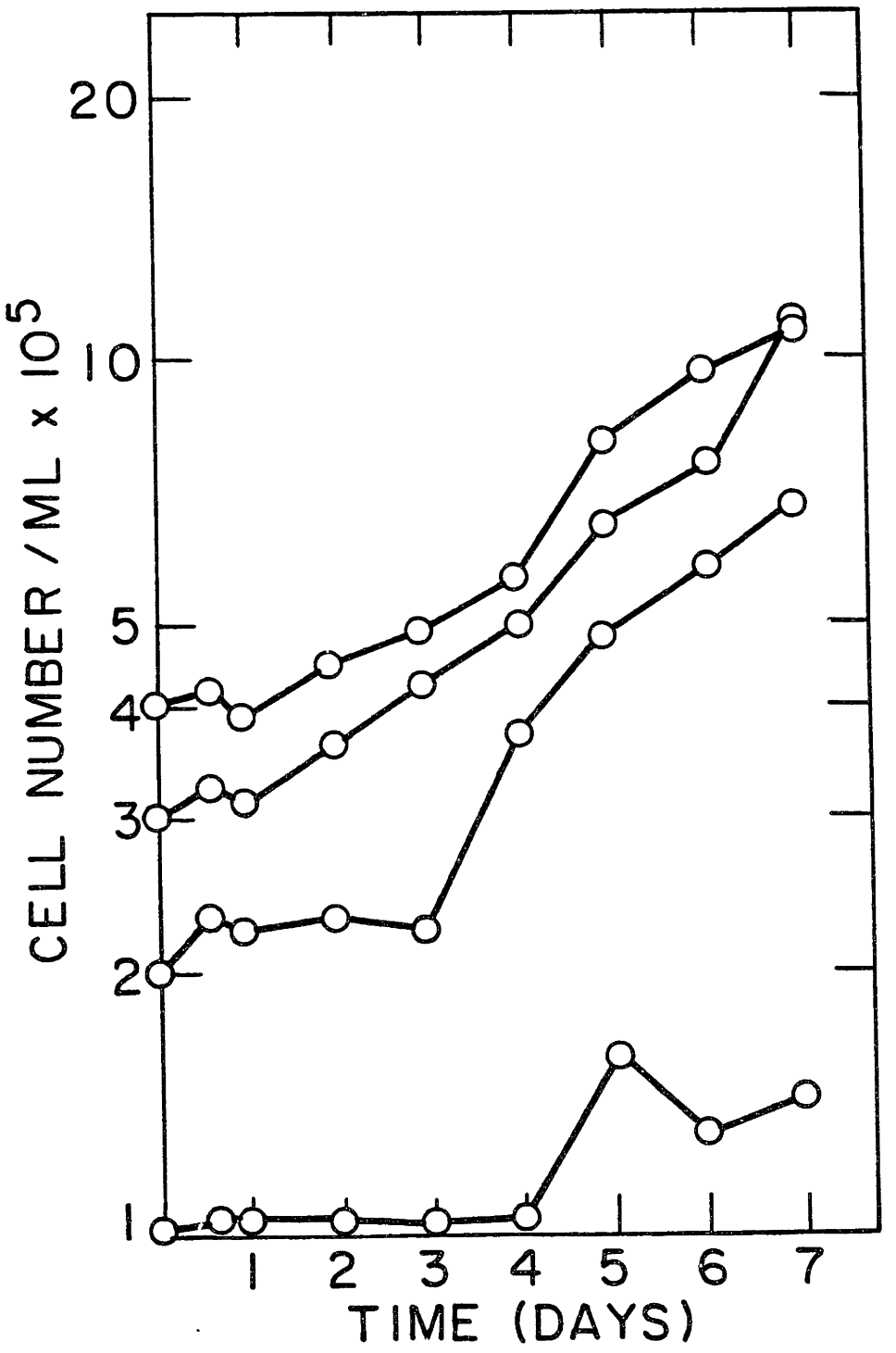
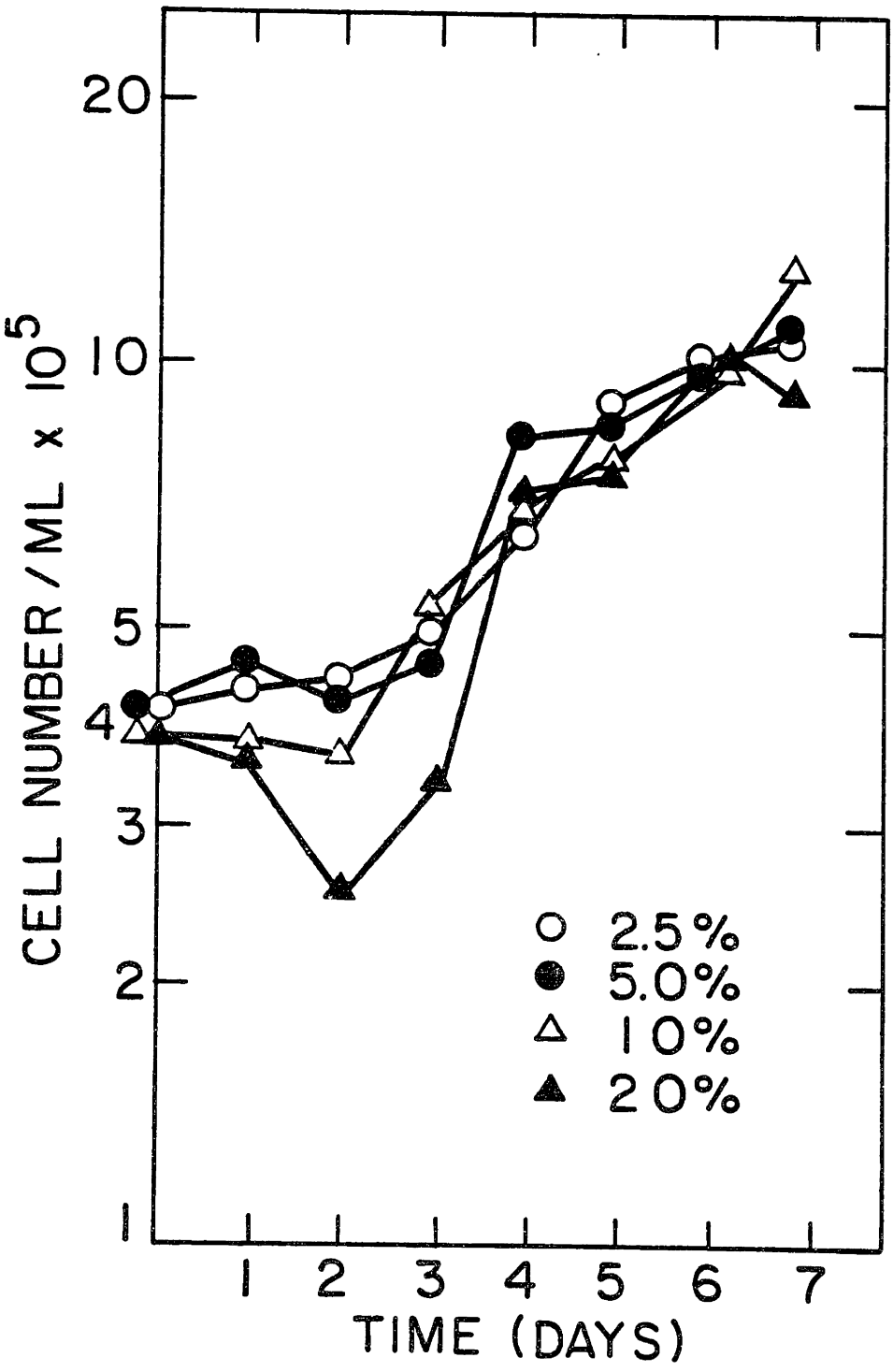

20
10
5
4
3
2
1
CELL NUMBER / ML x 10^5
1 2 3 4 5 6 7
TIME (DAYS)

Figure 22 Serum Concentration vs. FS-4 Cell Growth on Microcarriers

Cells from confluent monolayers grown in roller bottles were pooled and used to seed 100 ml cultures at varying concentrations of fetal bovine serum in DME. All cultures were seeded at an initial density of 4×10^5 cells per ml and cultures were set up in duplicate pairs.


20
10
5
4
3
2
1
CELL NUMBER / ML x 10^5
1 2 3 4 5 6 7
TIME (DAYS)
2.5%
5.0%
10%
20%

EFFECT OF SERUM CONCENTRATION ON CELL GROWTH AND INTERFERON YIELDS

A second parameter examined was the effect of serum concentration upon cell growth. Additionally, the capacity of these same cells to subsequently produce interferon was also examined. One of the initial problems with the growth of FS-4 cells on microcarriers was that they tended to fall off the beads about five to six days after inoculuation. It would appear, from the work of Ham's and Sato's groups (Ham, 1980; Bottenstein *et al.*, 1979), that the primary function of serum in the medium is to provide hormones and that the requirement for them by the cells is greatest during the initial phases of growth. Consistent with this assertion is the practice of reducing the serum concentration in the medium used to maintain cells in roller bottles culture following the initial seeding. In this experiment, a series of small spinners were seeded with FS-4 cells at a density of 4×10^5 cells/ml. The medium used ranged in serum concentration from 2.5 to 20% (v/v) FBS. The results are shown in Figure 22 on page 118. The growth rates and maximum cell densities observed for cells grown with 2.5, 5 and 10% serum were essentially the same: a lag of 2 days was seen after which the cells entered log phase and grew to a density of slightly more than 10^6 cells/ml. However, at 20% serum concentration, the cells showed a 3 days lag period and grew to slightly less than 10^6 cells/ml. Photomicrographs of the cells from these cultures were prepared on day 7 (see Figure 23 on page 122). Here, it can be seen that morphologically, the cells grown at the lower serum concentrations (2.5% and 5%) had the characteristic morphology with little cell detachment. On the other hand, cells grown in medium containing 10 and 20% serum, began to form clumps, the cells had a more rounded appearance and began to detach from the microcarriers.

These same cultures were then subsequently induced to produce interferon. In this experiment, induction and production were carried out with priming and at 37 °C. As seen from Table 6 on page 123, higher yields were obtained when cells were grown at low serum concentrations. An extremely low yield was obtained from cells grown in 20% FBS. The most probable explanation for these results is that those cells grown at the higher serum concentrations tended to detach from the microcarriers

Figure 23 Photomicrographs of FS-4 Cells Grown at Various Serum Conc.

Photomicrographs of FS-4 cells grown in 2.5, 5, 10, and 20% (v/v) FBS in DME were taken after 7 days of growth. It can be noted that the cells grown in the higher serum concentrations show poorer attachment.

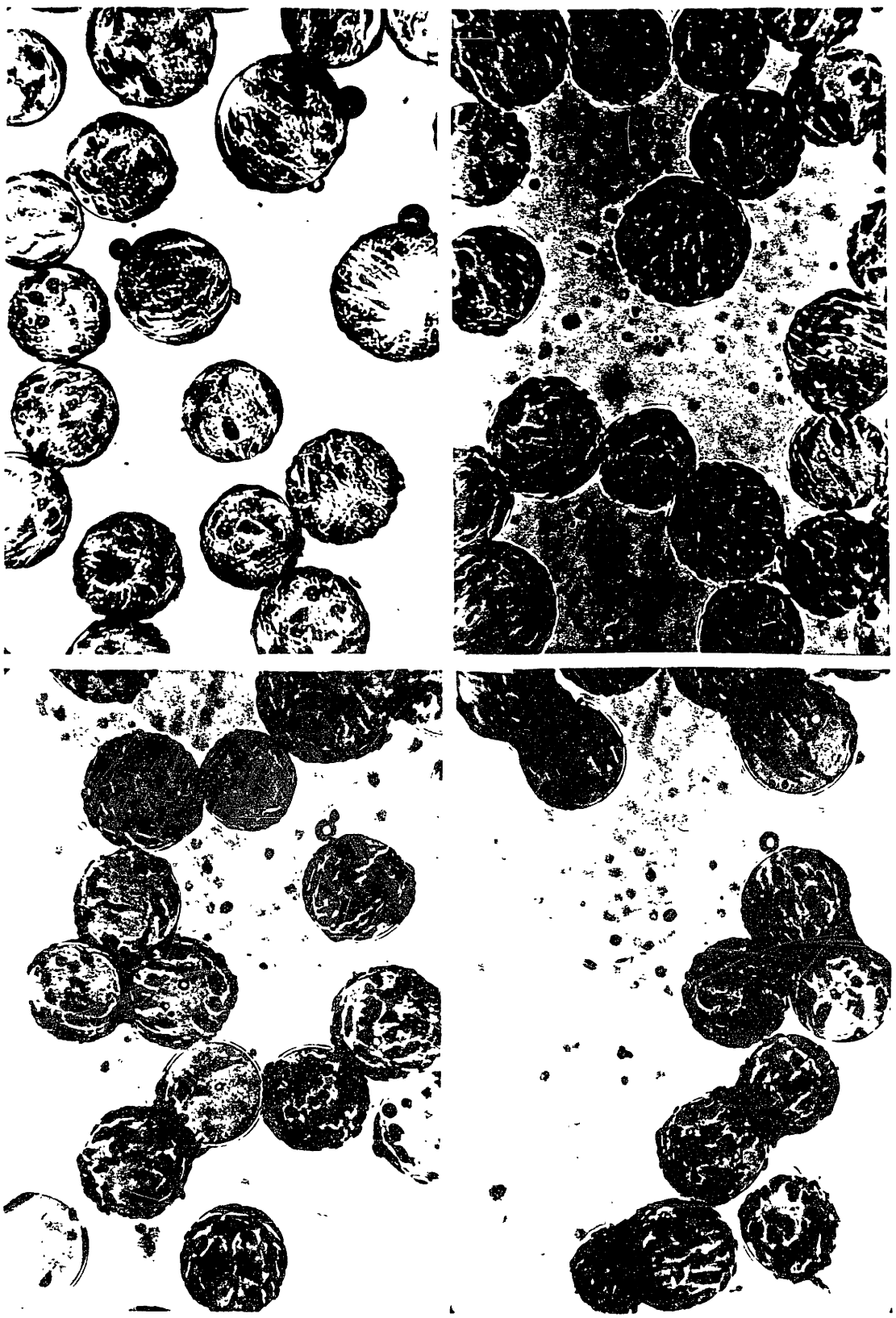

2.5
5
10
20

TABLE 6

SERUM CONC. DURING CELL GROWTH VS. INTERFERON YIELD

Serum conc. %	Interferon yield ($u/10^6$ cells)
2.5	11,000
5.0	14,000
10.0	6,600
20.0	310

during the induction and are thus unable to contribute in production. This view is strengthened by photomicrographs of the cultures taken one hour following the induction period (see Figure 24 on page 126).

DRY CELL WEIGHT AND PROTEIN CONTENT OF FS-4 CELLS.

To provide a basis of comparison for the data that would be obtained upon the oxygen utilization rate and energy metabolism of these cells, with other animal cells and also with data from the microorganisms, the dry cell weight and protein content of the FS-4 cells was determined. This was accomplished by lypholyzing cells that had been washed in an ammonium bicarbonate buffer. Ten roller bottles of confluent FS-4 cells (a total of 3.15×10^8 cells) were trypsinized and then washed extensively in an ammonium bicarbonate buffer (174 m *M*). This buffer has the property of evaporating during lypholyzation. Cell number was monitored during the washings and did not show a decrease or other signs of cell lysis. The cells were then aliquoted into 4 tubes and lypholyzed for 3 days. A control tube containing only buffer showed no weight gain after drying. The combined dry weight of the cells was 0.1396 grams. Thus, one gram of FS-4 cells (dry weight) corresponds to approximately 2.3×10^9 cells Additionally, the volume per cell is about 7×10^{-9} ml, as was determined by measuring the packed cell volume of cells grown to confluence in roller bottles.

To determine the protein content of these cells, the contents of one tube was hydrolyzed with 3 *M* NaOH in a boiling water bath for 10 min. after which the cell debris was removed by centrifugation. The protein concentration of the supernatant was measured using the Lowery assay. From these results, it was determined that 55% of the dry weight of FS-4 cells is protein.

CHANGES IN THE AMINO ACID COMPOSITION OF DME DURING THE GROWTH OF FS-4 CELLS

Figure 24 Photomicrographs of FS-4 Cells following Induction

Photomicrographs of the FS-4 cells grown with differing serum concentrations subsequent to induction for interferon. Note that cell detachment increased with increasing serum concentrations used during cell growth.

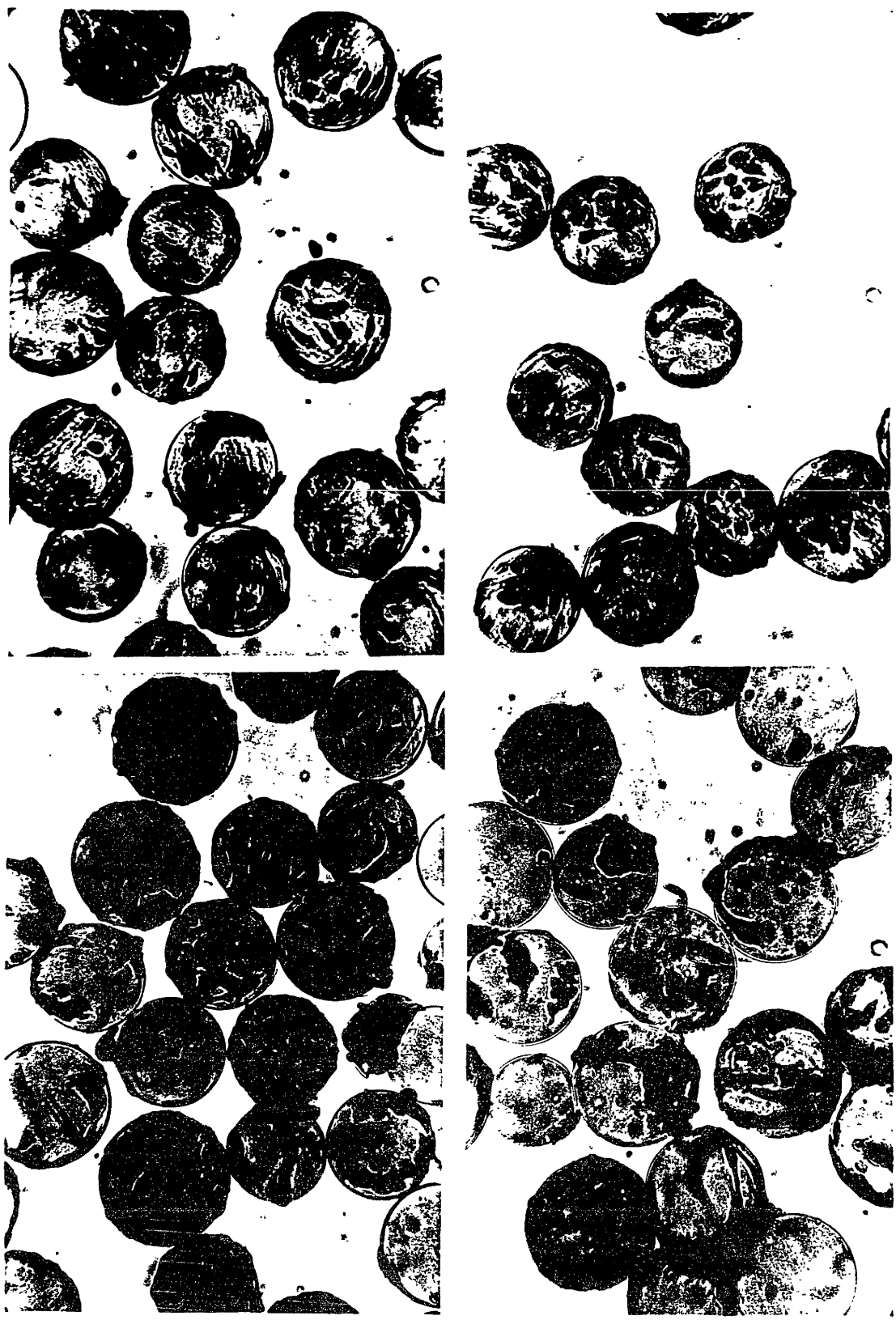

2.5
5
10
20

TABLE 7

AMINO ACID COMPOSITION OF DME VS GROWTH OF FS-4 CELLS

AA*/Time(hr)	0	24	62**	87
Arg	0.245	0.277	0.248	0.217
Gln	3.87	3.63	2.44	2.79
His	0.102	0.069	0.085	0.116
Ile	0.520	0.585	0.552	0.498
Leu	0.536	0.601	0.558	0.502
Lys	0.467	0.556	0.568	0.497
Val	0.555	0.646	0.614	0.556
Ala	0.057	0.111	0.159	0.121
Glu	0.486	0.666	0.707	0.619
Gly	0.258	0.326	0.422	0.388
Pro	0.021	0.054	0.102	0.089
Asp	0.011	0.028		0.018
Met	0.097	0.091	0.092	0.079
Phe	0.237	0.284	0.292	0.247
Thr	0.467	0.564	0.570	0.511
Thy	0.224	0.287	0.294	0.219

* Concentration of amino acids in m *M*

** Culture was fed (1/2 volume medium exchange) after time indicated

94**	120	134	160	185
0.210	0.212	0.178	0.196	0.170
2.17	2.54	1.85	1.58	1.19
0.099	0.142	0.121	0.132	0.078
0.494	0.544	0.456	0.487	0.444
0.492	0.547	0.451	0.476	0.429
0.499	0.543	0.497	0.541	0.528
0.558	0.592	0.516	0.560	0.532
0.129	0.130	0.117	0.161	0.194
0.583	0.558	0.578	0.691	0.679
0.345	0.455	0.440	0.468	0.478
0.081	0.105	0.102	0.185	0.267
	0.027	0.014	0.022	
0.083	0.102	0.078	0.094	0.081
0.258	0.280	0.249	0.277	0.267
0.514	0.547	0.496	0.564	0.547
0.265	0.246	0.220	0.267	0.265

The amino acid composition of the medium during the growth of FS-4 cells in microcarrier culture was also studied. This was to compliment the data that was to be obtained by examining the O_2 content and O_2 uptake rate, the glucose concentration, and the production of lactic acid. Two main objectives of this experiment were, first, does the availability of amino acids as nutrients represent a limitation to cell growth and second, that the role of glutamine in cellular energetics might be further illuminated. In this experiment, the FS-4 cells were grown on a 10L volume, the culture was feed twice (by exchanging 50% of the spent medium with fresh medium) and samples were obtained at the time indicated to be analyzed for their amino acid content, as presented in Table 7 on page 128.

The data is divided into three groups. The first group consists of seven amino acids showing a significiant rate of removal from the medium. Glutamine showed the most marked rate of removal, but it is not depleted. Also of interest is the casual observation that arginine, isoleucine, leucine, and valine show parallel concentration profiles over time. The second group consists of five amino acids whose concentrations increased with time, indicating net synthesis and secretion of these amino acids by the cells into the medium (especially alanine and proline). Finally, a third group of amino acids is listed whose individual concentrations showed little to no change with time.

Overall the amino acid composition of DME is not rapidly altered by the growth of FS-4 cells in microcarrier culture. This indicates that as yet the availability of amino acids does not present a limitation to further cell growth.

INTERFERON ASSAY

Interferon titers are assayed in our lab by a biological assay while referencing the results to an international standard. The objective of the experiments presented here was two-fold: one, to establish a calibration curve for the assay and two, to reexamine the 'appropriateness' of our use of the internal standard.

Figure 25 Calibration of Interferon Assay

Each point of the figure represents the titer of a single sample prediluted 1/8, 1/64, and 1/256 before the assay

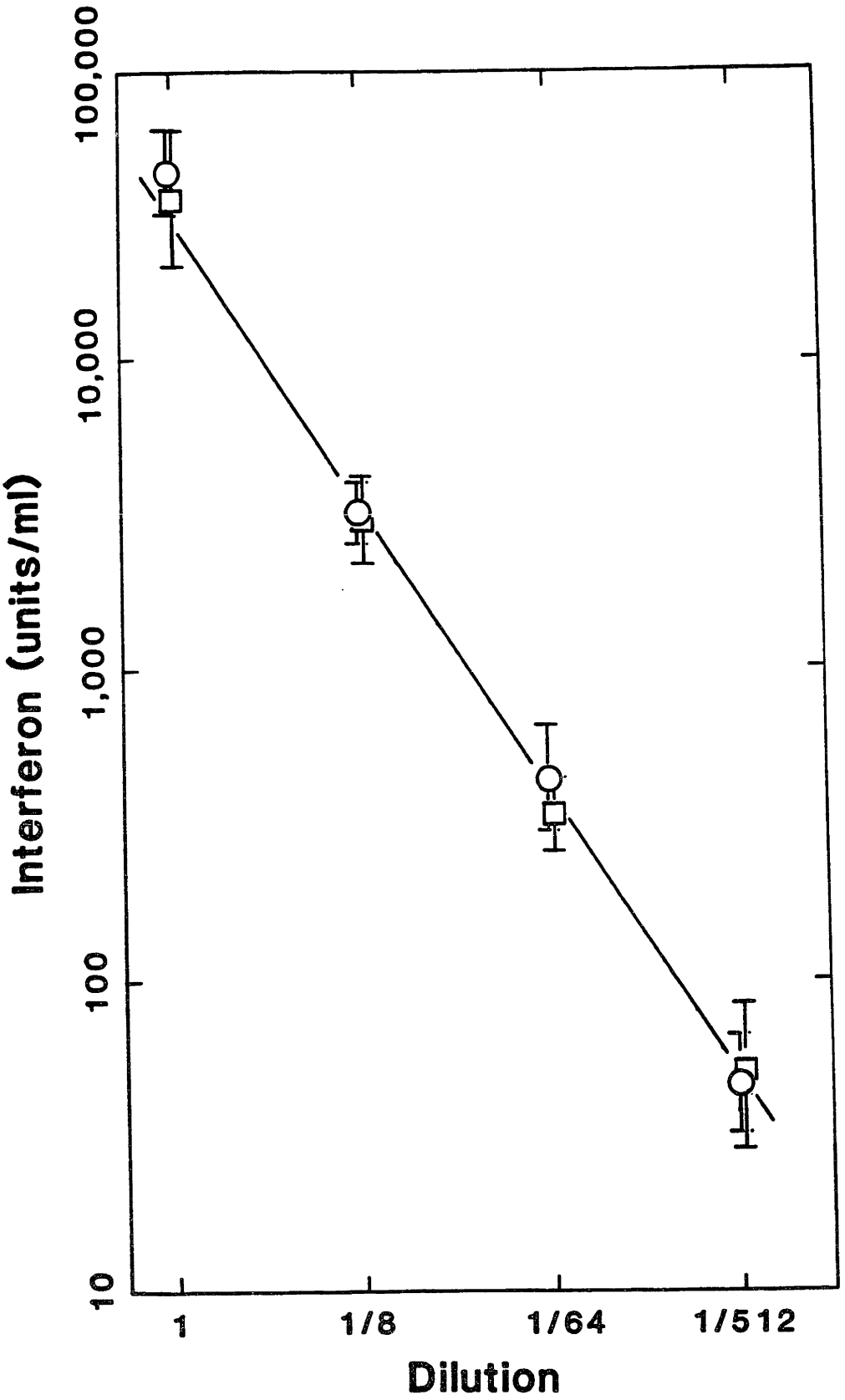

Interferon (units/ml)
100,000
10,000
1,000
100
10
1
1/8
1/64
1/512
Dilution

TABLE 8

USE OF STANDARDS IN THE INTERFERON ASSAY

Date	Observed Titers In Assay		Predicted Titer of In-house Ref.*
	IS-527	In-house Ref	
2/29/80	3,500	22,000	12,600
3/6/80	3,000	15,000	10,000
3/14/80	3,000	22,00	14,700
5/31/80	3,500	24,000	13,700
6/6/80	2,000	15,000	15,000
10/31/80	1,800	10,000	11,100
ave.	2,800	18,000	12,900
STD	±735	±5,500	±2,000

* Based upon the relative performance of IS-527 and the assignment of a value of 2,000 international reference units per ml to that material

USE OF INTERNAL STANDARD

The interferon titers are determined by measuring the inhibition of VSV-induced cytopathic effect (CPE). Quantitatively, the endpoint is judged to be the highest dilution of the sample showing 50% protection from the VSV. The variations that can be introduced by the day-to-day variations in cells, viruses, and medium are compensated for by including an internal standard in each assay. The internal standard will presumably be influenced by these factors in the same way as all of the other samples. We then challenged this assumption by assaying two standards (an international fibroblast standard, IS-527, and an in-house standard, FS4B) concurrently over an eight months period. The results are shown in Table 8 on page 132. Should the samples be affected in an unrelated fashion we would have predicted that the variance within the ratio of these two samples would be sizably greater than the individual variances. In contrast, what we see from this data is that the ratio of these two standards showed less variance than did the standards themselves, suggesting that the variations from one assay to another affected both samples equally (ie. the use of the internal standard is justified). Data is shown in Table 8 on page 132.

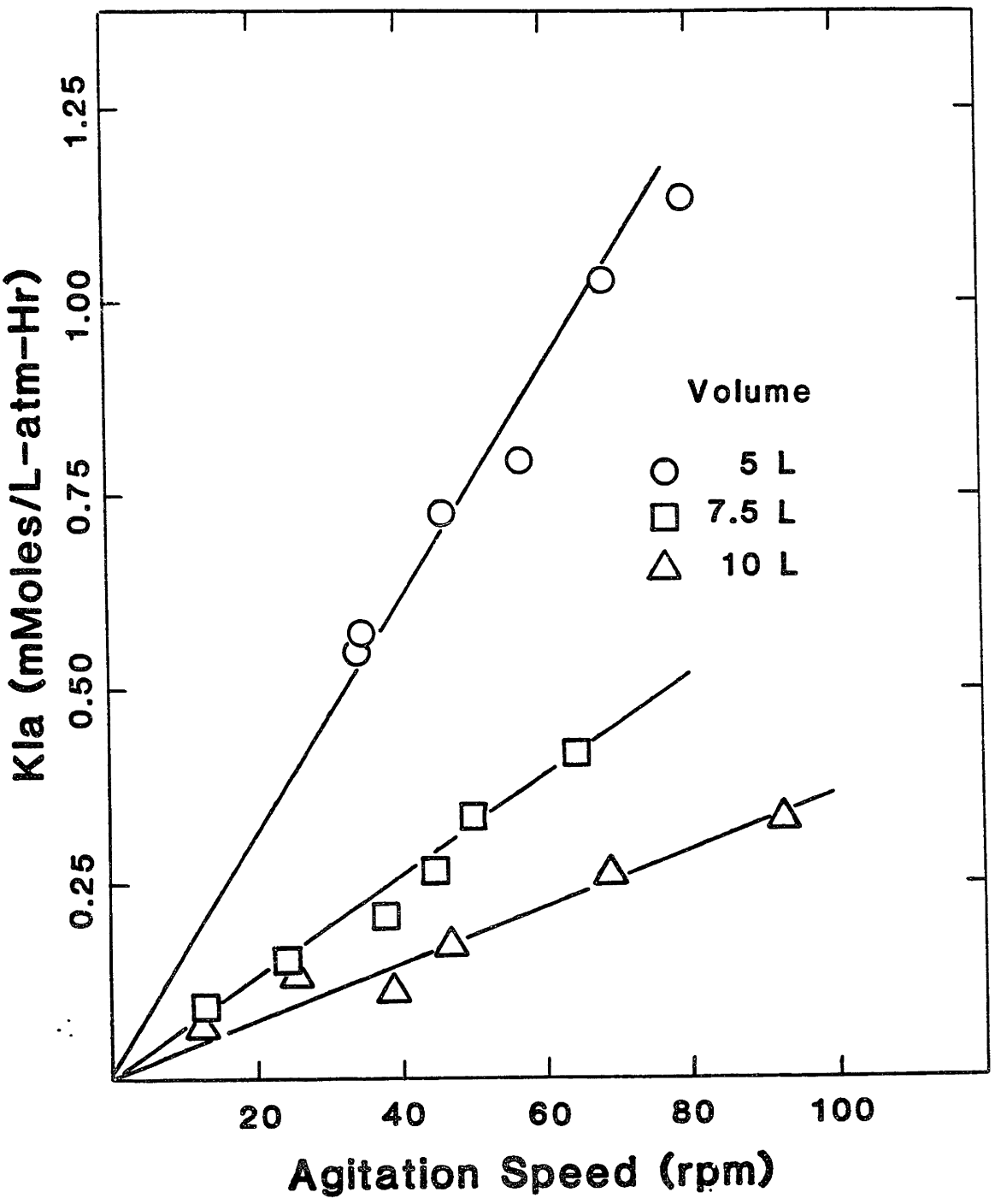
CALIBRATION OF THE INTERFERON ASSAY.

The linearity of the assay for interferon was evaluated by preparing several dilutions of a single interferon sample and subsequently assaying them (see Figure 25 on page 130). In essence, we were able to show that the observed titers corresponded linearly (1:1) with the predilution of the original sample. Furthermore, from the variances within these data points (not presented), it can be determined that the assay is not capable of readily distinguishing a difference between two samples of less than 35% (at a confidence level of 90%).

Additionally, the effect of changing the pipet tips used in performing the serial dilutions was examined. We saw no difference in the interferon titers whether one tip was used to serially dilute a sample across the microtiter plate or if the tip was changed every other dilution.

Figure 26 Characterization of Kla in 14-liter Fermentor

Dependence of the mass transfer coefficient of oxygen (Kla) across the surface of a 14-liter New Brunswick fermentor upon agitation speed and liquid volume.


Kla (mMoles/L-atm-Hr)
0.25
0.50
0.75
1.00
1.25
20
40
60
80
100
Agitation Speed (rpm)
Volume
5 L
7.5 L
10 L

PERFORMANCE AND CALIBRATION OF INSTRUMENTAL METHODS

To quantitate the energy metabolism of the FS-4 cells instrumental methods were developed to measure their oxygen uptake, their production of lactic acid, their production of carbon dioxide, their production of ammonia, and their consumption of either glucose or galactose. The performance and calibration of the methods we have developed is discussed in the following section.

OXYGEN DEMAND AND SUPPLY

Oxygen demand is determined by measuring the removal of oxygen from the culture medium during an induced transient. Silicone rubber tubing was used to provide an adequate supply of oxygen to the cells.

Mass Transfer of 14-liter Vessel

The mass transfer of oxygen across the surface of the 14-liter fermentor was determined for vessels filled with 5, 7.5, and 10 liters of medium and for agitation rates varied between 15 and 100 rpm (shown in Figure 26 on page 134). The results indicate that the mass transfer coefficient for a given volume (V) is nearly proportional to the agitator speed (N). The slopes of these curves ($\partial Kla/\partial N$) are not linearly related to the volume of the vessel, but rather exhibit an approximately inverse square relationship (shown in Figure 27 on page 138). In summary, the mass transfer coefficient for oxygen in this vessel can be described by the following equation:

$$Kla = 0.414\ N/V^{2.05}$$

Oxygen Transfer Potential of Silicone Rubber Tubing

The oxygen transfer potential of silicone rubber tubing was evaluated by examining the apparent Kla of a vessel fitted with 4.4 m of tubing as com-

Figure 27 dKla/dN vs. Volume

The relationship between the slope of the Kla vs. rpm (∂Kla/∂N) vs. liquid volume in a New Brunswick 14 liter fermentor.

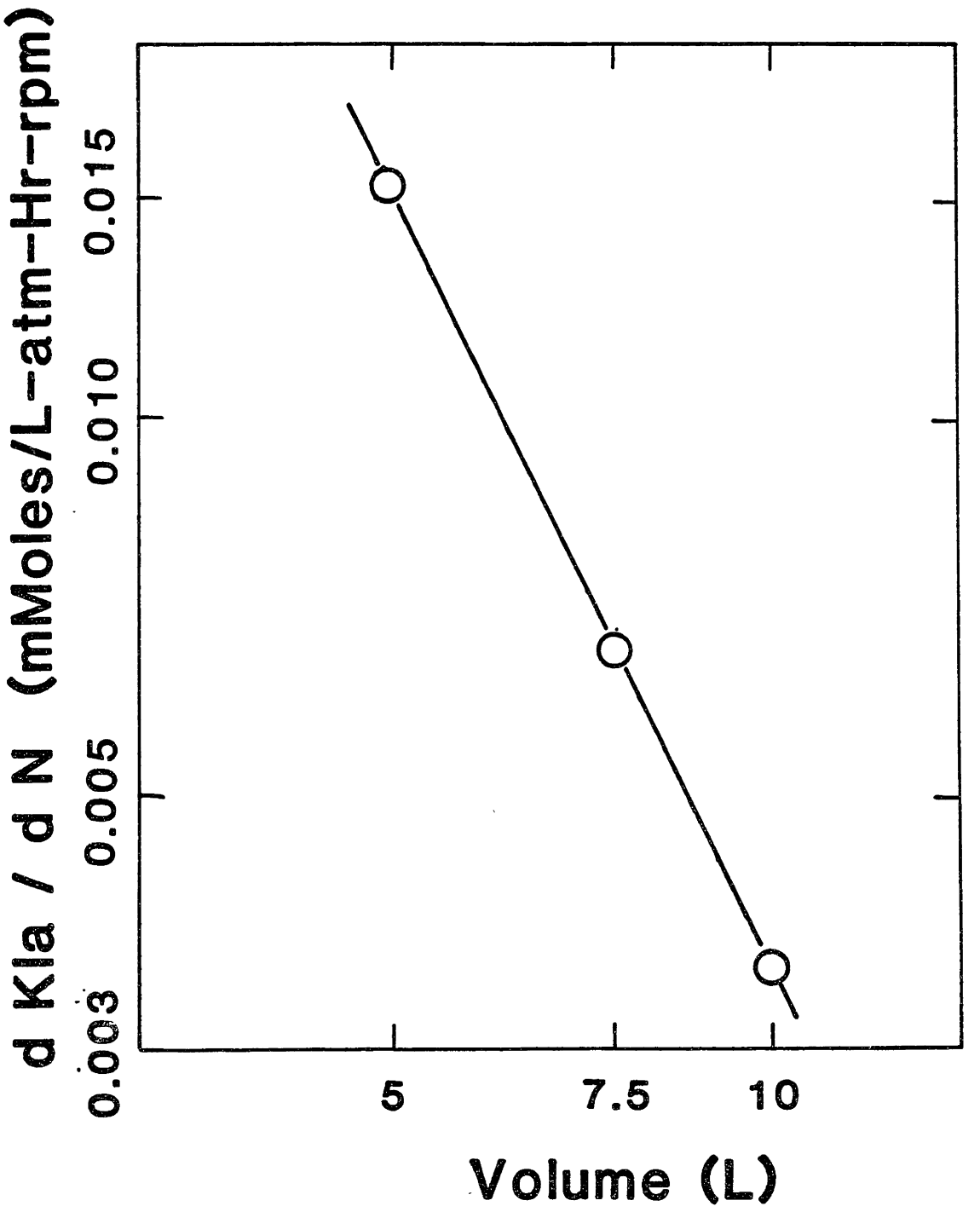

d Kla / d N (mMoles/L-atm-Hr-rpm)
0.003
0.005
0.010
0.015
5
7.5
10
Volume (L)

Figure 28 Oxygenation Potential of Silicone Rubber Tubing

Oxygenation of a 14-liter fermentor equipped with 4.4 m of silicone rubber tubing. The apparent Kla of the vessel with the tubing (liquid volume at 10 liters) vs. the agitation speed is contrasted with the Kla of the vessel alone.

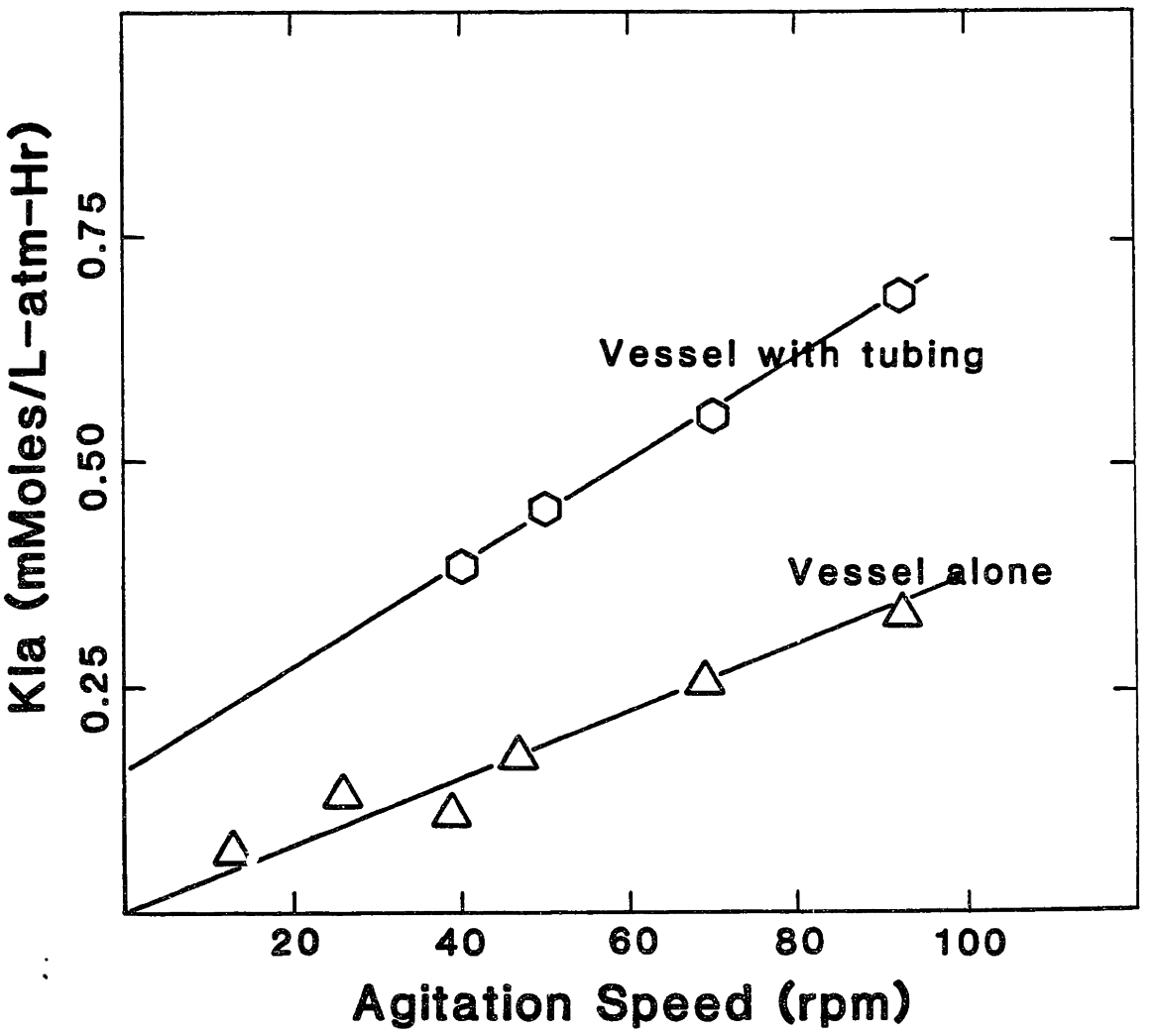
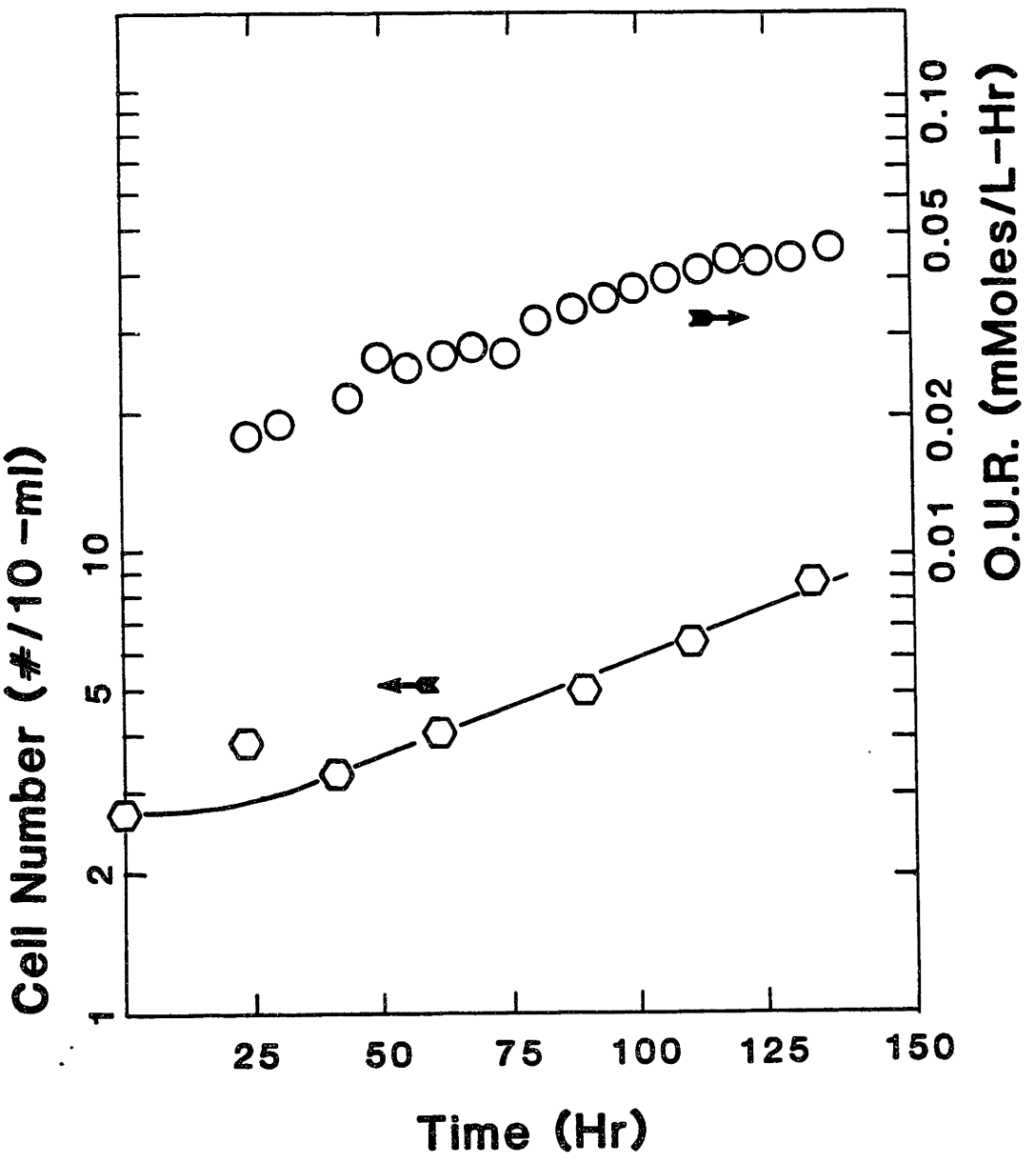

Kla (mMoles/L-atm-Hr)
0.25
0.50
0.75
Vessel with tubing
Vessel alone
20
40
60
80
100
Agitation Speed (rpm)

Figure 29 Oxygen Demand of FS-4 Cells

Relationship between oxygen demand (OUR) and cell number for the growth of FS-4 cells in microcarrier culture.


Cell Number (#/10⁵-ml)
1
2
5
10
25
50
75
100
125
150
Time (Hr)
0.01
0.02
0.05
0.10
O.U.R. (mMoles/L-Hr)

pared to the actual Kla of the same vessel (shown in Figure 28 on page 140). The difference between these two lines, that of the observed Kla and that of the actual Kla of the vessel corresponds to the oxygenation due to diffusion across the tubing. First, it should be noted that the oxygen transfer rate across the tubing is less sensitive to the mixing conditions of the fluid than is the transfer across the liquid surface, as can be derived from the more gradual slope of the curve. Secondly, the transfer of oxygen across the tubing is in itself quite substantial (0.6 mmoles O_2/atm-meter of tubing-h).

Oxygen Demand by FS-4 Cells in Microcarrier Culture

Using the methodology described previously in the materials and methods section, the oxygen demand of FS-4 cells in microcarrier culture was determined (Figure 29 on page 142). It can be seen that the OUR correlated well with the cell density of the culture.

MEASUREMENT OF BASE ADDITION

The formation of lactic acid was measured to quantitate the amount of energy being formed via glycolysis. Specifically, the addition of base to maintain a constant pH was used as a measure of the production of lactic acid by the cells. The actual amount of base added was measured using a loadcell.

Calibration of Loadcell

A calibration table for the loadcell was constructed by determining the output of the loadcell for a known weight. Each data point consisted of successively adding 15 ml of water to an erlenmeyer flask, the flask was weighed on a Mettler balance, and then the loadcell reading was determined. The data is shown in Table 9 on page 146 and the error curve is shown in Figure 31 on page 150. The output of the loadcell was linear with increasing weight. The full scale range corresponded to about 580 grams and the standard error in each measurement was ± 0.15 grams.

Figure 30 Titration of Lactic Acid Using pH Controller

The amount of lactic acid added to the 14-liter fermentor was estimated by measuring the amount of base used to achieve pH control. Base addition was determined using the loadcell to measure the weight of the base reservoir.

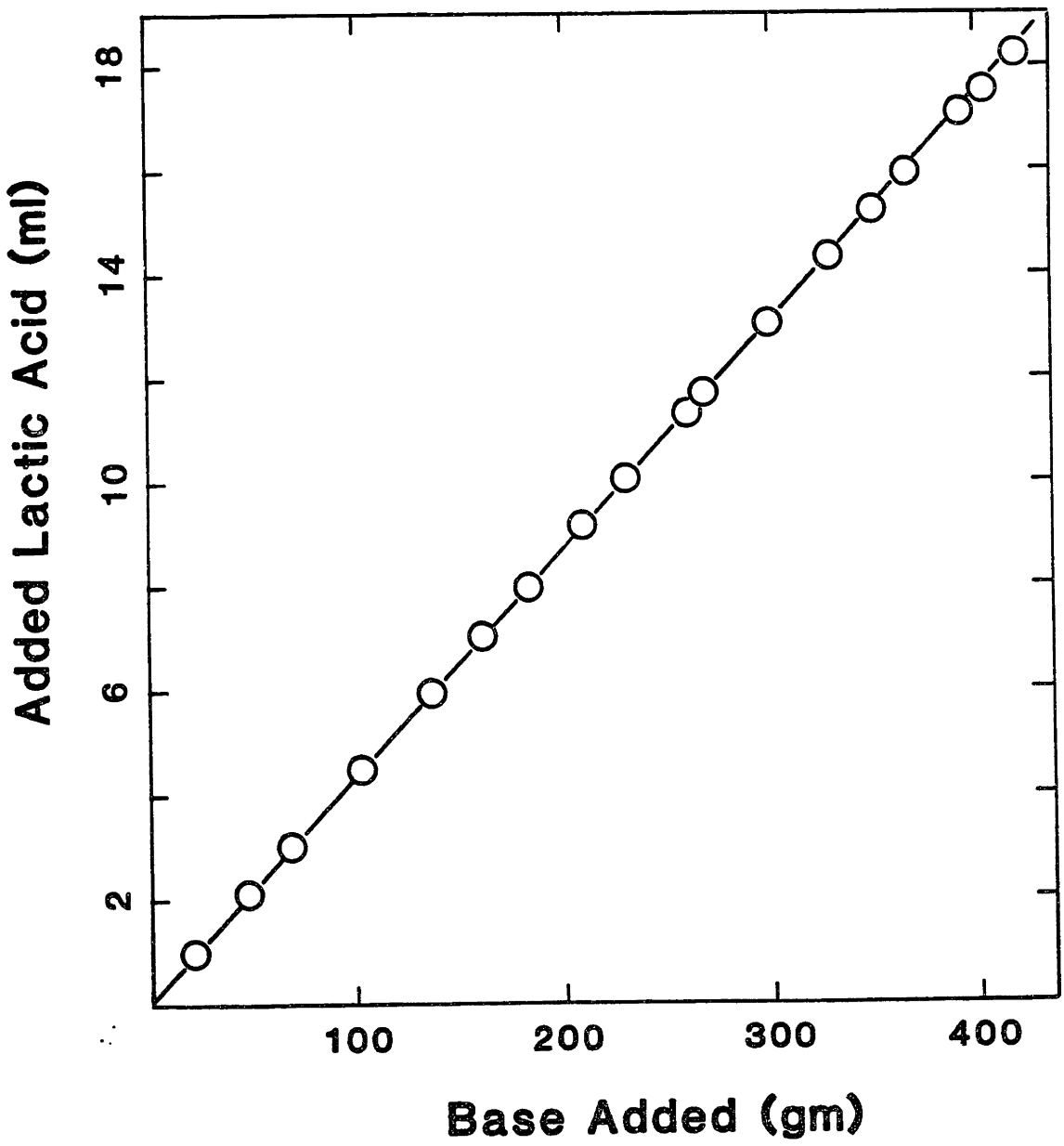

Added Lactic Acid (ml)
2
6
10
14
18
100
200
300
400
Base Added (gm)

TABLE 9
CALIBRATION OF LOADCELL

Load Weight (grams)	Loadcell Output (volts)
15.07	0.128
30.03	0.256
44.89	0.385
64.78	0.555
94.64	0.809
109.53	0.938
129.31	1.107
144.09	1.236
173.63	1.491
196.94	1.688
216.72	1.859
231.52	1.985
246.42	2.114
266.3	2.286
281.17	2.412
300.93	2.582
320.78	2.753
355.39	3.048
370.34	3.177
390.19	3.346
405.03	3.473
424.74	3.641
459.27	3.937
474.2	4.064
493.9	4.233
508.77	4.359
528.56	4.534

TABLE 10

TITRATION OF ACID USING PH CONTROLLER

Lactic Acid Added (ml)	Base Added (gm)
0.00	0.00
1.00	22.36
2.11	48.13
3.03	65.52
4.51	103.06
5.96	136.82
7.02	160.82
7.98	182.00
9.17	209.75
10.08	230.63
11.36	260.67
11.72	268.33
13.04	299.52
14.35	329.28
15.24	350.48
15.93	367.01
17.11	394.34
17.56	404.59
18.26	420.07

Titration of Lactic Acid Using pH Controller and Loadcell.

The 14-liter fermentor was filled with 10 liters of PBS. Lactic acid (approximately 5 *M* was added to the vessel with each addition. The pH controller maintained the pH at a value of 7.2. This was accomplished by adding a solution of NaOH (about 0.2 *M*). The weight of the flask containing the base was measured after each addition of lactic acid using the loadcell. The data is presented in Table 10 on page 147 and is shown in Figure 30 on page 144. It can be seen that the measurement of added base corresponds directly to the total acid content of the vessel.

DISSOLVED CO_2 ANALYSIS.

As a second measure of the energy formed via oxidative phosphorylation, the production of CO_2 by the cells was determined. We were able to show that the concentration of CO_2 in the gas exiting the silicone rubber tubing used for suppling the oxygen demand was determined to be a function of the dissolved CO_2 concentration of the culture fluid and rate of gas flow through the tubing.

Calibration of Flow Meter.

The flow meter was calibrated by the positive displacement of water in a 100 ml biurete. The volume of water displaced, the time required to displace that volume, and the difference in pressure of the air stream just prior to and after entering the fermentor was recorded and is presented in Table 11 on page 155. The gas flow rate is linearly related to the pressure differential across the silicone rubber tubing (see Figure 32 on page 152).

Influence of Gas Flow Rate on CO_2 Concentration in Exit Gas .

The content of CO_2 in the exit gas stream of the silicone rubber tubing is dependent upon the gas flow rate through the silicone tubing, generally decreasing with increasing flow.

Figure 31 Error in Loadcell Output vs. Load

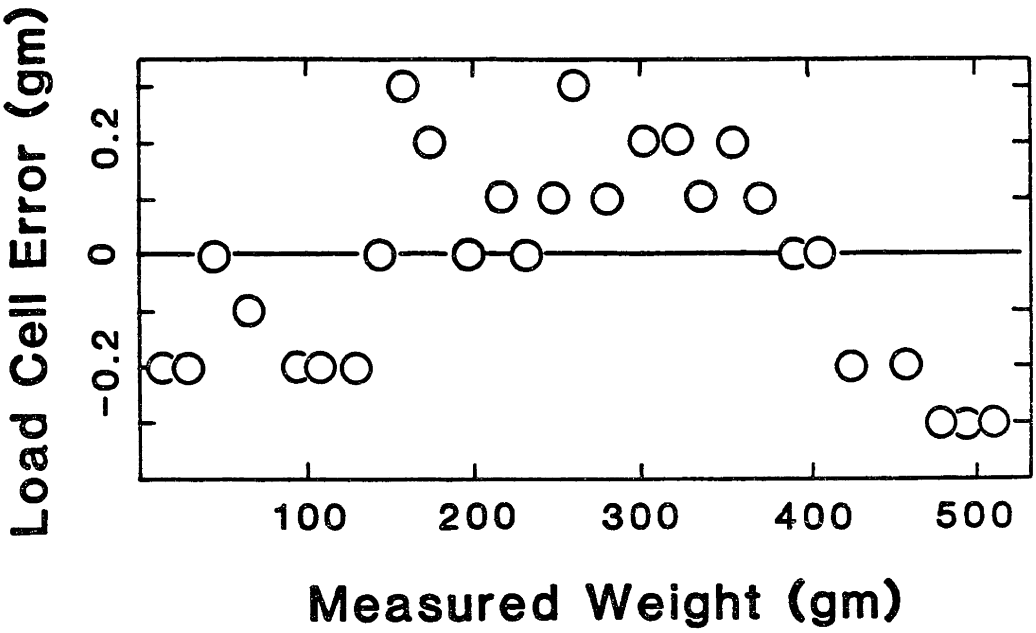

Load Cell Error (gm)
0.2
0
-0.2
100
200
300
400
500
Measured Weight (gm)

Figure 32 Gas Flow in Silicone Rubber Tubing vs Pressure Differential

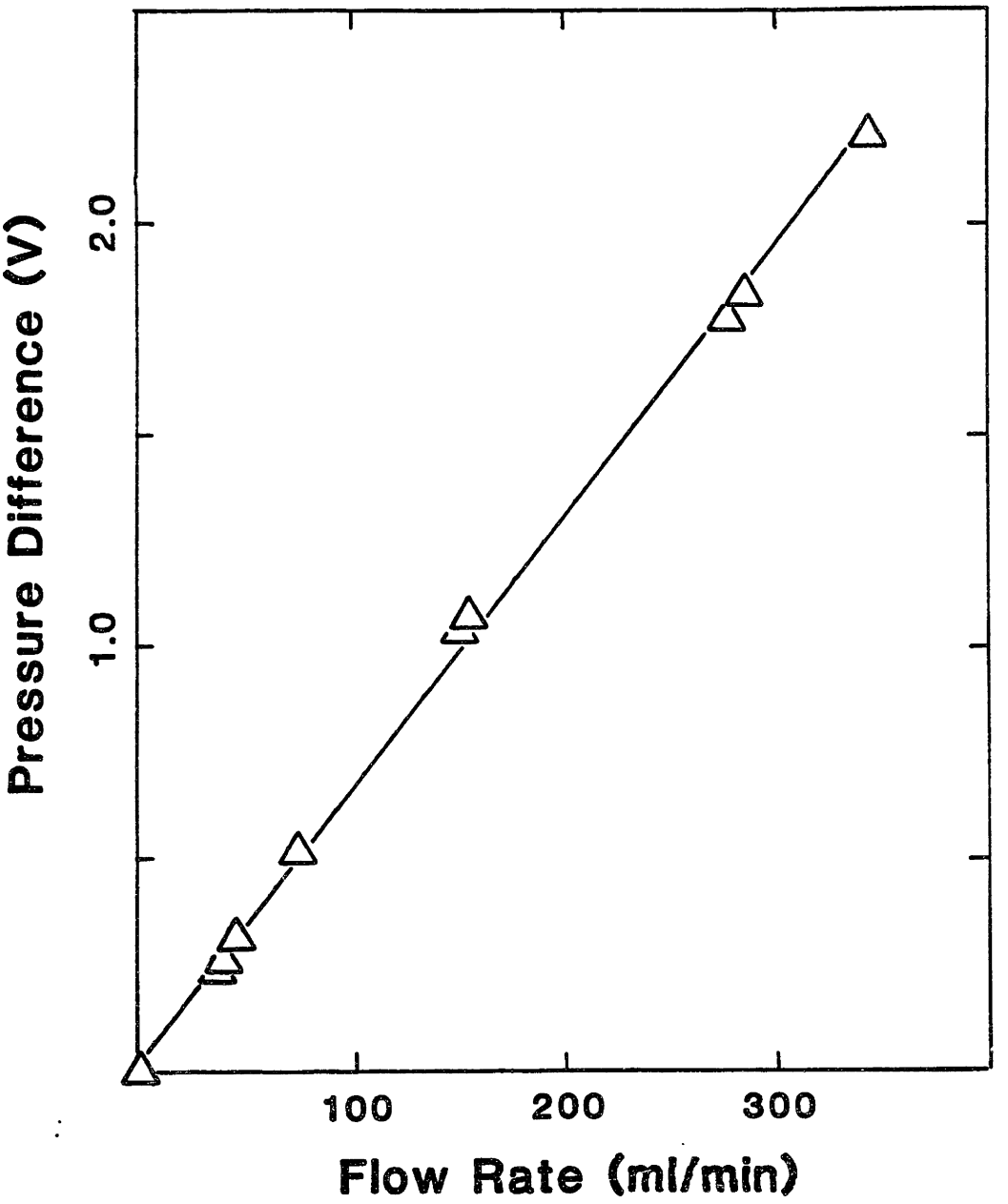

Pressure Difference (V)
2.0
1.0
100
200
300
Flow Rate (ml/min)

Figure 33 Dependence of CO_2 in Exit Gas Stream upon Gas Flow Rate

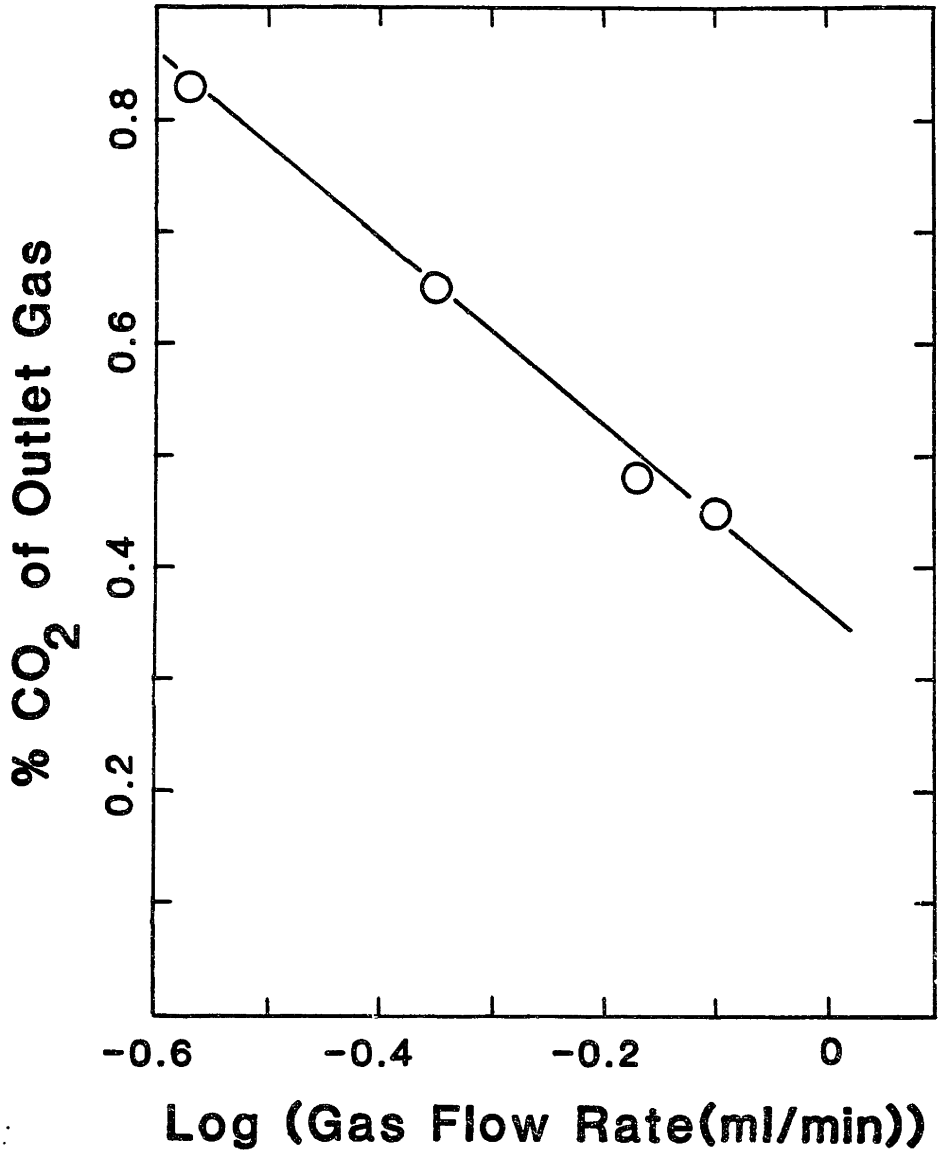

% CO_2 of Outlet Gas
0.8
0.6
0.4
0.2
-0.6
-0.4
-0.2
0
Log (Gas Flow Rate(ml/min))

TABLE 11
CALIBRATION OF FLOW GAUGE

Pressure Diff. (v)*	Δt To Displace 100 ml H_2O	Flow Rate cm^3/min
0.36	2.10	47.6
0.38	1.95	51.3
0.45	1.60	62.5
0.73	0.97	104
0.74	0.97	104
1.51	0.48	207
1.55	0.47	214
2.77	0.28	353
2.85	0.27	366
3.49	0.23	438

* 1 volt corresponds to approximately 0.36 psi

Sodium bicarbonate was added to the fermentor to give a final concentration of about 5 m *M* in 10 liters of PBS. The CO_2 concentration of the exit gas stream was then measured with the CO_2 analyzer at varying gas flow rates. The difference in CO_2 of the outlet gas versus the inlet gas (in this case, air) was plotted versus the gas flow rate on a log-log plot (see Figure 33 on page 154). The data indicates that the CO_2 content of the exit gas (for a constant dissolved CO_2 concentration) is approximately related to the inverse of the flow rate. Specifically, we observed the following relationship:

$$[CO_2]_{\text{exit gas}} \propto \Delta P^{-0.85}$$

Relation of CO_2 Conc. of the Exit Gas to the Bicarbonate Conc.

The CO_2 concentration of the exit gas stream of the the silicone rubber tubing was correlated to the bicarbonate concentration of the surrounding liquid. Using the 14-liter fermentor filled with 10 liters of PBS and maintained at 37°C, aliquots of a 10 *M* sodium bicarbonate solution were added to the vessel, while the pH, gas flow rate, and the CO_2 concentration of the exit gas stream was recorded. The data is shown in Table 12 on page 157. The increase of CO_2 in the exit gas stream was found to be proportional to the dissolved carbonic acid concentration[7] and the gas flow rate using the previously determined relationship. This is demonstrated in a plot of $\Delta CO_2\%/([\text{carbonic acid fraction}][\Delta P^{-0.85}])$ vs. the bicarbonate concentration as seen in Figure 34 on page 160.

---

[7] The fraction of the total dissolved CO_2 at a given pH can be approximated by the following function:
Fraction $CO_2 = 1/(1 + 10^{[pH - pKa]})$
where the pKa is 6.35

TABLE 12

BICARBONATE CONCENTRATION VS. CO_2 IN EXIT GAS

Bicarbonate Conc (m *M*)	pH	Flow (v)	CO2(%)
0.0	7.17	0.27	0.01
2.06	7.19	0.55	0.21
3.20	7.22	0.55	0.32
5.16	7.24	0.80	0.34
5.16	7.24	1.78	0.18
7.22	7.28	1.79	0.23
10.0	7.32	1.80	0.29

CALIBRATION OF AMMONIA ELECTRODE.

The iL ammonia electrode was calibrated by measuring the output of the electrode immersed in freshly prepared basic solutions of known ammonium ion concentration. The data is shown in Figure 35 on page 162. In essence, the data shows that the probe has a linear response in mv to the log of the ammonium ion concentration over the range 10^{-4} to 10^{-2} molar.[8]

SUGAR ANALYSIS USING O-TOLUIDINE.

To monitor the amount of sugar consummed by the cells, the sugar concentration of the fermentation broth was determined using the o-toluide assay. A calibration curve for glucose and galactose using the o-toluidine assay for aldohexose (Fring, C.S. *et al.*1970) was prepared. The data is shown in Table 13 on page 165. In addition, the effect of ammonia and DME components (using DME w/o glucose) was examined. The data shows a linear response between absorbance and sugar concentration as shown in Figure 36 on page 164. Secondly, the data indicates that the other components of the medium do not interfere with the assay. Finally, it was also determined (data not presented) that proteins (specifically, bovine serum albumin, human plasma proteins, and fetal calf serum) will not precipitate in the acetic acid reagent, even when the water content was increased to 50%, nor did they interfere with the assay.

---

[8] Concentrations greater that 10^{-2} *M* were not examined.

Figure 34 Bicarbonate Concentration vs. CO_2 in Exit Gas

The bicarbonate concentration of the fermentor is correlated to the concentration of CO_2 in the exit gas, using information concerning both the flow rate of the gas and the pH of the vessel.

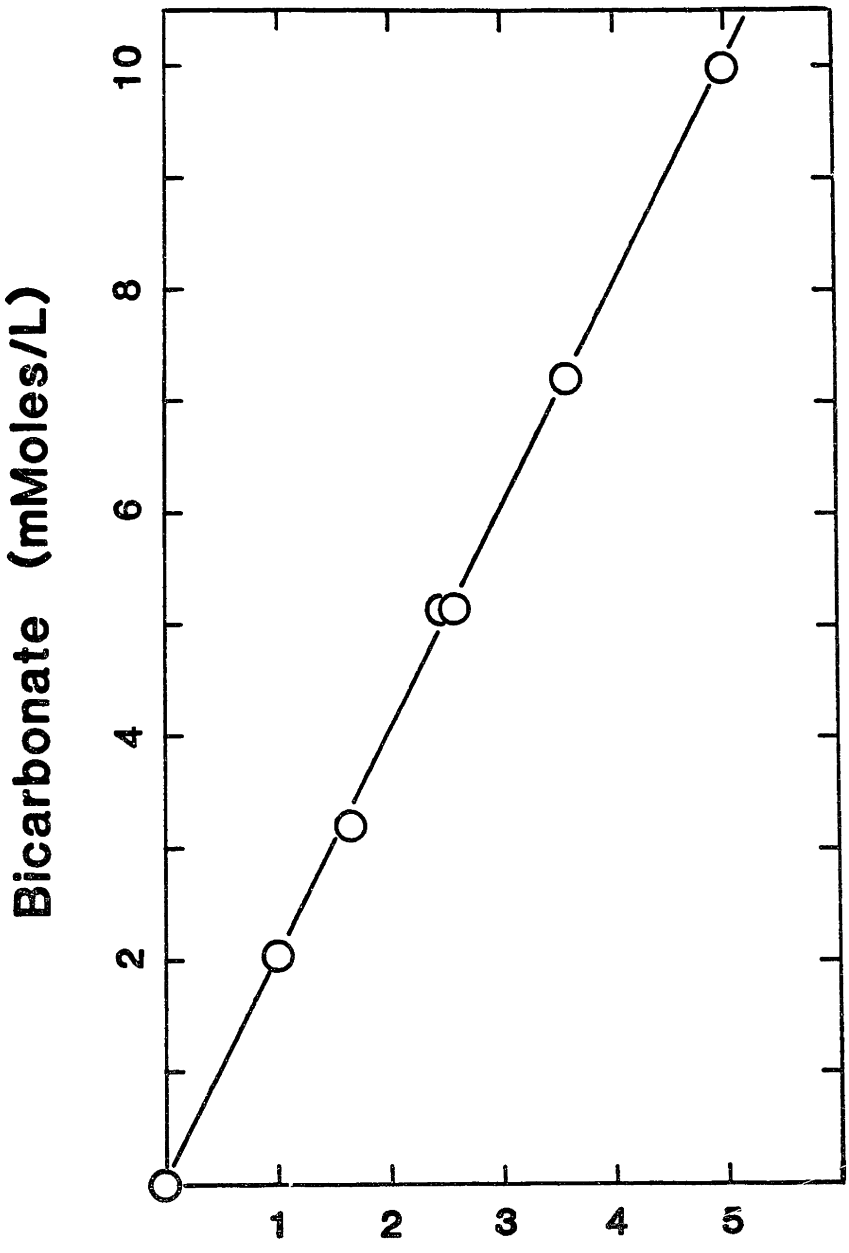

Bicarbonate (mMoles/L)
2
4
6
8
10
1
2
3
4
5
$CO_2\ \%/((1 + 10^{(pH-pKa)})(\Delta P - 0.85))$

Figure 35 Calibration of the Ammonia Electrode

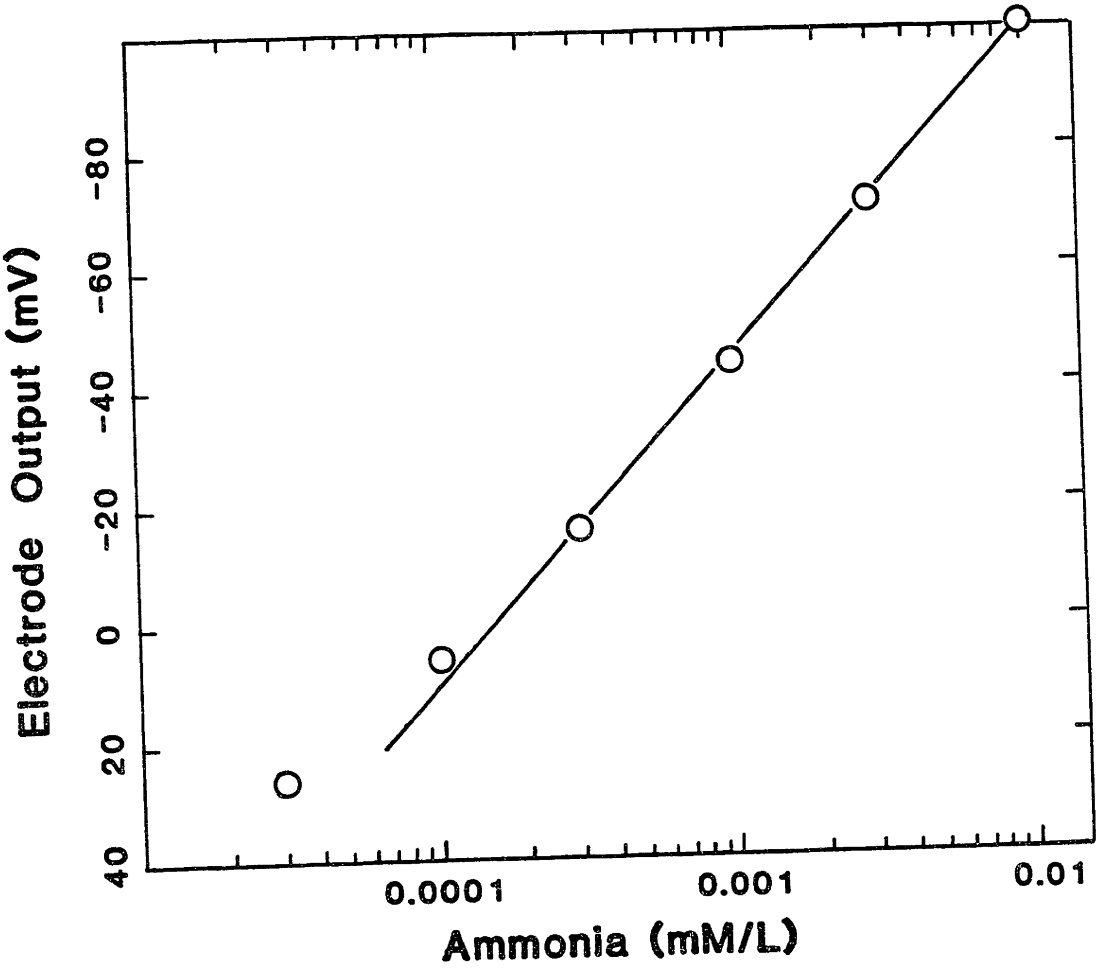

Electrode Output (mV)
40
20
0
-20
-40
-60
-80
0.0001
0.001
0.01
Ammonia (mM/L)

Figure 36 Sugar Analysis Using o-Toluidine

Sugars are assayed by measuring the formation of green color resulting from the reaction of aldohexoses with o-toluidine in acetic acid.

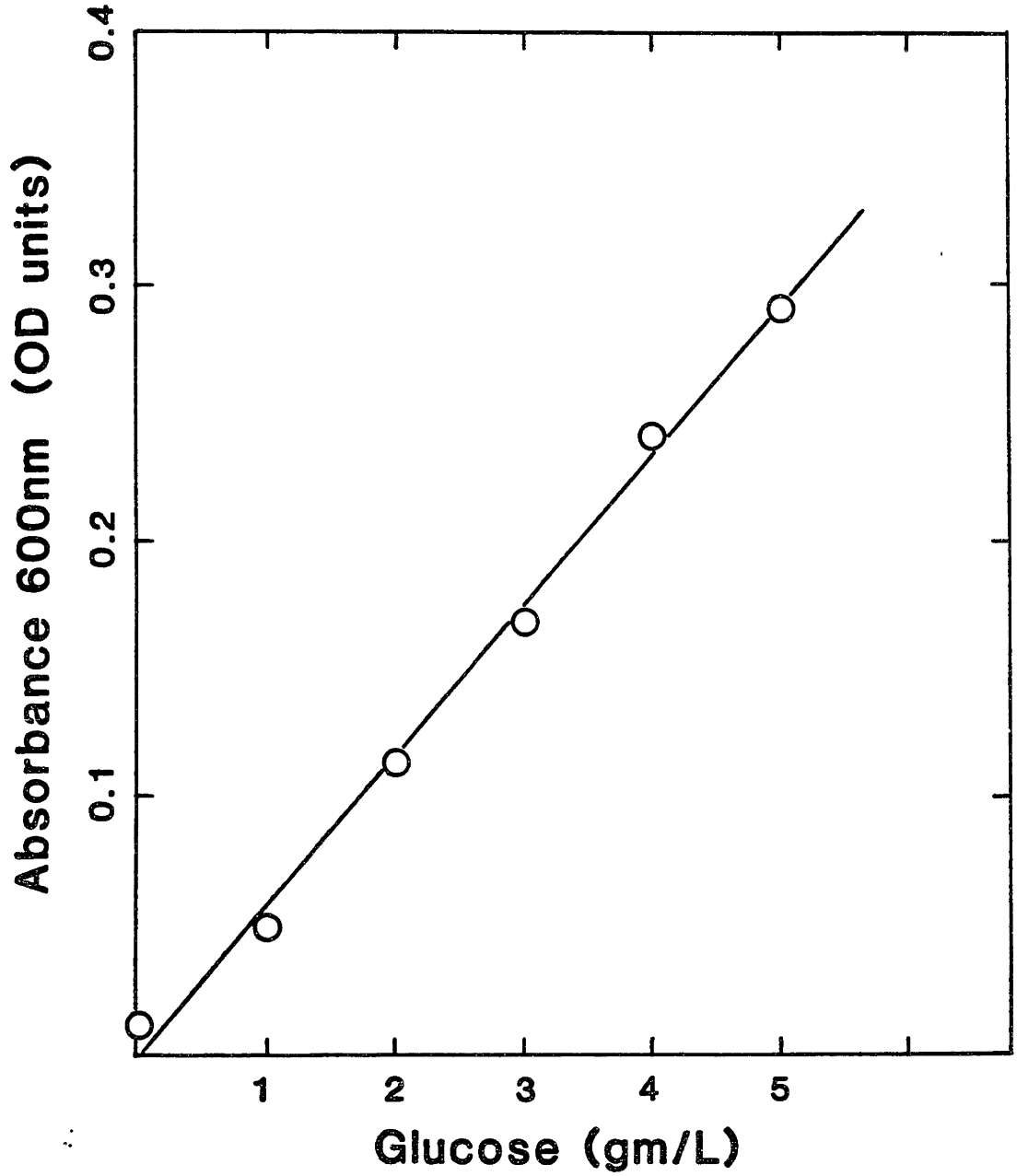

Absorbance 600nm (OD units)
0.4
0.3
0.2
0.1
1
2
3
4
5
Glucose (gm/L)

TABLE 13

ANALYSIS OF SUGARS USING O-TOLUIDINE

Sample	$Ab_{660\ nm}$
0 mg Glucose/100ml	0.003
100 "	0.049
200 "	0.112
300 "	0.169
400 "	0.241
500 "	0.290
0 mg Galactose/100ml	0.003
100 "	0.080
200 "	0.165
300 "	0.243
400 "	0.335
500 "	0.426
1 m *M* Ammonia	0.007
10 "	0.003
10 m *M* Glutamine	0.003
50 "	0.004
DME w/o Glucose	0.004

DISCUSSION

When this project was initiated, there were several obstacles confronting its development. First, although previous work had improved upon the efficiency of cell growth in microcarriers culture, the growth of the normal human diploid fibroblasts (eg WI-38, MRC-5, and FS-4) on microcarriers was unpredictable and troublesome[9]. In particular the FS-4 cells did not always grow following attachment. Were they to grow at all, they often showed a two or three day lag period and to complicate matters further, they tended to 'fall off' the beads after about six days in culture.

Second, the induction of interferon using these cells was not well characterized. Yields tended to be highly variable, with a sizable fraction of the inductions showing little or no production at all. This was further complicated by the poor growth and attachments of the FS-4 on microcarriers, necessitating induction on day five after inoculation regardless of the state of the cells.

Third, as indicated earlier, the use of instrumentation itself in cell culture was largely unexplored, leaving us with the burden of making both the initial analysis and subsequent construction of the more promising instruments.

The direction taken was a relatively direct one. Our first priority would have to be that of achieving good growth of these cells in microcarrier culture. Second, the process of interferon induction would have to be better characterized, the objectives being both to increase the overall

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[9] It is noted that Levine reported good growth of the cell strain HEL 299. However, HEL 299's, despite a normal karyotype, have the A type electrophoretic varient of Glu-6-P dehydrogenase (a HeLa marker). Moreover, some reseachers have cast serious doubt upon whether this cell strain is truly normal.

interferon yield and to make the induction a more reproducible process. Third, the means to monitor the energy metabolism of these cells was needed. It was felt that this provided the best means of obtaining information that was meaningful both in characterizing cell growth and the interferon induction process. This involved not only building the equipment to measure the oxygen uptake of the cells, the consumption of glucose, the production of lactic acid and the production of carbon dioxide, but also the methodology to measure and interpret this data.

This discussion will concern itself with the following topics:

- The characterization of FS-4 cell growth in microcarrier culture.
- The instruments used to measure the energy metabolism of the cells.
- The logic developed within the computer program to monitor and process the output of these instruments.
- An analysis of cell growth and energy metabolism in the FS-4 cells.
- The development and application of this system to the induction and production of interferon by these cells.

GROWTH OF FS-4 CELLS IN MICROCARRIER CULTURE

The growth of animal cells in microcarrier culture is typically more difficult than the more conventional monolayer techniques. Observationally, the cells appear to be more unforgiving. For example, often nearly identical conditions may result in little or poor growth on microcarriers while the same cells proliferate on plasticware. Secondly, cells on microcarriers are much less likely to recover from even minor insults. And third, no good general technique has been developed to prepare cell inocula from microcarriers.

In particular, the FS-4 cell line is more difficult than most cells to grow on microcarriers. Thus, to minimize unforeseen problems and thereby hopefully assure the routine growth of these cells for the term of this project, the following steps were taken. First, a large inventory of pre-tested medium salts and sera were secured. Second, to minimize cell loss upon entering the stationary phase of growth, the serum concentration of the medium was reduced. Finally, the inoculum was consistently prepared in a manner we determined to be most optimal.

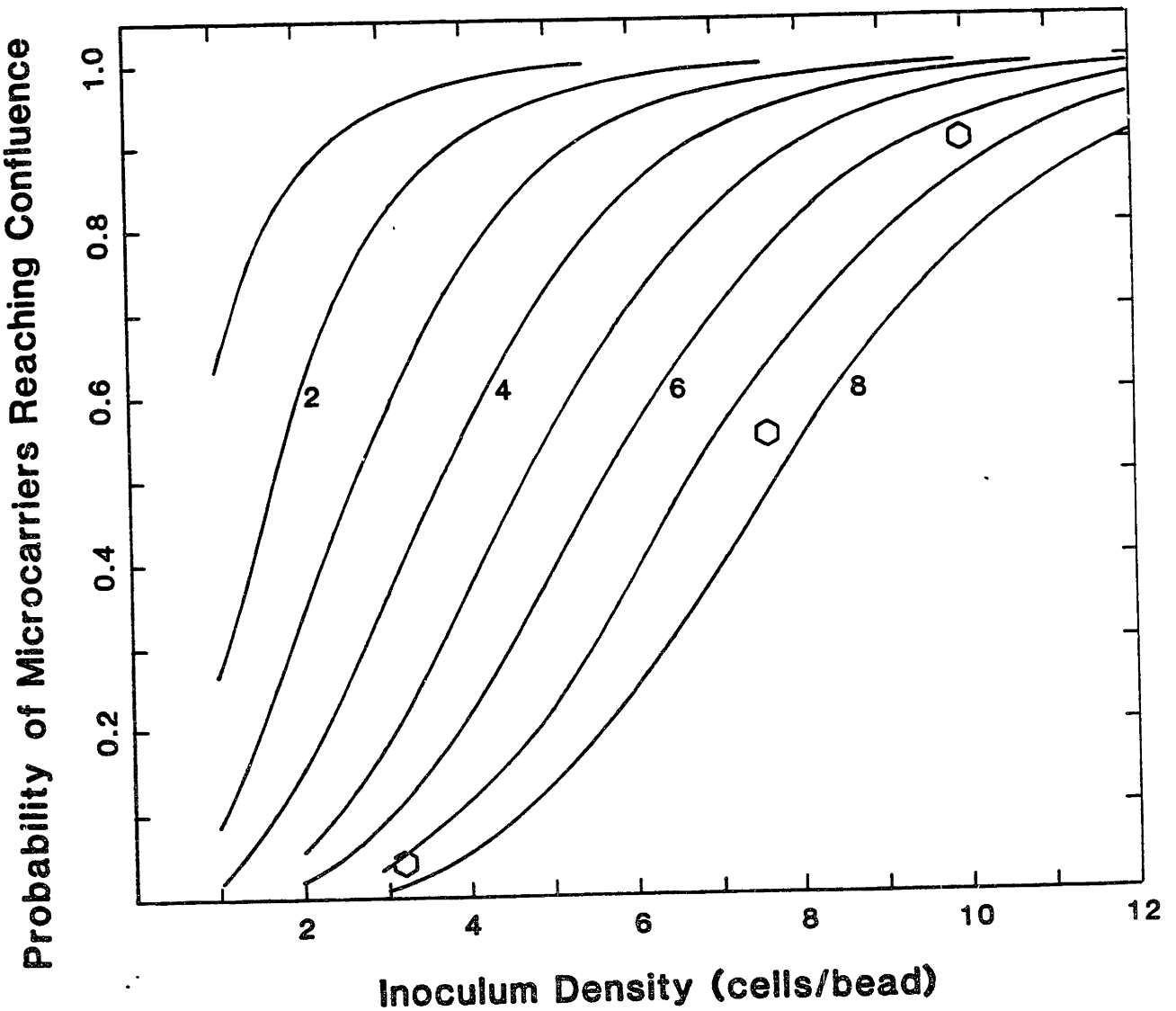
PREPARATION OF CELL INOCULUM

The inoculum used to seed microcarrier culture influences both the duration of the lag time before growth and the final cell density. First, it was determined that if cells were harvested from actively growing cultures, this period was remarkably shortened or eliminated. For production purposes this was achieved by subdividing the cell stocks (1:2) 48 to 72 hours prior to the initiation of the microcarrier culture.

Second, it was shown that the size of the inoculum seed directly influences the final density of cell growth in microcarrier culture. The results shown in Figure 21 on page 116, demonstrated that unless the seed density exceeded 3×10^5 cells/ml, the final cell density would be markedly reduced. Notably, however, the loss in final cell density did not correspond to microcarriers with less growth, but rather to fewer microcarriers which grew to confluency. This was most evident in the case when the seed density was 1×10^5 cells/ml. Here, only about one microcarrier in twenty was fully confluent. The data suggests two things, first, that cells once attached to a single microcarrier do not migrate with high frequency to other microcarriers, and second, that perhaps a minimum number of cells per microcarrier is essential for successful growth on microcarriers. The initial distribution of cells on microcarriers can be estimated using the Poisson distribution[10]. An effort has been made to demonstrate this in Figure 37 on page 170. Here the curve represents that fraction of microcarriers showing successful cell growth as a function of inoculum density. Each curve represents a different hypothetical minimum number of cells/bead essential for growth to occur on that bead.

Figure 37 Fraction of Microcarriers with Successful Growth vs Seed Density

Each curve represents a locus of points corresponding to the fraction of microcarriers showing successful growth given that a minimum of n cells/bead is required. The x-axis is the bulk average cell/bead ratio (which would increase with increasing inoculum density).


Probability of Microcarriers Reaching Confluence
0.2
0.4
0.6
0.8
1.0
2
4
6
8
10
12
2
4
6
8
Inoculum Density (cells/bead)

The closed circles represent the data from the experiment on seed density. Apparently, the data is consistent with the hypothesis that a minimum of about 7 cells/microcarrier is essential for successful growth.

REDUCTION OF SERUM CONTENT IN THE GROWTH MEDIUM

One of the initial problems encountered in growing FS-4 cells on microcarriers was that the cells tended to fall off the beads after about 5 days of growth. Also, the cells did not stick too well to the beads after induction to produce interferon. It was shown that these problems could be greatly minimized by reducing the serum concentration from 10%(v/v) FBS to 5%(v/v) FBS. Neither the growth rate nor the subsequent titer of interferon was reduced by this change, if anything they were improved. These results are presented in Figure 22 on page 118 and in Table 6 on page 123. Furthermore, we have subsequently shown that the serum concentration of the medium can be reduced from 5% to 1.25% during the course of growth with no adverse effects on the growth rate or subsequent Interferon production. Apparently, certain components of serum are most essential for cell growth at the time of subcultivation. This observation has been further supported by R. Ham and his co-workers. In the development of a defined medium for growth of human diploid fibroblasts they were able to show that cell maintenance is adequately provided for by those components already in DME (although their work was done using the MCDB series), plus certain minerals, micronutrients,

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[10] The fraction of beads with n cells per bead, when λ is the overall cell to bead ration is described by the following equation:

$$f_n = \lambda^n e^{-\lambda}/n!$$

This equation makes two implicit assumptions: One, cell attachment is a random event and two, the attachment of one cell to a microcarrier is not influenced by the previous attachment of other cells.

and the hormones insulin and transferin[11]. Cell growth requires epidermal growth factor (EGF). Additionally, the final cell saturation density can be further increased by platelet-derived growth factor (PDGF) (Phillips, personal communication). However, cell recovery from trypsin damage requires additional serum factors, as yet unidentified. Certainly, a completely defined medium would be highly desirable, but this development may be a ways off. On the other hand, using this information it does seem likely that a medium with a greatly reduced animal sera component will be formulated in the near future.

INSTRUMENTATION SYSTEM AND DEVELOPMENT

The instrumentation used to measure oxygen, carbon dioxide, glucose, ammonia, and sugar is discussed in the following section. Furthermore, the means that we used to provide an adequate and controlled supply of oxygen to the cells is also analyzed.

UTILIZATION OF OXYGEN IN CELL CULTURE

The measurement of oxygen demand by animal cells in culture has not been extensively pursued, particularly with regard to the change of demand with cell growth. Some available data is shown in Table 14 on page 173. First, it should be noted that the value of about 0.05 mmoles $O_2/10^9$ cells-h for the FS-4 cells grown on glucose, as determined by this study, agrees well with previous studies using skin fibroblasts. Second, the oxygen demand for the FS-4 cells is lower than values for other diploid fibroblasts. For example, a value of 0.15 mmoles $O_2/10^9$ cells-h has been determined for WI-38 cells, and for some other cell types, such as liver and intestine, oxygen demand values range from 0.3 to 0.4

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[11] Transferin can in some instances be replaced by an adequate supply of reduced iron.

TABLE 14

MEASURED OXYGEN DEMAND RATES OF HUMAN CELLS

Human Line	mmoles O_2/ liter-hour at 10^6 cells/ml	Reference
Lymphoblast (Namalwa)	0.053	Katinger *et al.* (1978)
Skin fibroblast	0.064	Danes *et al.* (1963)
AM-57 (amnion)	0.14	Green *et al.* (1958)
	0.45	Green *et al.* (1958)
	0.59	Green *et al.* (1958)
Diploid embryo		
WI-38	0.15	Cristofalo and Kritchevsky (1966)
MAF-E	0.38	Phillips and Andrews (1960)
Leukemia MCN	0.22	Phillips and Andrews (1960)
Lung To	0.24	Phillips and Andrews (1960)
Conjunctiva	0.28	Phillips and Andrews (1960)
HLM (liver)	0.37	Danes *et al.* (1963)
LIR (liver)	0.30	
Intestine	0.40	Phillips and Andrews (1960)
Detroit 6 (bone marrow)	0.43	Phillips and Andrews (1960)
Hela	0.47	Danes *et al.* (1963)
	0.097	Phillips and McCarthy (1956)
	0.10	Phillips and McCarthy (1956)
	0.39	Phillips and Andrews (1960)

mmoles $O_2/10^9$ cells-h. These oxygen demand numbers are not inconsequential, particularly with regard to the possible scale of microcarrier culture. Our data for the mass transfer of oxygen across the surface of the 14-liter fermentor suggest that 5 liters is the approximate limit for the growth of FS-4 cell in microcarrier culture without additional aeration. This is because the mass transfer of oxygen across the liquid surface (0.75 mmoles O_2/l-atm-h at 50 rpm) is unable to sustain the oxygen demand of confluent cultures of FS-4 cells (0.05 mmoles $O_2/ 10^9$ cells-h) and keep the dissolved oxygen concentration above 50% air saturation for volumes greater that 5 liters. Furthermore, it is obvious that with other cell types this limitation will be realized on an even smaller scale. Sparging is not the answer to this problem. In general, the presence of gas bubbles cause lysis of animal cell membranes and foaming of the medium (Kilburn and Webb, 1968). In particular, with microcarrier culture the microcarriers are often found concentrated in the foam (personal observation).

Based on the data in the literature on blood oxygenators (Kolobow *et al.* 1975), membrane oxygenation in cell culture using silicone rubber seemed quite promising. For our work, we chose to use commercially available silicone rubber tubing. The oxygen transfer potential of this material was entirely adequate for our purposes. One meter of tubing was found to be capable of aerating 10 liters of FS-4 culture, and this approach was utilized successfully to grow these cells in a 10 liter volume to a density greater than 10^6 cells/ml. It should be further noted that for cultures of FS-4 cells of less than 1,000 liters, silicone rubber tubing is entirely capable of providing adequate oxygenation. However, the use of tubing does not in all probability represent an optimal membrane design configuration. Thin sheets of silicone rubber supported on a porous matrix would certainly provide greater oxygenation with less surface area and perhaps in a more convenient physical configuration.

In the quantitation of oxygen demand by animal cells in culture, the dynamic means of measurement was utilized. The primary advantage of this method is greater sensitivity in the measurement of low rates of oxygen consumption. The disadvantages are that one must know accurately the solubility of oxygen in the medium and that, for small vessels (less

than 100 liters), one must also characterize the mass transfer rate of oxygen across the liquid surface. Additionally, if one is using silicone rubber tubing, the transfer across this surface must be taken into account. Nevertheless, the method can work well when properly utilized, as we have demonstrated. Surprisingly, for FS-4 cultures, the oxygen uptake rate was not strongly influenced by the growth rate of the cells and in fact the OUR is a good predictor of cell number (see Figure 38 on page 176). This would imply that a high fraction of the energy generated by these cells is utilized for maintenance requirements, which may be explained in part by the very low growth rate of animal cells.

In our methodology, the OUR measurements are determined over an hour interval or until the oxygen concentration falls below 25% of air saturation. Initially, we were concerned that the OUR might be influenced by the oxygen concentration, but on numerous occasions when the OUR measurement was divided into many smaller intervals of time (see Figure 39 on page 178), it was concluded that the OUR is independent of the dissolved oxygen concentration in the region between 25% and 75% of air saturation.

ANALYSIS OF SUGAR CONCENTRATIONS

The method of sugar analysis that we finally chose was the reaction of aldohexoses with o-toluidine in acetic acid. The reaction has the virtues of being very specific (but it can not distinguish between glucose and galactose), very sensitive (relatively dilute solutions can be accurately measured), and the presence of proteins or other serum components does not significantly interfere with the assay. During the course of the fermentation the sugar analysis of the broth is performed every five hours just before the oxygen uptake measurement. Specifically, a small stream of fluid is removed from the fermentor and subsequently diluted with the acetic acid o-toluidine reagent. It is then heated in a hot oil bath at 95°, the residence period of the flow stream in the bath is about 5 minutes. Subsequently, the fluid is briefly allowed to cool and the optical absorbance at 660_{nm} is then determined.

Figure 38 Oxygen Demand (O.U.R.) versus Cell Number

The data presented was obtained from three batch cultures of FS-4 cells grown on glucose.

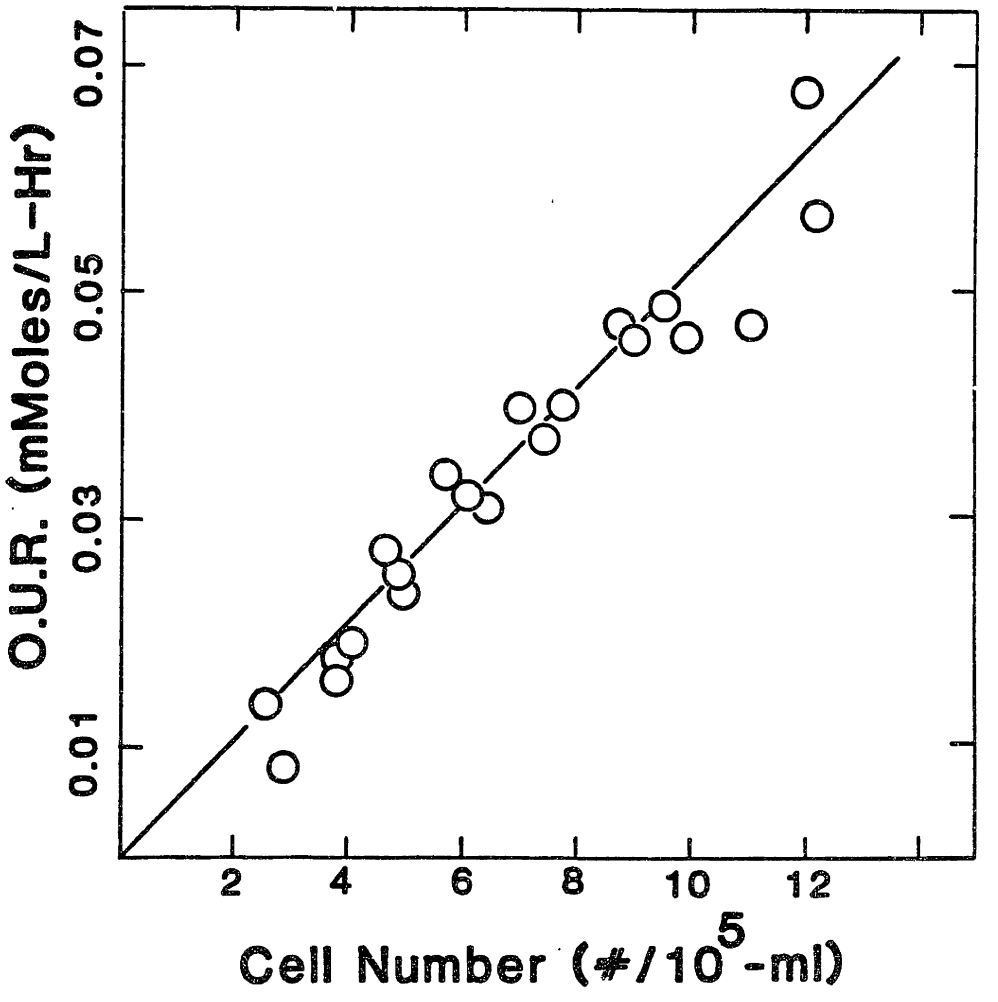
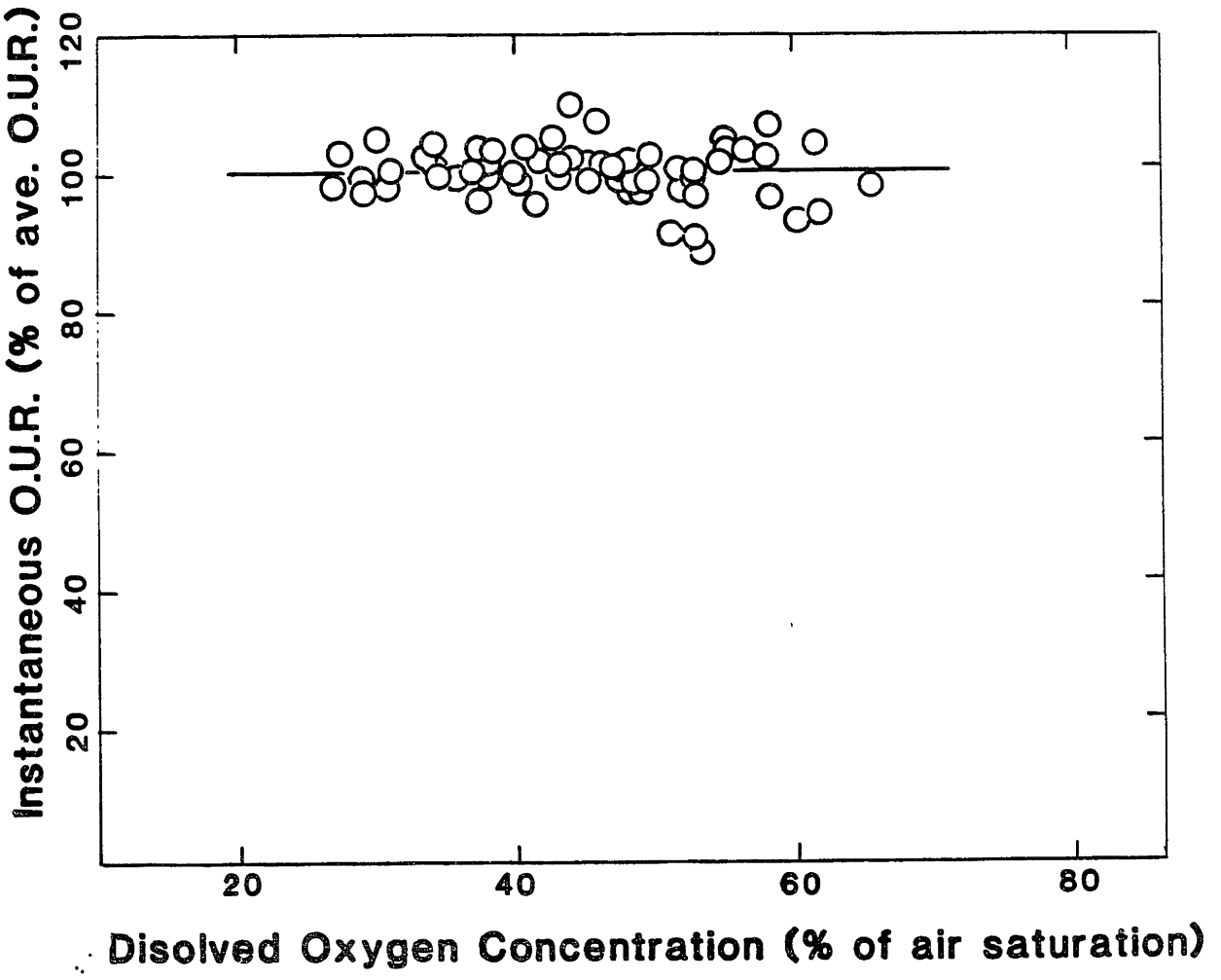

O.U.R. (mMoles/L-Hr)
0.01
0.03
0.05
0.07
2
4
6
8
10
12
Cell Number (#/10^5-ml)

Figure 39 The Instantaneous Oxygen Demand versus Dissolved Oxygen Concentration

The data is obtained by comparing the instantaneous O.U.R. and the average O.U.R. of a one-hour measurement. The data was obtained from 25 determinations with average O.U.R.'s ranging from 0.01 to 0.07 mmoles O_2/liter-hour.


Instantaneous O.U.R. (% of ave. O.U.R.)
120
100
80
60
40
20
20
40
60
80
Disolved Oxygen Concentration (% of air saturation)

The sample fluid from the broth is diluted at a ratio of about 1:100 with the reagent, consequently the measurement is very sensitive to changes in tubing dimensions. Such deformations are quite common when the tubing is in constant use over a period of several days, especially with the smaller diameter tubing. To overcome this problem, the computer is programmed to do an additional procedure. It selects the stream for sugar analysis, prior to mixing with the acid reagent, from one of three sources; the fermentor, and two sugar standard solutions (a high and a low value). By analyzing all of the three samples with each determination, the value of the sugar concentration in the culture broth can be estimated by interprelation between the two standards. This practice of constant recalibration has the added advantage of also compensating for changes in the lamp intensity (of the colorimeter) and batch to batch variations in the o-toluidine reagent. The two sugar standards are prepared in a solution of benzoic acid (2 g/l) to prevent microbial degradation.

In the development of this system two others were examined, the L&N glucose analyzer and an electrochemical technique for measuring carbohydrates developed by Schich *et al.* (1978). Neither was found to be satisfactory for this purpose. The L&N instrument, first of all, was only sensitive to glucose, and secondly seemed to have a number of unresolved problems; for example the baseline took an inordinate amount of time to reach a steady state and the amount of reagents consumed was excessive. The electrochemical technique of Schick *et al.*, 1978) is based upon the ability of nickel ions to catalyze the oxidation of carbohydrates in base. The oxidation occurs at the surface of a nickel or nickel-coated platinum electrode and is easily monitored electrically. Unfortunately, preparing stable electrode surfaces is a difficult art and we were plagued by a constantly changing electrode response, a problem further aggravated by the precipitation of serum components under basic conditions.

LACTIC ACID

Much of the glucose consumed by animal cells is converted to lactic acid and secreted into the environment. The result is a decrease in the pH of

the environment. There are a number of useful means of measuring lactic acid, the most convenient for our purpose is the measurement of base used to keep the pH constant. However, this requires the abandonment of the standardly used CO_2/Bicarbonate buffer system for pH control. To my knowledge, as this had not been done before with the FS-4 cells, I was compelled to first show that the cells could be grown in the absence of a CO_2 buffer before attempting to do so on a five- to ten-liter scale. To this end DME was prepared substituting HEPES (25 m *M*) for sodium bicarbonate in the routine medium composition and adjusting the osmotic strength to physiological values (about 295 mOs) by the addition of sodium chloride. In this medium FS-4 cells successfully grew and subcultivated, although the HEPES buffer did not appear to be as effective as the CO_2/Bicarbonate system. In retrospect it would have been more interesting to have examined the use of Lebowitz's L-15 medium. This medium was formulated by Lebowitz in 1963 to substitute for some of those media using the CO_2/Bicarbonate buffer. He accomplished this by substituting galactose, and a small amount of pyruvate, for glucose and increasing the concentration of certain basic amino acids to replace sodium bicarbonate. The second physiological concern was the possibility of altering the osmotic strength of the medium by the addition of the sodium hydroxide used for pH control, as cells grown on glucose would be expected to produce a considerable amount of acid. To this end the concentration of the base used for pH control with cell culture systems was prepared at a concentration of about 0.25 *M*.

A loadcell was used to measure the addition of base from a reservoir vessel to the fermentor. As can been seen from the data in Figure 31 on page 150 the load cell provides a very reliable means of weight measurement. By nature of it's strain gauge mechanism, it is relatively insensitive to vibrations and to variations in the ambient temperature. Secondly, coupling the load cell with the computer and a pH meter-controller yields a very reliable system for measuring added acid (see Figure 30 on page 144). Because, lactate and CO_2 are the predominate acid species produced by animal cells in culture, and much of the CO_2 produced is stripped away without a CO_2 overlayer, measurements of total acid are in fact reliable measurements of the lactic acid produced.

Initially, the use of conductance to detect the growth of animal cells was explored and did in fact show some utility (see Appendix P). However, the method was not used in the final instrumental setup and for two reasons. First, although it is possible to construct two chambers on a small scale (with one used as the determination chamber and the other as the reference chamber), it is not realistic to do so on a large scale. Much of our work have been on a five- to ten-liter scale. On these scales, the ability to devise a suitable reference chamber that is in temperature equilibrium with the determination chamber and undergo similar medium exchanges, is a non-trivial task. Second, as has been previously aluded, the information obtained from conductance measurements is somewhat "fuzzy", as there is no real certainty as to the exact chemical nature of the changing species. Presumably, in cell culture conductance would be largely a measurement of lactic acid formation, however, this was much more accurately (and conveniently) measured by quantitating the volume of base added to maintain pH control.

AMMONIA

The ammonium ion concentration of the culture broth was measured using a gas-sensitive electrode. This was accomplished by incorporating the electrode into the autoanalyzer system used for glucose analysis. Specifically, a portion of that fluid stream containing either the culture broth or the calibration standards is diverted and mixed with base prior to where the o-toluidine reagent is added. The fluid then passes across the gas-sensing membrane of the electrode inside a flow-through cap machined from a single piece of teflon and press-fit against the electrode. Because the air bubbles used to ensure the plug-flow fluid dynamics of the analytical stream rapidly reach equilibrium with the ammonia of the liquid phase and the stream itself is not open to the air, it is unnecessary to debubble the analytical stream before contact with the electrode.

Overall, the gas-sensitive electrodes are a reasonable means to measure ammonium ion concentrations. They are sensitive and fairly reproducible. They do, however, tend to drift and require periodic recalibration. Fortunately, because the analysis of glucose is unaffected by the presence of

ammonia, it was possible to prepare the internal standards used for the glucose analysis with differing concentrations of ammonia (10^{-2} *M* and 5×10^{-4} *M*) and use them concurrently in both assays. The major disadvantages with the gas sensitive electrodes are the concerns that the membrane may leak or that the membrane may become fouled (the precipitation of proteins under these basic conditions does not help the situation either).

CO_2

In the literature review section a number of means by which dissolved CO_2 concentrations might be measured were discussed. The 'tubing method' (see Figure 9 on page 60) was one of them. Note that the set-up used in the 'tubing method' closely resembles the apparatus used to oxygenate the culture fluid and in addition, that silicone rubber is very permeable to CO_2. Consequently, we chose to measure the CO_2 in the microcarrier culture fluid by analyzing the CO_2 content of the gas exiting the same silicone rubber tubing used to supply oxygen to the fermentor. To determine the CO_2 content of the culture fluid requires the determination of three variables: the gas flow rate through the tubing, the CO_2 content of the exit gas, and the pH of the culture fluid.

The gas flow rate through the immersed coil of silicone rubber tubing was determined by measuring the pressure drop across the coil. The flow versus ΔP calibration should be determined with each run as the tubing configuration may change. An example of such a calibration was presented in Figure 32 on page 152. It exhibits a linear relationship between gas flow and ΔP, which would be predicted by the classic Hagen-Poiseuille

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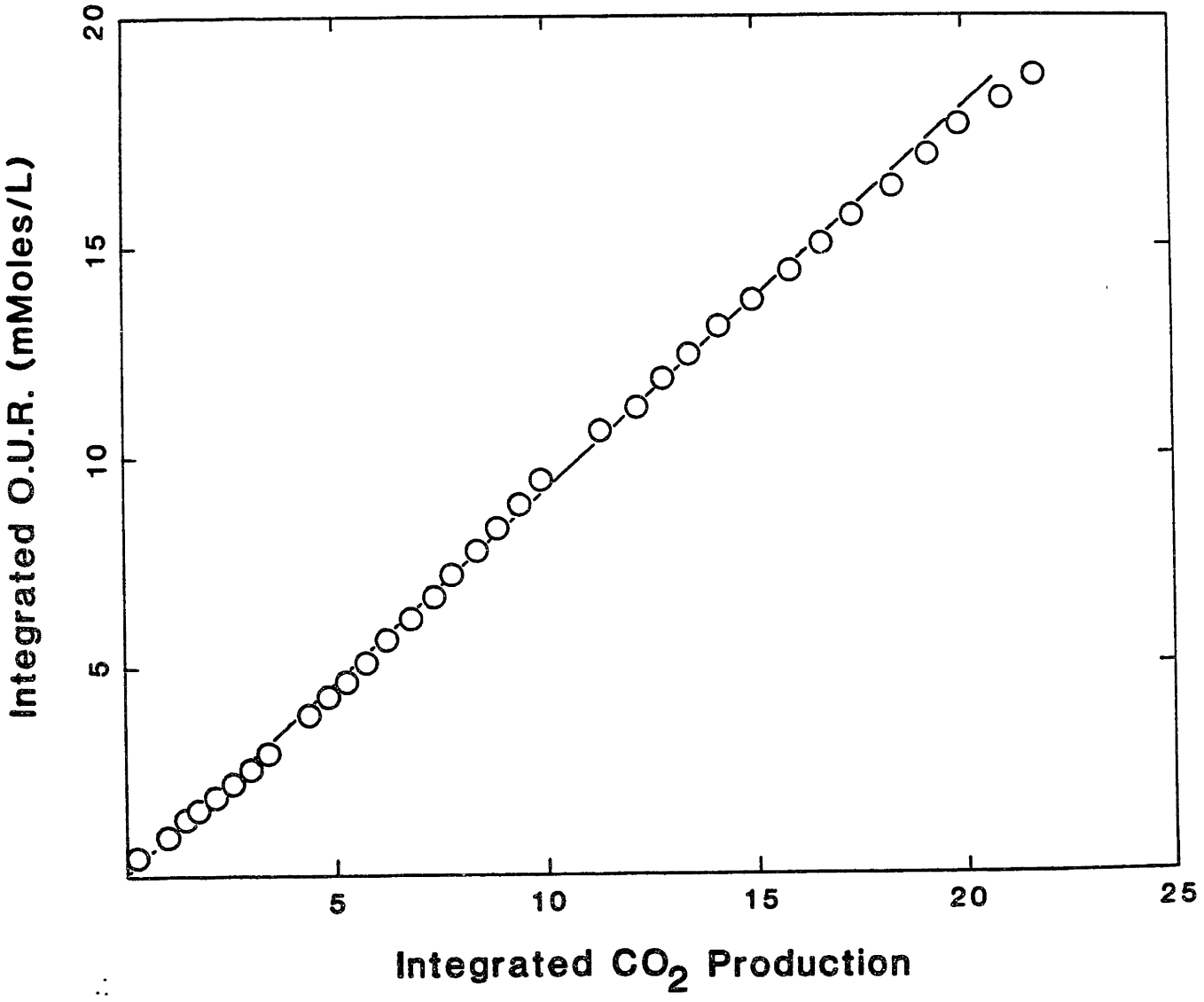
[12] Fluid flow (Q) is related to the tubing dimensions (r and l), the pressure drop (ΔP) by the following equation:

$$Q = \Delta P \, r^4 / 8 \, \mu \, l$$

Figure 40 Production of CO_2 vs. Cumulative Uptake of O_2

The integrated consumption of O_2 by FS-4 cells grown in microcarrier culture on galactose was plotted against the integrated production of CO_2.


Integrated O.U.R. (mMoles/L)
20
15
10
5
5
10
15
20
25
Integrated CO_2 Production

law[12]. Furthermore, using this equation and the data presented, the calculated viscosity of air flowing through the tubing agreed well with the accepted values, 0.0215 cp versus 0.019 cp.

The CO_2 in the exit gas stream was then shown to be both related to the carbonic acid content of the fluid and to approximately the inverse of the gas flow rate. Consequently, one can combine this information and predict the total CO_2 content of the fluid knowing the CO_2 concentration of the exit gas stream, the gas flow rate, and the pH of the fluid as was shown in Figure 34 on page 160.

Remarkably, however, the CO_2 content of the culture fluids showed little increase with time over the course of several microcarrier fermentations, whereas the oxygen uptake rates would have predicted a substantial production of CO_2 by the cells. This situation was resolved when it became apparent that the CO_2 removed by the silicone rubber tubing to the IR gas analyzer was roughly equivalent (~ 92%) to the production of CO_2 predicted from the oxygen data consumption (see Figure 40 on page 184). Two conclusions can be drawn from these results; first, as one would anticipate, that the CO_2 production is principally the result of carbohydrate consumption and is coupled to the oxygen uptake rate, and second, that the measure of base added for pH control is primarily a measure of the production of lactic acid. The later was confirmed by comparing the production of lactic acid (measured enzymatically) from glucose by the FS-4 cells to the production of titratable acid vs. glucose consumption. The data, which is presented in Figure 41 on page 188, shows that under these conditions lactic acid accounts for better than 95% of all acid formed by the cells.

SYSTEM PROGRAM

The primary function of the program is to orchestrate the acquisition of data and the *in-situ* calibration of the various instruments. The program is written in PASCAL and assembly language (Mostek 6502 microprocessor code). PASCAL is a structured, high-level language which facilitated the

Figure 41 Evidence That Lactic Acid Is the Major Acid Species Produced by the FS-4 Cells

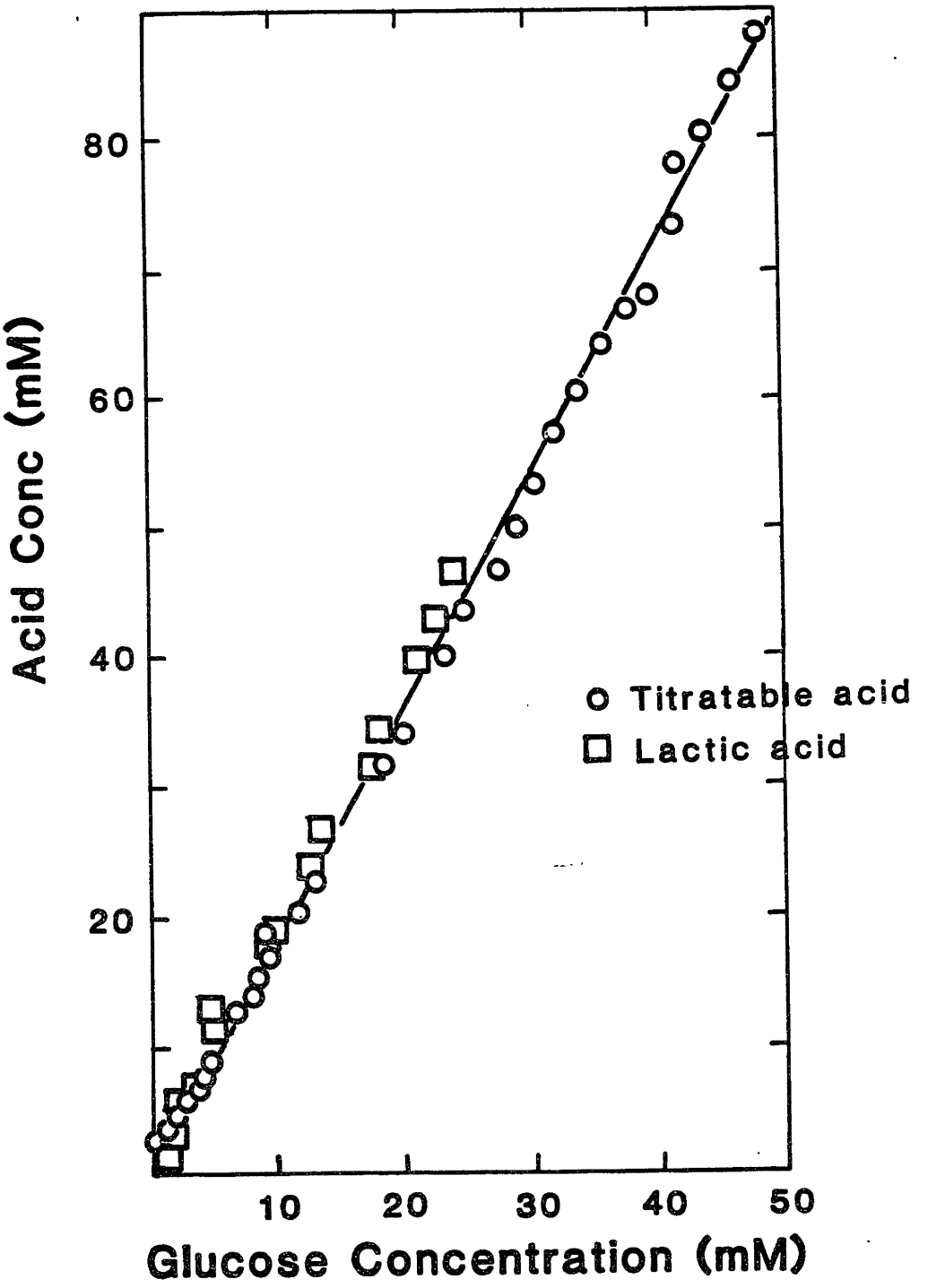

Acid Conc (mM)
80
60
40
20
10
20
30
40
50
Glucose Concentration (mM)
Titratable acid
Lactic acid

Figure 42 Sequence of Events in OUR Measurement

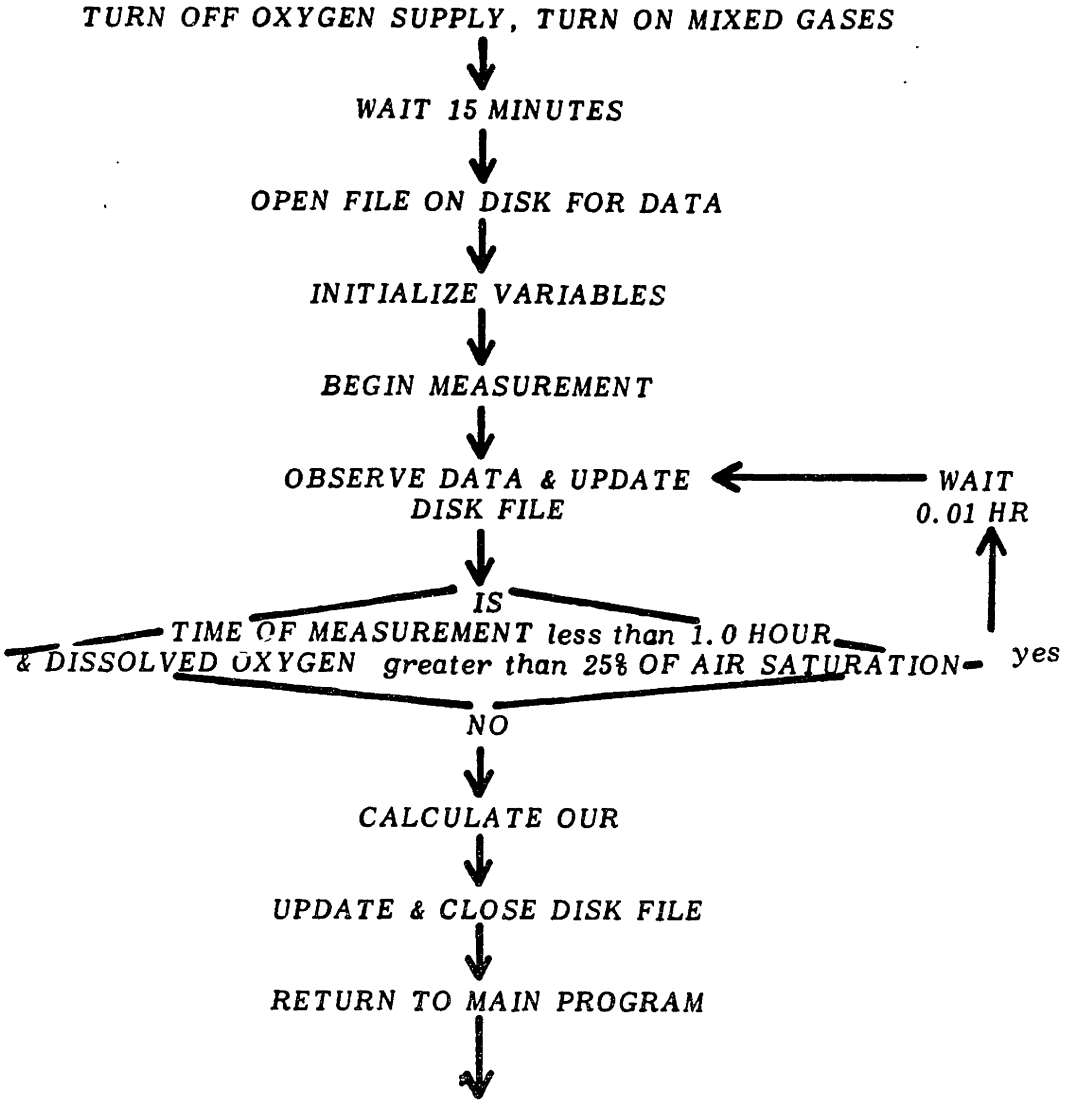

TURN OFF OXYGEN SUPPLY, TURN ON MIXED GASES
WAIT 15 MINUTES
OPEN FILE ON DISK FOR DATA
INITIALIZE VARIABLES
BEGIN MEASUREMENT
OBSERVE DATA & UPDATE DISK FILE
WAIT 0.01 HR
IS TIME OF MEASUREMENT less than 1.0 HOUR & DISSOLVED OXYGEN greater than 25% OF AIR SATURATION
yes
NO
CALCULATE OUR
UPDATE & CLOSE DISK FILE
RETURN TO MAIN PROGRAM

TABLE 15

SEQUENCE OF EVENTS IN DATA ACQUISITION CYCLE

Time (Hr)	Action
0 - 1.25	OUR determination
1.25 - 3.50	Wait
3.50 - 4.00	Determination of $\%CO_2$ in exit line
4.00 - 4.25	Calibrate Glucose and Ammonia analyzer [Full Scale]
4.00 - 4.50	Calibrate CO_2 analyzer [Zero]
4.25 - 4.50	Determine Glucose and Ammonia content of culture broth
4.50 - 4.75	Calibrate Glucose Ammonia analyzers
4.50 - 4.80	Calibrate CO_2 analyzer [Full Scale]

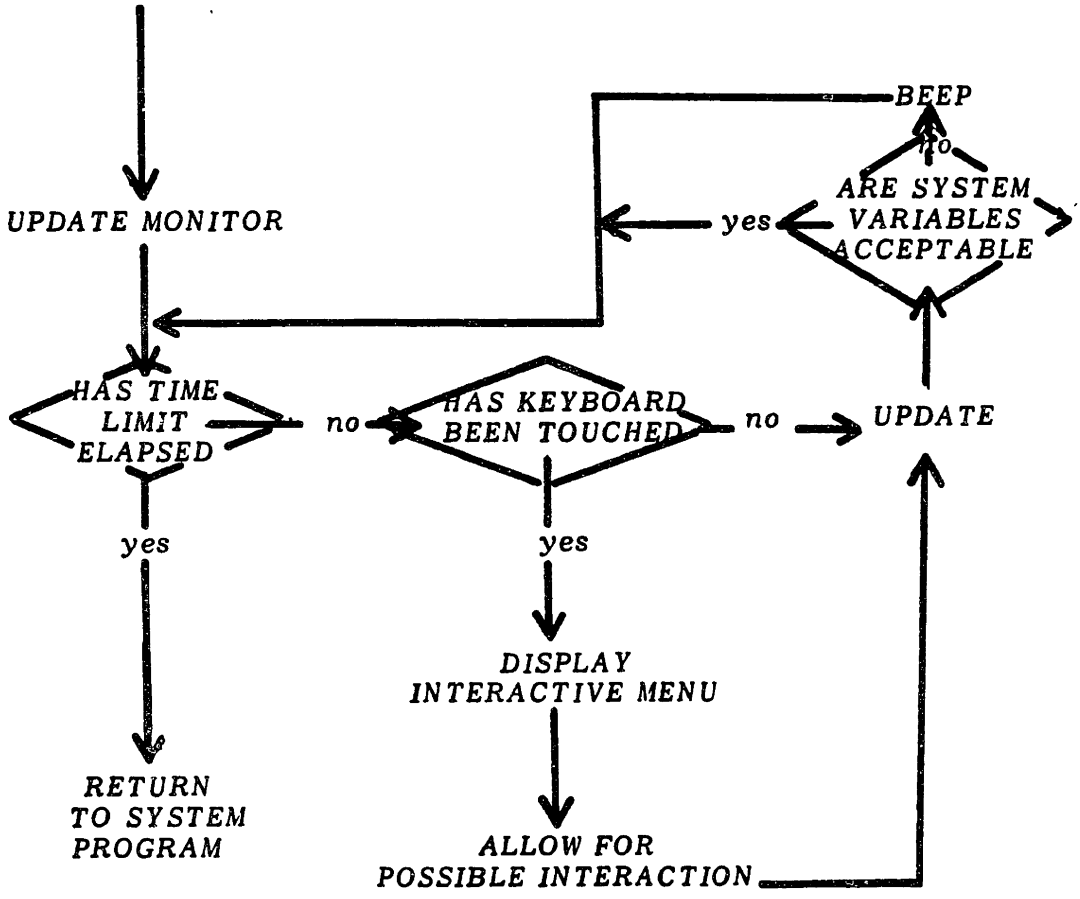
development of this program by allowing the process to be written in individual functional blocks.

The complete program listing is provided in appendix M. To understand the logic of the program it is most valuable to examine the main segment which begins on page 315. The main section has only two sequences of events, the first is a start-up sequence and the second is an interactive cycle of data acquisition. In the start-up sequence, the computer first sets the eight solid-state relays to the proper initial state. It then proceeds to initialize the system parameters and waits for about 5 minutes in an interactive mode with the operator to allow further changes of system variables before entering the data acquisition cycle. The data acquisition cycle is a 4.8 hour sequence during which the various instruments are calibrated and their signals recorded. The timing of this sequence of events is shown in Table 15 on page 191. It should be further noted that not all of this cycle is devoted to the actual data acquisition process and during the remainder, the computer, while waiting, is capable of interactively responding with the operator.

Because of memory constraints the program was written in 'segments'. A segment in the UCSD version of PASCAL is a program unit which is stored on disk until called for by the program. Only then is memory allocated for its function and when completed the memory is reallocated for another purpose. This dynamic use of memory by the PASCAL language is markedly different from the static use of memory by languages such as BASIC and FORTRAN. Three segments are defined in this program; an initialization sequence, the oxygen uptake measurement, and the interactive wait sequence.

The oxygen uptake measurement (see page 286 for the program listing) sequence is shown in block format in Figure 42 on page 190. Briefly, the sequence begins by turning off the supply of oxygen to the vessel and purging the vessel head space and silicone rubber tubing with a mixed gas mixture of 10% O_2/90% N_2). This has the net effect of inducing a transient during which the dissolved oxygen content of the culture decreases. After a 15 minute wait (time to equilibrate the gas in the head space with the 10% O_2 mixture) the program records the dissolved oxygen

Figure 43 Interactive Wait Sequence


UPDATE MONITOR
HAS TIME LIMIT ELAPSED
no
yes
RETURN TO SYSTEM PROGRAM
HAS KEYBOARD BEEN TOUCHED
no
yes
DISPLAY INTERACTIVE MENU
ALLOW FOR POSSIBLE INTERACTION
UPDATE
ARE SYSTEM VARIABLES ACCEPTABLE
yes
no
BEEP

level, the rpm, and the temperature every one one-hundredth of an hour for a one-hour period. It then uses this data to calculate the oxgyen uptake rate by the culture (see page 102 in the materials and methods section). This data is recorded onto disk for permanent storage. After this sequence, the computer prints the outcome of the measurement onto the printer along with the previous glucose, CO_2, ammonia, and lactic acid measurements.

The interactive wait sequence is shown in block format in Figure 43 on page 194. This procedure consists of a small loop, which over a short interval watches the keyboard to see if it has been touched. Given that the keyboard has not been touched it then updates the monitor screen by displaying the most current values of certain key parameters and then checks to see if any of these parameters are outside acceptable ranges (e.g. the temperature is either too hot or too cold). Should this occur the computer produces a warning beep. This loop is repeated for the time specified by the argument of the function.

Should the keyboard be touched the computer displays a menu of possible actions which the operator may take if he responds within a given period of time, otherwise the cycle resumes where it left off. It is by this process that the operator can change certain system parameters (such as the mixed gas concentration) or indicate to the program that the medium is being changed.

The assembly language programs provide the routines needed to read the real time clock, read the analogue to digital converter, control the solid state switches, and writes to the printer.

The analysis of data in this system is complicated by several factors; first is the addition of base (particularly if the sugar is glucose) , which dilutes the composition of the fermentation broth, second is the feeding routine used to replenish the cells with nutrients, and final is the removal of liquid from the system in the process of obtaining samples (and frequently not insignificant in volume). The approach taken here is to correct the data by means of a dilution factor, D, such that the concen-

trations correspond to those within an undiluted volume of medium. The details are outlined in appendix Q.

ENERGETICS

Quantitating the energy (ATP) flux of the FS-4 cells was the primary method used to monitor their growth and viability. In order to obtain a better understanding of what this ATP/cell growth relationship involved, cell growth was observed under very different metabolic conditions. This was achieved by manipulating the primary carbon source, which can have a profound effect upon the rate of glycolysis in animal cell culture, as will be discussed in this section.

METABOLISM OF GLUCOSE AND GALACTOSE

The metabolism of animal cells in culture growing on glucose is markedly different than that which occurs when those cells are grown on galactose. This has been known now for some time. Eagle *et al.* (1958) noted that cells grown on galactose produced much less acid than those same cells grown on glucose. He also noted that cell growth on galactose was somewhat variable unless the medium also contained a small amount of pyruvate (~ 1m *M*). Liebowitz also took advantage of this behavior in the formulation of the L-15 medium (1963). By substituting galactose for glucose, less acid was generated and the CO_2/bicarbonate buffer traditionally used in cell culture could then be replaced by simply increasing the concentration of certain basic amino acids.

Using the previously designed instrumentation, the growth of animal cells in microcarrier culture on glucose versus galactose was examined. This is shown in Figure 44 on page 198 and Figure 45 on page 200. First, it is fair to conclude that the growth curves in both circumstances were essentially identical with regard to the lag phase, the growth rate, and the final saturation density. Although it should be noted that the galactose growth curve did show some decline after reaching saturation, this will be treated separately. Second, as we anticipated, the amount of lactic acid

Figure 44 Metabolism of FS-4 Cells Grown on Glucose

The growth of FS-4 cells in microcarrier culture using glucose as the primary carbohydrate was evaluated using the instrumental system.

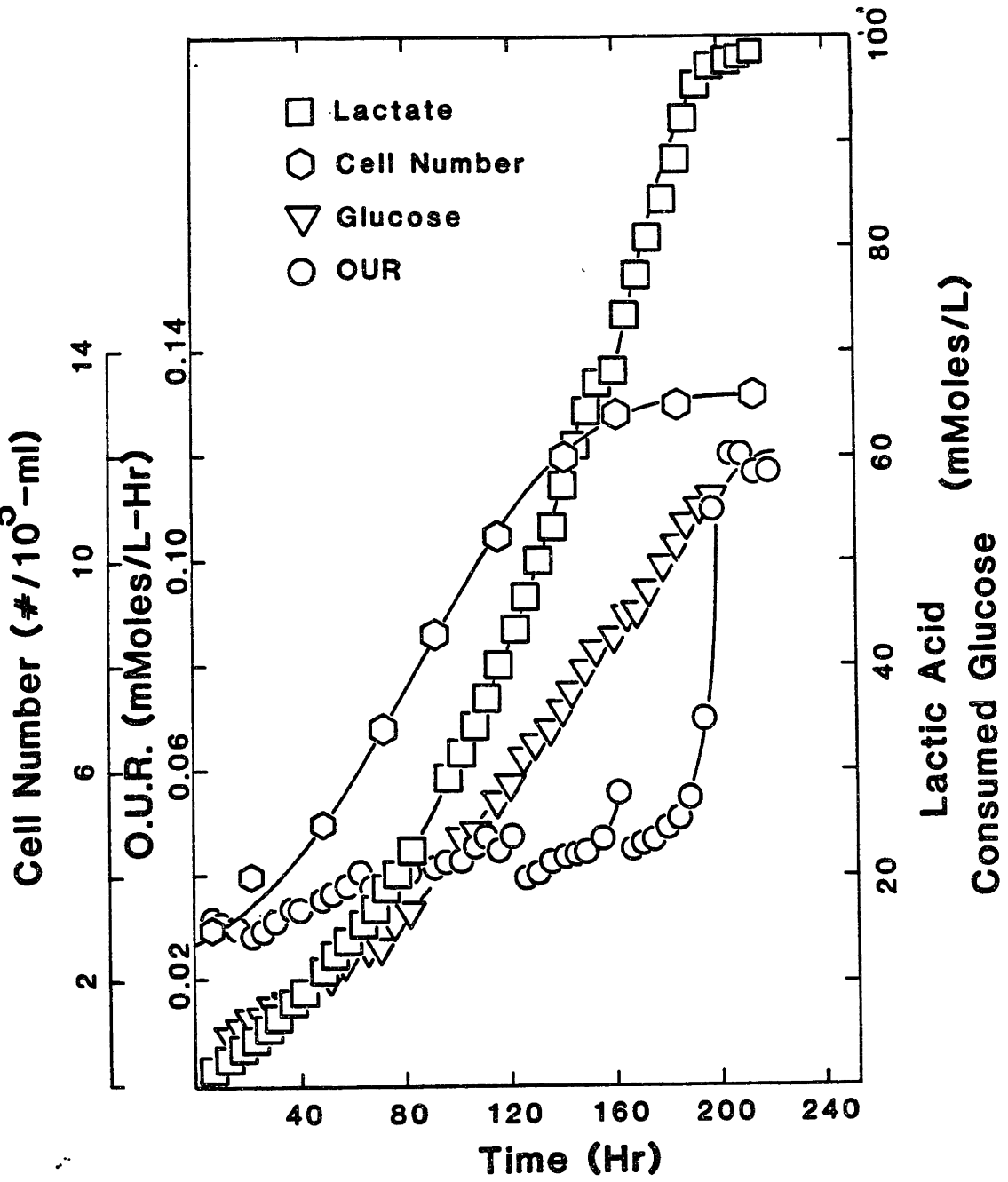
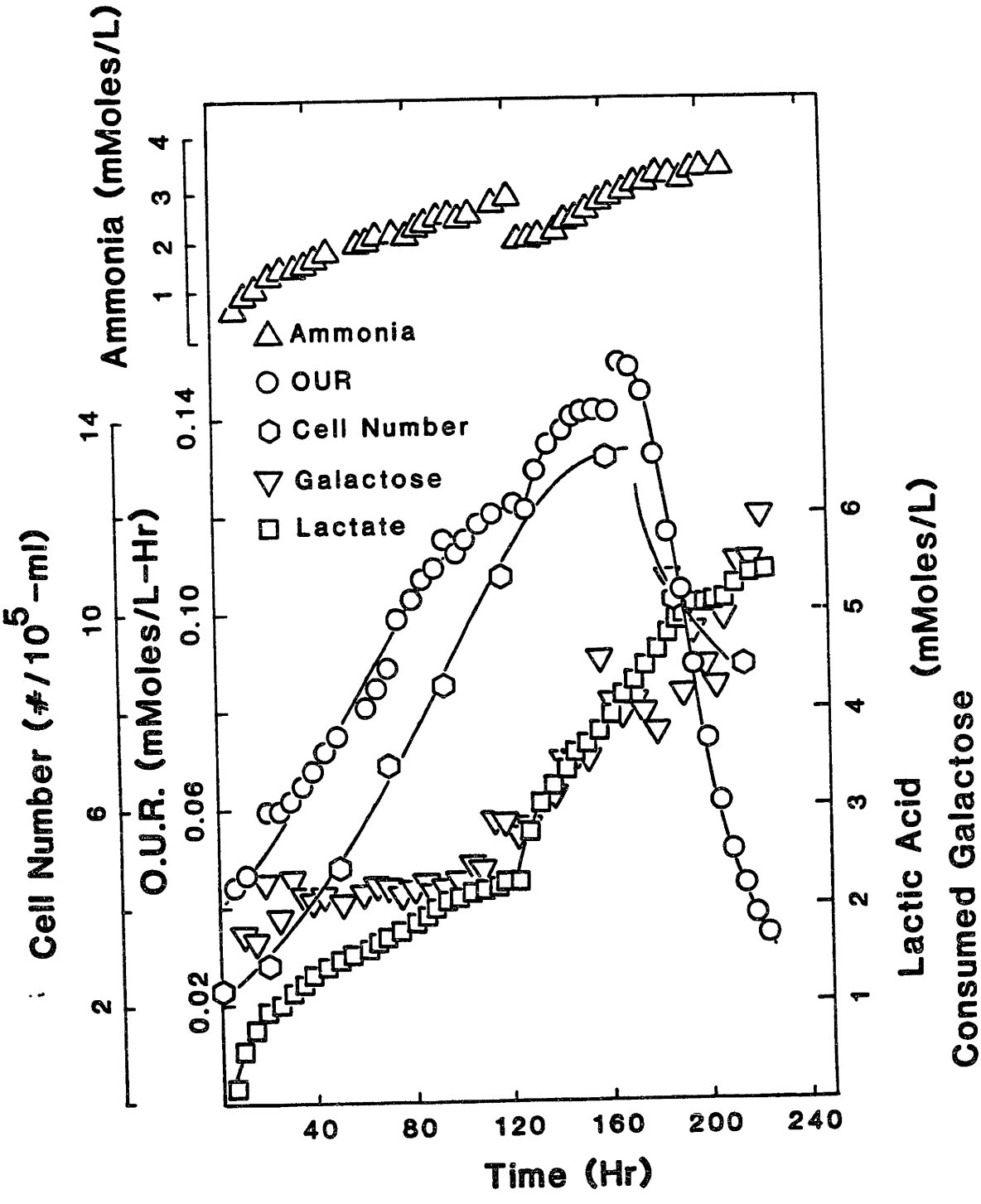

Lactate
Cell Number
Glucose
OUR
Cell Number (#/10^5-ml)
O.U.R. (mMoles/L-Hr)
Lactic Acid
Consumed Glucose
(mMoles/L)
Time (Hr)
2
6
10
14
0.02
0.06
0.10
0.14
20
40
60
80
100
40
80
120
160
200
240

Figure 45 Metabolism of FS-4 Cells Grown on Galactose

The growth of FS-4 cells in microcarrier culture using galactose as the primary carbohydrate was evaluated using the instrumental system.


Ammonia (mMoles/L)
Cell Number (#/10^5-ml)
O.U.R. (mMoles/L-Hr)
Lactic Acid (mMoles/L)
Consumed Galactose
Time (Hr)
Ammonia
OUR
Cell Number
Galactose
Lactate
0.02
0.06
0.10
0.14
2
6
10
14
1
2
3
4
1
2
3
4
5
6
40
80
120
160
200
240

produced by cells grown on galactose was much less than the amount of acid produced by cells grown on glucose. Additionally, not only was much less sugar consumed over the entire course of the fermentation, but the yield of lactic acid on galactose (0.84 mole/mole) was less than the yield of lactic acid on glucose (1.81 mole/mole). Third, and somewhat unexpectedly, the oxygen demand by the FS-4 cells grown on galactose (0.10 - 0.13 mmoles $O_2/10^6$-Hr) was much greater that that of the same cells grown on glucose (0.05 - 0.07 mmoles $O_2/10^6$-Hr). This metabolic data is summarized in Table 16 on page 206.

ATP FLUX

Theoretically we have sufficient data to estimate the energy consumption rates by these cells, given that for each mole of oxygen consumed 6 moles of ATP are generated and that for each mole of lactic acid produced 1 mole of ATP was generated. The results of this exercise are shown in Figure 48 on page 208. Here the calculated ATP flux (defined as mmoles ATP/gm dry cell weight-Hr) is ploted versus the time of fermentation. First, let me emphasize that the growth curves of these two fermentations were very similar (see Figure 46 on page 202), which is important, because the ATP flux for both fermentations are essentially identical. This, of course, has definite implications upon the regulation of ATP generation and also upon cell maintenance requirements and both will be addressed.

Maintenance Requirements

The rate of energy consumption by the FS-4 cells is highest at the beginning of the fermentation and drops rapidly during the first 48 hours. During the remainder of the run the energy consumption rate remains relatively constant, perhaps showing a very gradual decrease with time. It should also be pointed out that the cells were in exponential growth until 100-120 hours into the run and cell numbers did not plateau until the 160-170 hours time point. The importance of this is in noting that initial decrease in ATP flux is not accompanied by a decrease in growth rate and furthermore that a decrease in the growth rate was not accompanied by a decrease in the ATP flux. This again gives further credence to the earli-

Figure 46 Comparison of Cell Growth on Glucose vs. Galactose

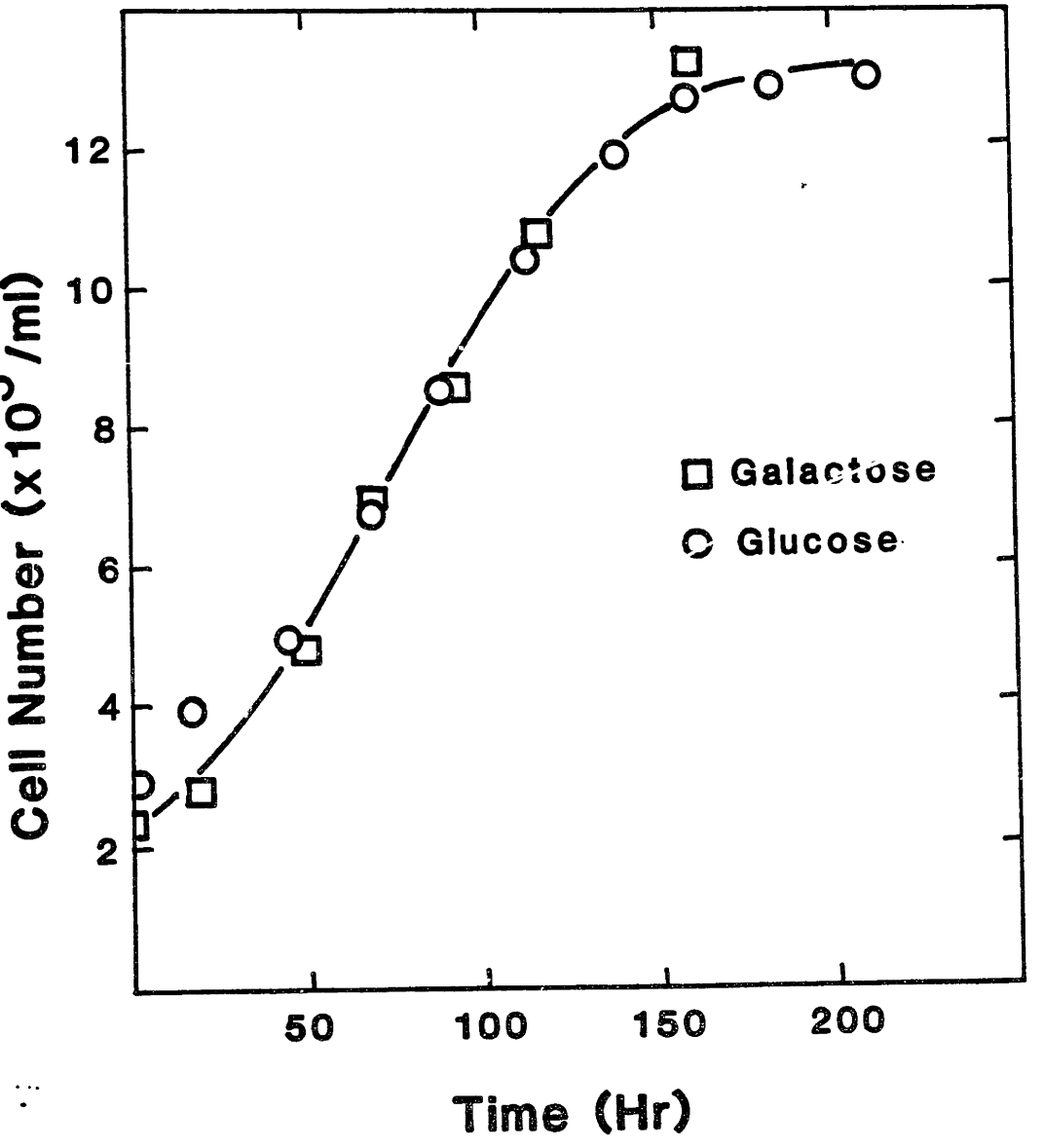

Cell Number (x10^5/ml)
12
10
8
6
4
2
50
100
150
200
Time (Hr)
Galactose
Glucose

Figure 47 Use of Calculated ATP Flux to Calculate Cell Number

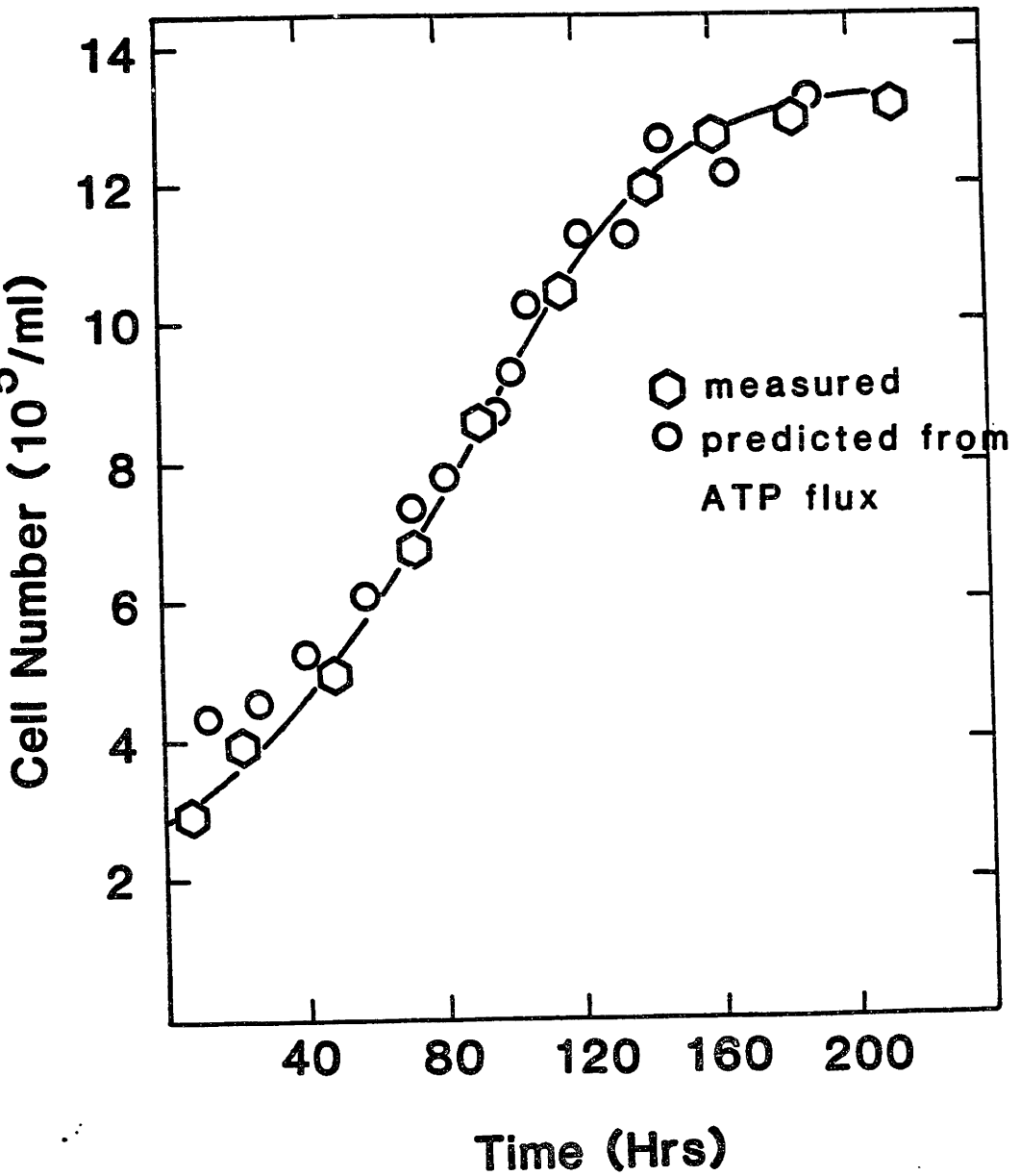

14
12
10
8
6
4
2
Cell Number (10^5/ml)
measured
predicted from
ATP flux
40
80
120
160
200
Time (Hrs)

TABLE 16

METABOLISM OF FS-4 CELLS GROWN ON GLUCOSE VS. GALACTOSE

	Glucose	Galactose
Carbohydrate consumption (mmoles/l)	~ 50	7
Lactic acid formation (mmoles/l)	~ 95	6
Oxygen consumption (mmoles/l)	8	22

er conclusion that most of the energy utilized by the cells is used for maintenance purposes, not for growth. This assertion was first proposed upon examining the oxygen uptake data. To further emphasize this point, the ATP flux data from the glucose fermentation was used to predict the cell number throughout the experiment (see Figure 47 on page 204). In this plot, the predicted cell number was obtained by simply assuming a constant maintenance requirement of 1.7 mmoles ATP/g DCW-Hr and is presented with the actual data. The close correlation between the predicted and actual cell number emphasizes the utility of this measurement for monitoring cell growth. Secondly, those changes in the ATP flux appear to represent changes in the maintenance requirements of the cells following the initiation of growth onto microcarriers. One can speculate upon several possibilities for such increased maintenance requirements: conditioning of the microcarrier surface, recovery from trypsin damage, and perhaps also the transition of the cell population from G_0 and G_1 into S phase.

Evidence that the Pathways are Coupled

Glycolysis and respiration are controlled not only by the principle carbohydrate but also by the relative activities of one another. This is strongly implied by the plot of ATP flux versus fermentation time for culture growth on either glucose or galactose (see Figure 48 on page 208). In summary, the data shows that the ATP generation rates follow the same course versus time despite the carbohydrate. This becomes more compelling when one recognizes that in the galactose situation little carbohydrate was consumed and about 95% of the ATP generated came from oxidative phosphorylation. In contrast, when glucose is the primary carbohydrate much more sugar was consumed and most of it was converted to lactic acid, while oxidative phosphorylation only accounted for about half of the ATP generated.

The argument of coupled metabolic activities for the generation of ATP is aided by two other experiments. First, it is important to recognize that the effect of glucose on respiration and glycolysis is not constant but is in fact a function of concentration: if the level of concentration is increased, further repression of respiration is observed (Crabtree

Figure 48 Calculated ATP Flux vs Fermentation Time for FS-4 Cells

The calculated ATP flux in FS-4 cells versus fermentation time is shown for cultures grown on either glucose or galactose.

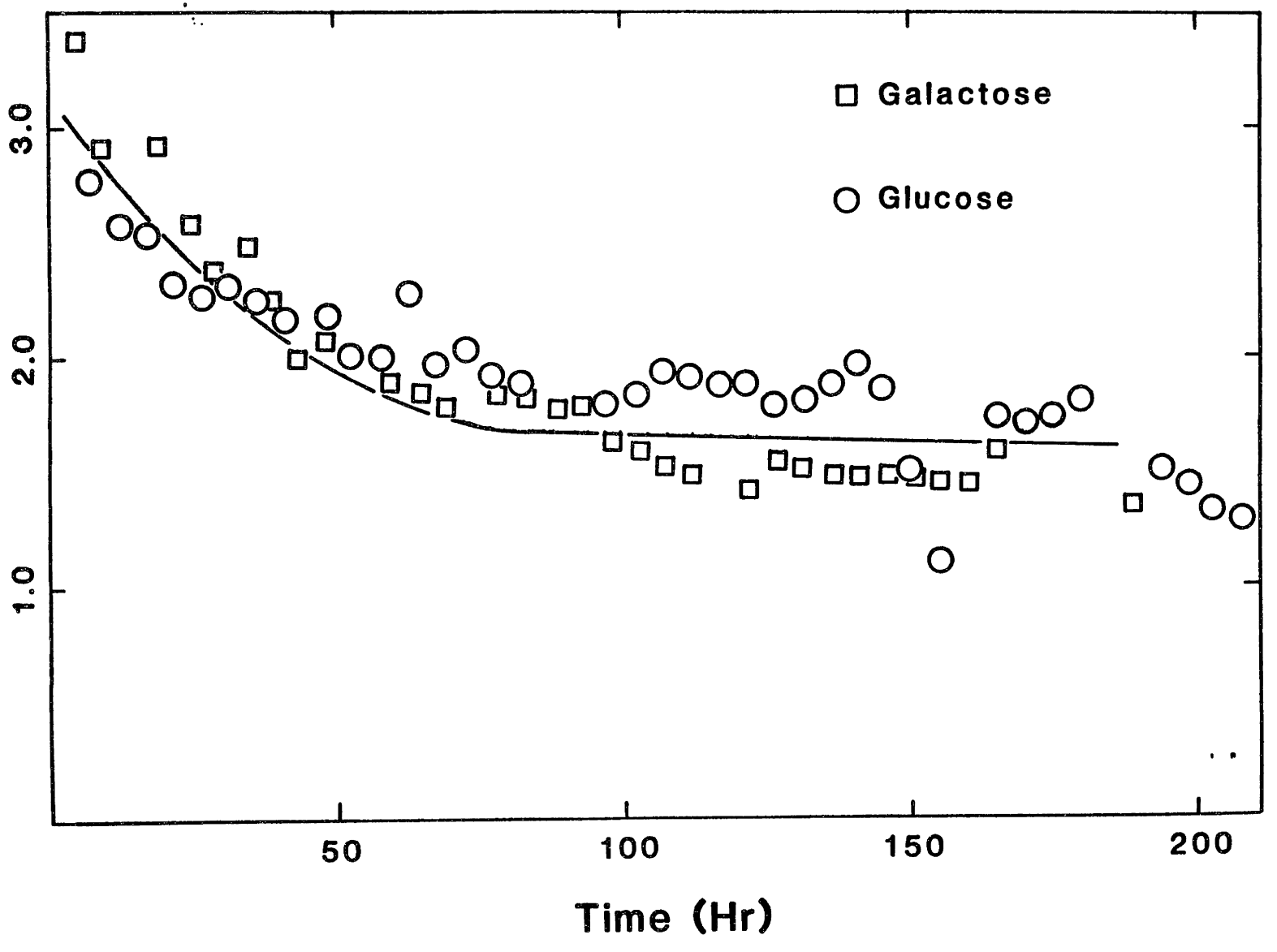
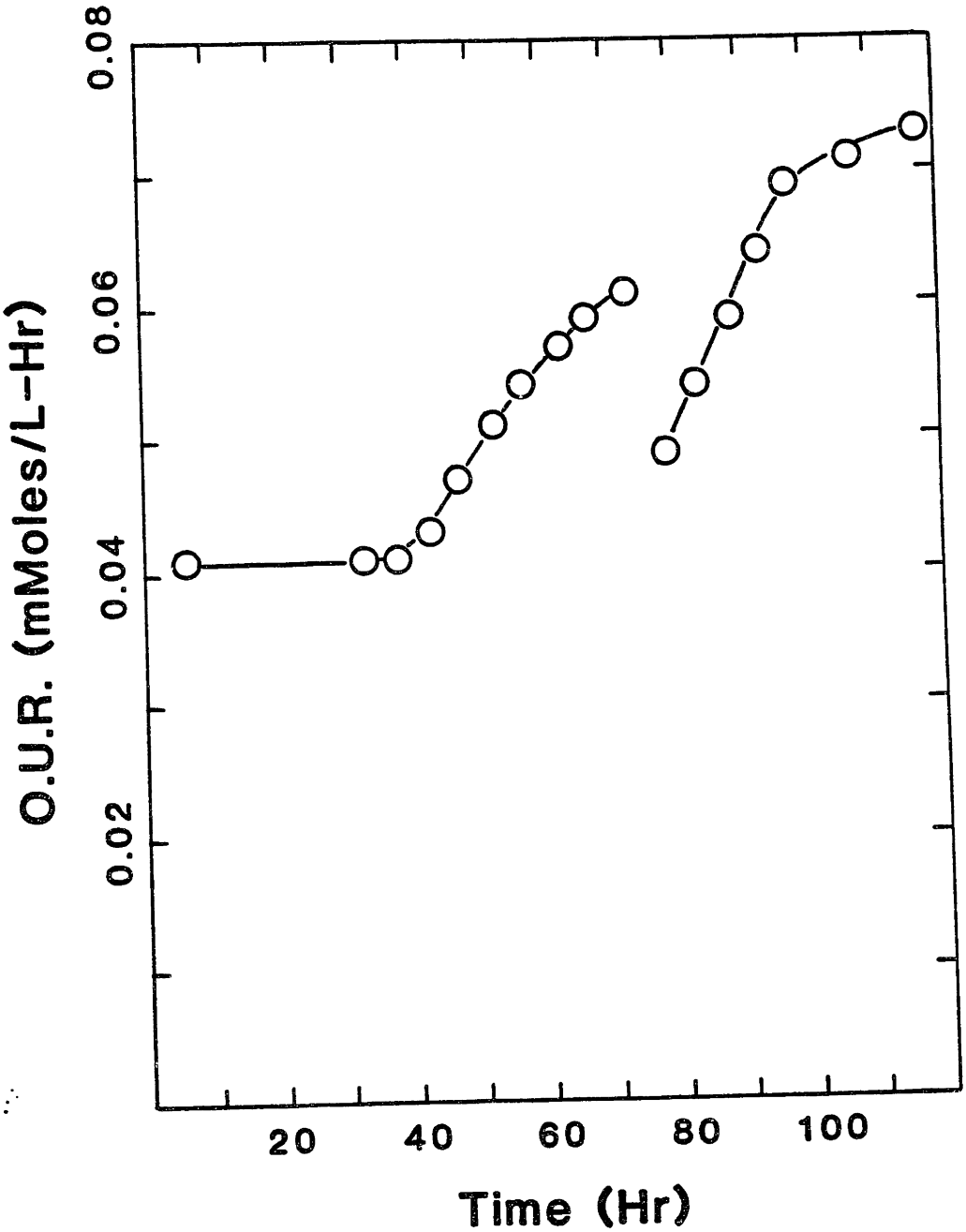

Galactose
Glucose
ATP Flux (mMoles/gm DCW-Hr)
3.0
2.0
1.0
50
100
150
200
Time (Hr)

Figure 49 Oxygen Uptake Rate by FS-4 Cells Following Glucose Feeding

The depression of oxygen uptake by the FS-4 cells following a feeding is shown as a function of time.


O.U.R. (mMoles/L-Hr)
0.02
0.04
0.06
0.08
20
40
60
80
100
Time (Hr)

effect)? Secondly, if the glucose level is very low the glycolytic pathway shows a reduced activty (Green *et al.*, 1974). Both effects have been observed using the instrumental system.

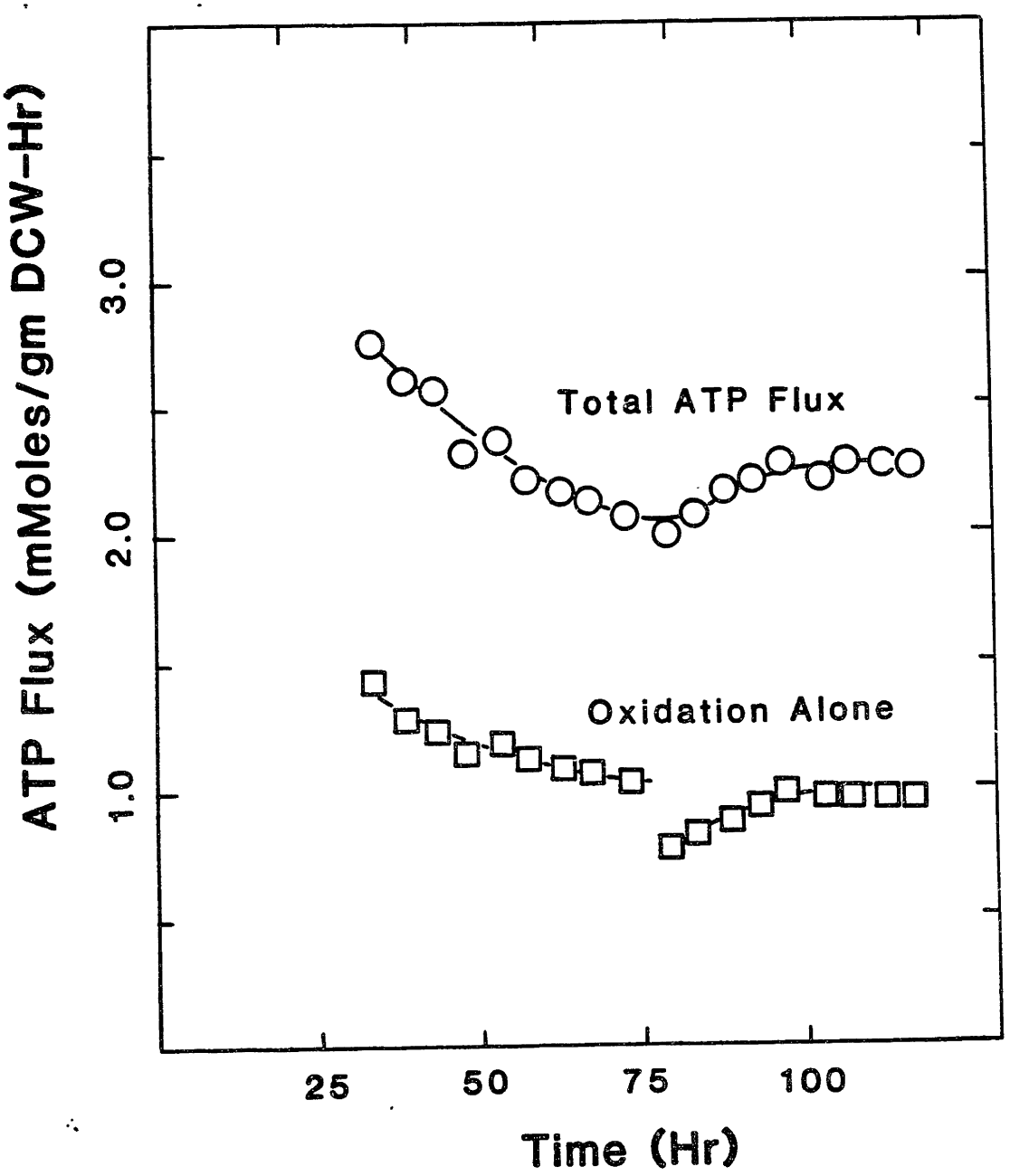
A sudden increase in glucose concentration was achieved by refeeding the actively growing cells with fresh medium. The change in the OUR is shown in Figure 49 on page 210. Clearly the increased glucose concentration repressed oxidative phosphorylation. Yet, if we also take into consideration the changes that occur in glycolysis and construct a plot of ATP flux (see Figure 50 on page 214), it becomes apparent from the results that the ATP flux is not affected by this transient.

In the second experiment, low glucose concentrations were achieved by simply allowing the culture to deplete the available glucose.. Here, in agreement with previous researchers, the rate of glycolysis at low glucose concentrations (below 1 m *M*) is dramatically reduced (about ten fold) and the oxygen uptake rate doubles (from about 0.05 - 0.07 mmoles $O_2/10^6$ -Hr to about 0.09 - 0.11 mmoles $O_2/10^6$-Hr), again emphasizing the coupled nature of these two pathways. Additionally one might also note that the metabolism of glucose at low concentrations is quite similar to the metabolism of galactose.

The relationship between glucose concentration and glucose metabolism that we have examined brings up another issue. *In vivo,* glucose is predominately metabolized by the citric acid cycle. The production of significant quantities of lactic acid is associated with muscle tissue under conditions of strenuous exertion or with certain metabolic disorders. Yet, in animal cell culture most of the glucose is converted to lactic acid even though the concentration of glucose presented to the cells is not atypical of those found in the blood. First, this further emphasizes that cell culture is not a perfect model of *in vivo* conditions. Second, it suggested that *in vivo* perhaps the cells are presented with glucose at a concentration much lower than that in the blood or that their metabolism is under a much different hormonal and chemical control than that mimicked in cell culture.

Figure 50 Calculated ATP Flux in FS-4 Cells during Glucose-Induced Inhibition of OUR

The calculated ATP flux for the FS-4 cells during the induced transient is shown plotted against fermentation time and also shown is that contribution of the ATP flux provided by oxidative phosphorylation.


ATP Flux (mMoles/gm DCW-Hr)
3.0
2.0
1.0
Total ATP Flux
Oxidation Alone
25
50
75
100
Time (Hr)

CONTROL OF GLUCOSE METABOLISM BY THE FS-4 CELLS

The objective of this experimental work was to demonstrate the utility of measuring energy metabolism, not only as a means to monitor cell function and cell growth but also towards achieving process control in cell culture. Specifically, the aim of this work was to utilize the measured ATP flux to control the metabolism of glucose by FS-4 cells such that the formation of lactic acid was minimized.

The justification for attempting to minimize the conversion of glucose to lactic acid is that this represents an undesirable and presumably unnecessary metabolic activity. Why cells should choose to produce ATP from glucose via glycolysis is not entirely understood, particularly when they have the capability of producing ATP from glucose via the citric acid cycle (oxidative phosphorylation). However, animal cells in culture will typically convert most of the available glucose to lactic acid. Glycolysis is not only less efficient than oxidative phosphorylation, but the rapid conversion of glucose to lactic acid is undesirable for several reasons. First, the excessive production of lactic acid rapidly lowers the pH of the culture. Cells function well only over a narrow range of pH and pH changes can interfere with cell growth. Moreover, these high rates of glycolysis often result in glucose being depleted from the medium much more rapidly than any of the other nutrients. Consequently, the culture medium must be changed more frequently than might otherwise be required in order to maintain an adequate supply of glucose.

Minimizing the formation of lactic acid in cell culture is not without some precedence. Eagle (1955) showed that substituting either fructose or galactose for glucose resulted in the formation of less acid. More recently, Rheinwald and Green (1974) have demonstrated that secondary sources of glucose, such as starch, can also lower the production of lactic acid. The function of these secondary glucose sources is to keep the concentration of free glucose at a low level. One explanation for these observations is that the metabolism of glucose is influenced by its concentration in the surrounding environment. Specifically, lower concentrations of glucose would tend to favor the production of ATP by means of oxidative phosphorylation. Data obtained from this thesis would

also tend to support this hypothesis. Specifically, I have observed oxygen uptake rates to increase and rates of lactic acid formation to decrease as glucose concentrations decrease. Notably, however, during these transients the estimated rate of ATP formation by these cells remains unchanged. This further suggests that glucose is the primarily energy source.

Thus, it is reasonable to assume that the formation of lactic acid from the utilization of glucose could be significantly reduced if the carbohydrate were fed to the cells in such a way that its concentration was always maintained at a relatively low level. However, enough glucose must always be available so that neither cell growth nor the cellular physiology is impaired. Such a control scheme can be implemented using three control loops: two feed-back and one feed-forward (see Figure 51 on page 218). The oxygen uptake rate and the rate of lactic acid formation can be used to predict the cell number and the corresponding glucose demand by these cells. That demand would be calculated on the basis of the desired ratio of glycolysis to oxidative phosphorylation. This feed-forward strategy for control is the basis of the first control loop (#1). However, to ensure that the glucose concentration remains within a certain range, a second control loop (#2) has been added. This feed-back loop adjusts the glucose feed rate to satisfy unaccounted for demands and to prevent adding glucose should the level be above the desired concentration. The trade-off in this scheme is that when presented with a high glucose level, the cells produce too much lactic acid. Alternately, too low a glucose level can interfere with their growth. This concern formed the basis of the third control loop (#3). The strategy was initially aimed at controlling the glucose concentration at a level known to support growth but would reduce the rate of glycolysis. The rate of glycolysis at that glucose level is then examined. Should the rate of glycolysis be too high, the glucose set point is then lowered.

Specifically, we have observed that the FS-4 cells grown on glucose usually form lactic acid at a rate of 1.3 mmoles/gm-DCW-h. In contrast, these cells when grown on galactose show a rate of approximately 0.1 mmoles/gm-DCW-h. Our control objective was to lower the rate of lactic acid formation to approximately 0.2 mmoles/gm-DCW-h, a rate comparable

Figure 51 Outline of Scheme Used to Control the Metabolism of Glucose by the FS-4 Cells

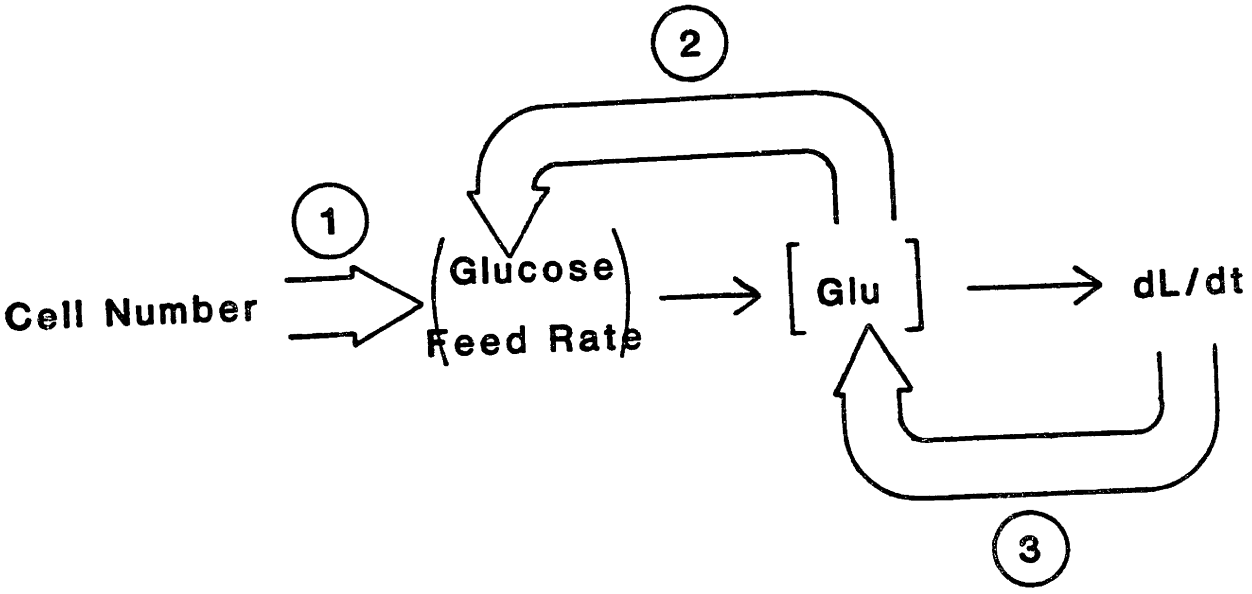
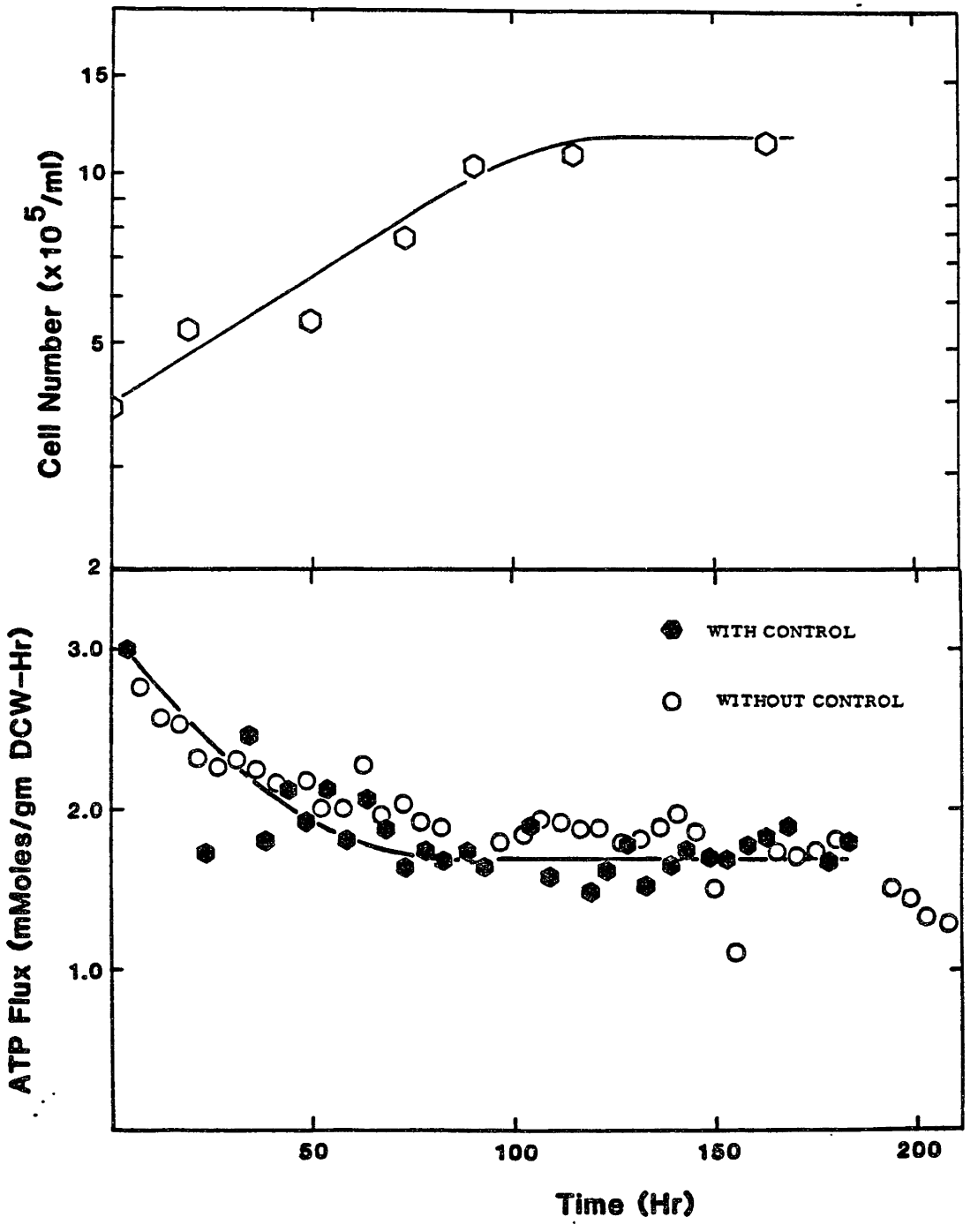

2
1
Cell Number
Glucose
Feed Rate
Glu
dL/dt
3

Figure 52 Plot of Cell Growth and Energy Metabolism vs. Time of Fermentation

FS-4 cells were grown in microcarrier culture (5gm/l bead concentration) in a 14 liter fermentor on DME containing only 0.5 m *M* glucose and buffered with 5 m *M* tricine and without bicarbonate. The inoculum was prepared using roller bottles. The initial culture volume was 7.5 liters.


Cell Number (x10^5/ml)
15
10
5
2
ATP Flux (mMoles/gm DCW-Hr)
3.0
2.0
1.0
WITH CONTROL
WITHOUT CONTROL
50
100
150
200
Time (Hr)

to that of cells grown on galactose. The glucose concentration in the cell culture medium (DME) is initially 25 m *M*. Previous data has indicates that the rate of lactic acid formation does not decrease significantly until the glucose level falls below 1 or 2 m *M*, so the initial glucose set point was chosen to be 0.5 m *M*.

The growth and metabolism of the FS-4 cells under the conditions of controlled glucose metabolism is presented in Figure 52 on page 220. In essence, the plot shows that the cells exhibited both normal growth and metabolism. Growth was judged both on the basis of the growth rate (doubling time of 65 hours) and the final saturation density (approximately 1.2 million per ml). The energy metabolism (as measured by the calculated ATP flux) of these cells was characteristic of cells grown without such control on either glucose or galactose. Yet, when the specific rate of lactic acid formation is examined (see Figure 53 on page 222) a notable difference between these cells and those grown without control is apparent. Controlling the glucose feed rate and glucose concentration resulted in the formation of significantly less acid. Additionally, the control objective, a rate of lactic acid formation of approximately 0.2 mmoles/gm-DCW-h, was achieved after corrective action was taken to lower the glucose set point. The original set point of 0.5 m *M* glucose was much too high and it was necessary to reduce the set point concentration to no greater than 0.1 m *M* glucose.

The lower curve of Figure 54 on page 224 is an effort to show this data in a slightly different format. Here, the fraction of the total energy metabolism derived from glycolysis is plotted against the glucose concentration of the medium. Thus, we see that FS-4 cells grown on glucose will maintain higher rates of glycolysis than cells grown on galactose unless the glucose concentration can be controlled at a level at or below 0.05 to 0.1 m *M*. Additionally, if we assume that the cellular energy metabolism is being derived principally from glucose metabolism, then for any level of glycolysis (dL/dATP) we can calculate an accompanying yield of lactic acid from consumed glucose (dL/dG) [this transformation is discussed in appendix R]. The solid curve in the upper half of figure 4 represents a calculated transformation of the lower curve. Here, we would predict that for glycolytic activities accounting for 40% or more of the total energy

Figure 53 Glucose Set Point, Glucose Conc., and Rate of Lactic Acid Formation by the FS-4 Cells vs. Time of Fermentation

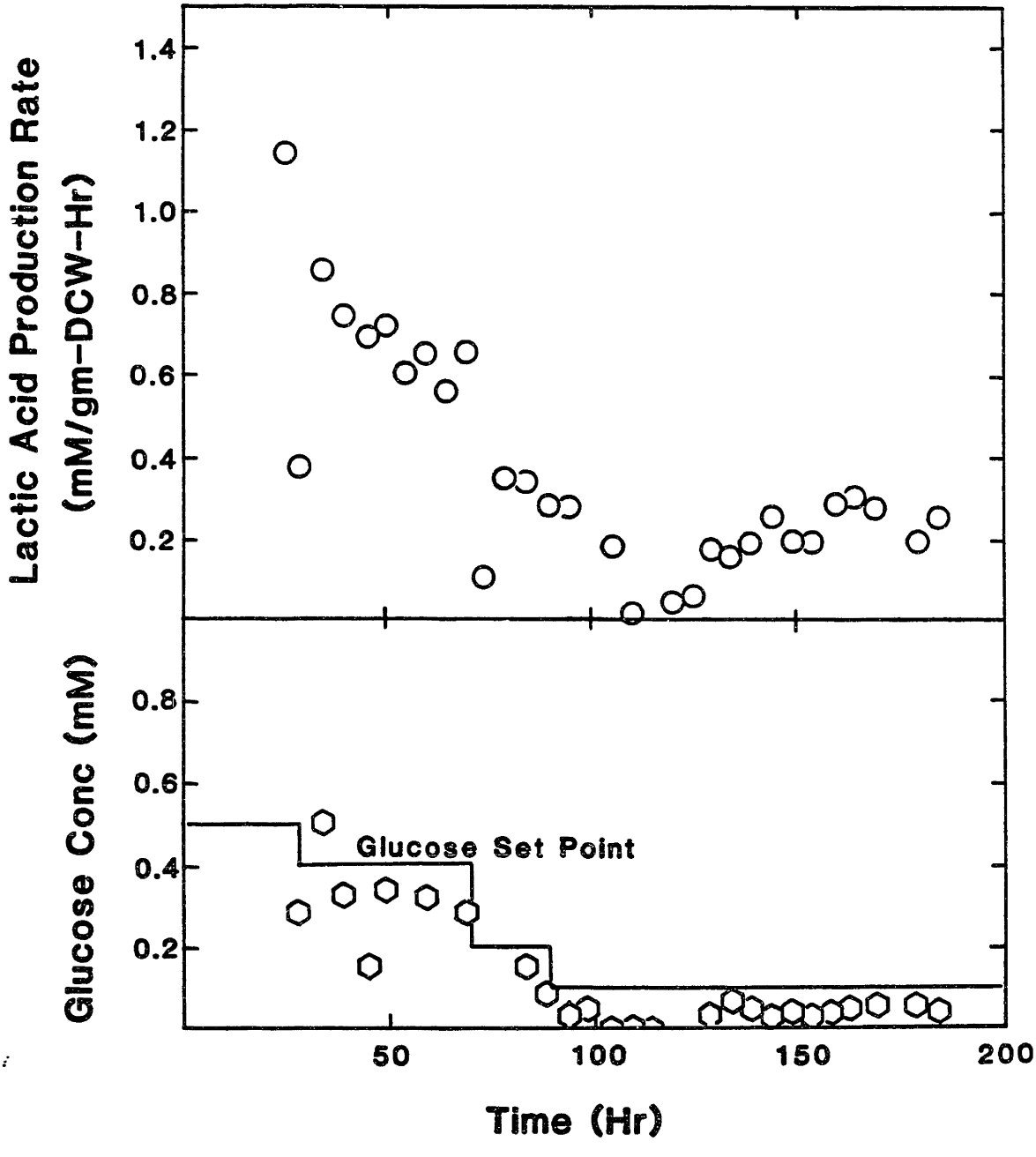
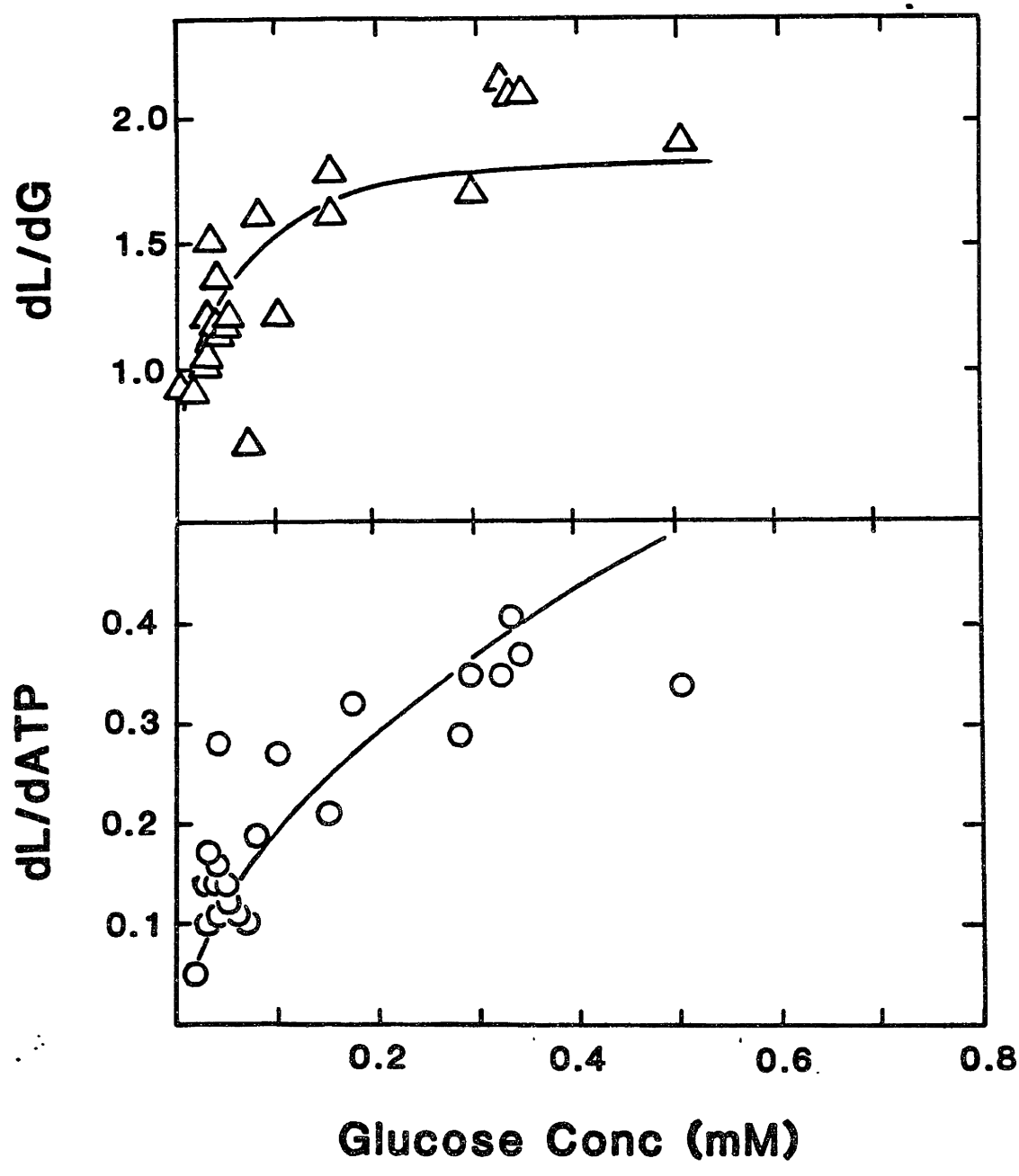

Lactic Acid Production Rate
(mM/gm-DCW-Hr)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Glucose Conc (mM)
0.8
0.6
0.4
0.2
Glucose Set Point
50
100
150
200
Time (Hr)

Figure 54 Fraction of Total Energy Metabolism Derived from Glycolysis and Yield of Lactate from Glucose Consumption


dL/dG
2.0
1.5
1.0
dL/dATP
0.4
0.3
0.2
0.1
0.2
0.4
0.6
0.8
Glucose Conc (mM)

metabolism, the yield of lactic acid from consumed glucose is approximately 2.0, and that this yield will not drop to less than 1.0 until the glycolytic activity is approximately less than 5% of the total energy metabolism. The triangles represent the experimentally determined points. Good correlation is observed between the yield of lactic acid upon glucose consumed predicted from the energy metabolism and that which was actually measured. This would suggest that most of the glucose consumed can be accounted for as the end products of the energy metabolism, i.e. lactic acid and carbon dioxide. This was true not only when the energy metabolism was principally derived from glycolysis, but also when it was principally derived from oxidative phosphorylation.

In summary this experiment was able to show that the measure of a calculated rate of ATP formation can be usefully applied to process control in cell culture as well as to monitor cell growth. Furthermore, I have also demonstrated that several of the original assumptions concerning the role of glucose in the metabolism of these cells were correct. Glucose was assumed to be primarily an energy source for these cells, though it was not being used very efficiently. Subsequently, I was able to control glucose metabolism such that less glucose is consumed, less acid is produced while at the same time altering neither cell growth nor their energy metabolism.

MODEL OF CARBOHYDRATE METABOLISM IN FS-4 CELLS.

Using the data previously discussed, it is possible to propose a model explaining the observed metabolism. This model is outlined in Figure 55 on page 228 and draws heavily upon previously published data concerning the enzymes and intermediates of the glycolytic and citric acid cycle pathways.

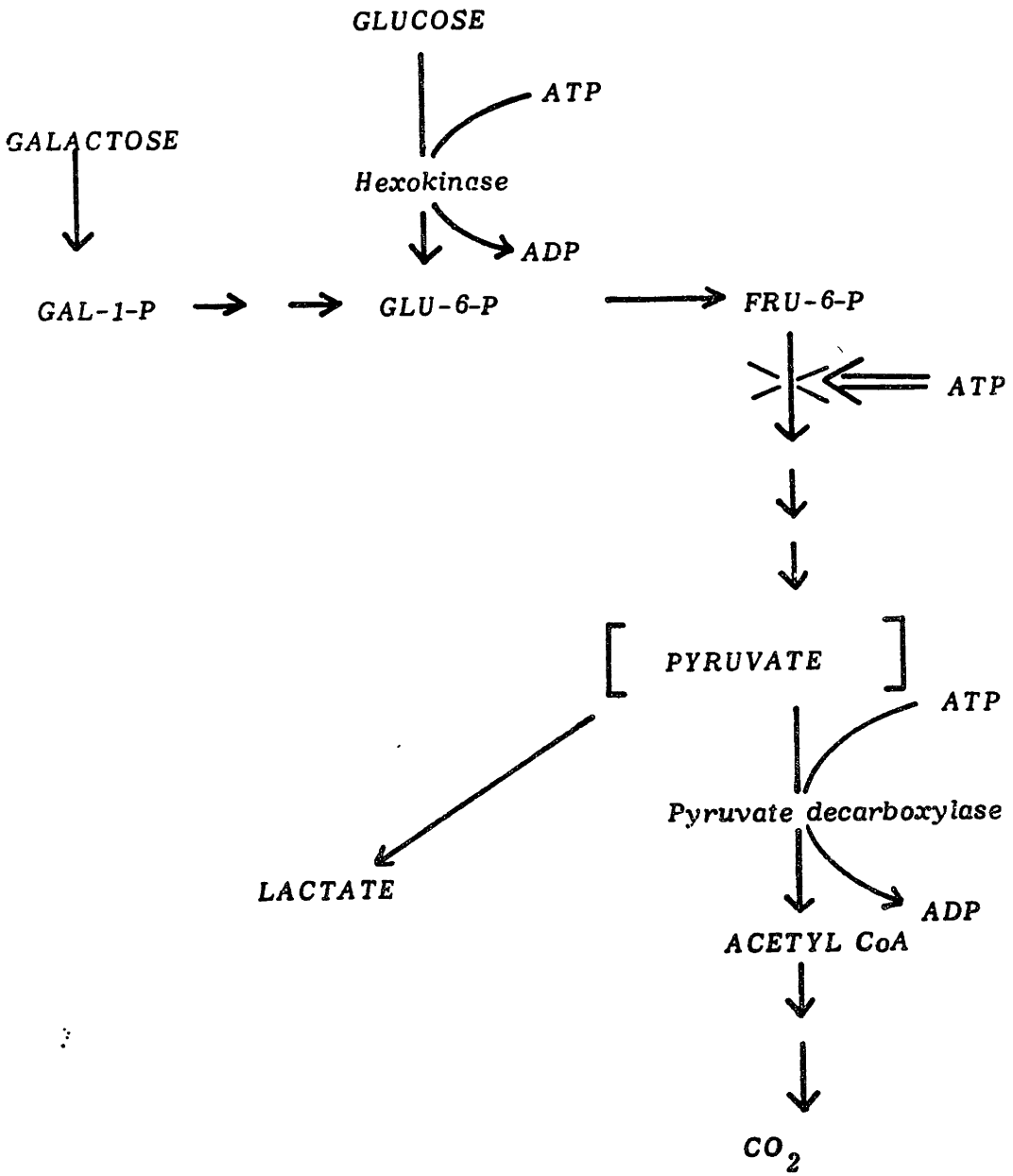
One, galactose enters the pathway by a mechanism slightly different than that used by glucose. Glucose is phosphorylated by hexokinase to Glu-6-P. Galactose on the other hand is first converted to Gal-1-P and then to Glu-1-P and then finally to Glu-6-P. In both instances, the overall velocity of these carbohydrates through the pathway is controlled at a

subsequent step, the phosphorylation of Fru-6-P, by the inhibition of fructose phosphate kinase by the intracellular concentration of ATP. Therefore, neither the conversion of pyruvate to lactate nor acetyl-CoA need be rate-limiting. Thus, as long as the utilization of ATP for cell growth and maintenance is less than the capacity of the energetic pathways to generate ATP, the ATP flux of these cells should be identical regardless of the carbohydrate used.

Two, the predominate conversion of pyruvate to lactic acid by these cells appears to be something of an anomalous behavior, resulting from excessive levels of readily utilized carbohydrates. This is substantiated if we examine the Km's of both lactate dehyrogenase and pyruvate dehydrogenase, and typical intracellular concentrations of pyruvate. The Km of lactate dehydrogenase (bovine) ranges from 10^{-4} *M* (heart) to 10^{-3} *M* (muscle) (Barnman 1969), whereas the Km of pyruvate dehydrogenase has been reported to be about 2×10^{-5} *M* (acsites tumor) (Barnman 1969) and intracellular concentrations of pyruvate have been measured to be about 5×10^{-5} *M* (erythrocytes) (Lehninger 1980). Thus, we would anticipate that the majority of the pyruvate would be consumed by the citric acid cycle. The divergence from this route can be explained by the requirement of pyruvate dehydrogenase upon ATP for activity. Given a great excess of glucose, hexokinase can shift the ATP/ADP ratio to the predominately ADP state. The activity of pyruvate dehydrogenase decreases causing the intracellular concentration of pyruvate to increase and thereby encouraging the formation of lactic acid.

Three, it should be noted that glucose and galactose appear to be the predominate energy substrates of these cells. This point is raised because some researchers (Reitzer *et al.* 1979) have concluded that with HeLa cells glutamine was the predominate energy source. Specifically, it was claimed that 98% of the energy utilized by these cells was derived from glutamine when either galactose or fructose was the primary carbohydrate and that more than half of the energy still came from glutamine even when glucose was available. Our data does not dispute that glutamine could be used as an energy source. However, we do not concur that in these cells it could be a major energy source. Given the amount of glutamine consumed and the amount of glutamic acid produced (which is

Figure 55 Model for Regulation of Energy Metabolism by FS-4 Cells


GLUCOSE
ATP
GALACTOSE
Hexokinase
ADP
GAL-1-P
GLU-6-P
FRU-6-P
ATP
PYRUVATE
ATP
Pyruvate decarboxylase
LACTATE
ADP
ACETYL CoA
CO_2

roughly in agreement with the measured ammonia production), then if all of the glutamine available to the cells as α ketoglutarate were used in the citric acid cycle, only about 14% of the total energy generated could be attributed to glutamine. Furthermore, not all of the glutamine removed from the environment is available to the cells, the formation of ammonium pyrrolidone carboxylate is a spontaneously reaction occurring at temperatures above 4 °C An estimate of 7-10% is probably more realistic. Additionally, I might add that about 95% of the glucose or galactose consumed can be accounted for by the production of CO_2 and lactic acid.

AMMONIA

One of the unexpected results from the growth of cells on galactose was that the medium needed to be changed long before the galactose itself was depleted. Previously, when the FS-4 cells had been grown on glucose, the glucose was consumed so rapidly that changing the medium to replenish the glucose was adequate for all of the cell needs. Certainly data concerning the amino acid uptake by these cells did not lead one to expect any problems concerning a medium limitation. However, upon growing the cells on galactose (see Figure 45 on page 200) it became apparent from the oxygen uptake data that the metabolism of the cells began to decline in an unexpected fashion, both before reaching saturation density and also in the presence of an adequate supply of galactose. Changing the medium promptly increased the metabolic activity of these cells. Furthermore, in this particular fermentation, the cells subsequent to having reached saturation showed decline as evidenced by cell detachment from the microcarriers and reduced oxygen uptake rates.

In both instances the observed decline in cell viability correlated with increasing concentrations of ammonia, particularly when the concentration exceeded 2.5 m *M*. Although the data is rather circumstantial it is reinforced by a similar observation in the microcarrier culture of chicken embyro fibroblasts (see Appendix O). In the 'feed-starve' experiment shown in Figure 59 on page 340 and Figure 60 on page 342, CEF cells that were more frequently fed showed more rapid growth and attained higher saturation cell densities. An effort was then undertaken to analyze the

declining growth rate of these cultures with regard to the concentrations of glucose, lactate, and ammonia. Here again the correlation with ammonia was striking, and a plot of growth rate (μ) versus the ammonia concentration is presented in Figure 56 on page 232. Much of the decline in cell growth occurred as the ammonia concentration increased from 2 to 3 m *M*.

Free ammonia is poisonous to most animal systems. The body detoxifies ammonia by converting it to urea, via the kidneys. Although, the actual mechanism of ammonia toxicity is not understood, that it is indeed toxic has been demonstrated by injecting urease into the blood of animals, which results in death. Additionally, increased serum concentrations of ammonia are frequently associated with certain hepatic comas.

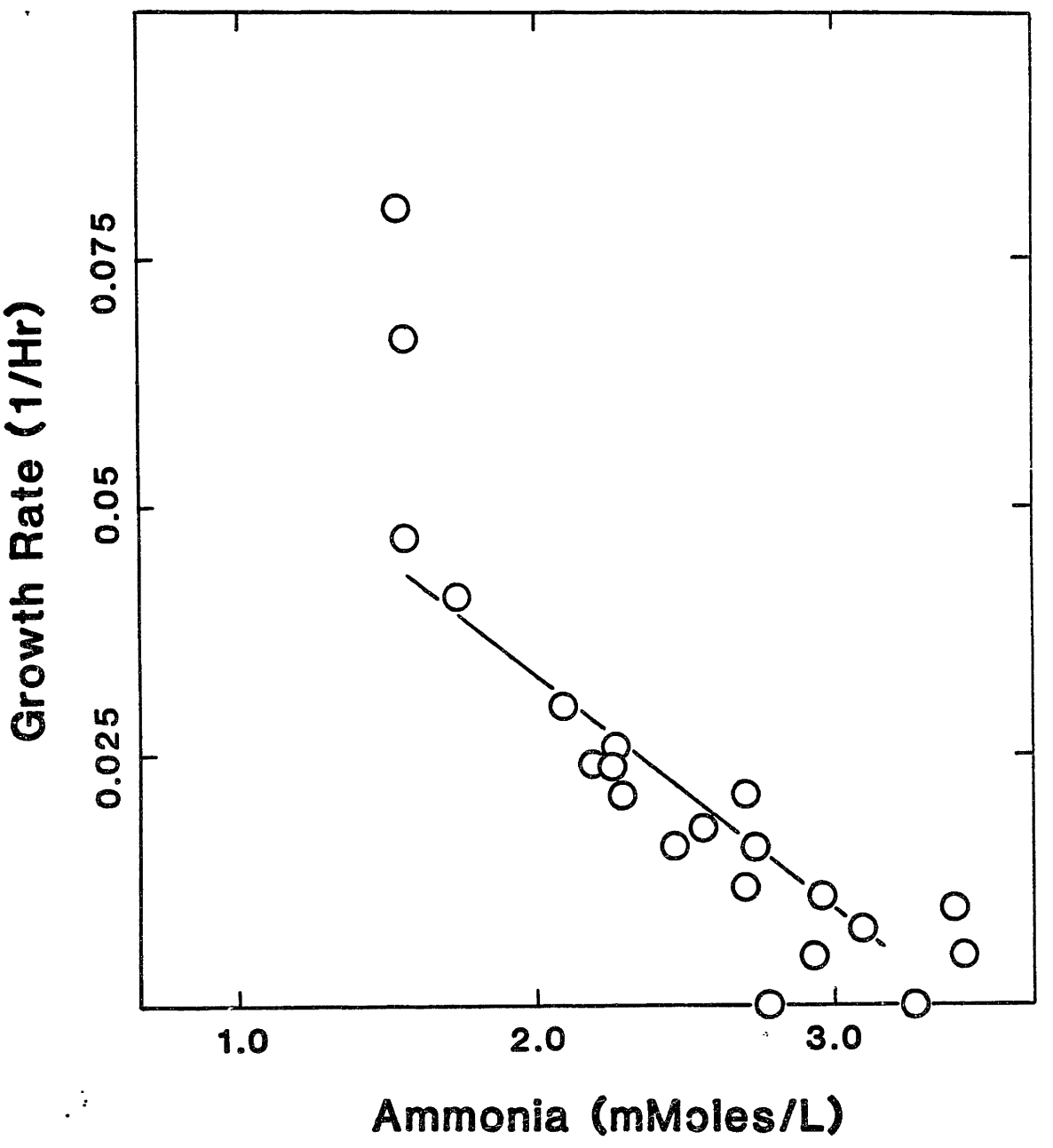
A number of possible procedures do exist to remove ammonia from biological fluids, such as binding to aldehyde starch or heavy metals ion exchange resins, electrochemical oxidation to gaseous nitrogen, and the enzymatic reduction of shift-base intermediates. The successful removal of ammonia from tissue culture fluids should certainly improve the growth of existing cells types and may aid in the establishment of cell types not previously grown *in-vitro*. The removal of toxic waste products is without question an issue of increasing importance in the growth of animal cells in high density cultures.

INTERFERON

Interferon is an induced cell product, as are many of the commercially useful products of animal cell culture. Subsequently, the conditions surrounding the time of induction can have profound effects upon the final product yield. The value of instrumentation in process should not be underestimated. It can aid in ensuring the cells are grown under conditions such that they will be favorably predisposed toward this event, it can aid in the timing of this event, it can monitor the cellular metabolism during the process to ensure that things proceed in an optimal fashion, and it can also be useful as a research tool in understanding how to optimize the induction process.

Figure 56 Growth Rate of CEF Cells vs Ammonia Concentration

The growth rate of CEF cells in 100 ml microcarrier culture as measured in the 'feed-starve' experiment is shown as a function of measured ammonia concentrations.


Growth Rate (1/Hr)
0.075
0.05
0.025
1.0
2.0
3.0
Ammonia (mMoles/L)

We have shown first, that the FS-4 cells can consistently produce respectable yields of interferon in microcarrier culture; second, that the induction of interferon is influenced by the growth rate of the cells, and third, that the instrumentation developed is useful in monitoring cell growth, and hence in determining when to begin the induction. Furthermore, that the information obtained concerning the energy metabolism of the cells during the induction and production phases of interferon production is useful in determining research directions to further optimize this process.

INDUCTION OF INTERFERON

The "superinduction" procedure of inducing cells to produce interferon was first developed by Vilcek and Havell (1972). However, the same process when initially applied to the microcarrier system was not altogether suitable. The principle problems were that the actual production of interferon was erratic and also that the results showed a less than desirable range of variance. Subsequently, an effort was made to optimize this process with the cells grown in microcarrier culture (see Appendix S) The principle objective was first to minimize the probability of total failure and second, to increase the actual interferon titers. The results are summarized in Table 17 on page 235. We first fine-tuned the time of exposure and concentration of the metabolic inhibitors used in the induction procedure. Of particular note is the reduction in the concentration of cycloheximide which showed a relatively narrow optimal range. Also the time of exposure to actinomycin-D, was beneficially extended. Secondly, it became apparent that the use of priming was particularly beneficial. Although it did not result in absolute higher titers it did appear to minimize the day-to-day variability in the induction we had experienced and thus resulted in substantially higher titers on average.

Finally, we looked at the affect of temperature on the induction process. What we learned was first, that the optimum temperature during the induction period while exposed to the antimetabolites was at 34 °C; second, that the kinetics of interferon production were most prolonged at 30 °C, and third, that the overall rate of productivity could be greatly

TABLE 17

OPTIMIZATION OF INTERFERON INDUCTION IN FS-4 CELLS

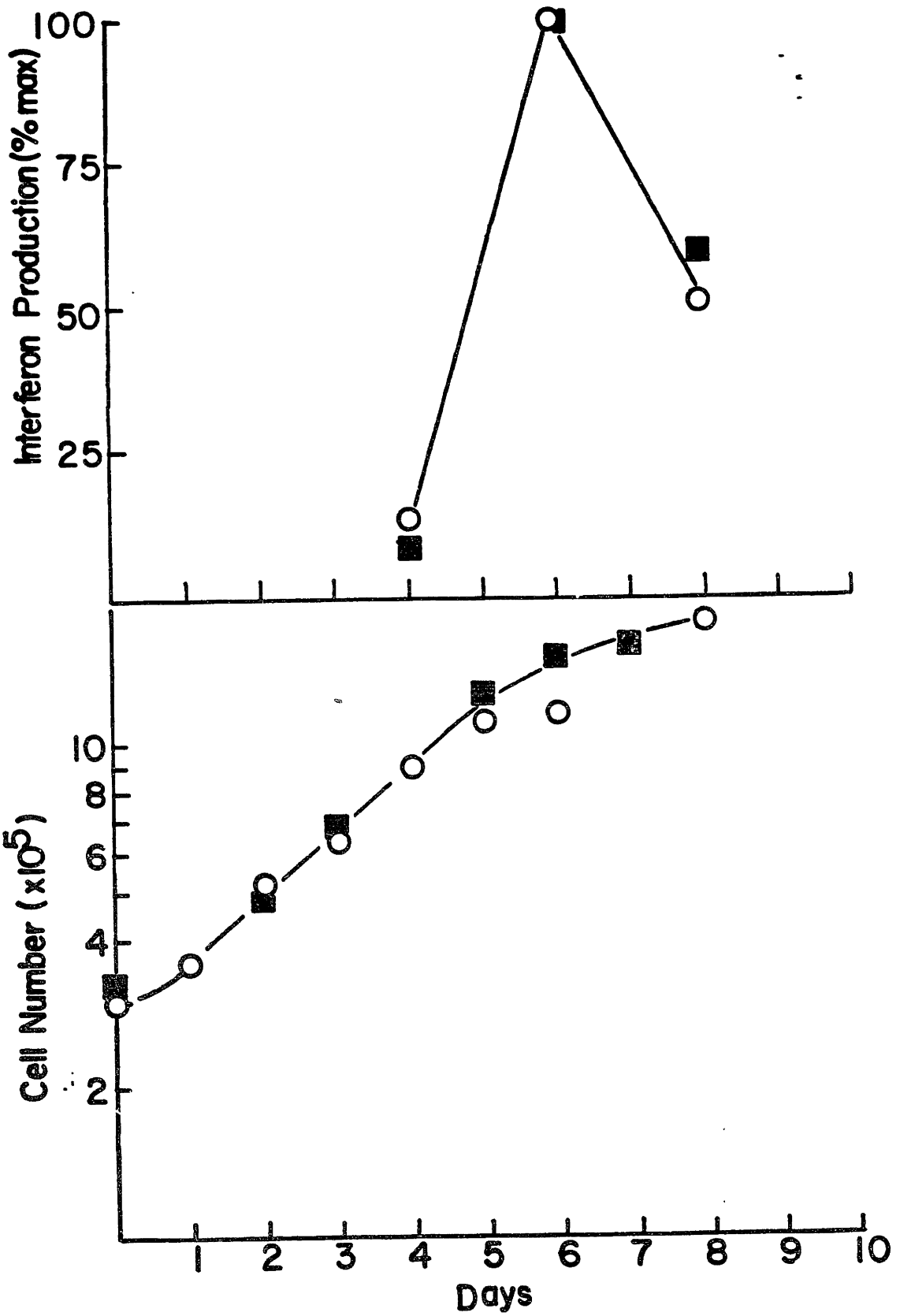
Method	IF Titer units/10^6 cells
Poly I • poly C alone (A)	60-100
Standard super induction (B)	1,000
Modified super induction (C)	5,000
C + priming (D)	15,000
D & T lower to 34 °C in induction and production	22,000
D & T lowered to 34 °C in induction + 37-30 °C temperaure shift during production (E)	44,000
E & extend production period to 48 hours	75,000

increased by first exposing the cells to 37 °C for a one-hour period at the beginning of the production period. Additionally, the temperature shift extended the duration of interferon production.

EFFECT OF CELL AGE ON INTERFERON PRODUCTION

The effect of cell age on the inducibility of the cells to produce interferon was investigated. The literature contained some reports that resting cells did better than growing cells (Vilcek and Havel, 1972) and more recently, it has been reported with lympoblastoid cells that cells in exponential growth produce less than cells that have begun to leave the log growth phase. The situation with microcarriers was completely unkown. Our initial experiments showed a 10 fold difference in per cell productivity between log phase growth cells and those entering the stationary growth phase. In these experiments,the induction and the production were performed at 37 °C and the cells were not primed prior to the exposure to poly I • poly C. In these experiments, one-liter microcarrier cultures were set up and samples of the culture were withdrawn during the exponential, the transition, and the stationary phases of growth (specifically the 4th, 6th, and 8th days of culture). The samples were induced and the titer, expressed as the % of maximum titer for each run, are shown in Figure 57 on page 238. The actual maximum titers of interferon were 18,000 and 32,000 units/million cells. Subsequently, work with the improved induction procedure with and without priming has also shown similar trends. The relative difference in yields between growth and early stationary phases is rather variable, but is typically at least three-fold. Furthermore, because cell mass correlates well with the total energy flux of the culture the ability to discern between log phase growth and cells entering stationary phase is greatly simplified with the instrumental system. The value of this becomes more obvious when one realizes that these instrumental methods with little modification would work well with other large-scale cell culture technologies such as packed glass beads, where it would be otherwise impossible to estimate the cell mass.

Figure 57 Interferon Production vs Growth Phase


Interferon Production (% max)
100
75
50
25
Cell Number (x10^5)
10
8
6
4
2
1
2
3
4
5
6
7
8
9
10
Days

CELL METABOLISM DURING PRODUCTION OF INTERFERON.

The induction of interferon is essentially a three-stage process: Cells are first primed, then induced, and subsequently they begin the actual synthesis of interferon. Optimizing the production of interferon with these cells has been an involved process, and we are probably still far away from achieving near optimal productivity. The energy metabolism of these cells during the induction process was then analyzed with the objective that the information learned could aid in the decision process of formulating new research directions (ie. what do we do next?).

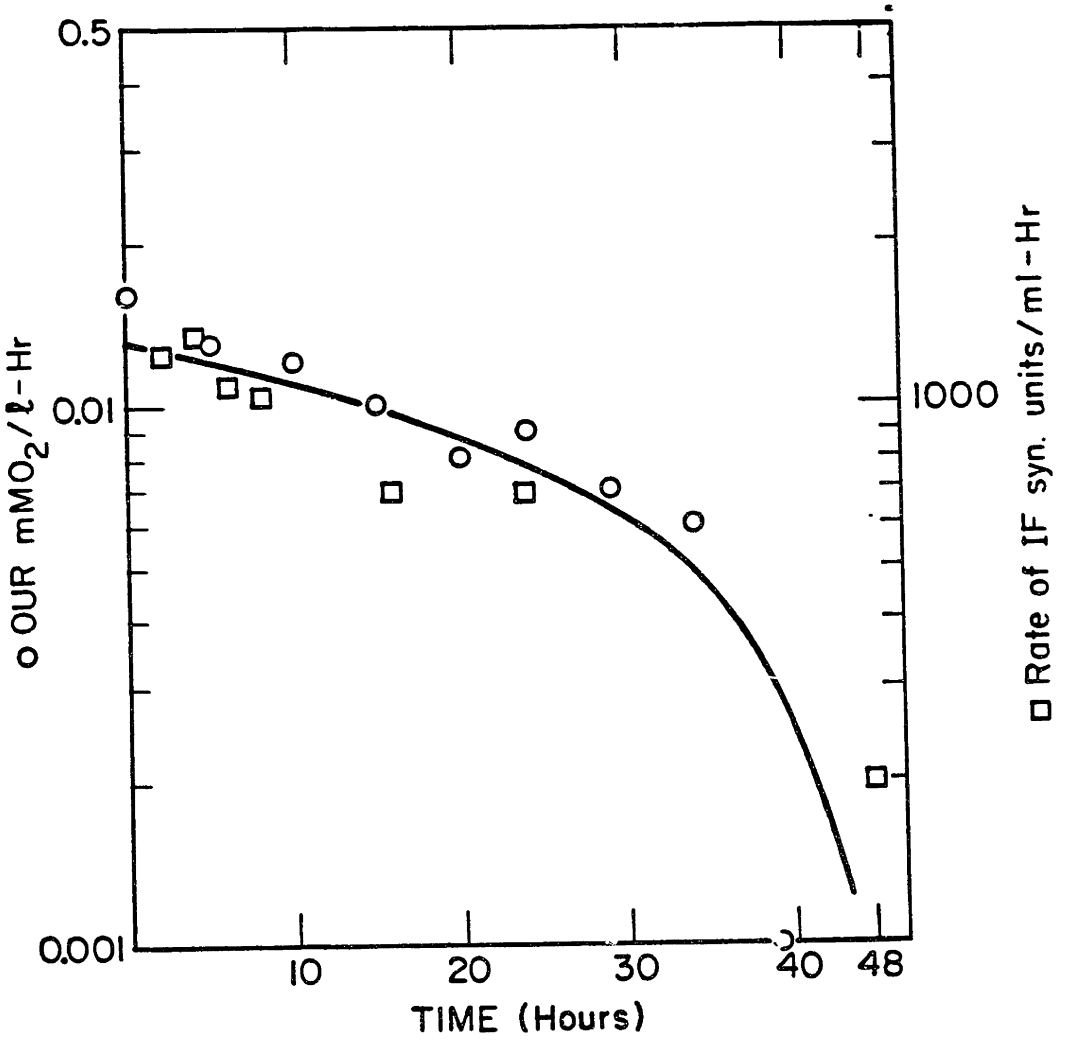
Cells grown on glucose were primed and then induced. What we learned can be summarized as follows; priming itself does not significantly affect cell metabolism. However, during the induction step there is a very large decrease in overall cell viability, the greatest portion of which follows the addition of actinomycin-D. Finally, in the production phase the metabolism of the cells shows first a greatly reduced but stable activity and then proceeds to decline. The rate of interferon synthesis during this period was also analyzed and is shown together with the oxygen uptake rate in Figure 58 on page 240. The strikingly parallel rate of decline would suggest that the rate of interferon synthesis is in fact presently limited by the viability of the cells themselves.

The implications for further research are as follows; One, though we've made considerable progress through the careful study and manipulation of temperature in the production phase, we seem to be approaching the limits of what these actions can achieve. Further work on this aspect is probably not going to be particularly rewarding. Two, if we are going to improve upon the overall productivity we must either achieve higher initial rates of synthesis (which would involve changing the conditions of priming and the induction) or by prolonging the production phase by reducing the toxicity of actinomycin-D (or finding a less toxic alternative).

Parenthetically, one might add that by monitoring the cellular metabolism during the production phase we also have a means by which to judge when the period of interferon synthesis has ended.

Figure 58 OUR and Rate of IF Synthesis During Production

The oxygen uptake rates of FS-4 cells in microcarrier culture was determined during the interferon production period and is plotted against time. Also presented is the rate of interferon synthesis.


0.5
0.01
0.001
o OUR mMO2/ℓ-Hr
1000
□ Rate of IF syn. units/ml-Hr
10
20
30
40
48
TIME (Hours)

SUMMARY AND CONCLUSIONS

In summary the following objectives have been achieved:

- Characterization of the growth of the FS-4 cells, a human diploid fibroblast cell strain, in microcarrier culture and those parameters essential to obtain reproducible and consistent cell growth.

- Characterization of the production of interferon by the FS-4 cells in microcarrier culture and having optimized many of the variables of this process in order to obtain reproducible and high titered yields.

- Development of a means to provide oxygen to large-scale high density cell culture, by the use of membrane oxygenation.

- Development of instrumentation to measure the energy metabolism of animal cells in culture.

- Developmet of a computer controlled system to monitor and process the output of these sensors.

- Determination that most of the energy produced by these cells is used for cell maintenance.

- Control of glucose metabolism in animal cells using computer control, such that lactic acid formation was reduced. This control scheme was base upon the availability of an on-line measurement of the energy metabolism.

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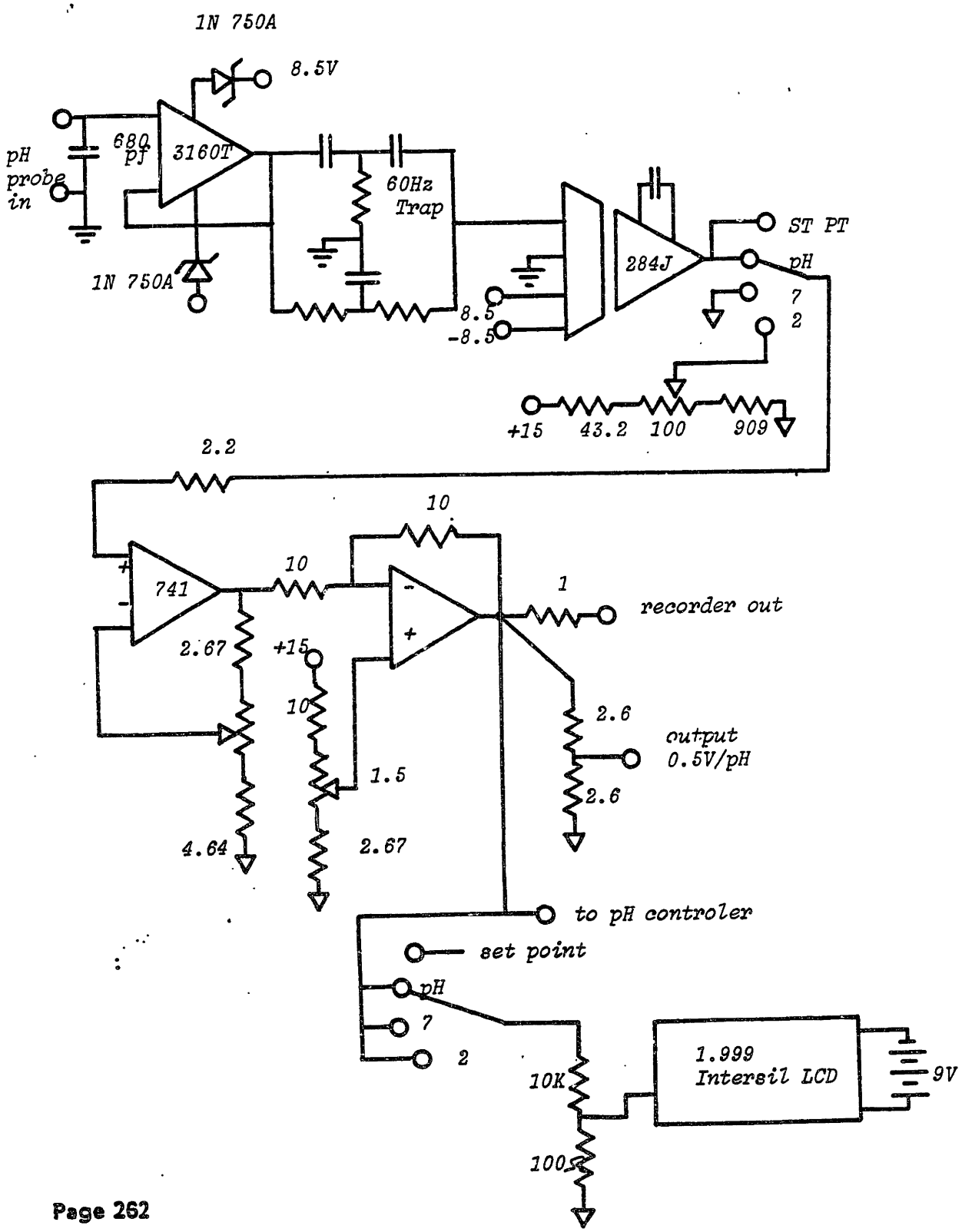
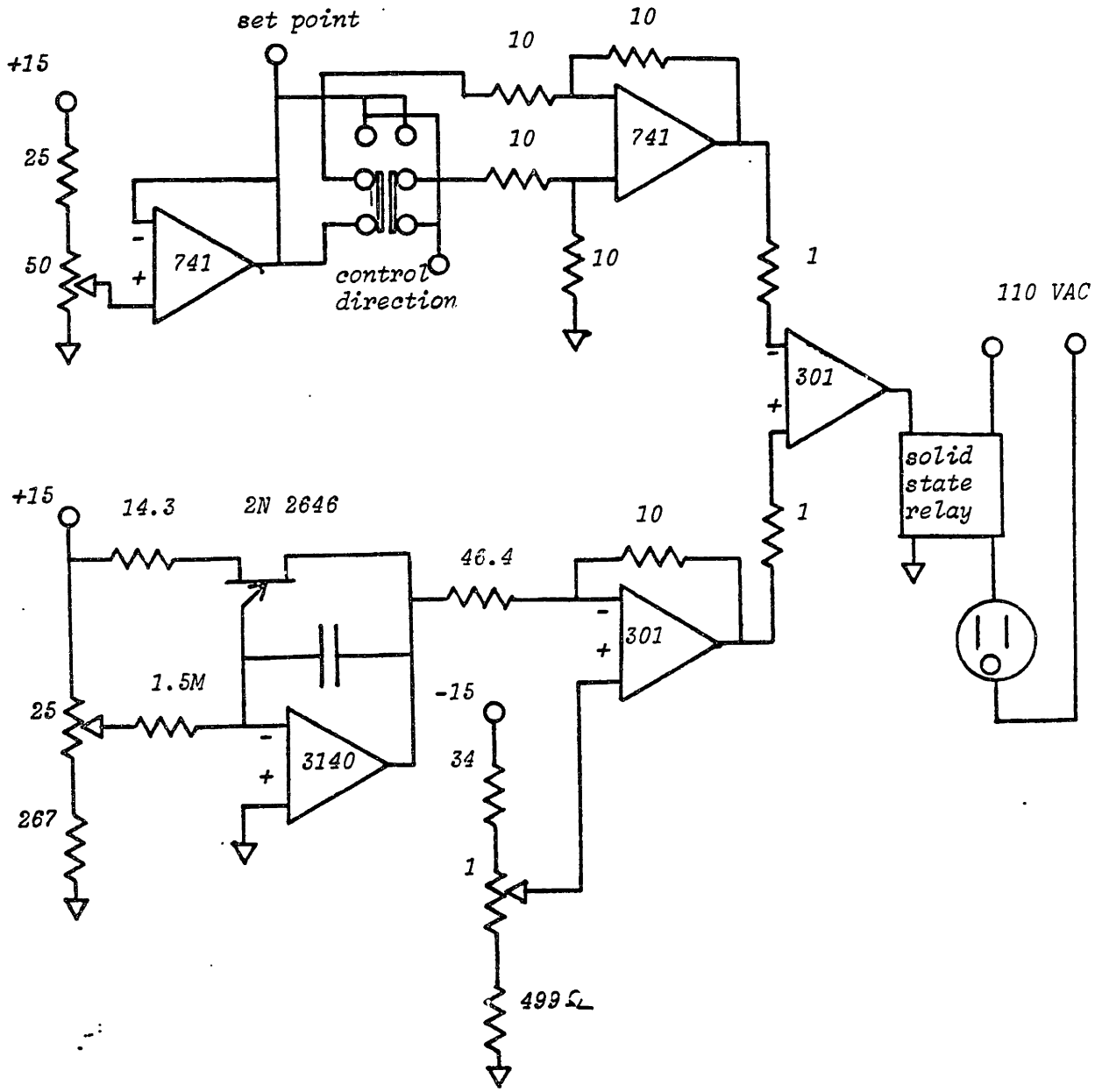
APPENDIX A: PH METER AND CONTROLLER

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741
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301
solid
state
relay
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25
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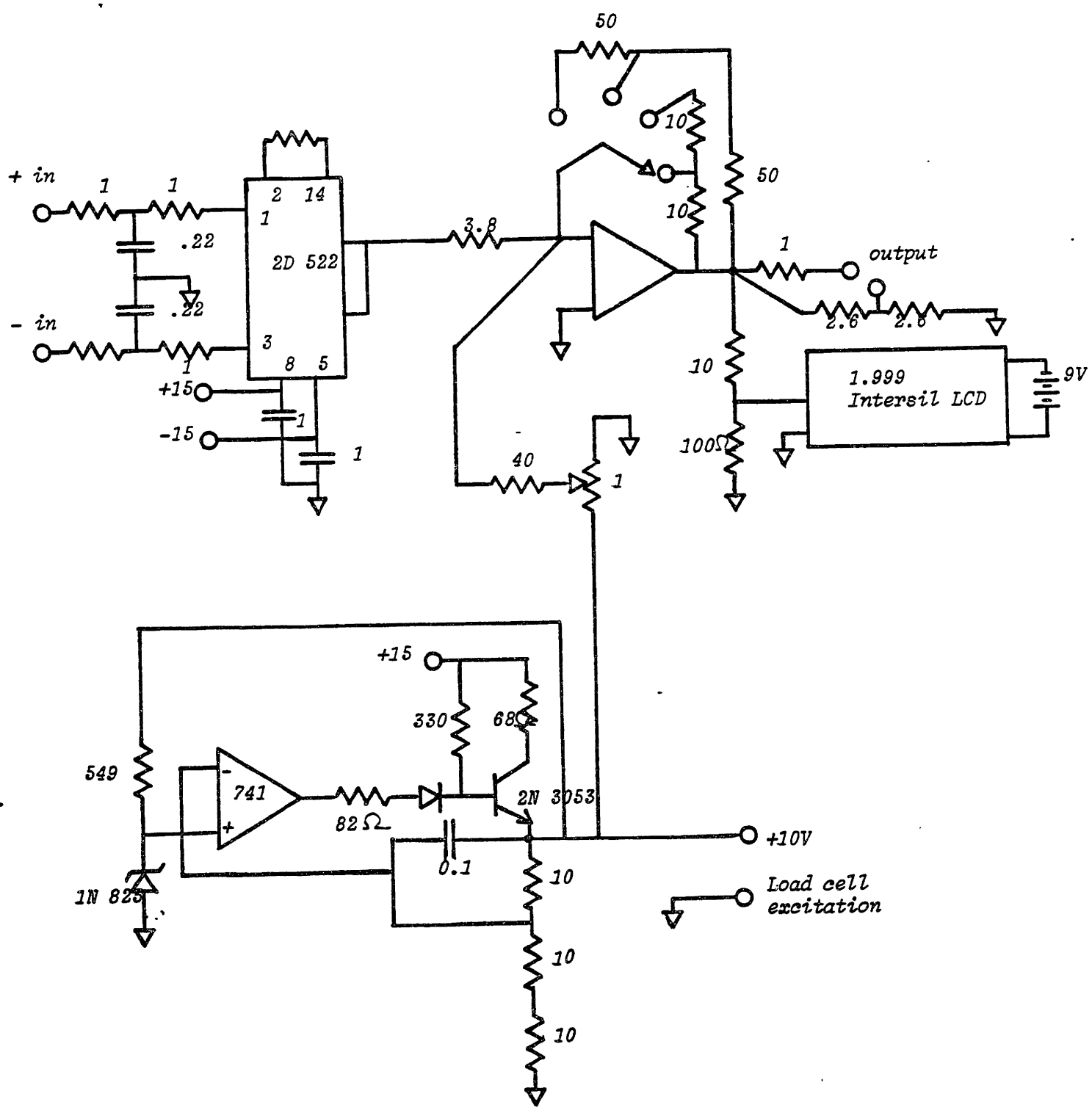
34
1
499Ω
301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14
1
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2D 522
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1
output
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2.6
2.6
- in
3
8
5
10
1.999
Intersil LCD
9V
+15
1
-15
1
1
40
1
100Ω
+15
330
68Ω
549
741
2N 3053
82Ω
+10V
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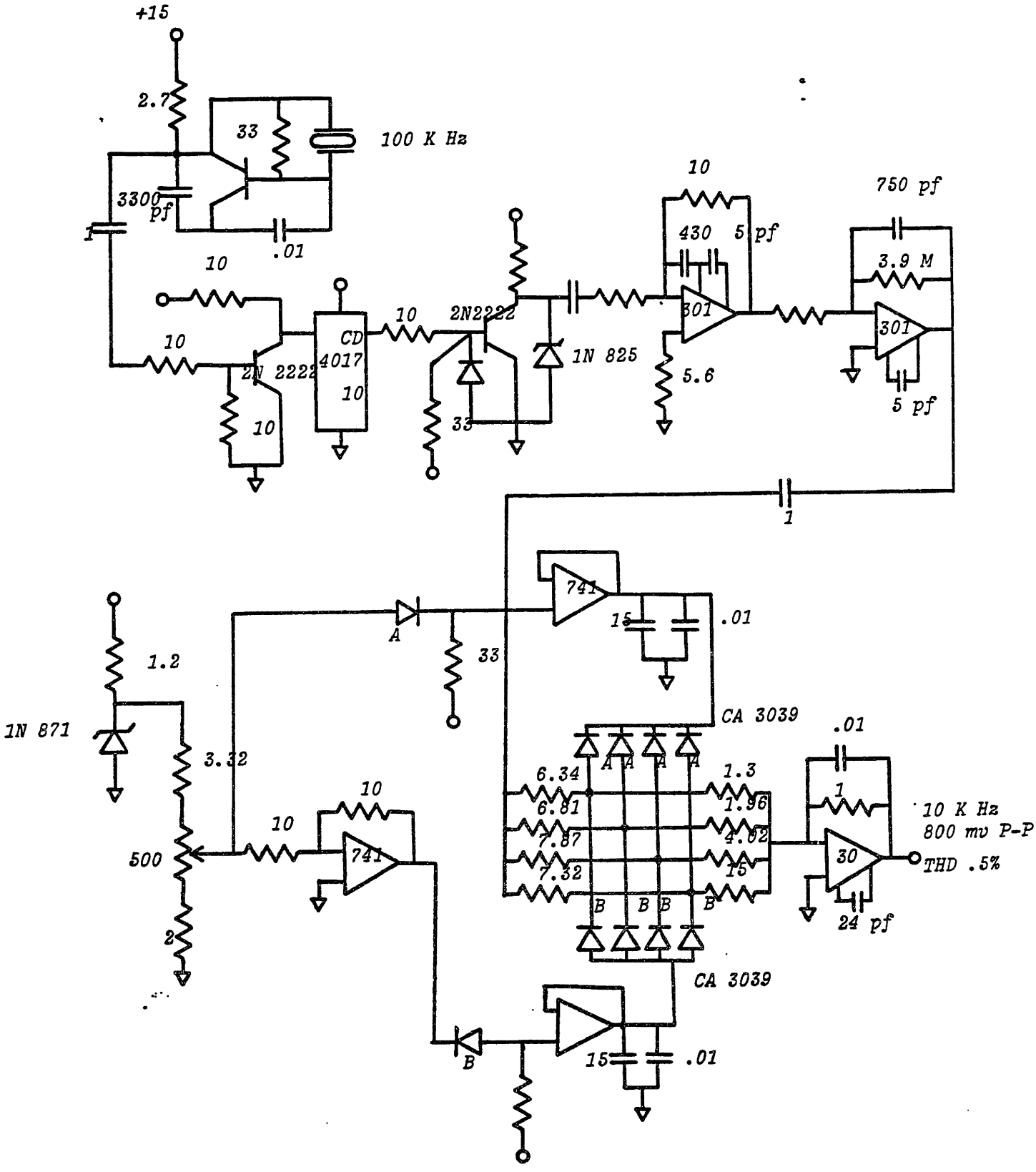
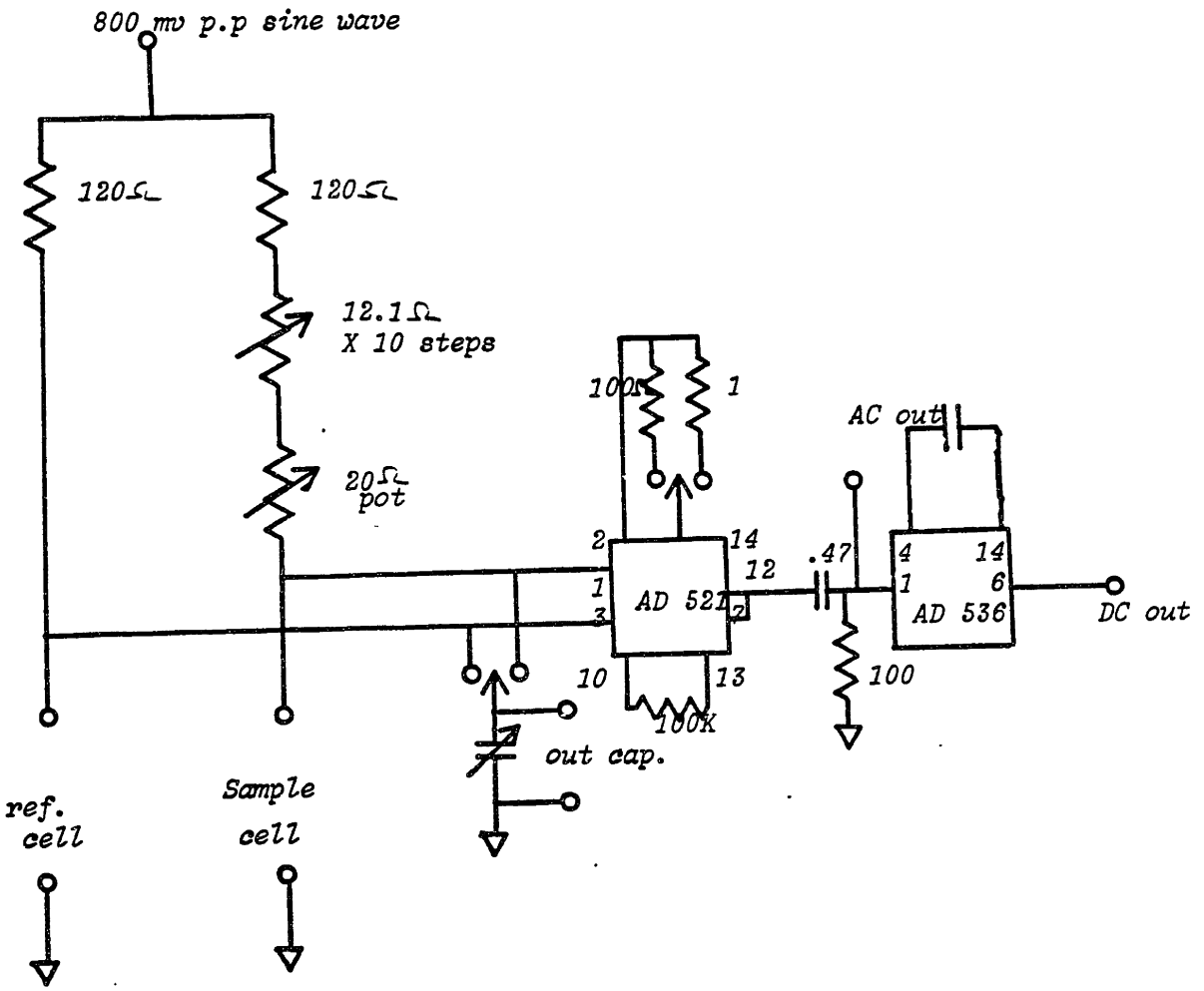
10
1N 825
Load cell
excitation
10
10

APPENDIX C: OSCILLATOR AND BRIDGE

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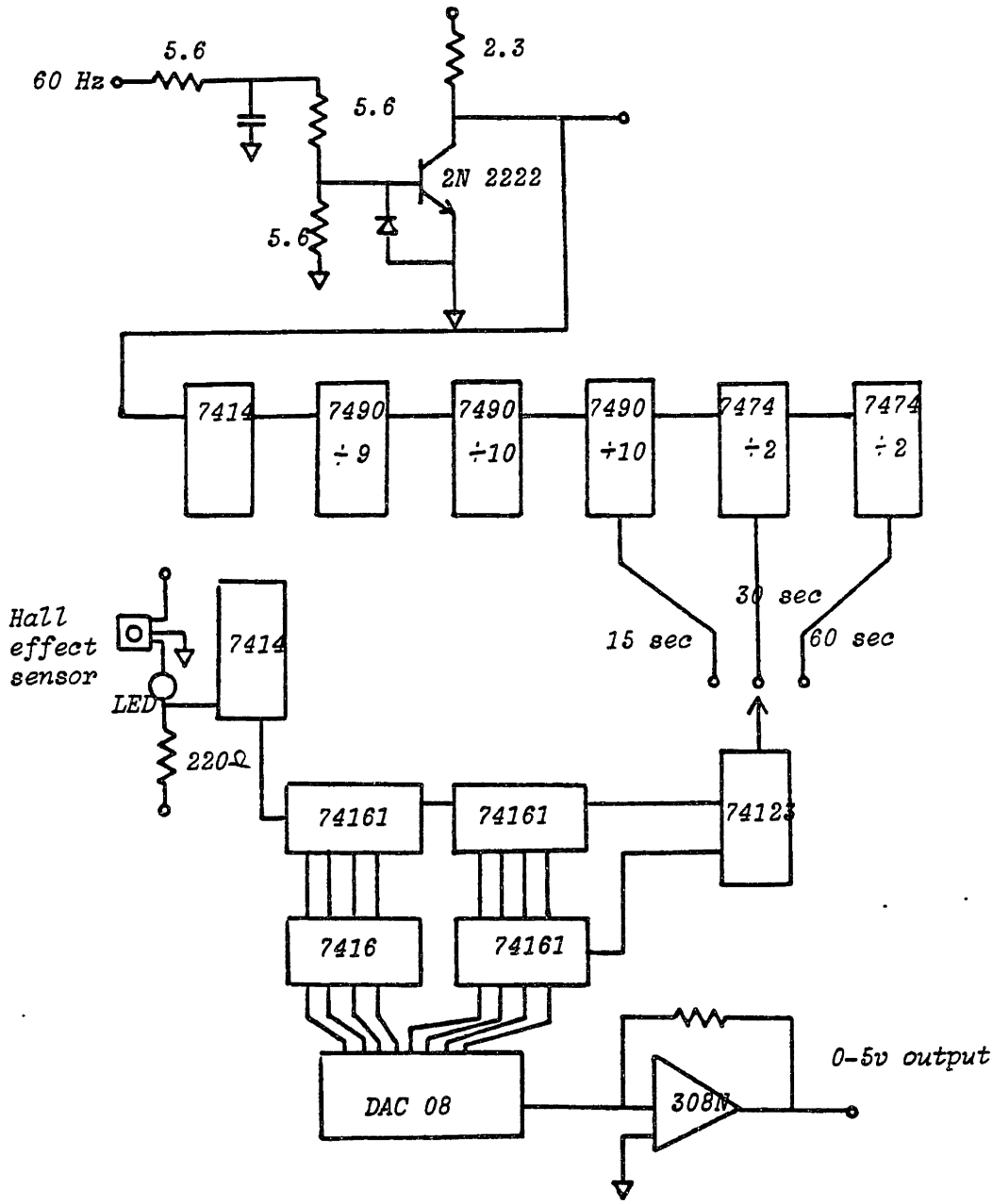
120Ω
120Ω
12.1Ω
X 10 steps
20Ω
pot
100Ω
1
AC out
2
14
12
1
3
AD 521
.47
4
14
1
6
AD 536
DC out
10
13
100K
100
out cap.
ref.
cell
Sample
cell

APPENDIX D: RPM METER



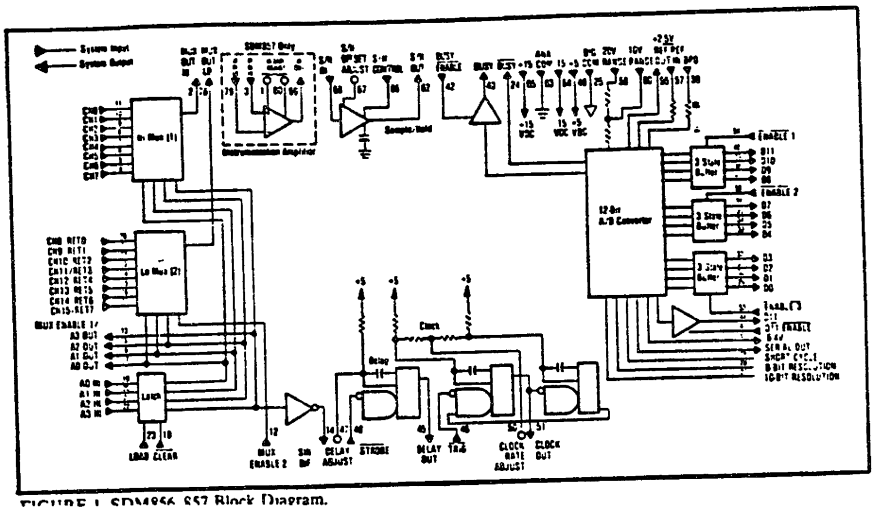
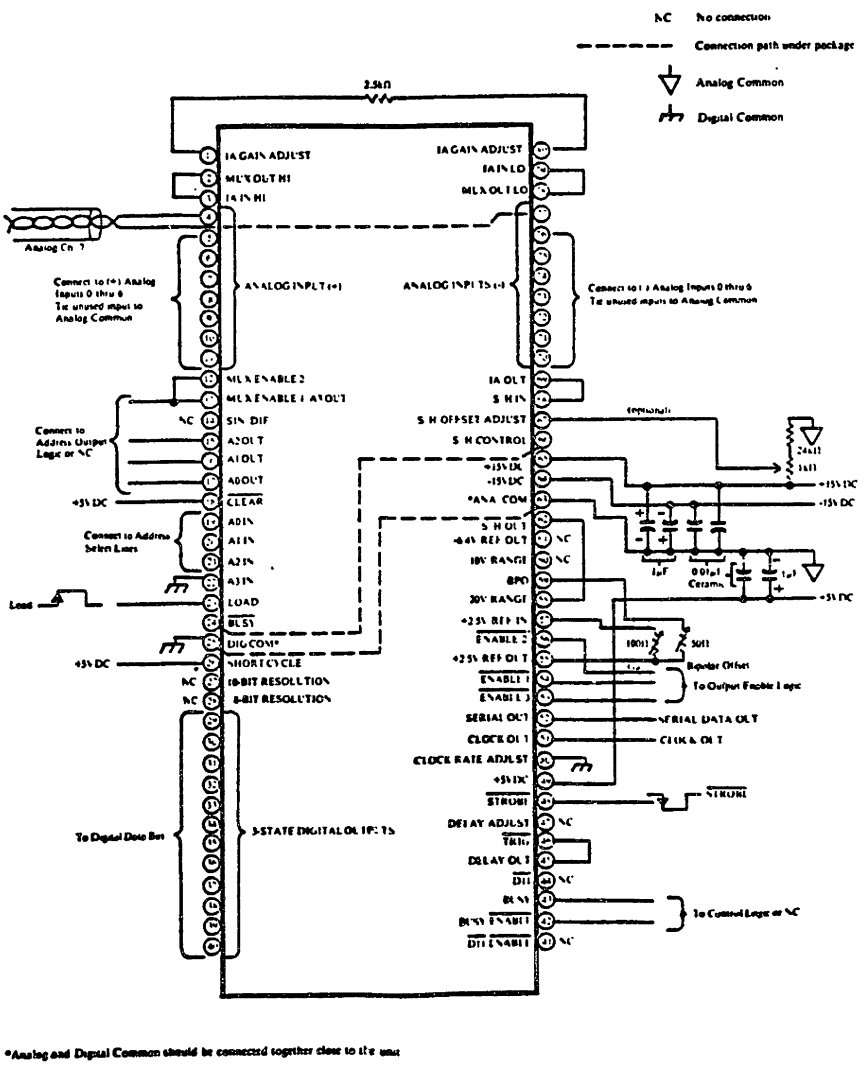


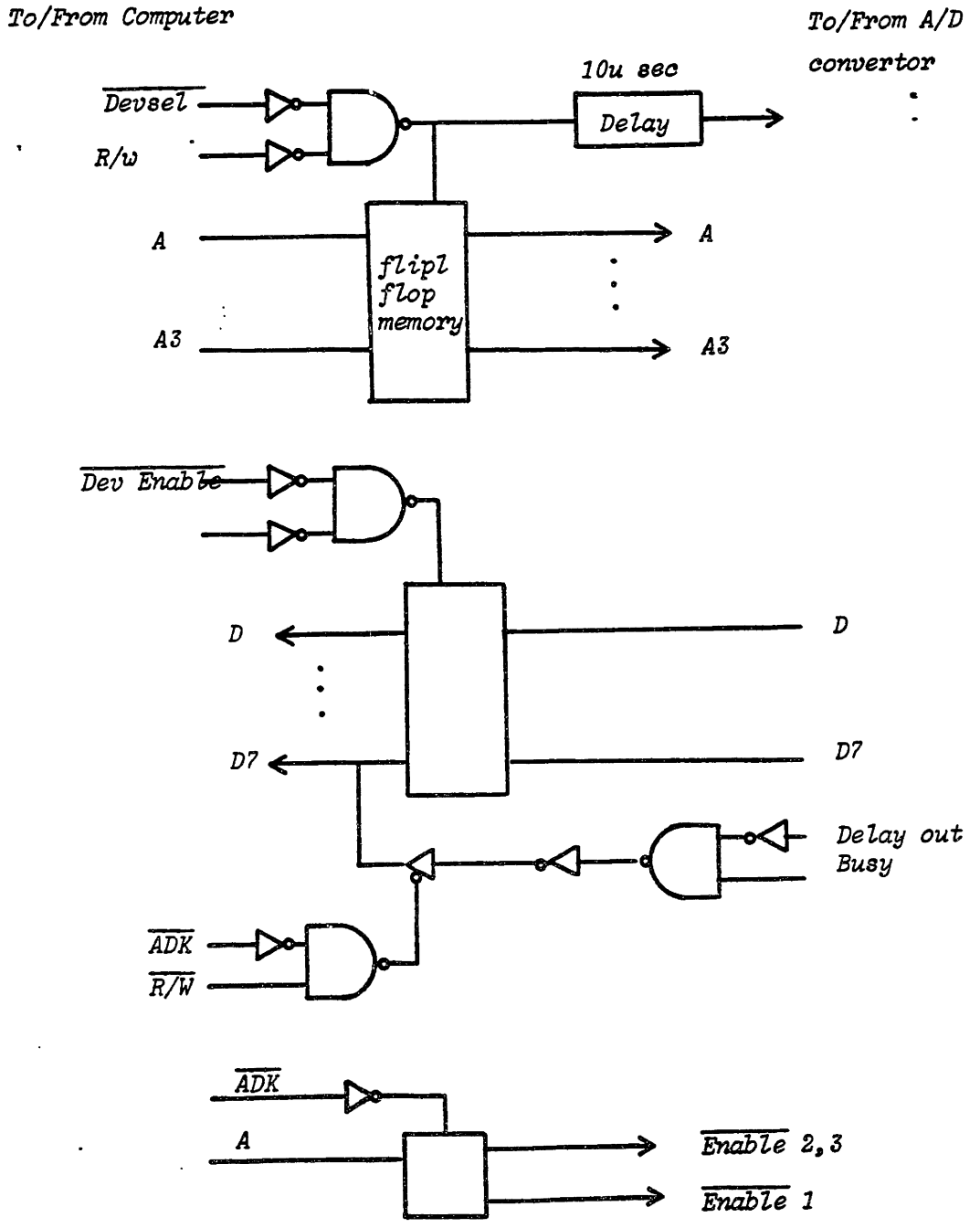
FIGURE 1. SDM4866 857 Block Diagram.

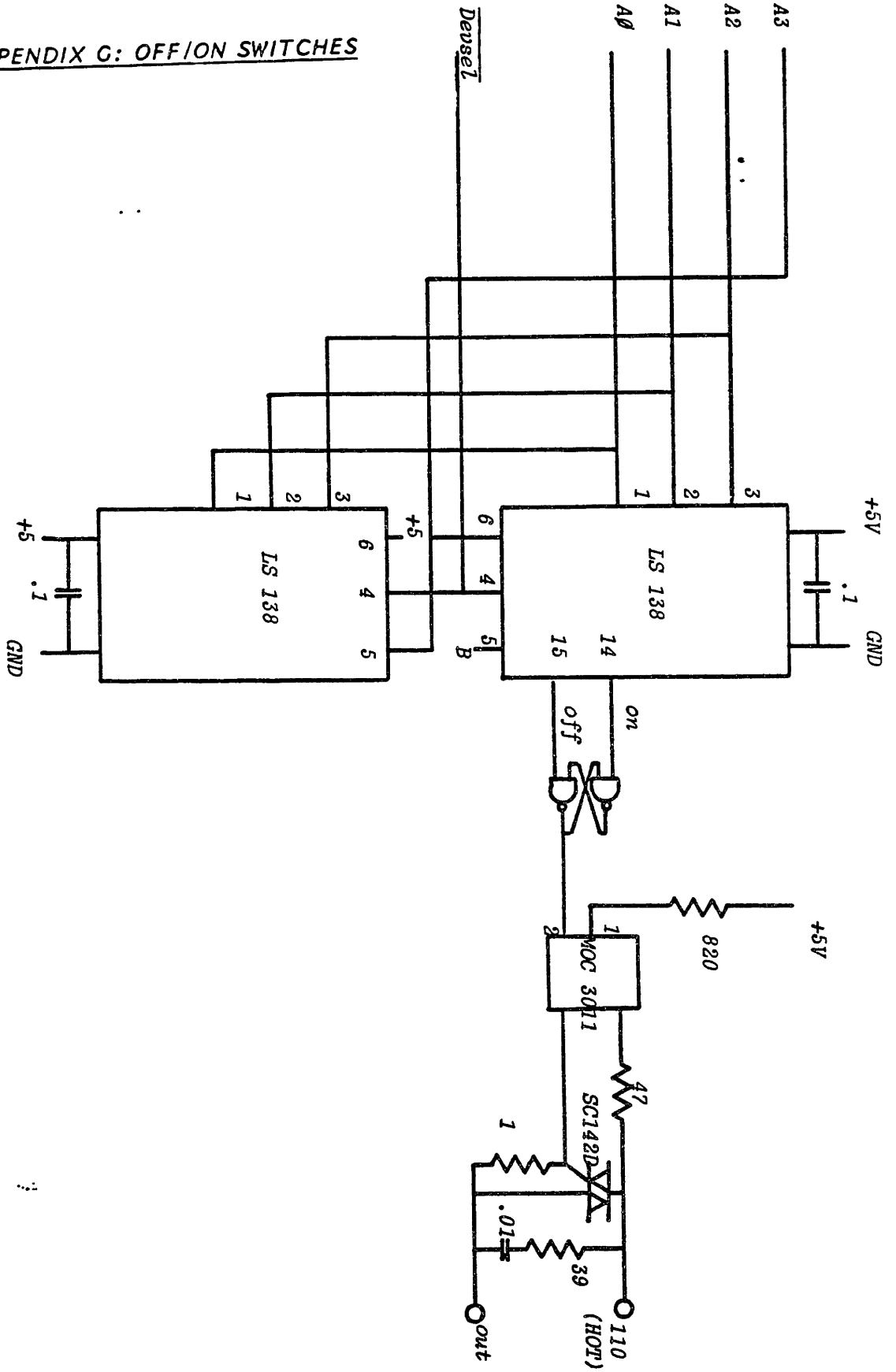


*Analog and Digital Common should be connected together close to the unit

FIGURE 2. Connection Diagram for SDM857 Operating Under These Conditions:
IA Gain = 10
Analog Input: Bipolar, differential
Reference Voltage: Internal
Resolution: 12-bits
Mode: Normal
Digital Output: Binary

APPENDIX F: A/D INTERFACE

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A0
DevSel
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.1
GND
LS 138
LS 138
+5
.1
GND
off
on
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MOC 3011
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SC142D
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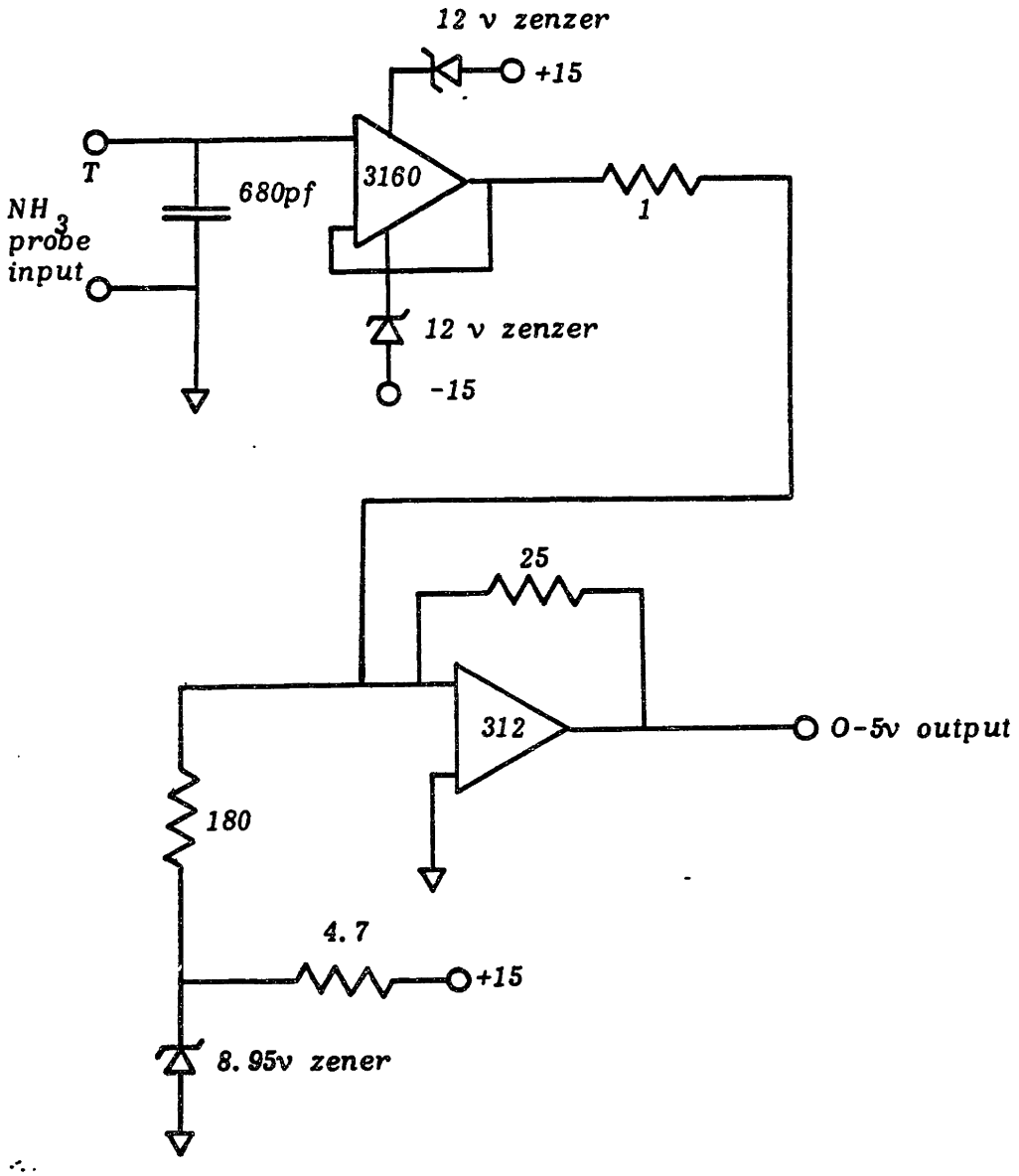
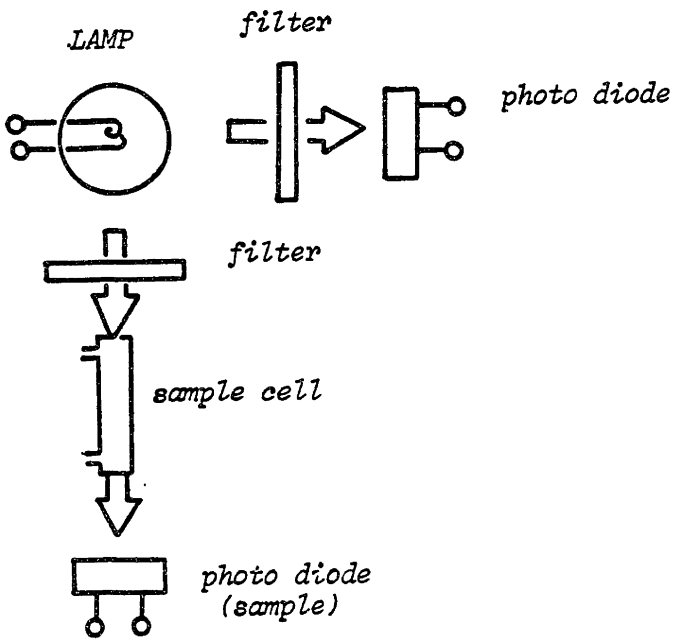
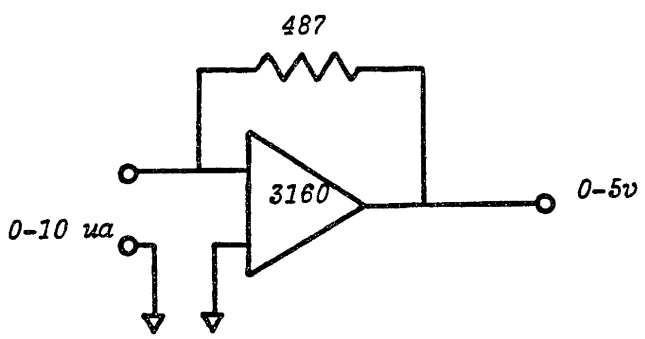
.01
39
out
110
(HOT)

APPENDIX H: AMMONIA PROBE

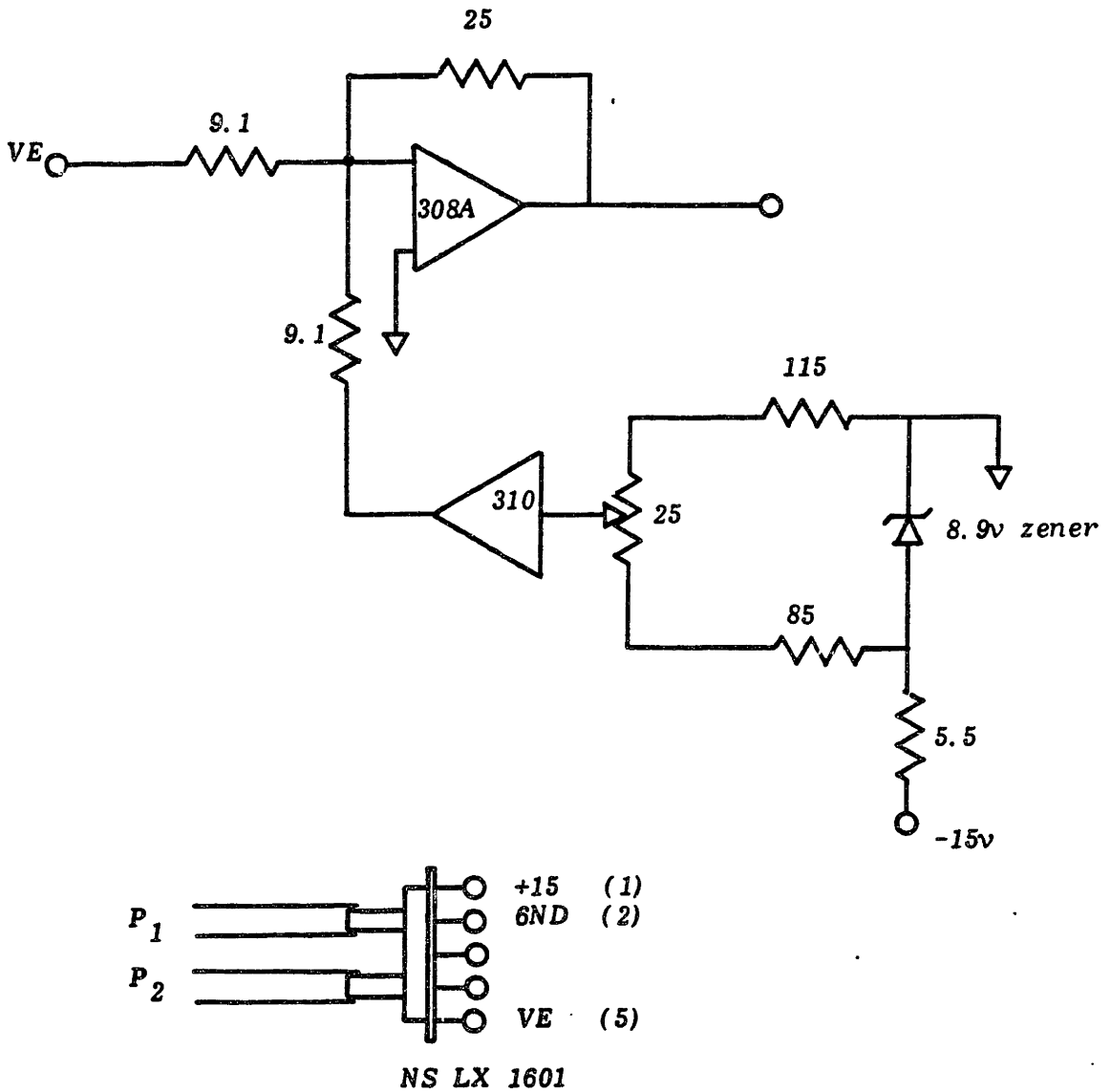


APPENDIX I: COLORIMETER





APPENDIX J: DIFFERENTIAL PRESSURE FLOW METER



National Semiconductor

Instrumentation Amplifiers

LH0036/LH0036C Instrumentation Amplifier

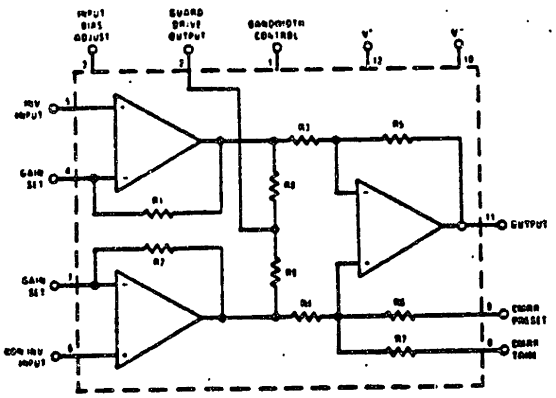
general description

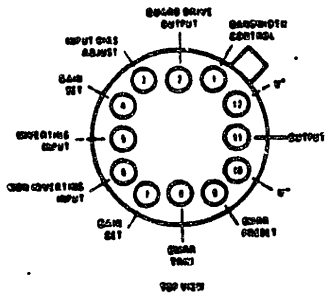
The LH0036/LH0036C is a true micro power instrumentation amplifier designed for precision differential signal processing. Extremely high accuracy can be obtained due to the 300 MΩ input impedance and excellent 100 dB common mode rejection ratio. It is packaged in a hermetic TO-8 package. Gain is programmable with one external resistor from 1 to 1000. Power supply operating range is between ±1V and ±18V. Input bias current and output bandwidth are both externally adjustable or can be set by internally set values. The LH0036 is specified for operation over the -55°C to +125°C temperature range and the LH0036C is specified for operation over the -25°C to +85°C temperature range.

features

■ High input impedance	300 MΩ
■ High CMRR	100 dB
■ Single resistor gain adjust	1 to 1000
■ Low power	90μW
■ Wide supply range	±1V to ±18V
■ Adjustable input bias current	
■ Adjustable output bandwidth	
■ Guard drive output	

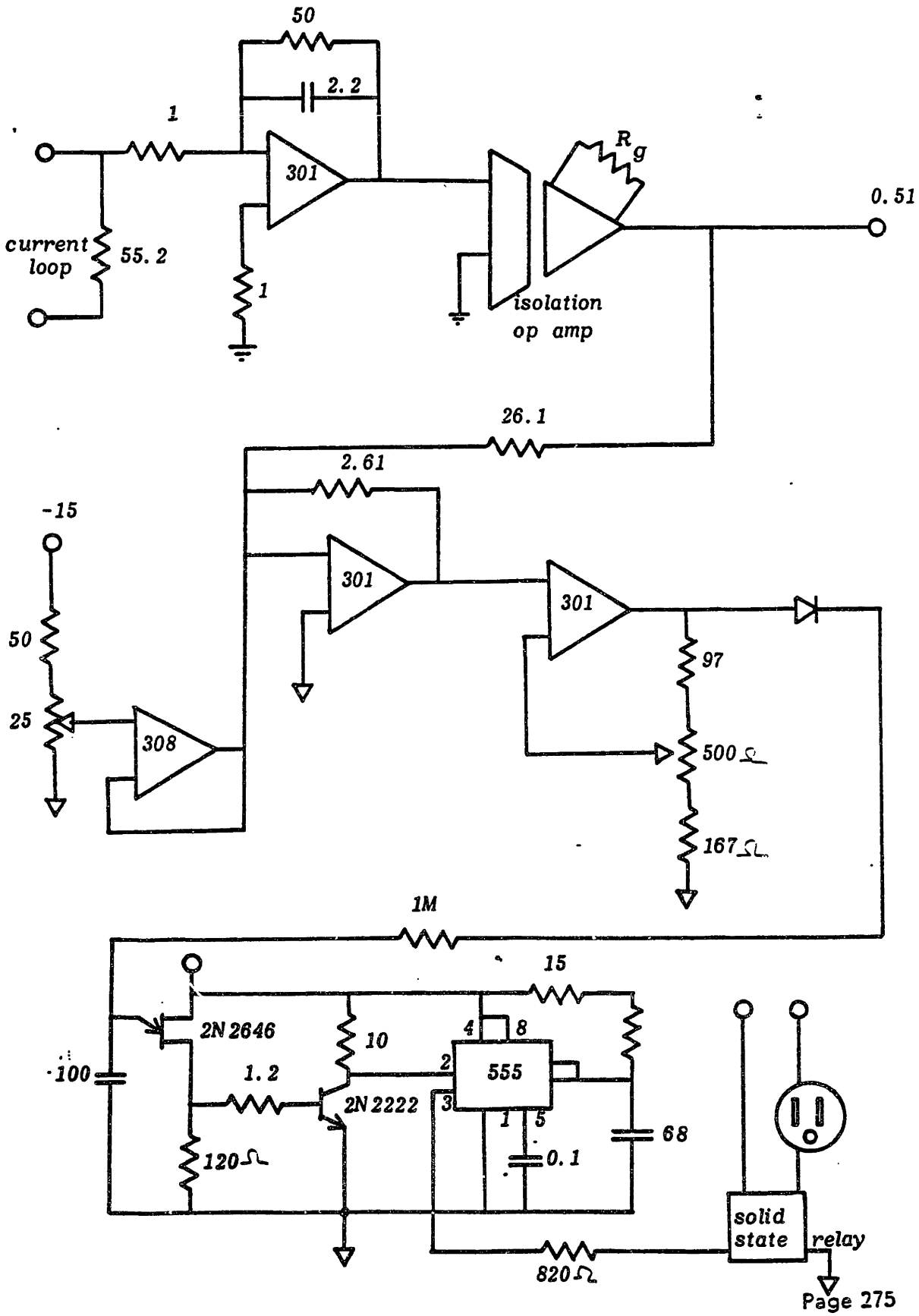
equivalent circuit and connection diagrams





Order Number LH0036 or LH0036C
See NS Package H12B

APPENDIX L: OXYGEN CONTROLLER



APPENDIX M: MAIN SYSTEM PROGRAM

```
(*$S+*)
(*$I-*)
Program ATPFLUX;

Uses turtlegraphics,transcend;

var
 cnsl,pntr:interactive;
 s,sdata,ssugar,sx:string;
   ami,amii,ams,baseconc,ccn,ccnp,CO2,CO2air,CO2cal,CO2f,
   CO2s,CO20,delay,dp,dpress0,fCO2,llt,lt,mgas,pcCO2,pv,
   sbc,sbrmc,sdsv,sf,sgr,slr,smc,sramm,srCO2,ssv,sugf,sugs,
   sug0,sug0conc,sxourp,tprime,tset,t0,t1,t2,volume0,
   weight0,xamm,xat,xCO2,xflow,xglu,xour:real;

 nave,ndisk,nfondisk,npoints,nsample:integer;
 xdata:array[1..50] of real;
 xsample:array[1..40] of
                  record
                    b:boolean;
                    n:real;
                    t:real;
                    v:integer;
                  end;


 Function A2D(select:integer):integer;
     external;

 Function time(select:integer):integer;
     external;

 Function keypress:boolean;
     external;
```

```
Procedure onoff(select:integer;data:boolean);
    external;

Procedure onoff1(select:integer;data:boolean);
    external;

Procedure hdump;
    external;

Procedure opr(select:integer);
    external;

Procedure note(pitch,duration:integer);
    external;


Procedure charsize(size:char); forward;

Function hours:real; forward;

Function et:real; forward;

Function ct:real; forward;

Procedure wait(interval:real); forward;

Function intadc(select,n:integer):real; forward;

Function DO2:real; forward;

Function rpm:real; forward;

Function pH:real; forward;

Function temp:real; forward;

Function loadcell:real; forward;
```

```
Function probe:real; forward;

Function ira:real; forward;

Function dpress:real; forward;

Function od:real; forward;

Function bdens:real; forward;

Function baseadded:real; forward;

Function vol:real; forward;

Function vprime:real; forward;

Function d:real; forward;

Function cglu:real; forward;

Function xtota:real; forward;

Function ctotap:real; forward;

Function xlac:real; forward;

Function clac:real; forward;

Function cCO2:real; forward;

Function cxamm:real; forward;

(*                                                    *)
(*                                                    *)
```

```
Segment Procedure our;
var i,n:integer;
    ns:string;
    f:interactive;
    cf,cg,c0,dc,df,dt,k,kla,k1,pDO2,r,t,t0,x,x1,y1,z:real;


(*                                                    *)
(*      graphic procedures used in our                *)
(*                                                    *)

(*                                                    *)
(*      draws notches on y axis                       *)
(*                                                    *)

      Procedure ynotch(n,x1,y1,y2:integer);
      var i,l:integer;
         begin

            for i:= 1 to n do
               begin

                  l:= (y2-y1) div (n+1);
                  pencolor(none); moveto(x1,y1+(i*l));
                  pencolor(white); move(4);
               end;
          end;

 (*                                                   *)
 (*      draws notches on x axis                      *)
 (*                                                   *)


      Procedure xnotch(n,x1,x2,y1:integer);
      var i,l:integer;
         begin
```

```
            for i:= 1 to n do
               begin

                  l:= (x2-x1) div (n+1);
                  pencolor(none); moveto(x1+(i*l),y1);
                  pencolor(white); move(4);
               end;
         end;


(*                                                    *)
(*      draws grid and labels axises                  *)
(*                                                    *)

      Procedure grid(sdata:string);
      var i,n,x,y:integer;
          ch:string;
         begin

            initturtle;

(*       draw box                                     *)
            pencolor(none);
            moveto(21,9); pencolor(white);
            moveto(21,191); moveto(279,191);
            moveto(279,9); moveto(21,9);

(*       notch y axis                                 *)
            turnto(0); ynotch(3,21,9,191);
            turnto(180); ynotch(3,279,9,191);

(*       notch x axis                                 *)
            turnto(90); xnotch(4,21,279,9);
            turnto(270); xnotch(4,21,279,191);
```

```
(*      number axises and label figure                    *)
        for i:= 1 to 4 do
           begin

              n:= 25*i;
              x:= 0;
              y:= (i*182 div 4)+1;

              pencolor(none); moveto(x,y);
              str(n,ch); wstring(ch);
           end;

        for i:= 1 to 4 do
           begin

              n:= 2*i;
              x:= (i*258 div 5)+10;
              y:= 0;

              pencolor(none); moveto(x,y);
              str(n,ch); ch:= concat('.',ch);
              wstring(ch);
           end;

        moveto(268,0); wstring('1.');

        moveto(150,180); wstring(sdata);

     end;


     Procedure plot(x,y:real);

  (*  x and y in range of 0 - 100                         *)

     var x1,y1:integer;
        begin
```

```
        x1:= round(x*2.58)+21;
        y1:= round(y*1.82)+9;

         grafmode;
         pencolor(none);    (* draw cross at x,y *)
         moveto(x1+2,y1); pencolor(white);
         moveto(x1-2,y1); pencolor(none);
         moveto(x1,y1+2); pencolor(white);
         moveto(x1,y1-2); pencolor(none);
      end;


   Procedure scaleour(x,y:real; var x1,y1:real);
   (* scale our data for plotting *)
      begin
         x1:= x*100;
         y1:= y;
      end;


(*                                                    *)
(*     function calculates solubility of oxygen      *)
(*      in the media at the present temperature       *)
(*                                                    *)

   Function O2sat(t:real):real;
   var a0,a1,a2,b,ch,ci,k,k1,lpv,n,p,pv:real;
      begin

         a0:= -4.0605;
         a1:= -5416.7;
         a2:= 1026100.0;
         t:= t + 273.15;

         k1:= a0 + (a1/t) + (a2/(t*t));
         k:= 1.0/exp(k1);
```

```
        p:= 0.99568 - (0.000335*(t-303.15));

        b:=1244142.0*p/k;
        n:=b/(82.05*t);

        lpv:= 8.0479 - (1718.75/(t-40.55));
        pv:= exp(lpv);

        ch:= (760.0-pv)/760.0;
        ci:= 0.973;

        O2sat:= n*ch*ci*0.2095;

      end;


(*                                                    *)
(*    function calculates change in our over the      *)
(*     last twenty hours                              *)
(*                                                    *)

    Function slope:real;
    var a,b,l,lys,xlys,x,xs,xxs:real;
        i:integer;
       begin

          lys:=0;
          xlys:=0;
          xs:=0;
          xxs:=0;

       if npoints >= 4 then
          begin

             for i:= 1 to 4 do
                begin
```

```
                if xdata[npoints-4+i] > 0.0
                    then x:= ln(xdata[npoints-4+i])
                    else x:= 0.0;
                lys:= lys+x;
                xlys:=xlys + (i*5*x);
                xs:=xs+(i*5);
                xxs:= xxs+(i*i*25);
              end;

            b:=(xlys-(xs*lys/4))/(xxs-(xs*xs/4));
            slope:=b;
          end

        else slope:=0;

      end;


(*                                                    *)
(*     procedure to print system parameters out       *)
(*      to the printer for hard copy record           *)
(*                                                    *)


    Procedure print;
    var k,n:integer;
        y,x:real;
      begin

        charsize('u');
        writeln(pntr);
        writeln(pntr,'data observed [time = '
                    ,et:5:2,'hrs]');
        writeln(pntr);
        writeln(pntr,'pH    ',pH:5:2);
        writeln(pntr,'temp  ',temp:5:2,'c');
        writeln(pntr,'DO2   ',pDO2:5:2,'%');
```

```
        writeln(pntr,'vol    ',vol:5:2,'l');
        writeln(pntr,'vol''  ',vprime:5:2,'l');
        writeln(pntr,'rpm    ',rpm:6:1);
        writeln(pntr);
        writeln(pntr,'==============');
        writeln(pntr);
        writeln(pntr,
              'parameters      [ ]      [ ]''       cum');
        writeln(pntr);
        writeln(pntr,'total acids  ',xtota:6:2,
                     (xtota/d):8:2,ctotap:8:2);
        writeln(pntr,'CO2          ',xCO2:6:2,
                     (xCO2/d):8:2,cCO2:8:2);
        writeln(pntr,'lactic acid  ',xlac:6:2,
                     (xlac/d):8:2,clac:8:2);
        n:= length(ssugar); k:= 13-n;
        writeln(pntr,ssugar,' ':k,xglu:6:2,
                     (xglu/d):8:2,cglu:8:2);
        writeln(pntr,'ammonia      ',xamm:6:2,
                     (xamm/d):8:2,cxamm:8:2);
        writeln(pntr,'oxygen uptake',xour:7:3,
        writeln(pntr,'slope        ',slope:7:3);
        x:= (6*sxourp)+clac; y:= 6*sxourp;
        writeln(pntr,'atp          ',x:21:2);
        writeln(pntr,'atp from oxid',y:6:2);
        writeln(pntr,'last cell #  ',ccn:6:2,ccnp:8:2);
      end;

(*    write system parameter to end of our file      *)

    Procedure writef;
      begin

        writeln(f,pH:5:2,temp:5:2,DO2:5:2,
                  vol:5:2,vprime:5:2);
        writeln(f,xtota:6:2,(xtota/d):8:2,ctotap:8:2);
        writeln(f,xCO2:6:2,(xCO2/d):8:2,cCO2:8:2);
```

```
        writeln(f,xlac:6:2,(xlac/d):8:2,clac:8:2);
        writeln(f,xglu:6:2,(xglu/d):8:2,cglu:8:2);
        writeln(f,xamm:6:2,(xamm/d):8:2,cxamm:8:2);
        writeln(f,xour:7:3,(xour/d):8:3,sxourp:8:3);
        writeln(f,slope:7:3);
        writeln(f,ccn:6:2,ccnp:8:2);
      end;


  begin   (*   begin our  *)

    writeln(' our measurement in progress');

    pDO2:= DO2;
    onoff(0,odd(2));   (*turn off O2 control *)
    onoff(1,odd(1));   (*turn on mixed gases *)

    tprime:= et;
    wait(0.25);     (*allow time for head space to *)
                    (*equilibrate with mixed gases *)
    textmode;
    llt:= lt; lt:= et+0.25;
    i:=trunc(lt);
    str(i,ns);
    sdata:= concat('#5:',sx);
    sdata:= concat(sdata,ns);

(*      create new file and makes the file as open      *)
(*       file of type 'sx'nn.text                      *)
(*       where nn is the elapsed time in hrs            *)

    rewrite(f,sdata);

    grid(concat(sx,ns));

(*      initialize parameters of our                    *)
```

```
        cg:= (mgas/20.95)*(100-CO2);
        c0:=DO2;
        kla:= 0.414/exp(2.05*ln(vol));
        n:= 1;
        r:= rpm;
        x:= DO2;
        z:= 0.0;

        t:= hours;
        t0:= t;

        write(f,(t-t0),x,r);

        while ((t-t0) < 1.0) and (x > 25.0) do
           begin

              wait(0.01);

              n:= n+1;
              t:=hours;
              r:= rpm;
              x:= DO2;

    (*      transfer across 10 liter surface        *)
              k:= kla*r;
    (*      transfer across si rubber tubing        *)
              k1:= 0.146 + (0.0022*r);
              k:= k+k1;

              df:= k * (cg - x) * 0.2095 / 100.0;
              z:= z + df;

              write(f,(t-t0),x,r);

              scaleour(t-t0,x,x1,y1);
              plot(x1,y1);
```

```
        end;

(*      write eol to file on disk                              *)

      textmode;
      writeln(f);

      cf:= x;
      z:= z/n;

      dc:= (cf-c0)*O2sat(temp)*10.0;
      dt:= (t-t0); if dt = 0.0 then dt:= 0.01;
      dc:= dc/dt;

      xour:= z-dc;

      writef;
      close(f,lock);

      textmode;

      npoints:= npoints+1;
      nfondisk:= nfondisk+1;
      xdata[npoints]:= xour/d;
      onoff(0,odd(1)); onoff(1,odd(2));
      print;
      sxourp:=sxourp + (xour*(lt-llt)/d);

  end; (* end of our *)


Segment Procedure initialize;
  begin
```

```
baseconc:= 0.250;
ccn:= 0.0;
ccnp:= 0.0;
CO2:= 0.0;
dpress0:= 0.0;
llt:= 0.0;
lt:= 0.0;
mgas:= 9.15;
nfondisk:= 0;
npoints:= 0;
nsample:= 0;
pv:= 0.015;
sbc:= 0.0;
sbrmc:= 0.0;
sdsv:= 0.0;
sgr:= 0.0;
slr:= 0.0;
smc:= 0.0;
sramm:= 0.0;
srCO2:= 0.0;
ssv:= 0.0;
sx:= 'fs4';
sxourp:= 0.0;
tset:= 37.0;
volume0:= 10.0;
weight0:= loadcell;
xamm:= 0.0;
xCO2:= 0.0;
xour:= 0.0;

s:= 'console:';
reset(cnsl,s);  (* initialize ctr     - file id: cnsl *)
s:= 'printer:';
reset(pntr,s);  (* initialize pinter - file id: pntr *)

t0:= hours;
```

```
    write(' input delay time in hours :');
    readln(delay);

    t1:= et;
    writeln('input sugar concentration of media');
    write(' in mmoles/l: ');
    readln(sug0conc);
    xglu:= sug0conc;

    writeln('input sugar type <eg glucose>');
    readln(ssugar);

    writeln('input CO2 calibration gas conc');
    writeln(' <eg 0.5%>');
    readln(CO2cal);

  end;


Segment Procedure waitdt(interval:real);
var t,t0,t10:real;


(*                                                    *)
(*      procedure to interactively change and/or      *)
(*        system varialbes                            *)
(*                                                    *)

      Procedure xsysparm(var x:real; s,su:string;
                         n1,n2:integer);
      var t1,t,z:real;
          c:char;
        begin

          page(cnsl);
          writeln;
          writeln('the system variable ',s);
```

```
            writeln(' presently equ. ',x:n1:n2,su);
            write(' do you wish to change (y/n)?');

            t1:= hours;
            t:= hours;

            while (not keypress) and ((t-t1)<0.01) do
                 t:= hours;

            if keypress then
               begin

                  read(c);
                  if c = 'y' then
                     repeat

                        writeln; writeln;
                        write('type new value :');
                        readln(z);
                        if ioresult<>0 then z:= 0.0;
                        x:= z;
                        writeln(s,' now equ. ',
                                x:n1:n2,su);
                        write(' is this correct (y/n)?');
                        read(c);
                      until c = 'y';
                end;

          end;


(*      displays last our plot                                  *)


          Procedure dlast;
             begin
```

```
            grafmode;
            wait(0.005);
            textmode;
          end;

(*      procedure records sample data                    *)


        Procedure xrecord;
        var t,t1:real;
            c:char;

              Procedure case1;
              var c:char;
                  iv:integer;
                  sv,t:real;
                 begin

                    t:= et;
                    writeln;
                    writeln(' enter sample volume in ml');
                    readln(iv);
                    if ioresult<>0 then iv:= 0;
                    writeln;
                    writeln(' a ',iv,' ml sample was');
                    writeln(' withdrawn at time = ',t:4:1,
                            ' hrs');
                    write('   this is correct (y/n)? ');
                    read(c);
                    if c = 'y' then
                       begin

                          nsample:= nsample + 1;
                          sv:= iv/1000.0;
                          ssv:= ssv + sv;
                          sdsv:= sdsv + (d * sv);
```

```
            xsample[nsample].t:= t;
            xsample[nsample].b:= false;
            xsample[nsample].v:= iv;
          end;
      end;



Procedure case2;
var c:char;
    i:integer;
    t,x:real;
   begin

      read(c);
      writeln;writeln;writeln;
      writeln(' do you wish to ',
              'record cell count(y/n)?');
      read(c);
      if c = 'y' then
         begin

            page(cnsl);
            writeln(' #    hr    ',
                    'vol   cell count');
            writeln;
            for i:= 1 to nsample do
                begin

                  t:= xsample[i].t;
                  if xsample[i].b = false
                  then writeln(i:2,t:7:1,
                         xsample[i].v:6,
                               '     nd')
                  else writeln(i:2,t:7:1,
                         xsample[i].v:6,
```

```
                                    xsample[i].n:9:1);
                            end;

                        writeln;
                        write('input sample number ',
                              'of cell count :');
                        readln(i);
                        if ioresult<>0 then i:= 0;
                        write('input cell count :');
                        readln(x);
                        if ioresult<>0 then x:= 0.0;
                        writeln(' cell count of ',
                           'sample ',i:3,' = ',x:4:1);
                        write(' is this correct ',
                              '(y/n)?');
                        read(c);
                        if c = 'y' then
                           begin

                              xsample[i].n:= x;
                              xsample[i].b:= true;
                              ccn:= x;
                              ccnp:= x/d;
                              wait(0.001)
                            end;
                      end;

                end;


        begin

           page(cnsl);
           writeln;
           writeln('do you wish to record ');
           write('   sample time or cell count (y/n)? ');
```

```
                t1:= hours;
                t:= hours;

                while (not keypress) and ((t-t1)<0.01) do
                      t:= hours;

                if keypress then
                   begin

                      read(c);
                      if c = 'y' then
                         begin

                            writeln;writeln;writeln;
                            writeln('to record sample time ',
                                    'enter - 1');
                            writeln('to record cell count ',
                                    'enter - 2');
                            write(' ?:');
                            readln(c);
                            case c of

                                '1': case1;
                                '2': case2;
                             end;
                         end;
                   end;
             end;

    (*                                                    *)
    (*      procedure manages bookkeeping in refilling    *)
    (*       base reservour on loadcell                   *)
    (*                                                    *)
```

```
Procedure xbaseres;
var c:char;
    dx,t,t1,x,x0,x1:real;
   begin

      page(cnsl);
      writeln;
      writeln(' do you wish to refill base ',
              'loadcell (y/n)? ');
      t1:= hours;
      t:= hours;

      while (not keypress) or (t-t1>0.01) do
            t:= hours;

      if keypress then
         begin

            read(c);
            if c = 'y' then
               begin

                  writeln;writeln;
                  writeln(' turn off base ',
                          'addition pump');
                  writeln('  and hit <ret>');
                  readln;

                  x0:= loadcell;
                  writeln;
                  writlen('fill reservour ',
                          'w/ base and hit <ret>');
                  readln;

                  wait (0.01);
                  x1:= loadcell:
                  dx:= x1-x0;
```

```
                    sbc:= sbc + dx;
                    writeln;
                    writeln('turn on base ',
                            'addition pump');
                    wait(0.01);
                  end;
              end;
        end;(* xbaseres *)

(*                                                    *)
(*      procedure manages bookkeeping for media change  *)
(*                                                    *)

      Procedure xmedia;
      var c:char;
          brmc,gr,mc,rCO2,t,totg,t1,vx,v1,v2,xta:real;
        begin

          page(cnsl);
          writeln;
          writeln('do you wish to change the ',
                  'media (y/n)?');
          t1:= hours;
          t:= hours;

          vx:= vol;

          while (not keypress) or (t-t1>0.01) do
               t:= hours;

          if keypress then
             begin

               read(c);
               if c = 'y' then
                  begin
```

```
writeln;writeln;
writeln('turn off base ',
        'addition pump');
writeln(' & hit <ret>');
readln;

repeat
   writeln;
   write(' enter volume of ',
         'media removed: ');
   readln(v1);
   if ioresult <> 0
      then v1:= 0.0;
   writeln;
   writeln(' volume is now = ',
             (vx-v1):4:2,'l');
   writeln(' is this correct ',
           '(y/n?)');
   read(c);
until c = 'y';

repeat
   writeln;
   write(' enter volume of ',
         'media added: ');
   readln(v2);
   if ioresult <> 0 then v2:= 0.0;
   writeln;
   writeln(' volume is now = ',
           (vx-v1+v2):4:2,'l');
   writeln(' is this correct ',
           '(y/n?)');
   read(c);
until c = 'y';
```

```
                    xta:= xtota;
                    mc:= v1-v2;
                    smc:= smc + mc;

                    totg:= (xglu * (vx-v1)) +
                                (sug0conc * v2);
                    gr:= totg - (xglu * vx);
                    xglu:= totg/vol;
                    sgr:= sgr + (gr/vprime);

                    brmc:= v1 * (xta);
                    sbrmc:= sbrmc + brmc;

                    rCO2:= (v1*xCO2)/vprime;
                    srCO2:= srCO2 + rCO2;
                    xCO2:= xCO2*(vx-v1)/vol;

                    sramm:= sramm + (xamm*v1/vprime);
                    xamm:= xamm*(vx-v1)/vol;

                end;

            end;

        end; (* xmedia *)

(*                                                    *)
(*    procedure check zero reading on flow gauge      *)
(*      to CO2 analyzer                               *)
(*                                                    *)


    Procedure zdpress0;
    var c:char;
    t,t1:real;
      begin
```

```
        page(cnsl);
        writeln;
        writeln(' do you wish to zero flow ',
                'gauge (y/n)?');
        t1:= hours;
        t:= hours;

        while (not keypress) and ((t-t1)<0.01) do
        t:= hours;

        if keypress then
           begin

              read(c);
              if c = 'y' then
                 begin

                    writeln('turn off flow ');
                    wait(0.01);
                    dpress0:= dpress;
                    writeln('zero pressure now ',
                            'set at ',dpress0:4:2,
                            'volts');
                    wait(0.001);
                 end;
           end;
     end; (* zdpress0 *)


(*                                                    *)
(*     send cell sample record to printer             *)
(*                                                    *)


     Procedure precord;
     var t:real;
```

```
    i:integer;
  begin

     charsize('u');
     writeln(pntr);
     writeln(pntr,' #     hr    vol   ',
                  'cell count');
     writeln(pntr);
     for i:= 1 to nsample do
         begin

            t:= xsample[i].t;
            if xsample[i].b = false
               then writeln(pntr, i:2,t:7:1,
                            xsample[i].v:6,
                            '       nd')
               else writeln(pntr, i:2,t:7:1,
                            xsample[i].v:6,
                            xsample[i].n:9:1);
         end;
  end;

(*                                                    *)
(*     procedure change disks and zero nfondisk
(*                                                    *)


  Procedure xdisk;
  var c:char;
  t,t1:real;
     begin

        page(cnsl);
        writeln;
        writeln(' do you wish to change disk (y/n)?');
        t1:= hours;
        t:= hours;
```

```
        while (not keypress) and ((t-t1)<0.01) do
        t:= hours;

        if keypress then
           begin

              read(c);
              if c = 'y' then
                 begin

                    writeln('change disk in drive 2');
                    nfondisk:= 0;
                    wait(0.001);
                 end;
           end;
     end; (* xdisk *)



Procedure reestablish;
var c:char;
    s:string;
    i:integer;
    t,t1:real;
   begin

      read(c);
      textmode;
      page(cnsl);

      writeln;
      writeln(' to change the system ',
              'variables type ');
      writeln('    the folowing number');
      writeln;
      writeln('0 - change media');
```

```
writeln;
writeln('1 - initial volume');
writeln('2 - CO2 conc (old)');
writeln('3 - CO2air constant');
writeln('4 - temperature set point');
writeln('5 - display last our measurement');
writeln;
writeln('6 - record sample time ',
        'or cell count');
writeln('7 - mixed gas concentration');
writeln('8 - to print last our plot');
writeln('9 - base concentration');
writeln;
writeln('a - refill the loadcell with base');
writeln('b - to obtain hard copy sample ',
        'record');
writeln('c - zero flow gauge for CO2 ira');
writeln('d - sample volume to pumps');
writeln('e - change disks');

t1:= hours;
t:= hours;
while (not keypress) and ((t-t1)<0.01) do
      t:= hours;

if keypress then
   begin

      read(c);
      case c of

         '0': xmedia;
         '1': xsysparm(volume0,
                'the initial volume','l',5,2);
         '2': xsysparm(CO2,'the CO2 conc.',
                '%',5,2);
         '3': xsysparm(CO2air,
```

```
                    'CO2 in air const.','v',5,3);
                '4': xsysparm(tset,
                    'the temperature setting',
                    'c',5,2);
                '5': dlast;
                '6': xrecord;
                '7': xsysparm(mgas,
                    'the mixed gas conc.',
                    '%',5,2);
                '8': hdump;
                '9': xsysparm(baseconc,
                    'the base conc.','m',5,3);
                'a': xbaseres;
                'b': precord;
                'c': zdpress0;
                'd': xsysparm(pv,'pump vol','l',5,3);
                'e': xdisk;

            end;

        end;

end; (* reestablish *)


    Procedure beep;
    var duration,i,pitch: integer;
      begin

        duration:= 20;
        pitch:= 20;
        for i:= 1 to 20 do
          begin

            note(pitch,duration);
            note(0,duration div 2);
```

```
            end;
        end;


    (*                                                  *)
    (*      procedure checks varialbes to for deviation  *)
    (*        from desired ranges                        *)
    (*                                                  *)

        Procedure warning;

            Procedure wpa(x,x1,x2:real;s:string;
                          n:integer);
              begin

                if (x<x1) or (x>x2) then
                  begin

                    gotoxy(1,n);
                    writeln(s,
                      ' is outside desired range');
                    beep;
                  end;
              end;
        begin

          wpa(pH,7.0,7.5,'pH',15);
          wpa(DO2,20.0,97.0,'DO2',16);
          wpa(temp,tset-0.5,tset+0.5,'temperature',17);
          wpa(loadcell,100.0,1000.0,'base level',18);
          wpa(nfondisk,0.0,22.0,
              '# of files to disk',19);
        end;
```

```
(*                                                          *)
(*      procedure outputs system variables to console       *)
(*                                                          *)

      Procedure update;
         begin

            page(cnsl);
            writeln;
            writeln(' et = ',et:6:2,
                    'hrs    cycle time = ',ct:4:2,'hrs');
            writeln;
            writeln('      pH    = ',pH:5:2);
            writeln('      temp  = ',temp:5:2,'c');
            writeln('      rpm   = ',rpm:4:1);
            writeln('      DO2   = ',DO2:4:1,'%');
            writeln;
            writeln(' last our measurement taken ',
                    lt:6:2);
            writeln('  consumption = ',xour:6:3);
            writeln;
            writeln('# of files to disk: ',nfondisk);
         end;



begin     (* waitdt  *)

   page(cnsl);
   update;
   t:= hours;
   t0:= t;
   while (t-t0) < interval do
        begin

           t10:= hours;
           t:= hours;
```

```
            while (not keypress) and ((t-t10)<0.01) do
                t:= hours;

            if keypress then
               reestablish;

            update;
            warning;
          end;

  end; (* waitdt *)



(*                                                    *)
(*        general functions and procedures            *)
(*          common to all of the segments             *)
(*                                                    *)


Procedure charsize;
(*      procedure sends control character to    *)
(*      printer to determnine size of           *)
(*      subsequent text sent to devise          *)
  begin

     case size of
        'u': opr(30); (* set printer to upper case mode *)
        'l': opr(31); (* set printer to lower case mode *)

     end;
  end;


Function hours;  (* function examines real time clock *)
                 (*  and returns time in hours since  *)
```

```
                    (*  begin of the year                *)
var sec:real;

   begin

      sec:= time(0)/1000+time(1)+time(2)*32768.0;
      hours:= sec/3600;
   end; (* hours *)


Function et;
   begin
      et:= hours-t0+delay;
   end; (* et, elapsed time *)


Function ct;
   begin
      ct:= et-t1;
   end; (* ct,cycle time *)


Procedure wait;          (* procedure waits for a given *)
                         (*  in hrs                      *)
var t,t0:real;

   begin

      t:= hours;
      t0:= t;

      while (t-t0) < interval do
      t:= hours;

   end; (* wait *)
```

```
Function intadc;    (* integrates  a/d converter output *)
var x:real;
    i:integer;

  begin

    x:= 0.0;
    for i:= 1 to n do
        x:= x+A2D(select);

    intadc:= x/n;

  end;


(* functions interfacing instrumentation output*)

      Function DO2;       (* range 0 - 100% *)
        begin

          DO2:= intadc(1,1000)/40.95;
        end;


      Function rpm;       (* range 0 - 171 *)
        begin

          rpm:= A2D(6)/24;
        end; (* rpm *)


      Function pH;
        begin

          pH:= intadc(2,100)/409.5 - 0.03;
        end; (* pH *)
```

```
Function temp;        (* range 25 - 45 degrees c *)
  begin

    temp:= 25.0+intadc(7,100)/204.8;
  end; (* temp *)


Function loadcell;        (* range 0 - 500 grams *)
  begin

    loadcell:= intadc(3,500)*538.09/4095.0;
  end; (* loadcell *)


Function probe;(* output of ammonia probe amplifier *)
               (*  range 0 - 5 volts                  *)
  begin

    probe:= intadc(10,250)/819.2;
  end;


Function ira; (* output from CO2 infrared analyzer *)
              (* range 0 - 5 volts                  *)
  begin

    ira:= intadc(9,250)/819.2;
  end;


Function dpress; (* output of gas flow meter to ira *)
                 (*  range 0 - 5 volts                *)
  begin
```

```
        dpress:= intadc(13,250)/819.2;
      end;


    Function od;     (* output of sugar autoanalyzer    *)
                     (*  range 0 - 5 volts              *)
    var ref,sample,x,x1:real;
      begin

        ref:= intadc(11,250)/819.2;
        sample:= intadc(12,250)/819.2;
        if ref <= 0.0  then od:= 0.0
          else if (sample-0.04) <= 0.0 then od:= 3.0
                 else od:= log(ref/(sample-0.04));
      end;


(*  Functions calculating system variables *)

     Function bdens;
(*   function calculating density of naoh soluns *)
(*  to convert loadcell reading from grams to ml *)
       begin

         bdens:= (baseconc * 0.0440) + 1.0002;
       end;


(* function determines ml of base added to *)
(*  the 14 liter vessel                    *)
     Function baseadded;
     var wghtadd:real;
       begin

         wghtadd:= weight0 + sbc - loadcell;
         baseadded:= wghtadd / bdens;
       end;
```

```
(* function determines volume of liquid in the *)
(*  14 liter culture vessel                     *)
     Function vol;
        begin

           vol:= volume0 - ssv - smc + baseadded/1000;
        end;


(* volume of vessel without base addtion *)
(*  or media changes                     *)
     Function vprime;
        begin

           vprime:= volume0 - sdsv;
        end;


(* dilution factor of media by base additions *)
(*  and media changes                         *)
     Function d;
        begin

           d:= vprime/vol;
        end;


(* cumulative sugar conumption (mmoles in v') *)
     Function cglu;
        begin

           cglu:= sug0conc - xglu/d + sgr;
        end;
```

```
(* cumulative acids produced, sum of CO2 and *)
(*  lactic acid                                *)
      Function xtota;
         begin

            xtota:= (baseadded*baseconc-sbrmc)/vol;
         end;


(* cumulative acids produced, sum of CO2 and *)
(*  lactic acid (mmoles in v')                *)
      Function ctotap;
         begin

            ctotap:= baseadded*baseconc/vprime;
         end;


(* estimated lactic acid conc (mmoles in v) *)
      Function xlac;
         begin

            xlac:= xtota - xCO2;
         end;


(* estimate cumulative lactic acid *)
(*  production (mmoles in v')      *)
       Function clac;
          begin

             clac:= ctotap - xCO2/d - srCO2;
          end;
```

```
(* cumulative production of CO2 (mmoles in v') *)
      Function cCO2;
         begin

           cCO2:= xCO2/d + srCO2;
         end;


(* cumulative production of ammonia  *)
(*  (mmoles in v')                   *)
      Function cxamm;
         begin

            cxamm:= xamm/d + sramm;
         end;




Procedure setswitches(select:integer);

   begin

     case select of

        1: begin

           onoff(0,odd(1)); onoff(1,odd(2));
           onoff(2,odd(2)); onoff(3,odd(2));
           onoff1(0,odd(2)); onoff1(1,odd(2));
           onoff1(2,odd(2)); onoff1(3,odd(2));
           end;

        2: begin
```

```
            onoff(0,odd(1)); onoff(1,odd(2));
            onoff(2,odd(1)); onoff(3,odd(2));
            onoff1(0,odd(2)); onoff1(1,odd(1));
            onoff1(2,odd(1)); onoff1(3,odd(1));
            end;

         3: begin

            onoff(0,odd(1)); onoff(1,odd(2));
            onoff(2,odd(1)); onoff(3,odd(2));
            onoff1(0,odd(1)); onoff1(1,odd(1));
            onoff1(2,odd(2)); onoff1(3,odd(2));
            end;

         4: begin

            onoff(0,odd(1)); onoff(1,odd(2));
            onoff(2,odd(1)); onoff(3,odd(1));
            onoff1(0,odd(2)); onoff1(1,odd(1));
            onoff1(2,odd(1)); onoff1(3,odd(2));
            end;

         5: begin

            onoff(0,odd(1)); onoff(1,odd(2));
            onoff(2,odd(1)); onoff(3,odd(1));
            onoff1(0,odd(2)); onoff1(1,odd(2));
            onoff1(2,odd(2)); onoff1(3,odd(2));

            end;
      end;
   end;



   begin (*main section*)
```

```
setswitches(1);
initialize;
waitdt(0.07);
while et<250 do
      begin

         t1:= et;
         our;
         while ct < 3.20 do
            begin
              setswitches(1);
              waitdt(0.05);
            end;

         CO2s:= 0.0;
         xflow:= 0.0;
         sf:= 0.0;
         nave:= 0;

         while ct < 3.70 do
              begin
                 setswitches(1);
                 nave:= nave+1;
                 CO2s:= CO2s+ira;
                 sf:= sf+dpress;
                 waitdt(0.01);
              end;

         CO2s:= CO2s/nave;
         fCO2:= 1.0/(1.0 + (2.303 * exp(pH - 6.35)));
         sf:= sf/nave;
         dp:= sf-dpress0;
         if dp = 0.0 then xflow:= 10.0
                     else xflow:= exp(-0.85 * ln(dp));

         writeln(pntr,CO2s,fCO2,xflow);
```

```
while ct < 3.95 do
   begin
     setswitches(2);
     waitdt(0.05);
   end;

sugf:= od;
ami:= probe;

writeln(pntr);
writeln(pntr,sugf,ami);

while ct < 4.20 do
   begin
     setswitches(3);
     waitdt(0.05);
   end;

CO20:= ira;
sugs:= od;
ams:= probe;
ssv:= ssv + pv;
sdsv:= sdsv + (d * pv);


writeln(pntr);
writeln(pntr,CO20,sugs,ams);

while ct < 4.45 do
   begin
     setswitches(4);
     waitdt(0.05);
   end;

sug0:= od;
amii:= probe;
```

```
        xglu:= 16.67 * (sugs-sug0)/(sugf-sug0);

        if (ami-amii) <> 0.0 then
           begin
             xat:= (1.301*(ams-amii)/(ami-amii))-0.301;
             xamm:= 2.303*exp(xat);
           end

           else xamm:= 0.0;

        writeln(pntr);
        writeln(pntr,sug0,amii);
        writeln(pntr,xglu,xat,xamm);

        while ct < 4.70 do
           begin
             setswitches(5);
             waitdt(0.05);
           end;

        CO2f:= ira;
        pcCO2:= CO2cal*(CO2s-CO20-CO2air)/(CO2f-CO20);
        xCO2:= 2.02*pcCO2/(fCO2*xflow);

        writeln(pntr);
        writeln(pntr,CO2f,pcCO2,xCO2);


        while ct < 4.75 do
           begin
             setswitches(1);
             waitdt(0.05);
           end;

    end;
```

```
end.
```

```
;-------------------------------------
;
; Macro pops 16 bit arguement
;

        .MACRO POP
        PLA
        STA     %1
        PLA
        STA     %1+1
        .ENDM


;-------------------------------------
;
; Macro returns 16 bit return address
;

        .MACRO RPOP
        LDA     %1+1
        PHA
        LDA     %1
        PHA
        RTS
        .ENDM

;-------------------------------------
;
; Macro discard 4 bytes stack from stack
; .     bias
;

        .MACRO PL4
        PLA
        PLA
```

```
        PLA
        PLA
        .ENDM

;-------------------------------------
;
; Macro creates delay to allow data to
;       settle before attemping to read
;

        .MACRO DLY
        NOP
        NOP
        NOP
        NOP
        NOP
        NOP
        NOP
        .ENDM

;-------------------------------------
;
; Macro shifts data to right 4 positions
;
;-------------------------------------


        .MACRO SR
        LSR A
        LSR A
        LSR A
        LSR A
        .ENDM


;-------------------------------------
;
```

```
; Macro shifts data to left 4 positions
;
;-------------------------------------


        .MACRO SL
        ASL A
        ASL A
        ASL A
        ASL A
        .ENDM


;-------------------------------------
;
; Macro clears, adds, and stores
;
;-------------------------------------


        .MACRO CAS
        CLC
        ADC     %1
        STA     %1
        .ENDM


;-------------------------------------
;
; Function for analog to digital converter
; Function A2D (select: integer):integer;
;

        .FUNC A2D,1     ;one word parameters
```

```
RETURN  .EQU    0       ;temp var for return address
DATA    .EQU    2       ;temp var for data
XSAVE   .EQU    3       ;temp var for x


        POP     RETURN
        STX     XSAVE
        PL4
        PLA             ;get lsb, channel address
        TAX
        PLA             ;discard msb of channel address

        STA     0C0B0,X ;initiate convertion
        DLY             ;allow delay for comunication

START   LDA     0C300   ;read busy signal
        AND     #80
        BMI     DONE    ;zero when done
        JMP     START

DONE    LDA     0C0B0   ;read in lsb
        STA     DATA
        LDA     0C301   ;select msb
        LDA     0C0B0   ;read in msb
        AND     #0F

        LDX     XSAVE
        PHA             ;push msb of return value
        LDA     DATA
        PHA             ;push lsb of return value

        RPOP    RETURN

;-------------------------------------
;
;  Procedure Onoff(select:integer; data: boolean);
;
```

```
;-------------------------------------
        .PROC ONOFF,2

RETURN  .EQU    0
YSAVE   .EQU    2

        POP     RETURN  ;save return address to pascal

        STY     YSAVE   ;save y index

        PLA             ;get lsb boolean data 1=true
        LSR A           ;save boolean in carry
        PLA             ;discard msb

        PLA             ;get lsb select
        AND     #03

        ROL     A       ;double and add carry

        TAY             ;put i/o strobe in y
        LDA     0C058,Y ;activate

        PLA             ;discard msb select

        LDY     YSAVE   ;restore y

        RPOP    RETURN  ;go back to pascal
        RTS

;-------------------------------------
;
; Procedure Onoff1(select:integer; data: boolean);
;
;-------------------------------------

        .PROC ONOFF1,2
```

```
RETURN  .EQU    0
YSAVE   .EQU    2

        POP     RETURN  ;save return address to pascal.

        STY     YSAVE   ;save y index

        PLA             ;get lsb boolean data 1=true
        LSR A           ;save boolean in carry
        PLA             ;discard msb

        PLA             ;get lsb select
        AND     #03

        ROL     A       ;double and add carry

        TAY             ;put i/o strobe in y
        LDA     0C0F0,Y ;activate

        PLA             ;discard msb select

        LDY     YSAVE   ;restore y

        RPOP    RETURN  ;go back to pascal
        RTS

;-------------------------------------
;
;       Procedure prints hires graphic
;
;-------------------------------------

        .PROC HDUMP,0

RETURN  .EQU 0
XFF     .EQU 2
PSW     .EQU 3
```

```
XSAVE   .EQU 4
YSAVE   .EQU 5
MSK1    .EQU 6
MSK2    .EQU 7
LINE    .EQU 8
TEMP    .EQU 9
BASE    .EQU 10
DATA    .EQU 12
XC0     .EQU 13
XBF     .EQU 14
TEMP1   .EQU 15


        STX     XSAVE   ;save index regs
        STY     YSAVE


;---------------------------------------
;
;       initialize printer
;
;---------------------------------------

        LDA     #0F
        STA     XFF
        ASL A
        ASL A
        ASL A
        ASL A
        ORA     XFF
        STA     XFF

        LDA     #0C
        ASL A
        ASL A
        ASL A
```

```
        ASL A
        STA     XC0

        LDA     #0F
        STA     TEMP1
        LDA     #0B
        ASL A
        ASL A
        ASL A
        ASL A
        ORA     TEMP1
        STA     XBF


        LDA     #00
        STA     0C091
        STA     0C093
        STA     0C090
        LDA     XFF
        STA     0C092
        LDA     #04
        STA     0C091
        LDA     #34
        STA     0C093


        LDA     XC0
        STA     PSW

        LDA     PSW     ;do hires screen dump
        AND     #01     ;20 column mode?
        BEQ     HDRIVE
        JMP     GETBACK ;yes ignore command
HDRIVE  LDY     #00     ;clear char index
HDR1    LDA     #7F     ;initialize masks
        STA     MSK1
        LDA     #01
```

```
        STA     MSK2
NXTCHAR LDA     XBF     ;start at bottom
        STA     LINE    ;left of screen
        LDA     #0A     ;output lf
        JSR     OUT1
        CPY     #28     ;done?
        BCC     SPACES
        JMP     GETBACK ;yes getback
SPACES  LDA     #0E
        JSR     OUT1    ;set not inverse
        TYA
        PHA             ;save y reg on stack
        LDY     #05
CENT    LDA     #20     ;output 5 spaces
        JSR     OUT1
        DEY
        BNE     CENT
        PLA
        TAY             ;restore y from stack
        LDA     PSW
        AND     #10     ;inverse mode?
        BEQ     SETCNT
        LDA     #0F     ;yes set inverse
        JSR     OUT1
SETCNT  LDA     #1C     ;set graphic mode
        JSR     OUT1
        LDA     XC0     ;set byte count to 192
        JSR     OUT1
GET     LDA     LINE    ;line number for calc
        JSR     HBASCALC;base addr to (base)
        TYA
        PHA             ;push y
        LDA     MSK1
        AND     (BASE),Y
        STA     DATA
        LDA     MSK2
        INY
```

```
        AND     (BASE),Y
        LDY     MSK2
        STY     TEMP



ALGN    ASL A
        ASL A   TEMP
        BPL     ALGN
        LDY     MSK1
        STY     TEMP
ALGN1   ROR     TEMP
        BCS     DONE
        ROR     DATA
        BCC     ALGN1
DONE    ORA     DATA
        JSR     OUT1    ;output byte to printer
        PLA
        TAY             ;restore y reg
        DEC     LINE
        LDA     XFF
        CMP     LINE
        BNE     GET
ADJMSK  ASL A   MSK1    ;adjust masks for new line
        ASL A   MSK1
        CLC
        ROR     MSK1
        SEC
        ROL     MSK2
        BPL     NEXTY
        INY
        INY
        JMP     HDR1
NEXTY   INY
        JMP     NXTCHAR
```

```
HBASCALCPHA             ;line number in a
        AND     XC0
        STA     BASE    ;for line = abcdefgh
        LSR     A
        LSR     A       ;calculte
        ORA     BASE
        STA     BASE    ;base = eabab000
        PLA             ;base+1 = pppfghcd
        STA     BASE+1
        ASL A           ;ppp = 001 page 1
        ASL A           ;ppp = 010 page 2
        ASL A
        ROL     BASE+1
        ASL A
        ROR     BASE
        LDA     BASE+1
        AND     #1F
        ORA     #20
        PHA
        LDA     PSW
        ROR  A
        ROR  A
        ROR  A
        PLA
        BCC     HBAS1
        ADC     #1F
HBAS1   STA     BASE+1
        RTS

;---------------------------------------
;
;       Out1
;       Procedure outputs character
;       to printer
;
;---------------------------------------
```

```
OUT1    PHA

BUSY    LDA     0C090
        BMI     BUSY
        PLA
        STA     0C092

TIME    LDA     #01
DELAY   ASL A
        BCC     DELAY
        LDA     #3C
        STA     0C093
        LDA     #34
        STA     0C093

        RTS

GETBACK LDX     XSAVE
        LDY     YSAVE
        RPOP    RETURN


;---------------------------------------
;
;       Procedure outputs character
;       to printer
;
;---------------------------------------

        .PROC OPR,1

RETURN  .EQU    0

        POP     RETURN
```

```
BUSY    LDA     0C090
        BMI     BUSY
        PLA
        STA     0C092

TIME    LDA     #01
DELAY   ASL A
        BCC     DELAY
        LDA     #3C
        STA     0C093
        LDA     #34
        STA     0C093

        RPOP    RETURN


;-------------------------------------
;
; Function Time (select:integer):integer;
;
;       select; choses range on clock
;               0, < 1 sec
;               1, >1 and < 18 hours
;               2, >18 hours and , 1 yr.
;
;-------------------------------------


        .FUNC TIME,1

RETURN  .EQU    0       ;allocate space for variables
TEMP    .EQU    2
TEMP1   .EQU    3
TEMP2   .EQU    4
SELECT  .EQU    5
```

```
        POP     RETURN
        PL4
        PLA             ;lsb of select
        AND     #3
        STA     SELECT  ;store select

        PLA             ;discard msb of select

        LDA     SELECT
        CMP     #1      ;evaluate which part to do
        BMI     ZERO
        BEQ     ONE
        BPL     TWO

        LDA     #0
        PHA
        PHA
        RPOP    RETURN

ZERO    LDA     0C0D4   ;cal t < 1 sec
                        ;read in 1 & 10 milisec
        AND     #0F     ;select 1's
        STA     TEMP
        LDA     0C0D4

        SR              ;select 10's digit
        STA     TEMP1
        ASL A           ;mult. by 10 (2+8)
        CAS     TEMP

        LDA     TEMP1   ;(8)
        SL
        LSR A
        CAS     TEMP
```

```
        LDA     0C0D3   ;100's
        AND     #0F
        STA     TEMP2

        STA     TEMP1   ;mult. by 25 (1+8+16)
        SL
        LSR A
        CAS     TEMP1
        LDA     TEMP2
        SL
        CAS     TEMP1   ;25-done in temp1

        CLC
        ASL A
        ASL A
        ADC     TEMP
        STA     TEMP    ;lsb

        LDA     #0
        ADC     #0      ;save carry
        STA     TEMP2

        LDA     TEMP1
        SR
        LSR A
        LSR A           ;mult by 4(100)
        CLC
        ADC     TEMP2   ;add in carry
        PHA             ;msb
        LDA     TEMP
        PHA             ;lsb
        RPOP    RETURN


TWO     JMP     TOO     ;because there are too many lines
```

```
ONE     LDA     0C0D1   ;1 sec <t <18 hr
        AND     #07
        SL              ;calc. msb
        STA     TEMP
        LDA     0C0D2
        SR
        CAS     TEMP

        LDA     0C0D2   ;calc. lsb
        SL
        STA     TEMP1
        LDA     0C0D3
        SR
        CAS     TEMP1

        LDA     TEMP    ;set up stack
        PHA             ;msb
        LDA     TEMP1
        PHA             ;lsb
        RPOP    RETURN

TOO     LDA     0C0D0   ;t>18 hr calc. msb
        LSR A
        LSR A
        LSR A
        AND     #03
        STA     TEMP    ;store msb
        LDA     0C0D0   ;calc. lsb
        SL
        ASL A
        STA     TEMP1
        LDA     0C0D1
        LSR A
        LSR A
        LSR A
        CAS     TEMP1
```

```
        LDA     TEMP    ;set up stack
        PHA             ;return msb
        LDA     TEMP1
        PHA             ;return lsb
        RPOP    RETURN

;-------------------------------------------------
;
;       Function Keypress: boolean; external
;
;-------------------------------------------------
;
        .FUNC KEYPRESS,0;0 words of parameters used

RETURN  .EQU    0       ;storage for return address
CONCKVEC.EQU    0BF0A   ;fixed address in bios
RPTR    .EQU    0BF18   ;fixed buffer pointer
WPTR    .EQU    0BF19   ;fixed buffer pointer
KEYBOARD.EQU    0C000   ;keyboard hardward
CONCK   .EQU    0FF5C   ;way to get conck in old system

        POP     RETURN
        PL4
        LDA     #0
        PHA             ;return msb zero
        LDA     KEYBOARD
        BMI     TRUE
        JSR     CONCK
        .DA     RPTR
        CMP     WPTR    ;char in buffer?
        BEQ     EMPTY
TRUE    LDA     #1      ;yes, return keypress=true
        BNE     KPDONE  ;always taken
EMPTY   LDA     #0      ;no, return keypress=false
KPDONE  PHA
        RPOP    RETURN
```

```
;-----------------------------------------
;
;       Procedure Note(pitch,duration); external;
;
;-----------------------------------------
;

        .PROC NOTE,2    ;two words of paramter
;
RETURN  .EQU    0
PITCH   .EQU    2
DURATION.EQU    3
XSAVE   .EQU    4
YSAVE   .EQU    5

        POP     RETURN

        PLA
        STA     DURATION;get and save lsb of duration
        PLA             ;discard msb of duration
        PLA             ;get lsb pitch
        STA     PITCH   ;and save it
        PLA             ;discard msb of pitch

        STX     XSAVE   ;save x register
        STY     YSAVE   ;save y register

A1      LDA     0C030
A2      DEY
        BNE     A3
        DEC     DURATION
        BEQ     EXIT
A3      DEX
        BNE     A2
        LDX     PITCH
        JMP     A1
```

```
EXIT    LDX     XSAVE
        LDY     YSAVE
        RPOP    RETURN
        RPOP    RETURN
        .END
```

APPENDIX O: GROWTH OF CHICKEN EMBRYO FIBROBLASTS

Chicken embryo fibroblasts were cultivated on microcarriers to examine the influence of the environment upon the growth characteristics of these cells. Two differing culture conditions were analyzed, first, one in which the culture was infrequently refed (condition A), and a second in which the culture was refed on an frequent basis (condition B). The growth curves are presented in Figure 59 on page 340 and in Figure 60 on page 342 In addition to the cell number the glucose, lactate, and ammonia concentrations were determined with time and are presented in Table 18 on page 340.

Figure 59 Growth Curve of CEF cells: Infrequent Refeedings

Figure 59 Growth Curve of CEF cells: Infrequent Refeedings

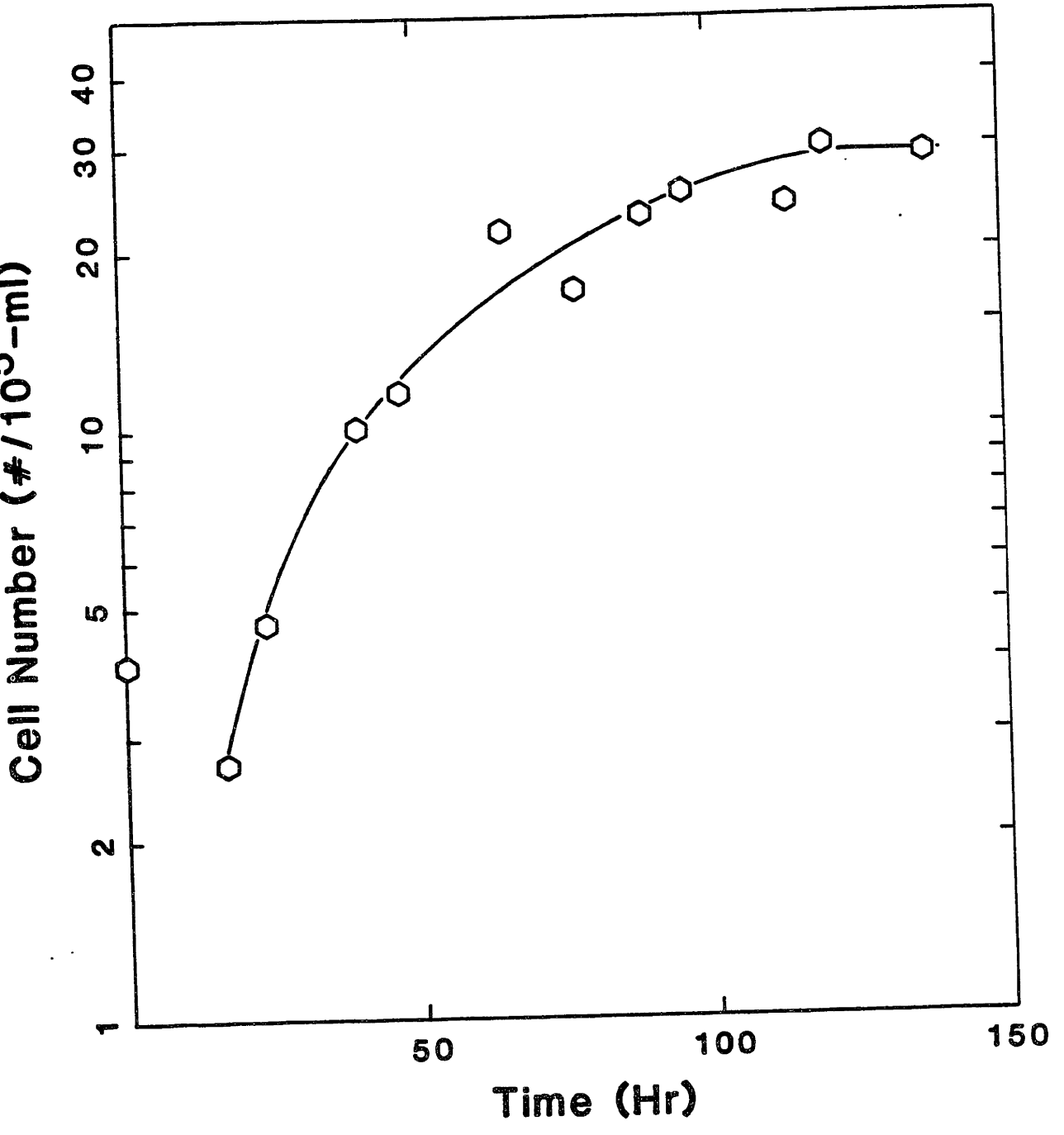

Cell Number (#/10^5-ml)
1
2
5
10
20
30
40
50
100
150
Time (Hr)

Figure 60 Growth Curve of CEF cells: Frequent Refeedings

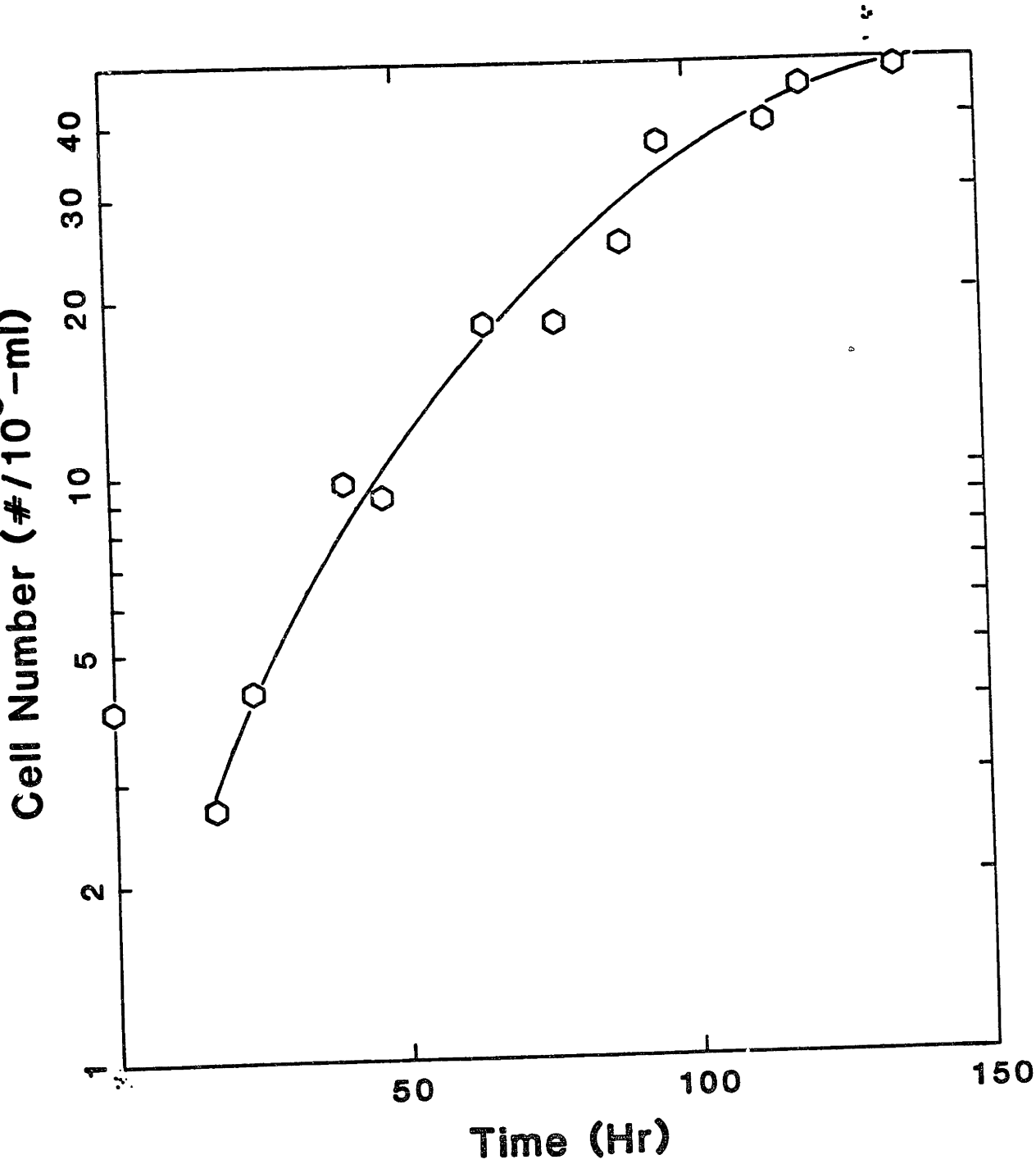

Cell Number (#/10^5-ml)
1
2
5
10
20
30
40
50
100
150
Time (Hr)

TABLE 18

CEF CELLS: CONCENTRATION OF CERTAIN COMPOUNDS VS GROWTH

Condition A, Infrequent Refeedings

Time (Hr)	Cell # 10^5	Glucose mM	Lactate mM	Ammonia mM
0	4.0	25.0	0.0	1.28
17	2.7	21.5	1.78	1.52
24	4.7	20.4	2.44	1.56
40.5	10.0	14.2	5.11	2.26
47	11.7	14.6	6.00	2.31
65	17	17.8	7.78	2.46
77	20.5	18.1	9.22	2.97
89	23.5	15.9	11.67	3.39
95.5	25	15.5	12.67	3.10
113.5	29	12.4	11.56	3.43
120	30	10.6	13.33	3.26
137	30	8.4	21.67	ND

Condition B, Frequent Refeedings

Time (Hr)	Cell # 10^5	Glucose mM	Lactate mM	Ammonia mM
0	4.0	25.0	0.0	1.28
17	2.7	22.3	1.44	1.58
24	4.3	21.2	1.89	1.72
40.5	8.4	19.5	4.33	2.11
47	10.2	16.8	5.22	2.27
65	16.2	18.9	7.33	2.18
77	21.5	17.5	9.11	2.71
89	27.5	17.6	9.78	2.57
95.5	31	17.2	11.22	2.73
113.5	41.5	14.0	14.22	2.70
120	45	13.5	16.0	2.92
137	49	12.0	16.56	2.77

APPENDIX P: CONDUCTANCE MEASUREMENTS

CHARACTERIZING THE CONDUCTANCE ELECTRODE

In order to develop a model of the electrode behavior, the frequency response of the conductance in a single chamber was evaluated. Specifically, the conductance of a single chamber containing DME was measured at room temperature, while the frequency of the oscillator used to drive the system was varied in increments of 10 kHz over the range of 10 kHz to 100 kHz. Furthermore, four different current densities across the electrodes were evaluated. The results are presented in Table 19 on page 348.

EVALUATION OF DIFFERENTIAL CONDUCTANCE MEASUREMENT CIRCUIT

The performance of the Wheatstone bridge circuit was evaluated with regard to three criteria:

- The ability of the circuit to measure an increase in the ionic strength of the fluid in the determination chamber.
- The response of the circuit to thermal changes in the measured fluids.
- The long term stability of the system.

Measurement of Changes in Conductance

The response of the circuit to changes in the conductance of the reference fluid was characterized in these experiments. Ammonia, lactic acid, and acetate were the electrolytes that were characterized. The first two because they are known cell waste products, and acetate, because it is similar to lactate in its ion mobility and is well characterized in the literature. Initially, the system was characterized using PBS as a reference solution which avoided the problems which might be associated with medium decomposition, particularly glutamine to glutamic acid and ammonia. Both chambers were immersed in a constant temperature water bath (36 ± .02 °C), and contained the reference solution. The ionic content of the determination chamber was then varied. The data is presented in Table

TABLE 19

CONDUCTANCE OF A SINGLE CHAMBER VS. FREQUENCY

Frequency	Current Density (ma/cm^2)			
kHz	3.5	4.8	11.6	17.0
10	127.9	130.7	136.3	130.3
20	124.8	127.2	133.1	126.0
30	123.7	126.1	132.0	125.3
40	123.1	124.7	132.0	125.3
50	122.8	124.4	131.2	124.2
60	122.8	124.3	130.3	124.0
70	122.7	123.8	130.2	123.9
80	122.8	123.9	129.5	123.7
90	122.6	123.6	129.2	123.4
100	122.5	123.4	128.3	123.5

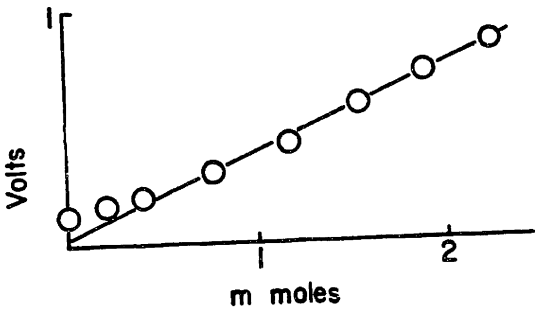
TABLE 20

CONDUCTANCE MEASUREMENTS: ADDITIONS TO PBS

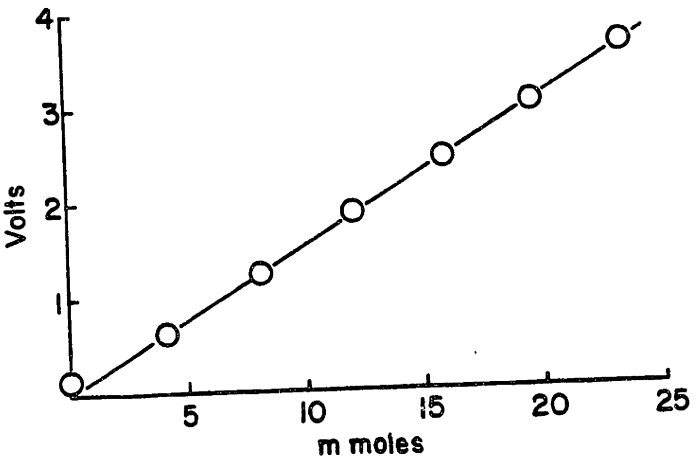
Lactic Acid		Ammonium Chloride		Sodium Acetate	
mM	volts	mM	volts	mM	volts
0.0	0.14	0.0	0.13	0.0	0.15
4.1	0.64	0.2	0.17	1.5	0.51
8.1	1.26	0.4	0.20	3.0	0.82
12.0	1.89	0.8	0.30	4.5	1.21
15.9	2.46	1.2	0.43	6.0	1.54
19.7	3.05	1.5	0.58	7.4	1.86
23.4	3.64	1.9	0.72	8.9	2.19
		2.3	0.84	10.3	2.52
				11.7	2.85
				13.1	3.15
				14.5	3.46

Figure 61 Conductance: Addition of Electrolytes to PBS

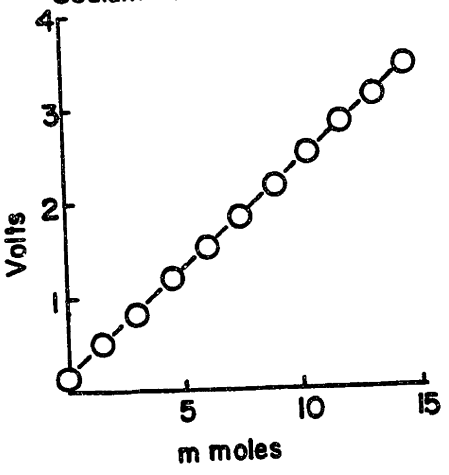
Ammonium Chloride



Lactic Acid



Sodium Acetate



20 on page 349 and the response of the system to the varying concentrations of these electrolytes in the phosphate buffer is shown in Figure 61 on page 350. Note that the conductance is expressed as the mv output of the amplifier rather that in absolute units. Ammonia was varied over the range of 0 to 2.5 m *M* and sodium lactate over the range of 0 to 25 m *M* . These ranges were chosen as representative of the concentrations of ammonia and lactic acid that would result from the growth of cells in microcarrier culture. The data is linear, with the standard deviation in the slope of the lactic acid data being about 1% and about 5% for the ammonia data.

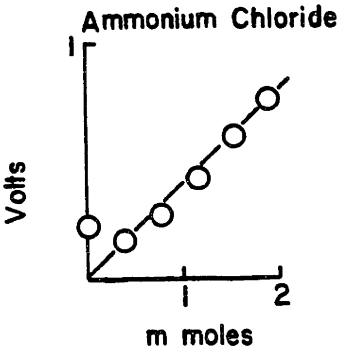
Second, an almost identical experiment was performed, except that DME, the cell culture medium, was used as the reference solution. The data is presented in Table 21 on page 355 and is shown plotted in Figure 62 on page 354. Again the linearity of the data is excellent.

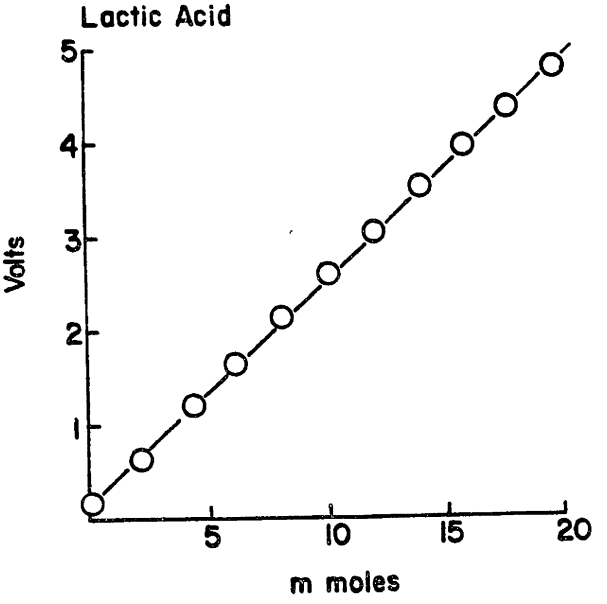
Finally, the system was analyzed with regard to its behavior as a linear operation, specifically, whether the addition of a mixture behaved as the sum of the individual components. In this experiment, a stock solution of lactic acid and ammonium chloride was prepared and added to the chamber. The effect of the addition of this mixed standard (data shown in Table 22 on page 356) was compared to a hypothetical result calculated from previous data. The stocks and additions were made in DME. The prediction versus the actual data is shown in Figure 63 on page 358. Note that the slope of the curve shows an almost exact 1:1 correspondence. The error (about 7%) is representative of those expected in the preparation of the combined standard.

Effect of Temperature upon the System

Because it is impractical to expect that the temperature of both the reference and determination chamber could be regulated to a specific temperature within 0.02 °C, the effect of temperature changes upon the measurement of conductance using the Wheatstone bridge circuit is an important consideration. In this experiment, two chambers, both filled with DME, were monitored as the temperature in the surrounding environment was altered by 5 degrees. The steady state output signal of the

Figure 62 Conductance: Addition of Electrolytes to DME


Ammonium Chloride
Volts
1
1
2
m moles


Lactic Acid
Volts
5
4
3
2
1
5
10
15
20
m moles

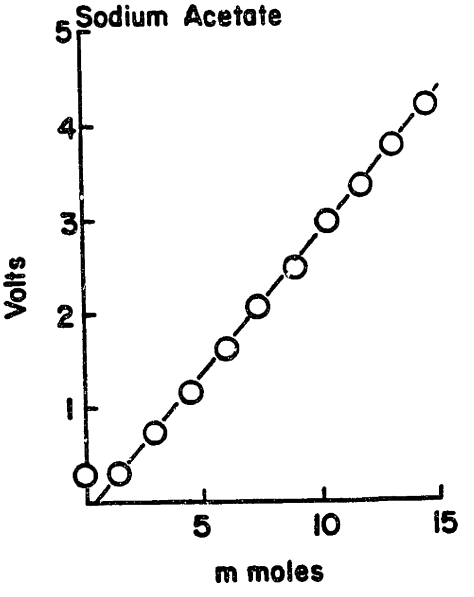

Sodium Acetate
Volts
5
4
3
2
1
5
10
15
m moles

TABLE 21

CONDUCTANCE MEASUREMENTS: ADDITIONS TO DME

Lactic Acid		Ammonium Chloride		Sodium Acetate	
mM	volts	mM	volts	mM	volts
0.0	0.18	0.0	0.22	0.0	0.30
2.1	0.65	0.4	0.15	1.5	0.30
4.1	1.14	0.8	0.27	3.0	0.73
6.1	1.63	1.2	0.42	4.5	1.17
8.1	2.13	1.5	0.60	6.0	1.64
10.1	2.59	1.9	0.76	7.4	2.09
12.0	3.05			8.9	2.50
14.0	3.52			10.3	2.97
15.9	3.94			11.7	3.39
17.8	4.35			13.1	3.80
19.7	4.78			14.5	4.22

TABLE 22

CONDUCTANCE: ADDITION OF MIXED ELECTROLYTES (IN DME)

Recorded Data volts	Predicted Data volts
0.20	0.46
0.45	0.74
0.74	1.01
1.04	1.29
1.33	1.56
1.63	1.81
1.92	2.08
2.21	2.34
2.50	2.60
2.79	2.86

system does not show any significant change with temperature, at least over this range. However, the system did demonstrate an initial transient response. The effect of temperature on the system was also examined when the composition of the two chambers was not identical. If the two cells are not identical in composition, then changing the temperature should have an unequal influence on the conductivity and hence, a net difference between the two cells would be expected. Experimentally, this was evaluated by adding acetate to the determination chamber (at a concentration of 15 m *M*). The temperature was changed from 36.3 °C to 37.9 °C and later to 39.7 °C. The conductance signal changed from 4.22 volts to 4.33, with the first temperature shift, and then to 4.42, with the second shift. Roughly, the measured differential conductance showed a change of 1.4%/°C.

Long Term Stability of the System

One of the anticipated problems with the use of this method is the instability of the medium, particularly with the degradation of glutamine to glutamic acid and ammonia. Secondly, these experiments take place over several days in time and the stability of the electronic over this period of time must also be examined. To address this problem a number of experiments were conducted.

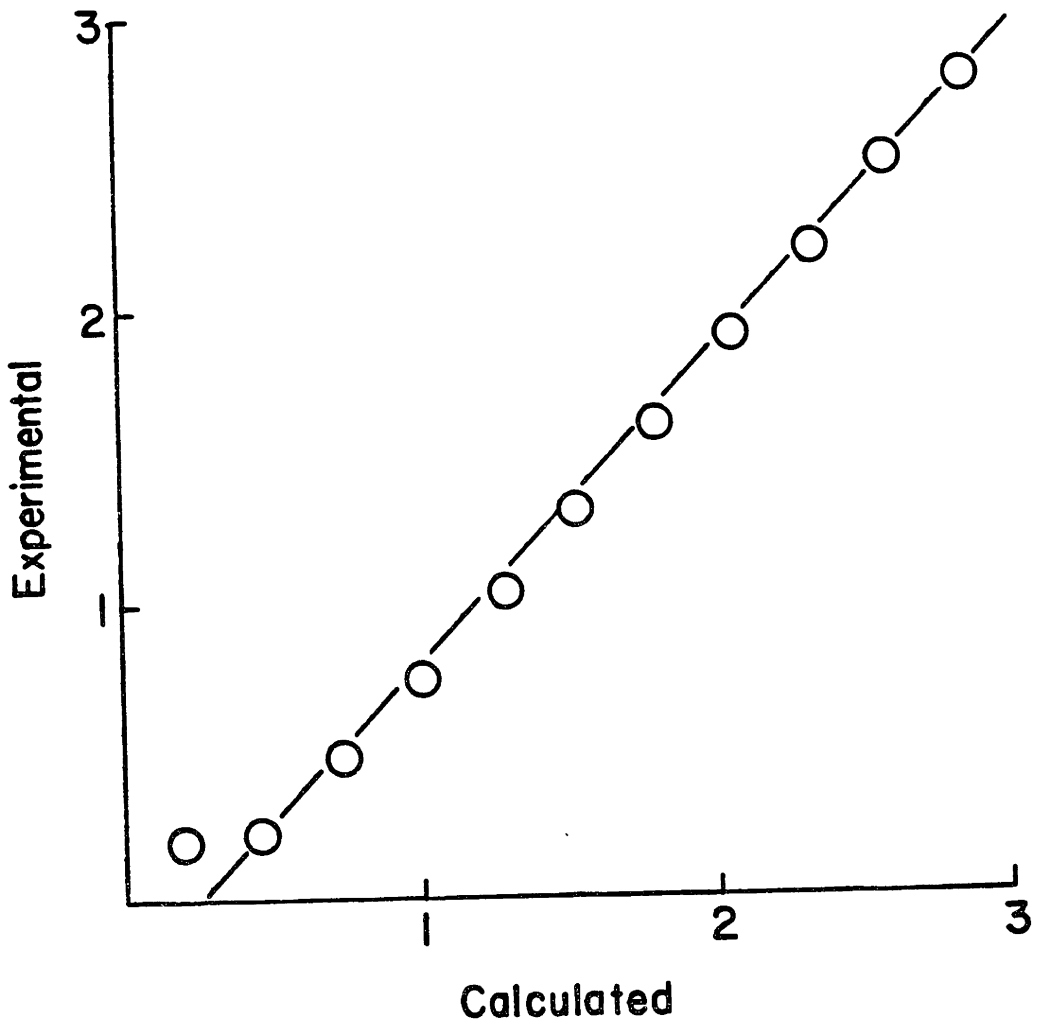
First, both chambers were filled with DME (without glutamine) and the stability of the system over three days was examined. No net change in conductance was observed.

Second, both chambers were filled with DME, and glutamine was added to the determination chamber and the conductance was examined over four days. No net conductance change was observed.

Third, both chambers were filled with DME containing sera, and glutamine was added to the determination chamber. The conductance of the determination chamber increased significantly with time.

Figure 63 Conductance: Addition of Mixed Electrolyte

The observed conductance resulting from the addition of a mixture of lactic acid and ammonia was compared to a predicted change in conductance based upon previous data


3
2
1
Experimental
1
2
3
Calculated

MEASUREMENT OF CONDUCTANCE CHANGES ACCOMPANYING CELL GROWTH

In this experiment, SV-80's (a transformed human fibroblast) were grown on microcarriers. They were grown in DME containing FBS and glutamine in a bicarbonate/carbon dioxide buffer. The SV-80's were used in this initial experiment because they grow well on microcarriers and do not require replacement of the medium after two or three days of growth. This simplified interpretation of the data. The results are shown in Figure 64 on page 362. It can be seen that the conductance measurements closely followed the change in cell number.

ANALYSIS OF CONDUCTANCE MEASUREMENTS

Characterizing the Conductance Electrode:

In order to understand the conductance measurement, developing a model for the conductance chamber and its electrode is very useful. Two models have been proposed in the literature, the series model and the parallel model. These models can be easily distinguished by their frequency responses.

In the series model the frequency response can be described by the following equation:

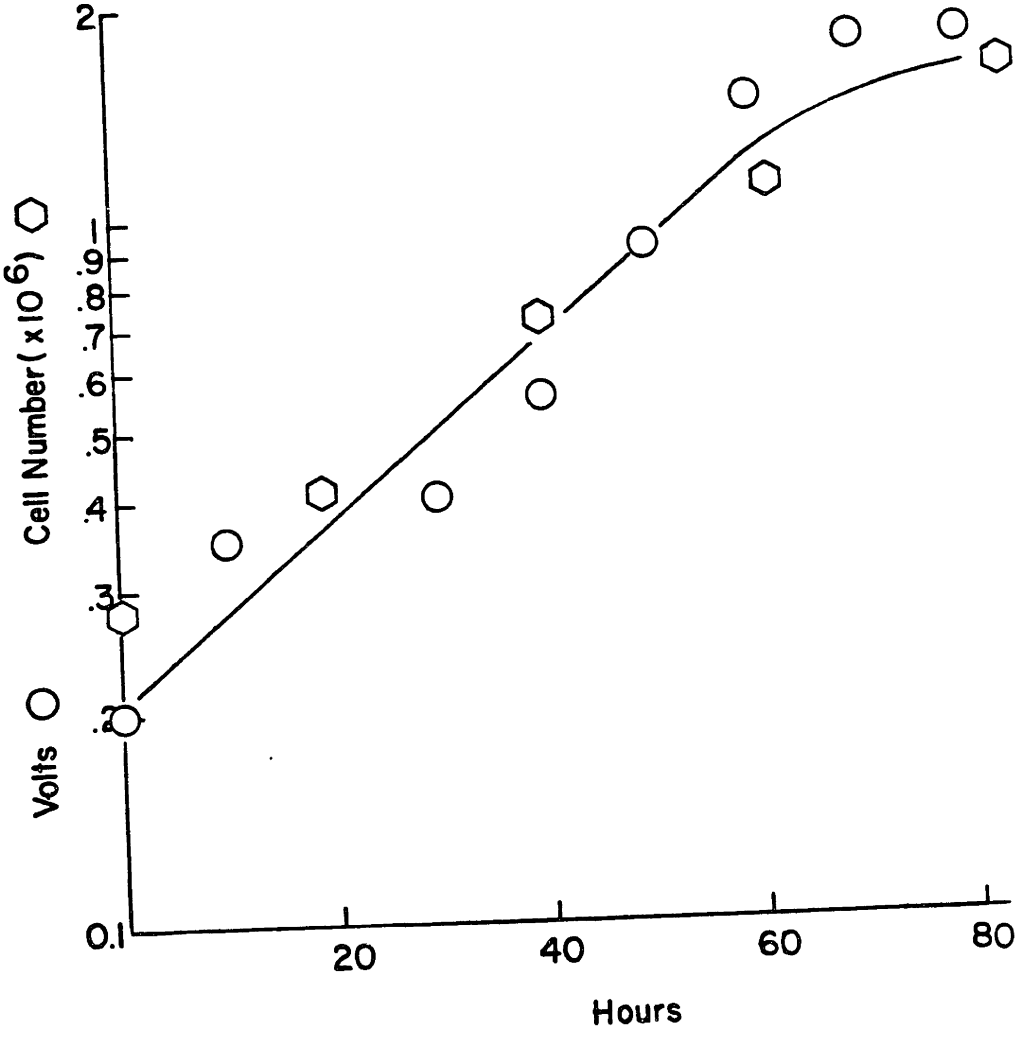
$$\zeta^2 = R^2 + 1/(C^2\omega^2)$$

where: ζ is the impedance of the chamber, R is the resistive component, C is the capacitive component, and ω is the angular frequency (2f). On the other hand, the frequency response of the parallel model is described by the following equation:

$$\zeta^2 = 1/R^2 + C^2\omega^2.$$

Figure 64 Conductance: Growth of SV-80 cells

SV-80 cells were grown in DME and the corresponding change in conductance of the medium was measured


Cell Number (x10^6)
2
.9
.8
.7
.6
.5
.4
.3
.2
0.1
Volts
20
40
60
80
Hours

If the series model were to correctly describe the electrode behavior, a plot of ζ^2 vs. $1/(\omega^2)$ will show a straight line. Alternately the parallel model predicts a linear relationship between $1/(\zeta^2)$ versus ω^2.

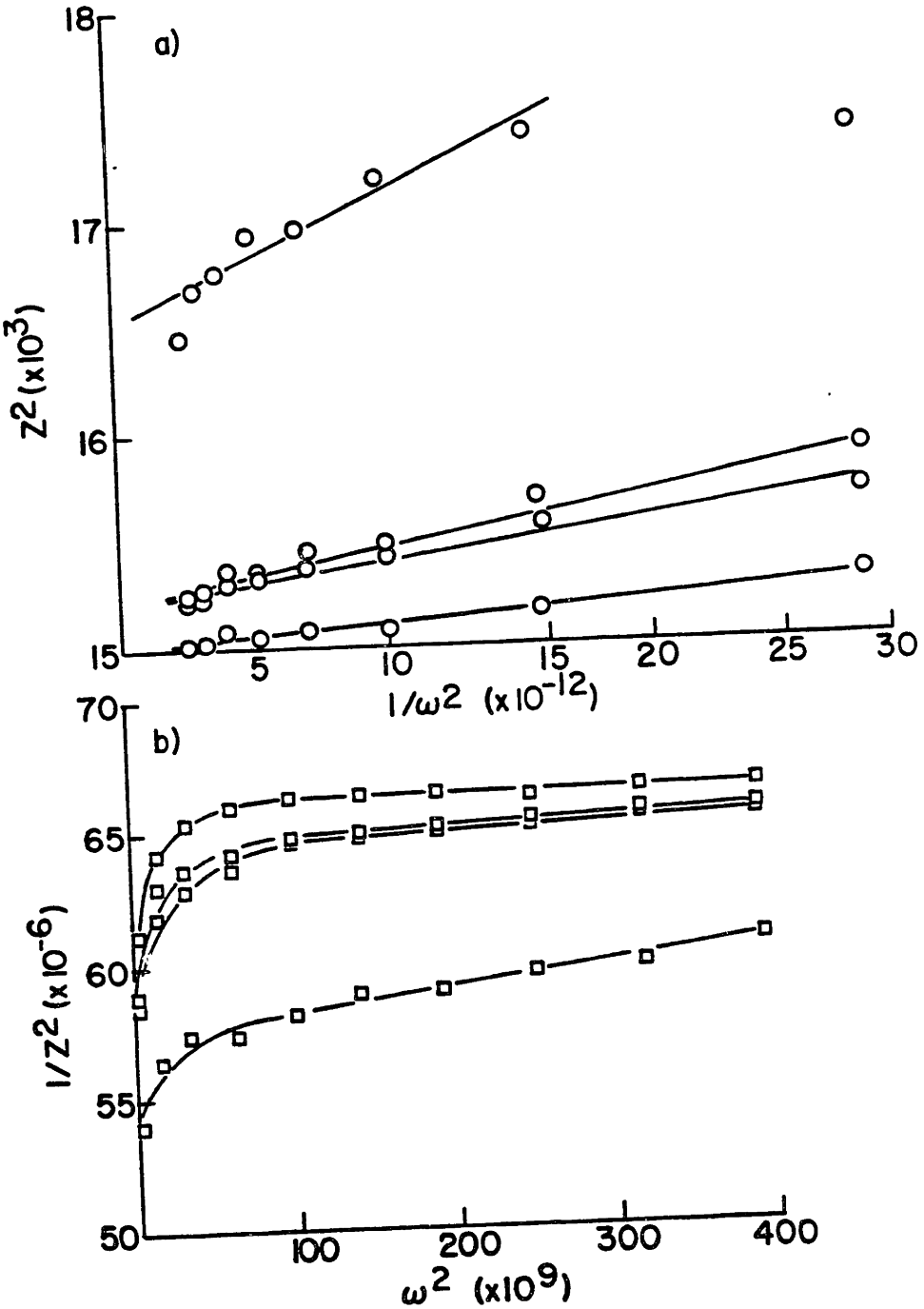
In evaluating these two models, the conductance of a single chamber containing DME was measured at room temperature, under condition of varying frequency and current density (see Table 19 on page 348). From the impedance data a plot of $1/\zeta^2$ versus ω^2 and a plot of ζ^2 versus $1/\omega^2$ were prepared (see Figure 65 on page 364 and Figure 65 on page 364). It would appear that the series model appears to more closely approximate the experimental data, although neither model demonstrated an ideal agreement with the observed results. The argument for using the series model approximation becomes more compelling when these plots are used to approximate the capacitance and resistance terms of the models. Though both models estimate the resistance to be approximately 130 ohms, the capacitance values are very different. The parallel model estimates a capacitance of approximately 3pf whereas the series model estimates a value of approximately 0.4 uf. This then allowed us to construct analogue models of the conductance electrode system from purely electrical components. These models were then compared to the actual system by virtue of their response to a 10 kHz square wave. This experiment showed that the series model was in fact a much closer approximation (although somewhat slower) of the actual electrode response.

The deviation between the series model and actual conductance chamber may be partially attributed to the fact that the conductance term of the electrode does not behave as an ideal electronic component and is probably somewhat leaky (i.e., has a resistive component associated with it).

That the series model of the electrode better fits the data would imply, as has been previously asserted by Richards *et al.*, that the capacitance element of the electrode impedance is derived from the metal/liquid junction of the electrode. Consequently, the capacitive element of the electrode is a likely source of error and problems in our efforts to measure the metabolic activity of animals cells in culture. Practical considerations allow this term to be minimized by reducing the surface area of the electrodes and increasing their separation. Additionally,

Figure 65 Frequency Response of Conductance Chamber: Analysis

The frequency response of the conductance chamber is analyzed first under the assumption that the electrode can be model as a resistor and capacitor in parallel. and second under the assumption that the electrode can be model as a resistor and capacitor in series.


a)
18
17
16
15
$Z^2 (\times 10^3)$
5
10
15
20
25
30
$1/\omega^2 (\times 10^{-12})$
b)
70
65
60
55
50
$1/Z^2 (\times 10^{-6})$
100
200
300
400
$\omega^2 (\times 10^9)$

phase lock amplifiers can be used to electronically eliminate that term by "locking" onto that portion of the output signal from the bridge circuit which is in phase with the input signal. In theory, the capacitance term of the impedance is 90° out of phase with respect to the input signal.

Evaluation of the Bridge Conductance Measurement Circuit

In examining the data from the calibration curves for conductance versus ionic content, it is first of all apparent that the conductance shows a linear change with corresponding additions of an ionic substance. However, It should also be noted that the slope of the curves in DME is different than the slopes obtained when the calibrations were conducted in PBS (see Table 20 on page 349 and Table 21 on page 355).

One explanation for this discrepancy comes in examining the circuit in greater detail. First, consider the bridge to be composed of resistive elements using the notation shown in Figure 66 on page 368. Then, make the following assumptions: R1 and R2 are equal, and that R3 and R4 (the reference and determination chambers) differ only by dR which is small, then one can relate the voltage drop (e) to the following equation;

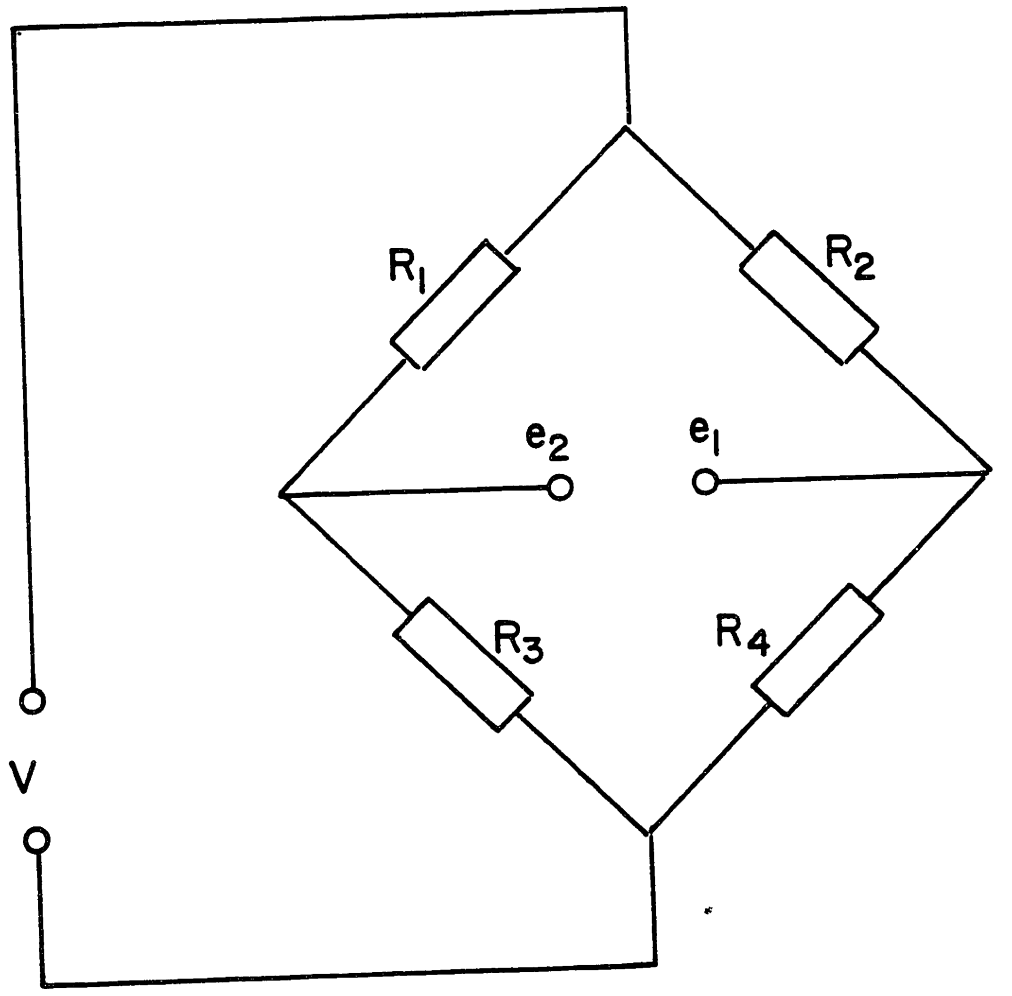
$$e = dR/(R1 + R4).$$

Clearly, (with R1 fixed) the larger the resistance of the supporting medium (R4) the less the voltage drop for a fixed addition of lactic acid or any other ionic species.

Effect of Temperature upon the System

If the two conductance chambers are identical in composition, then we expect the conductances would change equally with temperature, and this has been shown to be the case. The situation, however, is more complicated when the two chambers are not identical in composition, in this instance the conductance of each chamber will change with temperature but probably in a somewhat unequal manner. Experimentally, the effect of temperature upon the two chambers with unequal internal composition was examined by adding sodium acetate to one chamber (at a concentration

Figure 66 Wheatstone Bridge Model


R1
R2
e2
e1
R3
R4
V

of 15 m *M*) and changing the temperature of both the reference and determination chambers equally. The results of this experiment showed that the measured differential conductance of the determination chamber changed about 1.4% per °C. The principle difference between the two chambers is the presence of the sodium acetate ion, consequently changes in conductance due to changes in temperature should be attributable to changes in the ionic mobility of the two individual ion species (sodium and acetate). The mobility of both ions have been reported to change about 1.5 to 2% per °C, which would tend to confirm our observation. A second and perhaps more useful point to this experiment is that it demonstrates how effectively the differental bridge circuitry can be used to minimize the otherwise overwhelming effect of temperature on the system, and effectively allows us to use less tightly controlled temperature environments.

Long-Term Stability of the System

Most of the work utilizing conductance to analyze the growth or presence of bacteria has been accomplished over a short period of time (i.e. on the order of a few hours). That information can be rapidly obtained has been one the main attractions of the technique. Although the method boasts of high sensitivity, we were faced with a somewhat different concern. Due to the nature of animal cells in culture (i.e. very slow growth), we are forced to examine the usefulness of the method over a rather extended period of time (on the order of several days).

The long-term stability of the system was then examined in a series of three experiments; First, to examine the suitability of the electrical measurement system alone which we had developed, we filled both the reference and determination chambers with DME (prepared without glutamine). Second to allay the concern over the spontaneous degradation of the medium itself, the decomposition of glutamine to ammonia and other products being perhaps the most well known, both chambers were filled with DME and glutamine was only included in the determination chamber. Surprisingly no net change in conductance was observed. Subsequently, both chambers were filled with DME containing sera, and glutamine was added to the determination chamber. This time, a definite

change in conductance was observed, presumably due to the formation of ammonia. The more rapid degradation of glutamine in the presence of serum is probably attributable to the presence of certain enzymes whose presence is capable of catalyzing the degradation process of glutamine to ammonia and glutamic acid (and/or α-keto glutarate).

In the next logical step the system was used to examine the growth of cells on microcarriers (see Figure 64 on page 362). In this instance the growth of SV-80 cells was examined. This method did moderately well, the growth of the cells was accompanied by a change in the overall conductance of the medium.

APPENDIX Q: LOGIC OF DILUTION FACTOR

The purpose of the dilution factor is to provide a basis by which observed cell numbers and material concentrations at different time points during the culture can be compared. This is accomplished by adjusting the observed values to correspond to those wtihin a hypothetical undiluted culture using the dilution factor. The dilution factor (d) is defined as:

$d = V^0/V$

Thus, for example the observed cell number can be corrected using the following equation:

$V \cdot N = V^0 \cdot N'$.

V is the observed culture volume and for eperimental purpose is monitored by the computer by balencing the flux of materials entering and leaving the fermentor, as is shown the following equation:

$V = V_{int}$ + Baseadded - Samples - Medium volume changes.

V^0 is not affected by the addition of base or the medium changes, but is affected by sampling. Thus a sample of volume S will reduce V^0 by the volume $d \cdot S$.

where:
- V = observed volume
- V_{int} = initial volume of the culture
- V^0 = corrected volume (to remove changes caused by base addition and medium changes)
- N = observed cell number
- N' = corrected cell number
- d = diluiton factor
- S = sample volume

APPENDIX R: TRANSFORMATION OF DL/DATP TO DL/DG

It is possible to calculate for a given level of glycolsis, a corrsponding yield for lactic acid from the glucose consummed. If we assume that the cellular energy metabolism is being derived principally from glucose metabolism. Equation (1) divides the glucose metabolism into two parts, that being used through glycolysis and that though oxidative phosporylation.

$$dG = dG_{(gly)} + dG_{(ox)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

The corresponding yield of ATP from this metabolism is given in equation (2).

$$dATP = 2 \cdot dG_{(gly)} + 36 \cdot dG_{(ox)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

Moreover, each mole of glucose consummed via glycolysis will yield two moles of lactic acid as is indicated in equation (3).

$$dG_{(gly)} = dL/2 \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (3)$$

Thus it is possible to estimate a yield of lactic acid from the overall glucose metabolism as is done in equation's (4), (5), and (6). Specifically equation (6) relates this yield of lactic acid upon glucose metabolism to the level of glycolysis.

$$dATP - dL = 36 \cdot dG_{(ox)} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (4)$$

$$dG = dG_{(ox)} + dG_{(gly)} = (dATP - dL)/36 + dL/2 \quad \ldots\ldots\ldots\ldots\ldots \quad (5)$$

$$dL/dG = 1/([(1/x) - 1]/36 + 1/2) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (6)$$

where: $x = dL/dATP$

Reprinted from Volume 22 of DEVELOPMENTS IN INDUSTRIAL MICROBIOLOGY
· *A Publication of the Society for Industrial Microbiology, 1981*

CHAPTER 20

Large-Scale Production of Human Fibroblast Interferon

D.J. GIARD*, R.J. FLEISCHAKER, A.J. SINSKEY, AND D.I.C. WANG

*Cell Culture Center * and Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139*

A variety of parameters have been examined in an effort optimize conditions for interferon production with microcarrier-grown cells using a superinduction procedure. Control of concentration as well as the time-course of addition of antimetabolites has been shown to be important. Concentrations of 10 µg/ml of cycloheximide and 1 µg/ml of actinomycin-D appear to be optimal. Optimal exposure times to antimetabolites are 5 to 6 hours for cycloheximide and at least 2 hours for actinomycin-D. Two very critical parameters affecting interferon yields are cell priming and temperature control. Priming with 50 units/ml interferon combined with a reduction in temperature to 34 C during induction and production resulted in consistent yields of approximately 20,000 units/ml. Further temperature control, including a temperature shift from 37 C to 30 C during the production phase and an extended production period, has resulted in an additional fourfold increase in interferon yields. Currently, yields of 50,000 to 100,000 units/ml (10^6 cells/ml) are obtained routinely.

INTRODUCTION

Results of many clinical trials using interferon as an antiviral and antitumor agent have been promising (Merigan et al. 1973; Strander and Cantell 1974; Arvin et al. 1978; Horoszwicz et al. 1978). A continuing problem in these efforts has been the difficulty in obtaining sufficient quantities of interferon. The leukocyte/New Castle disease virus system has been used for most of the large-scale interferon production to date; however, the system has obvious disadvantages including risk of contamination with viruses and a limited supply of leukocytes.

Human diploid fibroblast strains which do not have the above disadvantages appear to be suitable substrates for human interferon production (Hayflick 1973). The use of superinduction procedures (Tan et

al. 1970; Havell and Vilček 1972; Billiau et al. 1973) has significantly increased interferon yields with human cells. This, coupled with the development of an improved microcarrier system for the large-scale production of anchorage-dependent cells (Levine et al. 1977, 1979), has provided the potential for a feasible means of mass producing high-titered, low-cost human fibroblast interferon.

In our laboratories, we have demonstrated the potential usefulness of microcarrier culture for interferon production (Giard et al. 1979). In an effort to increase interferon productivity, we have identified and controlled a number of critical parameters affecting human interferon production with microcarrier-grown human fibroblasts, including priming, temperature control, and time of exposure of antimetabolites.

MATERIALS AND METHODS

Cell culture. Human diploid foreskin cells (FS-4) were obtained from Dr. Jan Vilček, New York University School of Medicine, NY, and were used in all experiments. Cells were obtained at approximately the 18th population doubling. They were frozen viably in one batch for the entire series of experiments and were used between the 30th and 40th population doublings. Stock cultures were maintained in Corning plastic roller bottles in a walk-in incubator at 37 C. For experimental purposes, microcarrier cultures were seeded from these stocks. Cultures were seeded onto microcarriers in either 2-liter spinner vessels (Wheaton Scientific) in a 1-liter vol or a 14-liter fermentor (New Brunswick Scientific Co., New Brunswick, NJ) in a 5-liter vol. Cell density ranged from 3×10^5 to 4×10^5 cells/ml. A microcarrier concentration of 5 mg/ml was used and cultures were incubated in a humidified incubator supplied with 10% CO_2. Growth medium for stock cultures consisted of Dulbecco's Modified Eagle Medium (DMEM) (Flow Laboratories, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (Sterile Systems, Inc., Logan, UT). For microcarrier cell growth, DMEM was used with the serum concentration reduced to 5%. Antibiotics used were penicillin (100 units/ml) and streptomycin (100 µg/ml) and were obtained from Sigma Chemical Co., St. Louis, MO. Cultures were split using a trypsin-EDTA solution (Grand Island Biological Co., Grand Island, NY).

Microcarrier preparation and initiation. The procedure for microcarrier preparation has been described previously (Levine et al. 1977). The initiation of microcarrier cultures also has been described, but essentially consisted of the following: microcarriers were suspended in phosphate-buffered saline (PBS) at a concentration of 10 mg/ml and sterilized in glass bottles by autoclaving. Microcarriers then were dispensed into spinner flasks containing growth medium to give a final concentration of 5 mg/ml.

Interferon production. The superinduction procedure used was a modification of the method reported by Havell and Vilček (1972). The modified procedure developed in our laboratory consisted of the following: for most experiments cells were grown to confluency on microcarriers in a 1-liter vol to a density of approximately 1×10^6 cells/ml. On the fifth or sixth day, cells were washed two times with DMEM and 50-ml aliquots were transferred to 250-ml spinner vessels (Wilbur Scientific,

Inc., Boston, MA). Cells were then primed at 37 C for 16 h in DMEM + 50 units/ml of fibroblast interferon. Cells then were washed two times in DMEM, after which 50 ml of DMEM containing 50 mg/ml of poly I·poly C (PL Biochemical Co., Milwaukee, WI) and 10 µg/ml of cycloheximide (Sigma Chemical Co.) were added. After 4 h of incubation at 34 C, actinomycin-D (Sigma Chemical Co.) was added to give a concentration of 1 µg/ml. After 2 h of further incubation at 34 C, the medium was removed and cells were washed two times in DMEM. DMEM + 0.5% Plasmanate were then added and cultures were incubated for 1 h at 37 C, followed by a temperature shift to 30 C. Culture fluids were collected after 24 to 48 h, clarified by centrifugation at 2,000 rpm for 10 min, and either assayed immediately or frozen at -70 C until assayed.

Interferon assay. The interferon assay, described by Havell and Vilček (1972), consisted of the following: samples were assayed in duplicate in Co-Star 96-well culture dishes (Microbiological Associates, Walkersville, MD). Growth medium (100 µl) was added to each well and twofold dilutions of each sample were made in duplicate, using a P200 Gilson Pipetman (Rochester Scientific, Rochester, NY) or the Titertek Multipipetter (Flow Laboratories, Rockville, MD). Each well was seeded with 50×10^3 FS-4 cells in 100 µl growth medium and dishes were incubated for 20-24 h in a humidified incubator at 37 C supplied with 10% CO_2. Cells were then challenged with 1,000 PFU per well of VSV (Indiana Strain, obtained from David Baltimore, Center for Cancer Research, M.I.T., Cambridge, MA). Several wells in each dish served as cell and virus controls. Dishes were incubated at 37 C and scored microscopically after 48 to 72 h. The highest dilution of the sample showing 50% destruction of cells was considered the endpoint. Internal standards calibrated against the International Standards G023-901-527 and G023-902-527 (obtained from the National Institutes of Health, Bethesda, MD) were included with each assay.

Cell counts. Cells in microcarrier cultures were enumerated by counting nuclei, using a modification of the method of Sanford et al. (1951) as described by Van Wezel (1973). Roller-bottle cell counts were made by dispersion with a trypsin-EDTA solution, followed by counting with a hemacytometer.

Quality control. Cultures were screened thoroughly for the presence of mycoplasma using the culture method (isolation of PPLO colonies on artificial media), the uridine-uracil assay described by Schneider et al. (1974), and the DNA-staining method reported by Russell et al. (1975). All results were negative.

RESULTS

Antimetabolite Concentration

The effects of varying the concentrations of cycloheximide and actinomycin-D during induction were investigated in an attempt to establish optimum levels. The cycloheximide studies (shown in Table 1) indicated that a four- to eightfold reduction in the concentration of cycloheximide (from 50 µg/ml to 12.5 or 6.25 µg/ml) resulted in approximately a twofold increase in interferon yields. Yields obtained at concentra-

TABLE 1. Effect of cycloheximide concentration on interferon yields[a]

Cycloheximide Concentration (μg/ml)	Interferon Yield[b] (units/ml)[c]
0	750
6.25	22,000
12.5	24,000
18	9,000
25	13,000
50	11,000
100	11,000

[a] Induction was carried out under standard conditions described in Materials and Methods. Cells were grown on microcarriers in 250 ml spinner flasks.

[b] Results represent an average of two separate experiments.

[c] Figures also refer to the number of units/10^6 cells.

tions from 18 to 100 μg/ml were not significantly different. In a separate experiment (data not shown), the effect of varying the actinomycin-D concentration was measured. No significant differences were seen in interferon yields resulting from varying the actinomycin-D concentration between 0.25 and 2.0 μg/ml. Lowering the concentration to 0.125 μg/ml resulted in approximately a twofold drop in interferon yield.

Effect of Time of Exposure to Cycloheximide and Actinomycin-D

Havell and Vilček (1972) showed that the addition of cycloheximide later than 3 h after stimulation with poly I·poly C significantly decreased interferon yields. These experiments were done in small plastic dishes. In separate experiments, we decided to test the effect of varying (1) the time of addition of cycloheximide relative to poly I·poly C and actinomycin-D, and (2) the time of exposure of cells to actinomycin-D upon the interferon yields in microcarrier culture. Figure 1A shows the results of varying the time of addition of cycloheximide. Cycloheximide was added 1 h before and 1 to 4 h after the addition of poly I·poly C. Actinomycin-D was added at the standard time of 4 h after addition of poly I·poly C and left on for 1 h. Thus, the total length of exposure to cycloheximide ranged from 1 to 6 h. Interferon titers increased significantly with increased time of exposure of cells to cycloheximide. A plateau was observed at around 5 to 6 h of exposure when cycloheximide was added either with poly I·poly C (5 h) or 1 h prior to poly I·poly C (6 h). The data appear to indicate that for optimum interferon yields at least a 5-h exposure to cycloheximide is required.

Figure 1B shows the effect of varying the time of exposure of cells to actinomycin-D. In this experiment, poly I·poly C was added with cycloheximide, and actinomycin-D was added 4 h later. Cultures were then exposed to actinomycin-D from 10 min to 2 h. Results show that significant levels of interferon can be obtained after only 10 min. Exposure for more than 60 min (i.e., 90 or 120 min) can result in significantly higher yields. Additional studies (unpubl. data) indicated that maximum yields were obtained when exposure to actinomycin-D was extended to approximately 2.5 h.

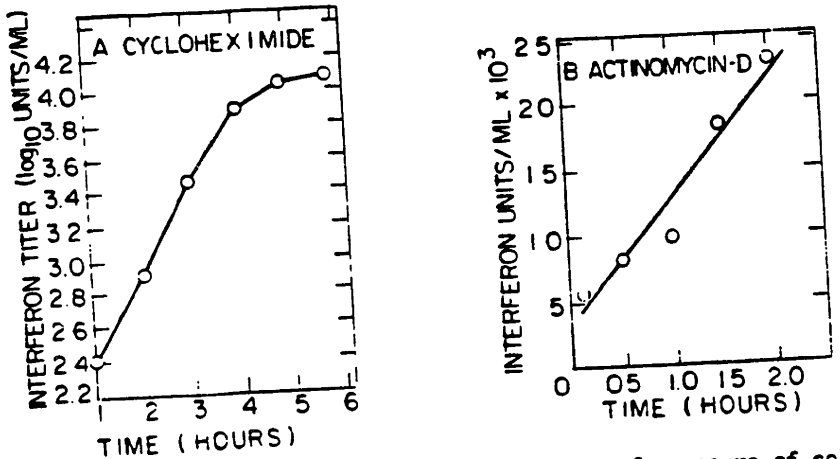


FIG. 1. Interferon yields as a function of time of exposure of cells to cycloheximide and actinomycin-D. FS-4 cells grown in 1-liter cultures were used to set up 50 ml cultures at 1 x 10^6 cells/ml for induction.

(A) Cycloheximide exposure--poly I·poly C was added 4 h prior to actinomycin-D in all samples and actinomycin-D was left on for 1 h. Cycloheximide (10 µg/ml) addition ranged from 1 h prior to 4h after addition of inducer, i.e., total time of exposure ranged from 1 to 6 h.

(B) Actinomycin-D exposure--Actinomycin-D was added 4 h after the addition of poly I·poly C and cycloheximide, and exposed to the cells for the times indicated (10 min to 2 h).

Comparison of Interferon Yields Using Standard and Modified Superinduction Procedures and the Effect of Priming

A series of experiments was carried out to compare the standard procedure reported by Havell and Vilček (1972) with a procedure modified by (1) reducing the temperature to 34 C during induction and production, (2) adjusting the concentrations and times of exposure of antimetabolites to optimal levels, and (3) priming. Results are shown in Table 2. The titers obtained using the modified superinduction procedure were considerably higher than those obtained with the standard procedure. However, the yields obtained with the modified procedure were lower and less consistent that expected (mean ±S.D. = 5,400 + 5,200 units/ml). The effect of priming the cells with 50 units of interferon per ml had the striking effect of giving higher and much more consistent yields (mean ±S.D. = 23,000 + 4,100 units/ml).

Kinetics of Interferon Production Using Different Temperatures During Induction and Production Phases

The kinetics of interferon production were examined using different combinations of temperatures. When the temperature was held constant (34 C) during production but varied during induction (30, 34, and 37 C), a significantly lower peak was observed at 30 C compared to the

TABLE 2. Comparison of interferon yields using standard and modified superinduction procedures

Treatment	Interferon Yield (units/ml)[a] Experiment No.[b]			
	1	2	3	4
Poly I · Poly C only	<100	N.D.[f]	N.D.	N.D.
Superinduction (Standard)[c]	1000	N.D.	N.D.	N.D.
Superinduction (Modified)[d]	3400	13,000	1000	4300
Superinduction (Modified) + priming[e]	25,000	17,500	22,000	27,000

[a]Since production was carried out at 1 x 10^6 cells/ml, the figures in this table also refer to the number of units/10^6 cells.

[b]The first experiment was carried out to compare yields obtained under a variety of different conditions. The next three experiments were done to determine the effect of priming cultures.

[c]Standard conditions were as follows: cycloheximide concentration was 50 µg/ml; actinomycin-D was left on cultures for 1 h and the temperature was maintained at 37 C.

[d]The standard procedure was modified as follows: cycloheximide concentration was 10 µg/ml; actinomycin-D was left on cultures for 2 h and the temperature for the experiments was maintained at 34 C.

[e]Priming consisted of exposing the cells to 50 units of interferon/ml for 16 h prior to induction on day 6. Cells were washed 2X with DMEM after which DMEM + 0.5% plasmanate + interferon was added.

[f]N.D. = not done.

higher temperatures, and the rate of decline (production essentially complete by 16 h) was approximately the same at all temperatures (Fig. 2A). Figure 2B shows the kinetics of interferon production when the temperature was held constant (34 C) during induction and varied during the production phase 30, 34, and 37 C. The highest peak (approximately 6,000 units/ml/h) seen at 37 C was approximately threefold higher than those observed at 30 and 34 C. Although a typically rapid decline in the production rate was observed at 34 and 37 C, the rate of decline at 30 C was less sharp and production was still significant after 24 h (400 units/ml/h). These kinetic experiments suggested the possibility that the use of a combination of temperatures during the production phase might enhance overall yields of interferon.

Determination of the Optimal Time for Temperature Shift During Production Phase

In order to determine the effect of a temperature shift during the production phase, duplicate cultures were induced at 34 C and either held at 30 or 37 C, or switched from 37 to 30 C after 1, 2, 3, and 4 h. As shown in Table 3, higher yields were obtained using the temperature switch (two- to threefold) regardless of the time of shift in comparison to control cultures at either 30 or 37 C. The yield was slightly higher with the 1-h shift (although probably not significantly different) than with the 2-, 3-, or 4-h shifts. Repeated experiments using a 1-h shift have consistently given high yields.

Results of Large-Scale Production Runs Using Temperature Control

The use of a temperature shift to increase interferon yields was fur-

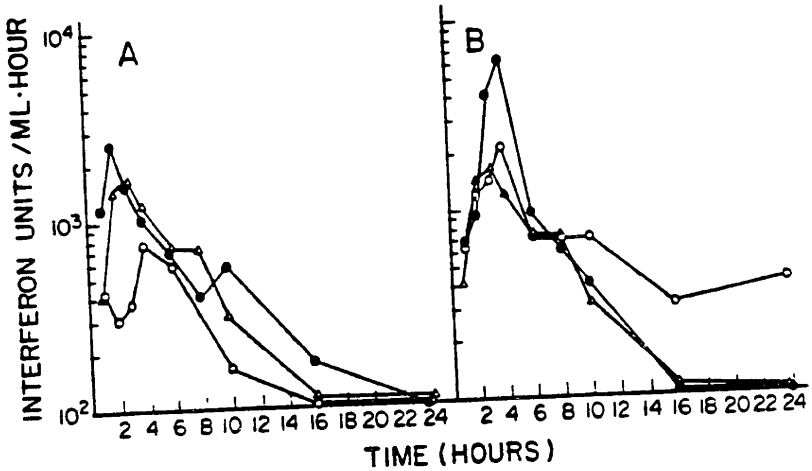


FIG. 2. A. Kinetics of interferon production using different temperatures during induction. Duplicate cultures of FS-4 cells were induced at temperatures of 30 C (o), 34 C (Δ), and 37 C (•) during induction. After inducer and antimetabolites were removed and production medium (DMEM + 0.5% Plasmanate) added, the temperature was held at 34 C throughout the production period. At each of the indicated times, medium was removed from each culture vessel and fresh production medium was added.

B. Kinetics of interferon production using different temperatures during production period. Duplicate cultures of FS-4 cells were induced at 34 C. Following the induction phase, production was carried out at 30 C (o), 34 C (Δ), and 37 C (•). Production medium (DMEM + 0.5% Plasmanate) was changed (50 ml per culture vessel) when samples were taken.

ther examined in 5-liter production runs using a 14-liter fermentor. The fermentor was seeded at a density of approximately 4 x 10^5 cells/ml with cells grown in roller bottles. The cells were primed on day 6 and induced on day 7. Table 4 gives the results of two separate 5-liter production runs. In each experiment a twofold increase in yield was

TABLE 3. Determination of optimal time of temperature shift during production phase

Temperature Conditions (°C)		Interferon Yield (units/ml)[a]
Induction	Production	
34	37	15,800
34	37 → 30 (1 h)	43,400
34	37 → 30 (2 h)	37,500
34	37 → 30 (3 h)	28,100
34	37 → 30 (4 h)	36,300
34	30	12,300

[a]Since cell density was 1 x 10^6 cells/ml, figures also refer to units/10^6 cells.

TABLE 4. Interferon production in 5-liter microcarrier cultures

Experiment	Interferon Yield (units/ml)[a]	
	24 h	48 h
1	24,000	48,000
2	65,000	110,000

[a]Since cell density was 10^6 cells/ml, figures refer also to number of units/10^6 cells. The data are cumulative and represent yields over the entire production period.

obtained by extending the production period from 24 to 48 h. Data for 48 h are cumulative, representing the total yields obtained over that period and ranged from 48,000 to 110,000 units/ml.

DISCUSSION

Tne rationale for experimenting with a range of concentrations of antimetabolites was that a different concentration (preferably a lower concentration which might result in a lower level of toxicity) might result in higher yields. The discovery that a lower level of cycloheximide (approximately 10 µg/ml) is optimal for interferon production differs from some previous results. However, it is in good agreement with the findings of Billiau et al. (1973) who also found 10 µg/ml to be optimal.

The effect of varying the time of exposure of cells to cycloheximide (Fig. 1A) is in good agreement with Havell and Vilček (1972) in showing that 4 to 6 h of exposure are required to give optimal interferon levels. Increasing the time of exposure of actinomycin-D from 1 h to 2 h (Fig. 1B) gave a significant increase in interferon yields. Further experimentation has demonstrated that even higher yields can be obtained by further extending the time of exposure to actinomycin-D to 2.5 h (unpubl. data).

We conducted a series of experiments in order to measure the effect of our modification of the superinduction procedure reported by Havell and Vilček (including priming and temperature reductions to 34 C). Results are presented in Table 2. Experiments were conducted (data not shown) which revealed the optimal concentration of interferon for priming to be approximately 50 units/ml. Titers obtained with the modified superinduction procedure were lower and less consistent than expected (mean ±S.D. = 5,400 + 5,200). However, the addition of priming to the procedure had the effect of giving consistently high yields (mean ±S.D. = 23,000 + 4,100). Under these conditions, the yield for any given experiment would be expected to fall between 29,700 and 16,300 (90% confidence level). The overall effect of priming in this series of experiments was a greater than fourfold increase in the amount of interferon produced. However, since yields in excess of 20,000 units/ml have been achieved many times without priming, the actual effect of priming probably had more to do with somehow giving consistency to interferon production with microcarrier-grown cells (perhaps by sensitizing cells to the induction procedure) than to imparting cells with the ability to produce increased amounts of interferon.

Our kinetic experiments allowed us to compare production rate over a 24-h period at different temperatures during induction and production

phases. The data (Figs. 2A and 2B) confirm the importance of keeping the temperature above 30 C during induction (Fig. 2A). In addition, it is apparent from Fig. 2B that when different temperatures are used during production, a higher initial peak is obtained at 37 C compared to either 30 or 34 C. This observation, coupled with the sustained, low-level production observed at 30 C, suggested the possible value of using a combination of temperatures, i.e., 37 C initially followed by a shift to 30 C. In subsequent experiments establishing the optimal time for temperature shift (Table 3) and demonstrating production in large-scale microcarrier culture (Table 4), it has been shown repeatedly that the use of a shift in temperature from 37 to 30 C during the production phase results in significantly higher interferon yields. Furthermore, yields are further increased by extending the production period from 24 to 48 h.

Our rationale for using temperature reduction was an attempt to increase interferon yields through stabilization of interferon mRNA. It is possible through a reduction in temperature that a stabilizing effect and hence a lower decay rate could occur during (a) induction, resulting in a greater amount of active interferon mRNA at the start of the production period, and/or (b) production, resulting in a greater amount of interferon mRNA being available for translation throughout the production phase. A reduction in temperature also could exert a stabilizing effect in other ways including (a) interference with an inhibitor which inactivates interferon mRNA; (b) a decrease in the rate of cellular deterioration allowing production over a longer period. The latter possibilities were suggested by Sypula and Zielinski-Jenczylik (1978) who worked with normal human fibroblasts, using NDV as inducer. The principal objective of their study was to preserve the inducer and the cells through temperature reduction. When increased interferon yields were obtained, it was speculated that the higher yields resulted from one or both of the above mechanisms.

We have made no attempt in these studies to compare microcarrier-cultured cells to other culture methods (including roller bottles and plastic dishes) with regard to the effect of temperature on interferon yields. It is likely, however, that temperature control can also be used with other culture systems to increase interferon yields, even though optimization of individual systems may be required.

In addition to the control of various critical parameters already discussed, there are a number of other important considerations pertaining to successful interferon production with microcarrier-grown cells. Among these are: (1) careful preparation of cell inoculum, avoiding overexposure to trypsin; (2) seeding microcarrier cultures with a minimum concentration of 3×10^5 cells/ml; (3) reduction of fetal bovin serum in FS-4 growth medium to approximately 5%, which allows cells to remain more firmly attached during induction, resulting in higher interferon yields; and (4) selection, by prescreening, of a satisfactory lot of poly I·poly C. Careful control of all the parameters discussed here have enabled us to currently obtain, in fermentor-size production runs, yields which consistently range from 50,000 to 100,000 units/ml.

ACKNOWLEDGMENTS

We acknowledge the technical assistance of Michele Fabricant in these studies. This work was supported by the National Science Foundation Grant PCM 78-19497 and the Lewis and Rosa Strauss Memorial Fund. Robert Fleischaker was supported by N.C.I. Training Grant No. 1-T32 CA09258-01.

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