AD_____

LARGE SCALE FERMENTER GROWTH OF ANIMAL CELLS FOR VIRUS VACCINE PRODUCTION: CONTROL OF SEED STOCKS AND GROWTH CONDITIONS (U)

ANNUAL PROGRESS REPORT

by

Donald J. Merchant, Ph.D.
Professor, Department of Microbiology

February 1969
(For the period 1 January 1968 to 31 December 1968)

Supported by

U. S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Office of the Surgeon General, Washington, D. C. 20315

Contract No. DADA-17-67-C-7073
The University of Michigan
Ann Arbor, Michigan 48104

DDC AVAILABILITY STATEMENT

FOREWORD

In addition to the author the following persons have contributed to this work:

Gregory Baumann
Barbara Brown
Lloyd L. Kempe, Ph.D.
William Kerr, Jr.
Fannie R. Parker
Jens Pedersen
Howard Stockdale, Ph.D.
Gary Stoner

TABLE OF CONTENTS

LIST OF FIGURES

SUMMARY

The purposes of the program are to achieve control of seed stocks of animal cells to be used for production of virus vaccines in fermenters and to achieve control of the fermentation process to give a high quality and a reproducible product.

Attention has been concentrated on LM mouse cells as a prototype of a heteroploid animal cell line capable of growth in suspension in a simple medium, and on BHK 21 hamster fibroblasts. The latter cells are near diploid and are closer to the type of cell ultimately desired. Work also has been done with HCAAT (a calf serum adapted HeLa cell line) and BSC-1.

During the year, work was completed on the equipment for maintaining and control of pH, ORP, and dissolved oxygen in the fermenter and several improvements have been included. Runs have been made in which all three parameters were monitored through several growth cycles and other runs were made where one or more parameter were controlled to observe the effect on viability, growth rate, and growth yield.

Additional work was done on colony morphology, isozyme variants, and chromosome changes as genetic markers for following seed stocks as well as for following growth in suspension.

An additional line of investigation was initiated. This deals with nutritional requirements and the composition of growth media for fermenter runs. Using a line of cells grown in chemically defined medium, a unique approach is being made to establishing a well balanced defined medium capable of supporting rapid and sustained growth. Another approach is aimed at development of an inexpensive medium utilizing readily available additives.

INTRODUCTION

The purposes of this project are (1) to evaluate methods of handling and expansion of cell seed stocks from frozen ampoules to large fermenters to be used for virus vaccine production, and (2) to determine optimal conditions of fermenter operation. The primary concern is to determine the degree of drift in cell populations, the major causes of such drift and to explore control procedures for minimizing these variables.

As stated previously this is being done by two approaches. We are using multiple genetic markers to follow population changes during seed stock build-up and to identify the nature and cause of change when possible. With use of automated recording and control equipment we are seeking to determine and maintain optimal growth conditions.

During the year 1968 we continued to approach these problems in the following ways: (A) Completion of Equipment for Monitoring and Control, (B) Analysis and Control of Fermenter Growth, (C) Refinement of Genetic Marker Systems, (D) Analysis of Population Changes, (E) Refinement of Analytic Procedures, and (F) Development of Improved Nutritional Systems.

It should be mentioned that this work was unavoidably disrupted for over a month late in 1968 due to unexpected changes in the building construction schedule at The University of Michigan. It was necessary for us to relocate the major part of the work in the Phoenix Memorial Laboratory from the East Medical Building.

A. COMPLETION OF EQUIPMENT FOR MONITORING AND CONTROL

The control unit which was described in detail in the progress report for 1967 has been completed and is in operation. In addition to the equipment previously described a dual timer has been installed to assure a more flexible control action of the gas mixing setup by allowing for continuous addition of either nitrogen or oxygen-nitrogen for up to sixty minutes.

A condensed description of the control unit and gas control equipment follows to permit the reader to more readily interpret the results presented in the next section.

1. The control unit is a wooden box with access from front and back to facilitate service. It is mounted on a metal cabinet with rollers (Equipto #236-2) to enable sequential service of several fermenters. The unit contains the following equipment:

(a) Two Beckman Model 900 pH analyzers of which one is modified to furnish current output for recorders and controllers in order to save investment in expensive potentiometric equipment.

(b) One Lee Scientific oxygen analyzer model 100 providing for the full-scale measuring ranges 0-21% oxygen and 0-100% oxygen. (The dissolved oxygen electrode has not performed successfully due to problems with condensation of water inside the plug that connects the probe stem with the cable inside the fermenter. This condition has resulted in unstable output to the dissolved oxygen analyzer. Therefore the observed values for dissolved oxygen are only qualitative. A procedure has now been adopted comprising a sealing of this connection with O-rings, Teflon tape, and heat shrinkable tubing.)

(c) Two CRI Pantam Messocontactor Controllers (optical meter relay) model PX2 M11 matched to the microampere output from the Beckman analyzers. The controller for pH has the range 5 to 9 corresponding to a meter input range -61.2 microamps to -2.9 microamps (not including zero). The controller for ORP has the range +400 mV to -400 mV corresponding to a meter input range -127.3 microamps to +63.3 microamps.

(d) One CRI Pantam Messocontactor Controller Model PX2 M11 matched to the 0-1 mA output from the Lee Scientific oxygen analyzer.

(e) Two Rustrak recorders model 88 specific for the same input range as the controllers.

(f) One Rustrak Recorder model 88, 0-1 mA to record the output from the oxygen analyzer.

(g) A Sola voltage transformer to furnish constant voltage for the controllers and recorders.

(h) Four percentage timers (Industrial Timer Corporation Recycling Timers series PC (two 60 sec and two 30 sec)) to prevent over-compensation of the controlling action from the controllers.

(i) One dual repeat cycle timer from Automatic Timing and Controls, Inc., Model ATC 306-C-017-A-00-PX. This timer provides for constant addition of two different gas mixtures in repeat cycles of up to one hour duration each.

(j) Four relays (Potter and Brumfield KRP11A DPDT) rated to carry the current that is necessary to activate the solenoids for electrolyte addition.

(k) A switch to select either ORP or DO control option.

(l) A switch to select either percentage timer or dual timer option in the ORP or DO control.

(m) A rotary switch to select between two different metal electrode inputs to the ORP analyzer.

(n) A series of switches to accomplish automatic or manual option of operation and a display of pilot lights to indicate mode of operation.

2. A design for the gaseous control of ORP and pH has been made. It provides for mixing of several streams of metered gases of various compositions of air, nitrogen, oxygen, and carbon dioxide. The ORP controlling part of the design has been executed. This provides for a continuous flow of carrier gas with a composition close to the operating conditions. One of three control gas stream options is then determined by the mode of the controller.

3. A conventional 5-liter New Brunswick fermenter is used. It has been modified with a Teflon electrode holder to accommodate up to six different electrodes and a conduit for the leads through the fermenter head.

A stainless steel sampler with a silicone rubber diaphragm as described in the addendum to the previous progress report has been installed. It facilitates the sample taking via long stainless steel needles with a pointed tip and has performed successfully.

B. ANALYSIS AND CONTROL OF FERMENTER GROWTH

Run NB5

Objectives

1. Steam sterilization of fermenter filled with distilled water and with all electrodes installed.

2. Check effect of sterilization on DO probe performance.

3. Calibration of DO probe in the fermenter by sparging with air and nitrogen.

4. Correlation of DO and ORP in water.

5. Charge fermenter with medium 199 + peptone and calibrate the DO probe by sparging with air and nitrogen.

6. Correlate DO and ORP while monitoring pH.

Results

Sterilization of the fermenter and accessories caused only a 1% change in the DO electrode span.

A correlation of the OR potential (expressed as E_S*) and DO for medium 199 + 0.5% peptone and 0.12% methyl cellulose was found to be, at pH 8.5 DO = 100% saturation with air, E_S = 182 mV. DO = 0% of saturation with air E_S = -110 mV.

Run NB6

Objectives

1. Autoclave ferementer with 900 ml 0.625% peptone water with all electrodes in place and immersed.

2. Calibrate DO probe by sparging with air and nitrogen.

---

*E_S is defined as the observed potential between an inert electrode and the silver-silver chloride reference electrode and as measured under the prevailing pH and temperature.

3. Remove portion of peptone water and add 5 X 199 to the remaining portion to obtain 800 ml 1 X 199 + 0.5% peptone and 5% FeCaS in the ferementer.

4. Inoculation of log phase L-M cells from spinner culture and monitoring of pH, ORP, and DO over several growth cycles. Initial pH to be adjusted to 7.2 by addition of acid or base. Final volume approximately 1 liter.

Results

The DO probe performed inadequately during this run.

Initial E_S and pH prior to inoculation were 150 mV and 7.19, respectively.

During the first growth curve the E_S declined to 102 mV, pH to 6.6 with a cell concentration of 2.2 X 10^6/ml and a viability of 99.5%. The population doubling time was 32 hours.

After dilution of the culture bacterial contamination occurred and the run was terminated.

Run NB7

Objectives

Same as Run NB6.

Results

The DO probe was calibrated with nitrogen and air giving readings of 2% and 100%, respectively. However, at the time of the first medium change the probe malfunctioned due to condensation of water inside the probe cable.

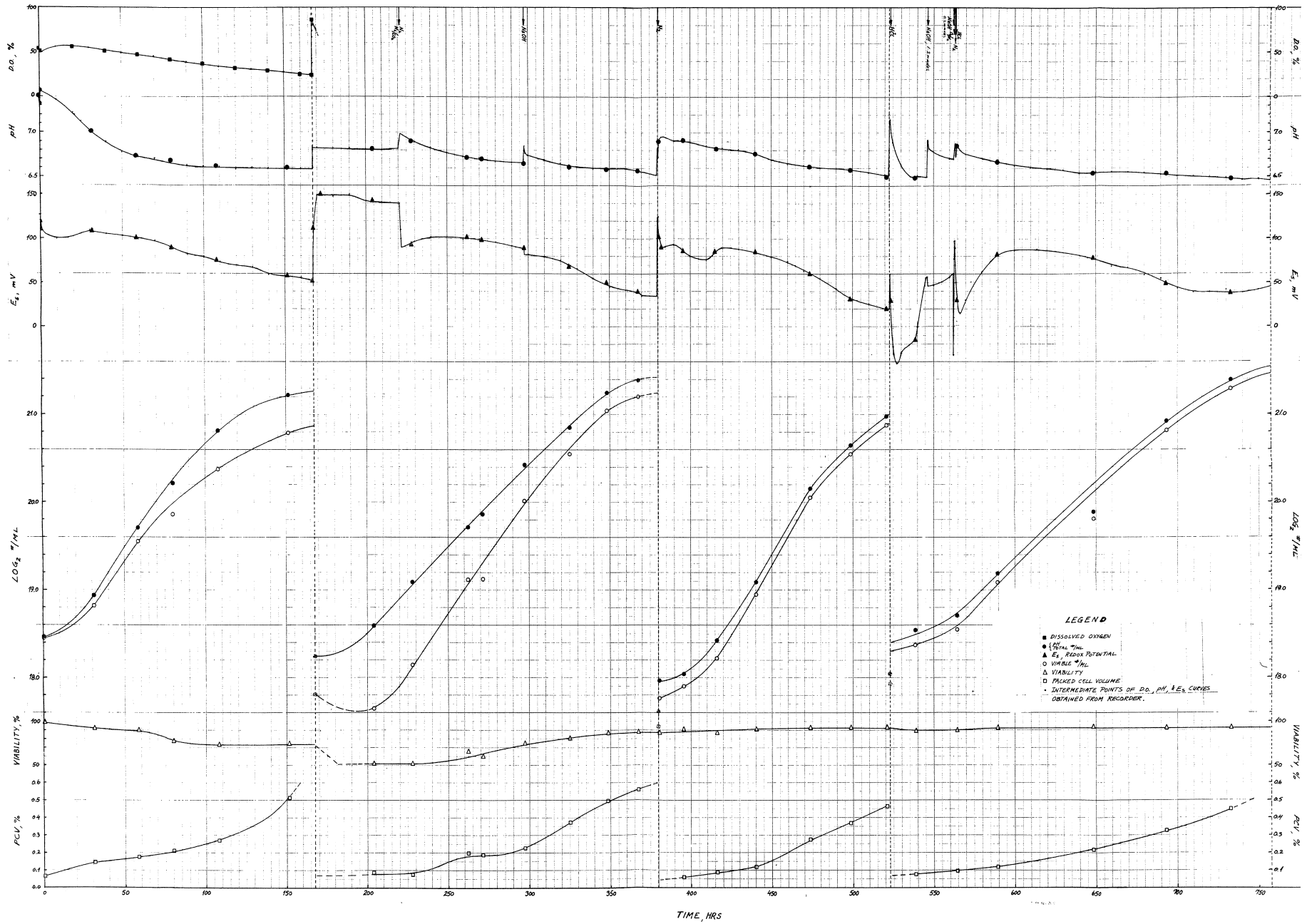
It was discovered that the thermocompensator in the ferementer has shorted, very likely due to water condensate inside the compensator. It was disconnected from the control unit and instead a precision resistor: 4.34 kohms ± .1% was installed across the thermocompensator terminals in the pH analyzer.

The population doubling times for Run NB7 during the 4 growth curves that were obtained showed the following values: 34, 50, 32, and 60 hours. These were all measured on the basis of total #/ml.

The following variables and parameters were obtained and plotted in the following figure.

Total and viable:	#/ml	DO:	% saturation
Viability:	%	pH:	
Pcv:	%	E_S:	mV.

NB7 LM 199 PSP FCS$_5$



The DO, pH, and E_s values outside the sample times have been obtained from the recorder charts with appropriate corrections for drift and deviation from controller readings. The culture was maintained for 30 days without contamination and was at that time transferred to a larger ferementer.

There is a definite overall pattern in the traces of pH and ORP through a growth curve. The differences are merely quantitative and in some cases due to manual control action with gases, acid, and base.

The ORP curves are S-formed and the inflexion points coincide reasonably well with similar points on the growth curves.

Disturbances in the ORP and pH patterns by manual control seems to even out with time such that the general trends of the curves are analogous. This run gives a good basis for deciding ranges for ORP, pH, and DO for controlled runs.

Run NB8

Objectives

Growth of BHK cells with ORP and pH monitoring and later control. The medium used was the same as described in Section F in this report.

Results

The cells were carried through two growth curves.

The pH history is similar to that of LM cells. The ORP starts off at a level similar to that of LM cells, but towards the end of the log phase ORP dropped to -50 mV, much lower than observed with LM cells which showed 40 mV.

During the second growth curve an attempt was made to maintain an ORP at about 100 mV corresponding to the value at mid log phase by gassing the overlay with air. However, this resulted in an increase in population doubling time from 18.5 hours to 24 hours.

Due to deterioation of the culture the run was terminated.

During this run the DO probe was not operating.

Run NB10

Objectives

Growth of the LM cells in medium 199 P + 5% FeCaS with pH control at 6.8 -

6.9 and DO control at 35% saturation with air and monitored ORP with the restriction on ORP range: 100-20 mV.

In addition: to demonstrate that the population density in the New Brunswick fermentor could reach similar levels as in spinner flasks, namely 3.5 - 40 X 10^6 cells/ml.

Results

Due to erratic performance of the DO probe, it was decided to carry out the run with ORP control in the range E_S = 100 to 20 mV.

During three growth curves it was observed that increased growth occurred when the ORP control range was maintained between 85 and 110 mV. This was the case even at high population densities. It was demonstrated that the population density could reach 3.2 - 4.0 X 10^6 cells/ml.

The run was maintained uncontaminated for 2 months. During the last 14 days correlation between pH, ORP, and DO was made on spent medium and fresh medium. This was possible since the DO probe at the end of the run bacame serviceable again.

During the successful runs, samples were taken and frozen for subsequent analysis of glucose, lactate, inorganic phosphate, nucleic acids, protein, and phospholipids. Part of these analyses have been initiated.

Future experiments have been outlined in which the effect of selected ORP control levels on the growth rate of LM cells in 199 P and FeCaS at constant pH and monitored DO will be investigated. Chemical analyses will be carried out concurrently in order to calculate glucose uptake rate and lactate production.

C. REFINEMENT OF GENETIC MARKER SYSTEMS

Unexpected difficulties were encountered in two of the systems for analysis of genetic markers. For several months we were unable to repeat previous results on isozyme separation by starch gel electrophoresis. Much time was spent on analysis of all known variables in the system including buffers, pH, ionic concentrations, methods of preparing cell hemogenates, and preparation of starch slabs. Several different batches of starch were tried. Finally it was learned that only two batches of starch have been manufactured in the past two years which are satisfactory. We are now to receive samples of one of these batches. In the meantime, alternate systems have been developed using agar and sephadex gels.

A similar situation developed in the analysis of colony variants. For most of the year we simply were unable to locate a lot of horse serum adequate for our needs. This finally has been accomplished and a sufficient supply is available to complete our studies. To further complicate this picture, changes in formulation of plastic dishes have occurred from time to time. These now seem to be largely resolved.

During the last year, extensive progress has been made in the application of computer methodology to the study of the chromosome complement of mixoploid cells. Chromosome measurements are made using a semi-automatic digital coordinate recorder. A number of computer programs have been developed which examine chromosome lengths in a number of different forms. Examination of the data so far obtained has shown that the mean length of telocentric chromosomes in a given metaphase spread and the mean length of biarm chromosomes are quite characteristic of the population. Although we are looking for more sensitive population characteristics, we have shown that using the information we are currently extracting from chromosome measurement data, one can quantitatively describe a population and can make statistical comparisons of populations which cannot be separated positively on the basis of other methods of chromosome analysis.

D. ANALYSIS OF POPULATION CHANGES

A study of population changes as monitored by chromosome changes was made. This involved comparison of monolayer and suspension cultures of small spinner and fermentor systems. The information is presented in the form of a formal report submitted by Mr. Gregory Baumann. Work on this aspect is continuing.

1. Population Selection in the Adaptation of an Established Animal Cell Line to Suspension Culture

Although mammalian cells have been successfully grown as monolayers on the surface of glass or plastic since 1922,[1] it was not until 1953 that successful proliferation of such cells in suspension was achieved.[2] Since that time, a variety of different methods have been employed to maintain cells in suspension. Rotating tubes, Erlenmeyer flasks on a rotary shaker, vessels equipped with rotating teflon coated bars, and stainless steel or glass fermentors with motor driven impellors have all been used.[2-8] Interest in these methods of cell proliferation *in vitro* is great. Suspension culture, according to Kulcher and Merchant[5] is extremely useful in the study of growing, homogeneous, cell populations where each cell is uniformly exposed to its environment. Such methods also make possible the growth of large volumes of cells for the production of virus-vaccines, hormones, and other biologically active materials. These cells can also be used in large quantities for biochemical and antigenic analysis.[4,6-8]

Ease of proliferation in suspension culture is, by no means, characteristic of all mammalian cells. In the cases where growth is possible, cells may produce sheets, form clumps and rimming in the culture vessel, or may grow as single, isolated cells in which case the culture exhibits growth patterns similar to those of microbial systems.[3] The transition which occurs when cells are changed from a monolayer type growth system to a suspension system is not fully understood but is thought to involve the processes of variation and selection.[9] It is becoming increasingly important to determine what does actually occur inasmuch as large suspension cultures of mixoploid cell lines grown in fermentors are now being considered for the production of virus-vaccines. If such cultures are to be used for this purpose it will be important to know what type of cell transformations occur when cells are adapted to and maintained in suspension and how permanent the changes are.

In this laboratory fermentor cultures are established by building up a cell population in a number of steps.[10] Cells are first grown in monolayers which are used to start small suspension cultures. These suspension cultures

are then used to seed the fermentor when a sufficient volume of cells is obtained. The purpose of this study has been to obtain an indication of the population stability and the selection processes which occur in the establishment of fermentor cultures by following the chromosome complement of the cells as they are carried through the developmental stages.

MATERIALS AND METHODS

The chromosome patterns of two very similar established mammalian cell lines were followed in this study. The LM strain is a subline of strain L originally derived from a C3H mouse.[11] Cells of the LMa strain, which was derived from the LM strain by passage through a C3H mouse,[12] were also used. Both cell strains are grown in modified medium 199[13] supplemented with peptone and fetal calf serum (in concentrations of up to 5% of the total volume of media). The LMa cells, when they were carried in suspension, were also supplemented with 0.12% methylcellulose (15 CPS, reagent grade, Dow Chemical Co., Midland, Michigan) which exhibits a protective effect on cells carried in suspension.[14] Both lines are mixoploid and their chromosome complements under normal culture conditions in monolayer have been studied extensively.[15] The chromosome complement of a large percentage of L strain cells includes one or both of the marker chromosomes shown in Figure 1. Derivatives of these markers missing

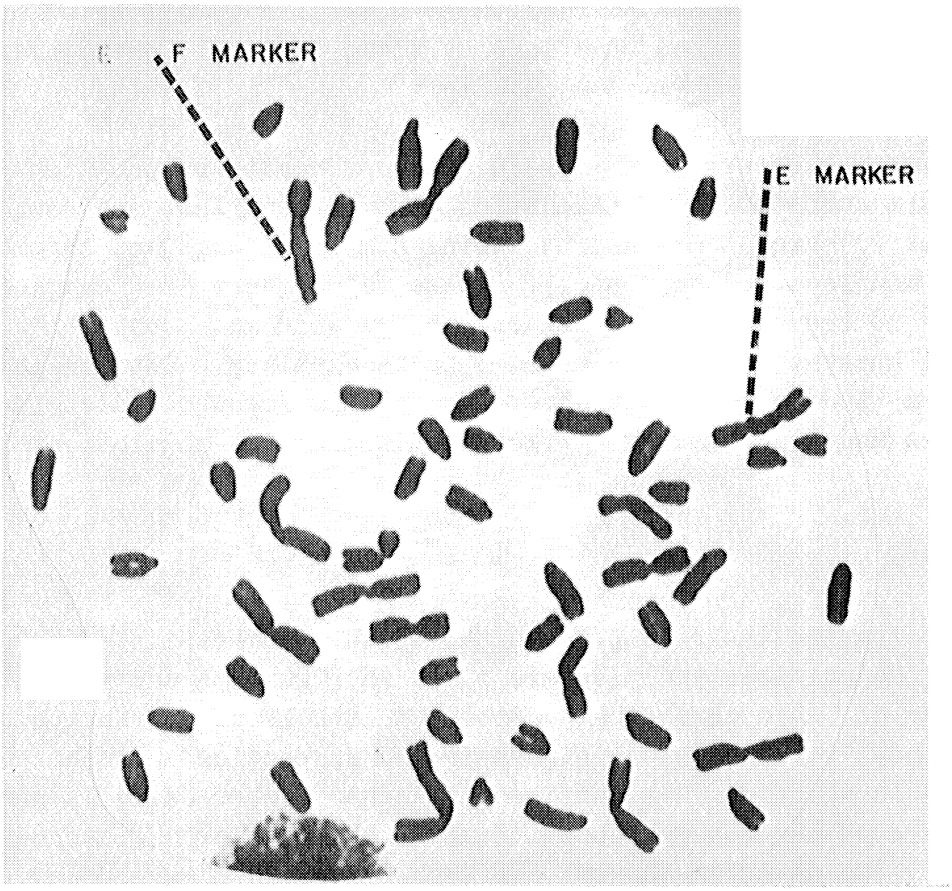


Figure 1. A typical chromosome spread from the LMa strain with the E and F marker chromosomes indicated.

one or two of the central, secondary constrictions associated with the markers are also found. The significance of these derivatives is questionable since the appearance of secondary constrictions is thought to be highly dependent on the method of preparation used.(16)

Although original plans called for studying the chromosome complement of cells in monolayer and at a number of times during suspension culture in both a small culture vessel and in the 5-liter fermentor, time and problems encountered in the preparation of samples made the study of complete sequences of samples impossible. The chromosome complement of the LM strain was studied once during culture in monolayer (in media containing 2.5% fetal calf serum) and during culture in the 5-liter fermentor (in media containing 5% fetal calf serum). Analysis of the LMa strain, which was carried in media with 5% fetal calf serum for the duration of the experiment, was carried out during culture as a monolayer and as a small suspension culture (150 ml). The treatment of these cells during the time they were being studied is described in Figure 2.

Spreads of metaphase chromosomes were made using a modification of the technique described by Merchant, Kahn and Murphy.(17) Two mcg of Colcemid (Grand Island Biological Co., Grand Is., New York) was added for every 10 ml of culture fluid to a culture of cells in midlog phase. After being incubated 12 hours with Colcemid, the cells were harvested, expanded in a hypotonic solution consisting of 0.7% sodium citrate in distilled water, fixed in 1:4 acetic acid: methanol, and spread on slides by flame drying. Chromosomes were stained with Giemsa stain.

Fifty metaphases were examined in each chromosome preparation and the numbers of biarm chromosomes (metacentrics, submetacentrics, and subtelocentrics) and telocentric chromosomes and the total number of arms and chromosomes were determined and recorded separately. These values were then checked against one another to reduce counting errors. Notes were made describing any abnormal chromosomes present in the metaphase spreads examined. Data collected by counting was then used to construct chromosome number frequency distributions and to determine modal chromosome characteristics for the population.

RESULTS

Plots comparing the results obtained by the enumeration of chromosomes in the LM and LMa cultures analyzed are shown in Figures 3 and 4. Although the modal number of biarm chromosomes present in the LM cells remains constant, the other modal values are one higher in the suspension culture than in the monolayer. In addition, a significantly larger number of cells contain the modal numbers of chromosome types. Similar but smaller differences are also exhibited by the data for the LMa cells.

Marker frequencies for the populations examined are shown in Figures 5 and 6. Because only two analyses are available for each population, and due to

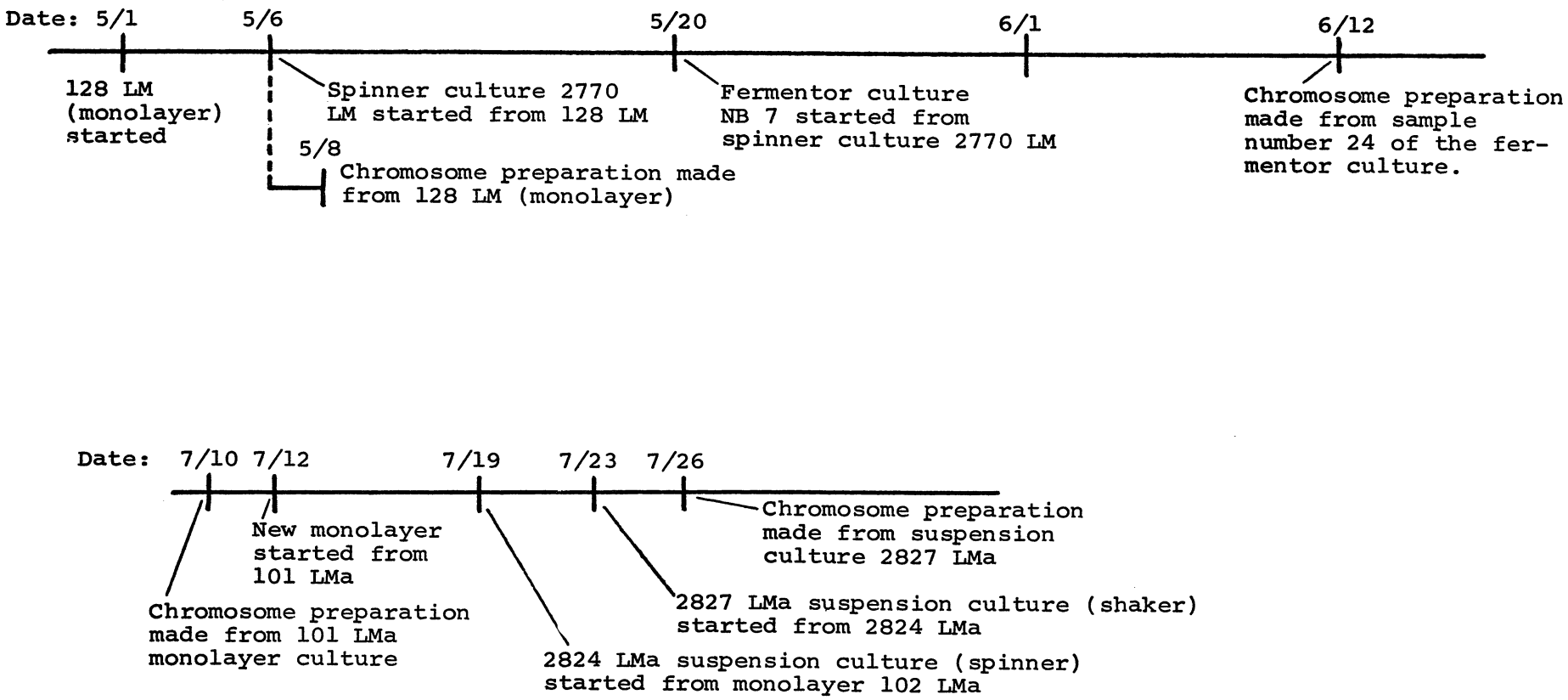


Figure 2. Time lines describing the handling of the LM and LMa strain cells for the duration of the experiment.

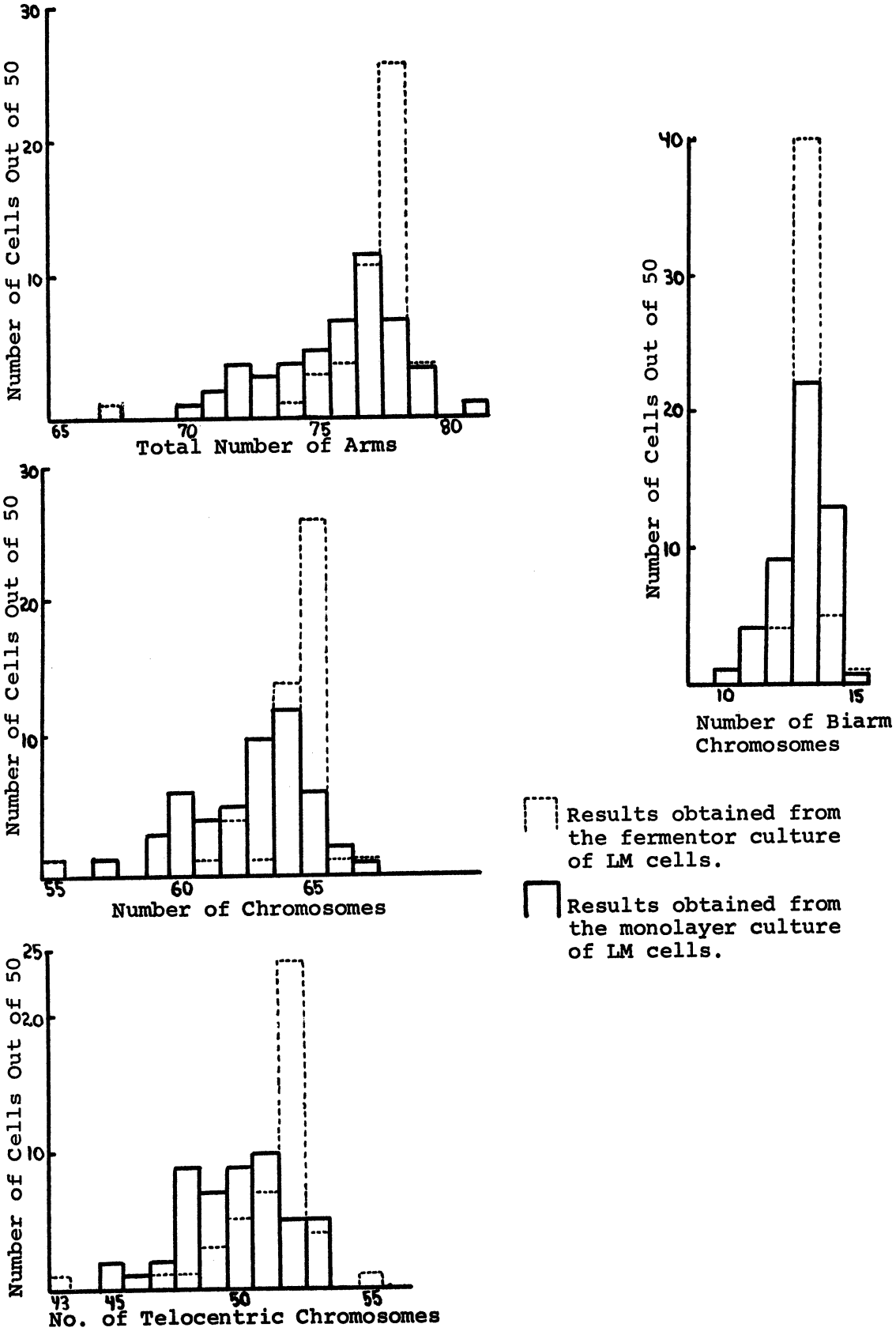


Figure 3. Results obtained by the enumeration of chromosomes of the LM strain in monolayer and suspension.

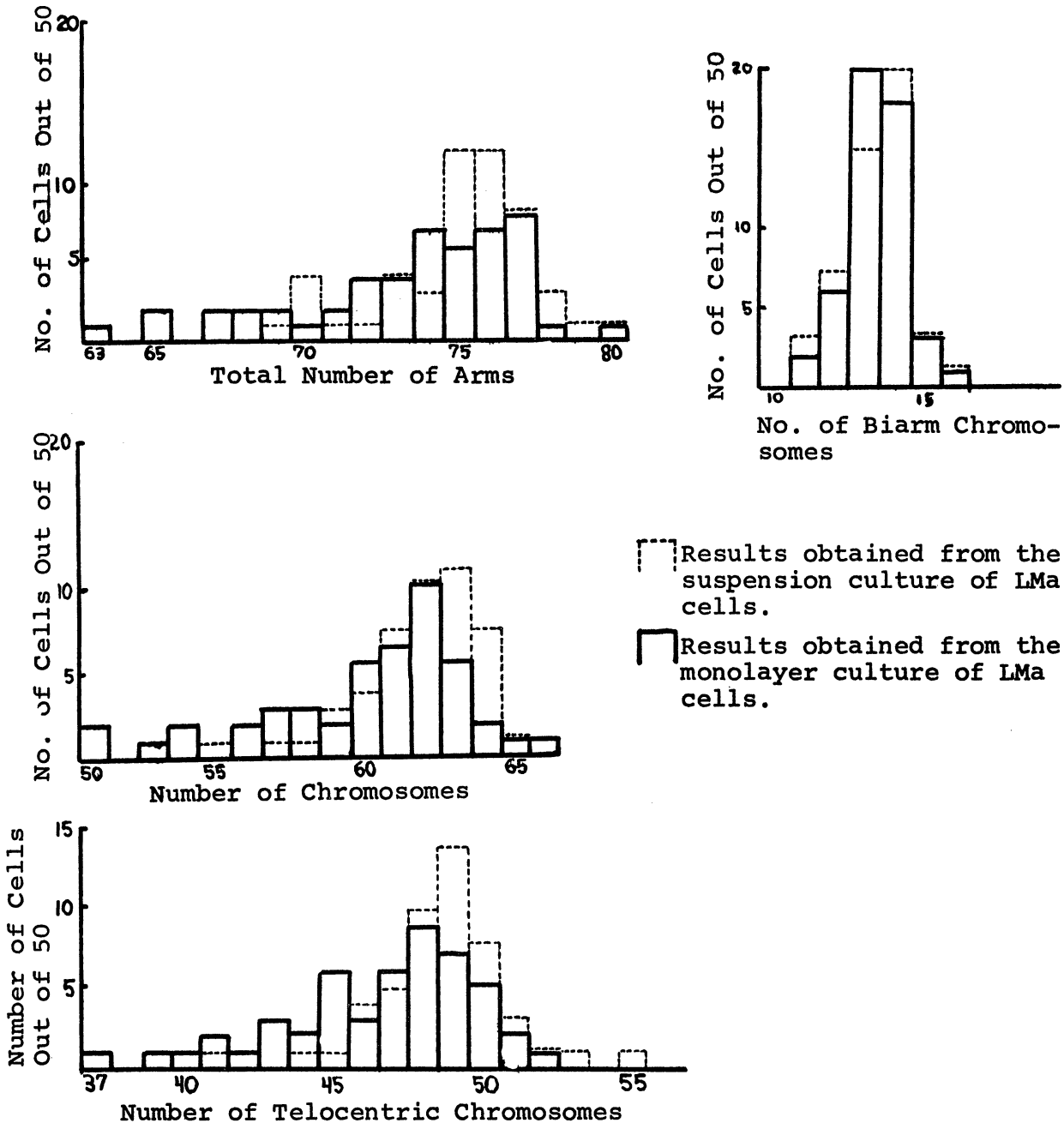


Figure 4. Plots comparing the results obtained by the enumeration of the chromosomes of the LMa strain in monolayer and suspension.

Marker Frequencies for the LM Strain in Monolayer (128 LM)

	E Marker Present	E Marker Absent
F Marker Present	10%	4%
F Marker Absent	36%	50%

Marker Frequencies for the LM Strain in Suspension (NB 7/XXIV)

	E Marker Present	E Marker Absent
F Marker Present	2%	6%
F Marker Absent	28%	64%

Figure 5. Marker frequencies obtained for the LM strain in monolayer and suspension culture in the 5-liter fermentor (50 cells counted in each population).

Marker Frequencies for the LMa Strain in Monolayer (101 LMa)

	E Marker Present	E Marker Absent
F Marker Present	28%	18%
F Marker Absent	28%	26%

Marker Frequencies for the LMa Strain in Suspension (2827 LMa)

	E Marker Present	E Marker Absent
F Marker Present	14%	26%
F Marker Absent	14%	46%

Figure 6. Marker frequencies obtained for the LMa strain in monolayer and suspension culture (50 cells counted in each population).

the extreme subjectivity involved in discerning marker chromosomes, the differences shown in these results are not thought to be significant.

Growth records for the LMa suspension culture show a period of initial slow growth. The different doubling times obtained for successive dilutions of the culture (Figure 7) seem to indicate that some sort of adaptation process was occurring.

DISCUSSION

Results obtained from the enumeration of the chromosomes of both the LM and LMa cell lines seem to indicate that suspension culture exerts a strong selection pressure on the cell population. In both cases there is evidence of selection of a modal cell type having chromosome characteristics different from the modal cell present in monolayer cultures. It is interesting to note that the selection is evident even after the short period of culture in suspension which passed before analysis of the LMa cells (see Figure 2). The data obtained for the total number of arms, total number of chromosomes, and number of telocentric chromosomes gives fewer different numerical values in analysis of the suspension cultures than in the analysis of monolayers.

The fact that LM cells studied were grown in 2.5% fetal calf serum in monolayer and 5% fetal calf serum in suspension in the 5-liter fermentor would seem to negate the validity of the results obtained. However, preliminary results from work being done in this laboratory on the effects of different serum concentrations on variability in the chromosome complement of LM cells indicates that the presence of calf serum in culture media contributes to increased variability in the population.

Although it is possible that such shifts might occur spontaneously, past experience indicates[15] that such changes are usually much more gradual than what was seen here. For example, a shift in the modal number of chromosomes of one chromosome was observed to occur over a four year period in the LM strain.[15] The homogeneity in conditions to which all cell surfaces are exposed in suspension culture[5] may contribute greatly to the short range effects observed here.

Other evidence that some sort of adaptation or selection occurs when cells are put in suspension can be seen in the growth curve for the LMa cells (Figure 7). For the first two growth cycles slow doubling times (36 and 60 hours) were observed. However, the next dilution provided a growth cycle which resulted in a much higher final cell concentration (2.01×10^6) and a much shorter doubling time (24 hours).

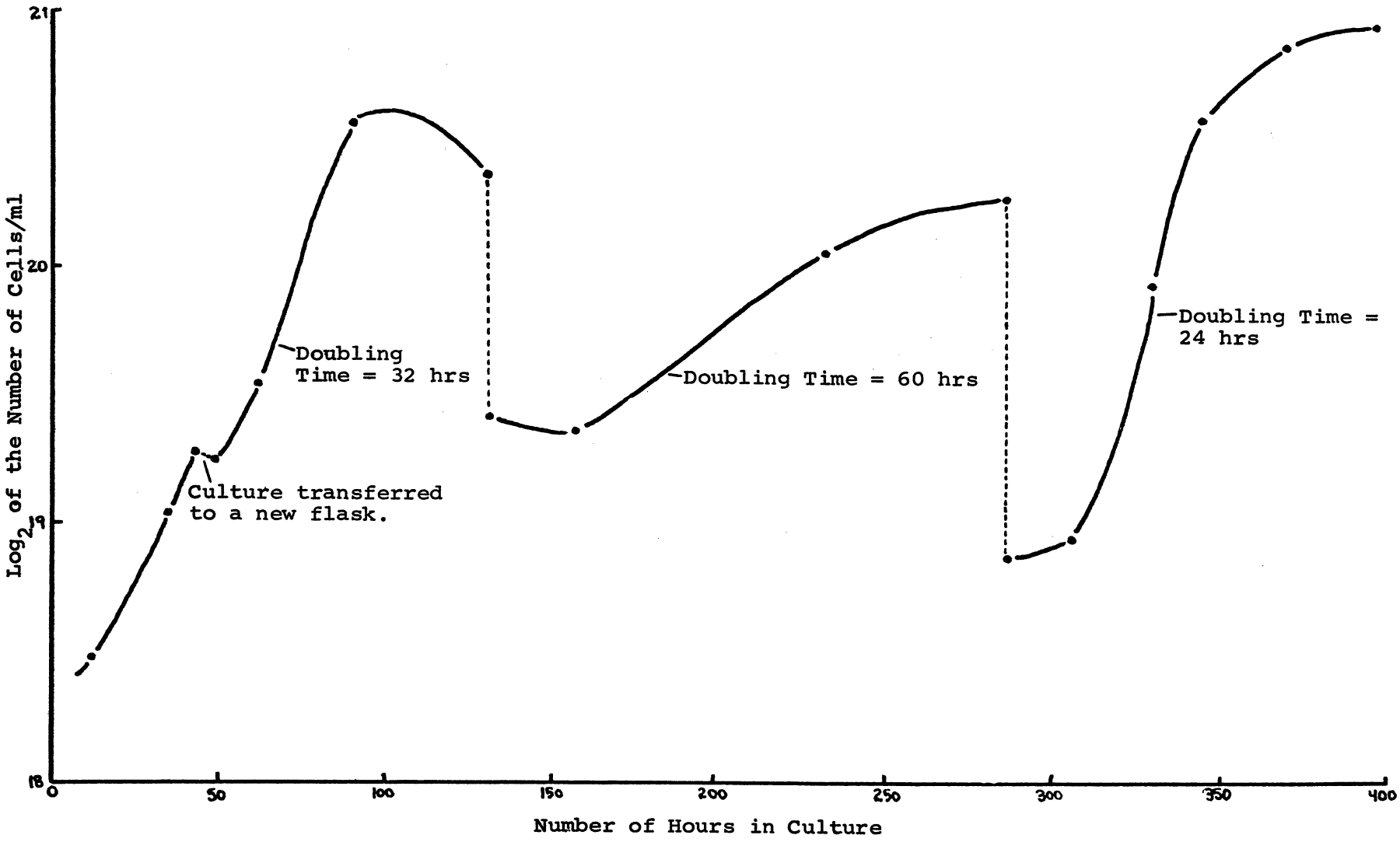


Figure 7. Growth curve for the LMa cells during the first 400 hr in suspension showing the initial period of adaptation present when the cells were placed in suspension directly from the monolayer.

CONCLUSIONS AND SUMMARY

The chromosomes of two mixoploid, mammalian, cell lines, LM and LMa, have been examined under conditions of growth as a monolayer and as a suspension culture. Although a more complete series of analyses will be necessary to substantiate the results obtained, date indicates that culture in both small suspension cultures and large fermentor cultures places a great selection pressure on the culture. Observations of changes in modal counts, ranges of the counts obtained, and number of cells having the modal chromosome numbers were taken to be an indication of the existence of a selection process. The lag period before LMa cells would grow well in suspension is further evidence of the need for some sort of adaptation.

Work on this project will continue. Attempts will be made to obtain more complete sequences of samples in the series of events from culture as a monolayer to fermentor growth. Results of this work will be quantified in terms of a computer analysis of the distribution of chromosome lengths in the populations examined and the mean lengths of biarm and telocentric chromosomes using a computer system developed here.[18] Cultures are now being grown in the 5-liter fermentor with conditions of pH, oxidation-reduction potential, and dissolved oxygen monitored and partly controlled using a system being developed by Jens U. Pedersen, a graduate student in the Department of Chemical Engineering at The University of Michigan. Cultures grown under these conditions will also be monitored for changes in chromosomal complement.

ACKNOWLEDGMENTS

Thanks are due to J. U. Pedersen, who supplied a number of samples of cells being used for fermentor growth, and to E. M. Lunk and R. L. Platte, Jr. for assistance in completing the necessary chromosome preparations and analyses.

REFERENCES

1. Carrel, A. and Eberling, A. H. Pure culture of large mononuclear leukocytes, J. Exp. Med., 36:365, 1922.

2. Owens, O. von H., Gey, M. K., and Gey, G. O. A new method for the cultivation of mammalian cells suspended in agitated fluid medium (Abstract). Proc. Am. Assoc. Cancer Res., 1:41, 1953.

3. Bryant, J. C., Schilling, E. L., and Earle, W. R. Massive fluid-suspension cultures of certain mammalian tissue cells. I. General characteristics of growth and trends of population. J. Nat. Cancer Inst., 21:331-348, 1958.

4. Hellman, A., Regan, J. D., and Martin, D. H. Large scale cultivation of mammalian cells in vitro. Applied Microbiol., 15:201-202, 1967.

5. Kuchler, R. J. and Merchant, D. J. Growth of tissue cells in suspension. Univ. of Mich. Med. Bulletin, 24:200-212, 1958.

6. McLimans, W. F., Giardinello, F. E., Davis, E. V. Kucera, C. J., and Rake, G. W. Submerged culture of mammalian cells: The five liter fermentor. J. of Bacteriology, 74:768-777, 1957.

7. Rightsel, W. A., McCalpin, H. and McLean, I. W. Studies on large-scale methods for propagation of animal cells. J. of Biochem. and Microbiological Tech. and Engineering, 2:313-325, 1960.

8. Ziegler, D. W., Davis, E. V., Thomas, W. J. and McLimans, W. F. The propagation of mammalian cells in a 20 liter stainless steel fermentor. Applied Microbiol., 6:305-310, 1958.

9. Harris, M. Cell culture and somatic variation. Chicago, Holt, Rinehart, and Winston, 1964.

10. Pedersen, J. U. Personal communication.

11. Hsu, T. C. and Merchant, D. J. Mammalian chromosomes in vitro XIV. Genotypic replacement in cell populations. J. Nat. Cancer Inst., 26:1075-1083, 1961.

12. Keefe, S. A., Merchant, D. J., and Kelsey, W. H. Alkaline phosphatase activity of L-M mouse cells and variants. Proc. Soc. Exp. Biol. and Med., 118:1031-1037, 1965.

13. Eidam, C. R. and Merchant, D. J. The plateau phase of growth of the L-M strain mouse cell in a protein-free medium. I. Patterns of protein and nucleic acid synthesis and turnover. Exp. Cell Res., 37:132-139, 1965.

14. Bryant, J. C., Evans, V. J., Schilling, E. L., and Earle, W. R. Effect of chemically defined medium NCTC 109 supplemented with methocel and of silicone coating the flasks on strain 2071 cells in suspension cultures. J. Nat. Cancer Inst., 26:239-252, 1961.

15. Giles, R. E., Merchant, D. J. and Masselink, E. Chromosomes of L-M mouse cells and variants. J. Nat. Cancer Inst., 37:663-373, 1966.

16. Gofman, J. W., Minkler, J. L., and Tandy, R. K. A specific common chromosomal pathway for the origin of human malignancy. Electronic scanning and computer chromosome measurement. UCRL-50356 (Nov. 1967).

17. Merchant, D. J., Kahn, R. H., and Murphy, W. H., Jr. Handbook of cell and organ culture, 2nd ed. Minneapolis, Burgess Publishing Co., 1964.

18. Baumann, G. W. and Merchant, D. J. in preparation.

2. Histochemical and Cytochemical Localization of Alkaline Phosphatase in the LM Cell

A second study aimed at elucidating the nature of the alkaline phosphatase marker was done as a Master's degree thesis problem by Miss Barbara Brown. This report is below.

INTRODUCTION

In recent years cell culture research has found it necessary to have techniques available which assess the changes in cells grown in vitro. Genetic markers, stable, heritable, characteristics or the cell, are a suitable technique. Many genetic markers are available to the researcher: antigens, karyotype, enzymes, etc.

Due to the ease and applicability of the enzyme alkaline phosphatase as a genetic marker, it seemed desirable to know more about this enzyme in cultured cells. A great deal of information about alkaline phosphatase in cultured human cells is available in the literature; very little information is available about alkaline phosphatase in cultured mouse cells. This work describes histochemical and cytochemical studies of the localization of alkaline phosphatase in LM mouse cells, and subsequent electrophoresis studies of isolated cell fractions.

LITERATURE REVIEW

Alkaline Phosphatase: Its Potential as a Genetic Marker

Genetic markers (as applied to the cell culture system) are stable, heritable characteristics of a cell or of a population of cells. They should persist despite sustained exponential growth, and, ideally, remain even after repeated cloning. Origin of markers include (1) normal cellular characteristics (antigens, karyotype) and/or nutritional requirements, (2) clones that have spontaneously acquired unique cellular characteristics, (3) donors of cell strains who themselves have abnormal cellular characteristics—galactosemia (Krooth and Weinberg, 1960), acatalasia (Krooth et al., 1962), G-6-P deficiency (Gartler et al., 1962), crotic aciduria (Krooth, 1965).

Other genetic markers (Gartler, 1967) in common use today include antigens (Korngold, 1962; Merchant et al., 1966; Watkins and Grace, 1967), karyotype (Moorehead, 1962; Harris, H., 1967; Weiss and Green, 1967), enzymes (DeLuca and Nitowsky, 1964; Weiss and Ephrussi, 1966), virus susceptibility (Chessin

and Hirschhorn, 1961; Melnick, 1962), and clonal morphology (Murphy et al., 1962). These markers have been in use for the past few years; preliminary work is being done on newer markers such as temperature resistance (Harris, 1967), and surely with time more markers will be found.

Most human cells grown in vitro have been found, unfortunately, to be similar in enzyme composition, nutritional requirements, and virus susceptibility, properties useful as genetic markers (Cox and MacLeod, 1964). These similarities may be due to (1) a selection of a ubiquitious cell type which is best able to multiply in vitro, and/or (2) subsequent development of common nutritional and metabolic patterns in different cell types due to in vitro culture conditions.

The choice of a genetic marker for any system depends on a number of factors including stability, applicability, and ease of detection. Enzymes may be particularly useful as markers because they may be studied at the cellular level using various histochemical and biochemical techniques.

The enzyme alkaline phosphatase has good potential as a genetic marker. It is one of the few enzymes showing induction and repression in cells of higher organisms while in cell culture (Cox and Pontecorvo, 1961). In vitro most enzymes, even with induction and repression, show little difference between maximal activity and control activity (Demars, 1958; Lieberman, 1957). One of the advantages of the alkaline phosphatase system is that it does show a great difference in the enzyme levels by induction and by clonal selection (Maio and DeCarli, 1962). Alkaline phosphatase has also shown correlations with other genetic markers as clonal cell type (Walker, 1965), and with karyotype (DeCarli et al., 1963).

However, as a genetic marker alkaline phosphatase is not without its disadvantages: (1) its physiological substrate(s) and function(s) are unknown, (2) it is a heterogenous group of enzymes with at least several molecular variants.

Alkaline phosphatase, like other markers, only recently has been used as a tool to study questions of monitoring long term growth in vitro, hybridization, differentiation, dedifferentiation, and senescence. Work to correlate changes in one genetic marker to changes in another marker has barely begun.

Detection of Alkaline Phosphatase

Numerous histochemical and cytochemical methods for the detection of alkaline phosphatase are available. A detailed discussion of the histochemical methods is available in Pearse (1960). Historically, the first method of alkaline phosphatase histochemical detection was worked out independently by Gomori (1939) and Takamatsu (1939). The method depends on the depositions of calcium phosphate at the sites of enzyme activity. Though the technique is

easy to use the interpretation of results may be deceptively simple as warned by Danielli (1950). Variations of the original Gomori method are commonly used today (Gomori, 1952).

Presently the histochemical staining methods are divided into coupling and noncoupling dye procedures. Menton et al. (1944), devised the first dye-coupling method called a simultaneous coupling method; Burstone (1958, 1961) and Rutenburg et al. (1958), followed with variations. These methods demonstrate by precipitation the alcoholic part of the phosphate ester used as substrate. In Burstone's method as with others, the substrate employed is an aryl phosphate, rather than the alkyl phosphate which is usually employed in the Gomori technique. The dye coupler is Fast Red RR salt, a diazotized amine; other couplers such as Fast Red RR, Fast Violet, etc., are acceptable. The reaction product formed at the site of alkaline phosphatase activity is a highly insoluble blue complex formed by the immediate coupling of the naphthol, released by hydrolysis of the substrate, and the diazonium salt.

Both substituted and unsubstituted naphthols may be used as substrate. Menton (1944) used an unsubstituted naphthol, β-naphthol phosphate; Burstone used a substituted naphthol, naphthol AS-E phosphate. The AS phosphates have the advantage of good stain localization because of the low solubility of their naphtholic reaction products, and are stable for long periods at alkaline pH. However, some inhibitory effects are seen with the diazonium salts. Other histochemical coupling procedures include Henrichsen (1956), Grogg and Pearse (1952), Maggi and Riddle (1965).

Loveless and Danielli (1949) designed a noncoupling azo dye methods in hopes of overcoming the disadvantages of the coupling procedure. The substrate used is a colored phosphate ester, p-nitrobenzene-azo-4-naphthol-1-phosphate, which on hydrolysis yields an insoluble, highly colored base which has no tendency to crystallize in the tissues. Unfortunately, the method gives unusually intense staining in the nucleus. For this reason the method is not widely used today. Recently a fluorescent substrate for alkaline phosphatase has become available (Land and Jackim, 1966).

Quantitative assays for alkaline phosphatase usually employ the method of Bessy, Lowry, and Brock (1946) as reported in the Sigma Technical Bulletin (1961). This method permits any type of cell disruption: sonication, homogination, extraction, etc., and measures the release of p-nitrophenol from the substrate p-nitrophenylphosphate. The yellow color of the p-nitrophenol is measured spectrophotometrically. Other quantitative methods more recently worked out include a fluormetric technique to measure activity in a single cell (Tiernay, 1964) and a microassay (Martin, 1964a) and a quantitative histochemical assay (Taft and Scott, 1958).

With the widespread use of electrophoresis in enzyme studies staining procedures for the detection of alkaline phosphatase in starch and acrylamide are available. The standard procedure for staining starch is that of Boyer

(1961). He uses the substrate α-naphthyl phosphate, and a dye coupler in Tris buffer. Epstein (1967) has recently published a method of an indigogenic reaction for alkaline phosphatase detection in acrylamide. The substrate, a synthetic para-toluidine salt of 5-bromo-4-chloro-3-indolyl phosphate, gives a precise localization with little or no diffusion and eliminates the need for a coupling reaction.

The electrophoretic study of alkaline phosphatase has presented several problems (Kaplan, 1963)—particularly slow migration or lack of migration in starch. Paul and Fortrell (1961) compare migration patterns from extracts of primary tissue samples from a variety of species and found unique migration patterns for each species. However, Keefe et al. (1965), using cultured mouse LM cells find no migration of alkaline phosphatase in starch. Other investigators routinely use the butanol extraction procedure of Morton (1954) to obtain migration of the enzyme in starch. The butanol extraction is believed to free the enzyme in the hydrophobic layer from the lipids in the microsomal or membrane portion of the cell (Wolman and Bubis, 1966). Extraction with Triton X-100 has been shown to increase the migration of some of the slow bands of acid phosphatase and esterase in rat liver (Allen et al., 1965). Likewise, Triton X-100 increases the mobility of previously immobile alkaline phosphatases and esterases from the digestive glands of Rhynchosciara angela in acrylamide (Coutinho et al., 1966). Perhaps the extraction procedure or enzyme treatment would be useful for the study of mouse alkaline phosphatase in starch and acrylamide.

Alkaline Phosphatase—Chemical and Physical Properties

The function of alkaline phosphatase in vivo is not known. Like most enzymes, it is very sensitive to changes in its environment. The usefulness of alkaline phosphatase as a genetic marker will be enhanced as more becomes known about the cell culture system and the relationship of the enzyme to changes in the invitro system.

In recent years Martin (1964b) and Herz and Nitowsky (1962) published data concerning the chemical and physical properties of alkaline phosphatase. The former used "the established cell cultures, of typical "fibroblastic" morphology, derived from the foreskins of normal male infants", the latter used established lines of HeLa, Chang liver, Zimmer liver, intestine and heart. Martin (1964) finds a pH optimum of 10.1; Herz and Nitowsky find a pH optimum of 10.6 in 1 M AMP buffer. 10% of the optimal activity of the alkaline phosphatase is present at pH 8.0 and 70% is present at pH 10.0. Switching to 0.1 M glycine buffer or 0.5 M ethanolamine buffer and the pH optimum remains at 10.6 but the maximal activity is only 50-60% of that in AMP. However, as Martin points out, increasing the molarity of AMP buffer from 0.04 to 0.83 M definitely enhances the enzyme activity.

Alkaline phosphatase is considered to be a nonspecific group of enzymes which will hydrolyze a variety of organic phosphate esters. Nitowsky and Herz (1962) show that Chang liver cells hydrolyze a variety of phosphomonoesters, except o-carboxyphenlyphosphate; the most rapid hydrolysis is observed with substrates β-naphthyl phosphate and p-nitrophenylphosphate. No hydrolysis is seen with ATP. The relative activities of alkaline phosphatase from different human tissue sources fall into two classes on the basis of "substrate spectra." The intestinal phosphatases form one class and those of bone, liver, kidney, and spleen form the other. The intestinal phosphatases hydrolyze adenosine-5-phosphate about twice as fast and hydrolyze p-nitrophenylphosphate and β-glycerophosphate about half as fast as the other group. This division by substrate spectra is also supported by immunochemical studies of Schlamowitz and Bodansky (1954).

Landau and Schlamowitz (1961) show that when two organic substrates are simultaneously available to alkaline phosphatase no additivity of activity is seen as might be expected if separate enzymes were present. Cox et al. (1967), show that "organic" alkaline phosphatase and "inorganic" pyrophosphatase activity are carried out by the same enzyme, indicating that the enzyme can hydrolyze both organic and inorganic substrates. Herz and Nitowsky (1962) demonstrate the effect of temperature on the rate of enzyme hydrolysis of p-nitrophenylphosphate. With Chang liver cells the maximal rate of enzyme activity is observed at 38°C with a sharp decline in activity at higher temperatures; human intestinal cells show maximal activity at 56°C.

Herz and Nitowsky (1962) find that alkaline phosphatase has interesting patterns of thermal stability and heat inactivation. The enzyme from the cell lines with high specific activity is relatively heat labile; the enzyme from lines with low specific activity is heat stable. A similar relationship between specific activity and hormonal induction also exists. Time course inactivation studies with Chang liver sonicates show uniform loss of activity; about 50% inactivation is evident during 18 minutes at 56°C. However, other cell lines (Chang liver clone 8A, human heart) show a diphasic inactivation curve. Heat stability studies of enzyme mixtures rule out the presence of an enzyme inhibitor or stabilizer as cause of the diphasic curve. Heat stability, substrate specificity, and optimal temperature data indicate intrinsic differences in the enzyme molecules, probably molecular variants.

In cell culture the alkaline phosphatase system may conveniently be studied under conditions of induction, repression, and inhibition. Even several types of induction systems are available; hormonal induction with prednisolone, substrate induction with phenylphosphate, and self-induction.

Prednisolone, a glucocorticoid hormone, in concentrations of 0.5 to 1.0 μg/ml has variable effects on cells in vitro. In some lines including HeLa it causes a pronounced increase in alkaline phosphatase (Cox and MacLeod, 1961) also in isolated clones of EUE (maio and DeCarli, 1963); in other lines it has little if any effect (Cox and MacLeod, 1961, 1962). The inducing effect is

specific for alkaline phosphatase; levels of other cellular enzymes remain unchanged (Cox and MacLeod, 1962; Nitowsky and Herz, 1963). Melnykovych (1962) studied the inducing effects of a large number of different steroids and finds that only glucocorticoids increase the alkaline phosphatase activity. Nitowsky and Herz (1963) and Maio and DeCarli (1963) claim the induction factor (specific activity of induced/specific activity of uninduced) with prednisolone and hydrocortisone is inversely proportional to the initial or constitutive enzyme level of the line. Constitutive levels above three fail to be induced and often show repression in the presence of the hormones. The prednisolone induction may be blocked by puromycin and actinomycin D (Griffin and Cox, 1966a).

Alkaline phosphatase is inducible in bacteria. But unlike bacteria, cultured cells show little increase in enzyme activity during the first 18-24 hours of induction; after 24-30 hours a sudden rise in activity occurs until 60 hours when induction is maximal. The increased activity may be diluted out by cell multiplication in 4-6 generations (Cox and MacLeod, 1964).

Prednisolone induces the heat stable alkaline phosphatase while the heat labile form is uninduced or decreases (Cox and MacLeod, 1964). The mechanism of hormonal induction is unknown though Melnykovych (1967) believes prednisolone affects the cell membrane permeability by partially preventing leakage of ninhydrin positive substances from the cell and prolonging cell survival under a variety of adverse conditions.

Nitowsky, Herz, and Geller (1963) demonstrate alkaline phosphatase induction by changing the medium osmolarity. Human skin fibroblasts do not respond to such osmolarity induction. Unlike hormonal induction, osmolarity changes induce both the heat stable and the heat labile alkaline phosphatase. The osmotic and prednisolone induction effects are additive. Miedema and Kruse (1967) show under perfusion conditions, in contrast to stoppered monolayer cultures, prednisolone induction appears to be regulated by inherent cellular control mechanisms which are sensitive to the contact phenomena and to population density changes.

So in fact, the effects of prednisolone in human cell cultures is not well defined (Miedema and Kruse, 1967). Its induction potential has been most widely studied; other effects include repression (Erwin and Whitehead, 1964); altered protein conformation (Griffin and Cox, 1966); and effects on the cell membrane (Melnykovych, 1966). Tissue of origin, morphology, and nutritional factors generally determine the type of response prednisolone produces (Cox and MacLeod, 1964).

Substrate induction of alkaline phosphatase is evoked by phenylphosphate; only a few epithelial lines show substrate induction, but most skin fibroblasts are inducible (Cox and Pontecorvo, 1961). The mechanism of substrate induction differs from hormonal induction; substrate induction is much slower. During substrate induction, inducible epithelial cells show an increase in enzyme activity in 3-5 days while the human fibroblasts require 7-12 days (Maio and

DeCarli, 1962; Cox and MacLeod, 1964). The kinetics of substrate induction divide human skin fibroblasts into three catagories: constitutive, noninducible; negative, noninducible; and inducible (Martin, 1964d; Cox and MacLeod, 1964). As yet unanswered is the question, alkaline phosphatase substrate induction, it is caused by the genotype of the donor or by selection of a given cell type?

Alkaline phosphatase will self-induce itself in cells allowed to multiply without medium change for 20-40 days. The ability to self-induce is related to substrate induction; one does not occur in the absence of the other (Martin, 1964c). The time required for self induction may be reduced to 3-7 days if preconditioned medium is used (Cox and MacLeod, 1964). Conditioned medium speeds up substrate induction, while frequent medium changes during induction abolishes or greatly reduces the alkaline phosphatase induction. Probably the conditioned medium removes or modifies a repressor molecule present in the fresh medium.

In addition to induction, alkaline phosphatase activity is subject to both repression and inhibition, where repression refers to interference with enzyme synthesis and inhibition refers to interference with the activity of the preformed enzyme. Cox and MacLeod (1963) show that unlike microorganisms (Torriani, 1960) mammalian cells are not repressed by inorganic phosphate and are independent of the concentration of inorganic phosphate in the medium.

Alkaline phosphatase levels in human cell cultures appear to be regulated by the L-crysteine and L-cystine concentrations in the medium. Studies by Cox and MacLeod (1964) suggest the control mechanism is repression, L-cysteine being the repressor. However, in vitro studies have shown L-cysteine is also an effective inhibitor of alkaline phosphatase (Albers, 1935; Herz and Nitowsky, 1962; and Cox and MacLeod, 1963). The mechanism and structural requirements of this inhibition are described by Agus, Cox, and Griffin (1966).

Cox and MacLeod (1964) show that substrate induction of alkaline phosphatase in skin fibroblasts is prevented or greatly reduced by supplementary cyst(e)ine, while induction in epithelial cells in independent of cyst(e)ine. Small amounts of cysteine enhance alkaline phosphatase levels, but as the concentration exceeds 0.4 mM the enzyme activity progressively decreases. Despite the rapid cellular uptake of cysteine (within less than 30 minutes) depression of alkaline phosphatase activity is not seen for about 40 hours. These data fit a mechanism of repression better than one of inhibition. Certain analogs of L-cysteine show interesting effects of alkaline phosphatase. Analog L-penicillamine is a better inhibitor than L-cysteine but is an ineffective repressor; mercaptopropionic acid cannot inhibit alkaline phosphatase but is an effective repressor.

Cysteine and other sulfhydryl compounds are known to inhibit alkaline phosphatase activity of crude and partially purified preparations. L-histidine is also a known inhibitor of the enzyme (Bodansky and Schwartz, 1963). Agus

et al. (1966), describe the structural requirements of the sulfhydryl inhibition. Two main groups of inhibitors are distinguishable: (a) analogs with free amino and sulfhydryl groups are good inhibitors, (b) analogs with blocked amino or sulfhydryl groups and compounds lacking these groups are poor inhibitors. The pH strongly influences the action of the inhibitors; the amino and sulfhydryl groups must be in their basic form. Little inhibition is seen at pH 8.1 but at least 50% inhibition is evident at pH 10.5. The inhibition mechanism is zinc chelation at the site of enzyme activity, a chelation in situ rather than a removal of the metal from the apoenzyme. Other chelators are effective inhibitors of alkaline phosphatase; DTPA (Brunk and Skold, 1966) and EDTA (Conyers et al., 1967). The inhibitory effect of L-cysteine and sulfhydryl chelation is reversible with excess zinc; the inhibition by EDTA has not yet been shown to be reversible.

Thus, the study of alkaline phosphatase in cell culture affords the application of one system to the study of induction, repression, and inhibition. Most of the variability in the system depends on the cysteine content of the medium and secondarily on factors like pH, temperature, etc. In contrast to bacteria where a clear cut relationship exists between the enzyme and the end-product repressor (as with aspartate transcarbamylase), the relation of the mammalian cell alkaline phosphatase to the cysteine repressor is unknown. In fact the in vivo function of the enzyme itself is still unknown; further work with the induction, repression, and inhibition systems may lead to a better understanding of the enzyme's function and enhance its usefulness as a marker.

Alkaline Phosphatase in the Cell Culture System

Localization studies of alkaline phosphatase, both histochemical and cytochemical, have led to confusing results. Most of the localization work has been done with HeLa and the mouse L cell. Two distinct systems are involved.

With the histochemical studies of HeLa Griffin et al. (1966), find alkaline phosphatase principally in the nucleus and the nucleoli, and on the chromosomes during division, though they allow that the latter may be an artifact of staining. Regan (1966) also finds localization of alkaline phosphatase near the nucleus and an apparent increase in dividing cells, which by other methods proves to be an artifact of histochemical staining caused by the rounding up of the cell. Hugon et al. (1967), made studies of glutaraldehyde fixed HeLa cells. They report the alkaline phosphatase in the cells with no freezing localizes in darkly stained bodies at the external part of the cellular membrane, none intracellularly. After freezing the densely stained bodies are found on the membrane and also intracellularly. No mention was made of nuclear staining. Cox et al. (1967), by histochemical staining saw alkaline phosphatase activity rather diffusely spread throughout the cytoplasm of HeLa cells but marked activity in the nucleus. Enzyme activity was highest in the nucleolus.

Cytochemical studies of the localization of alkaline phosphatase in Chang

liver clone 8A by Herz and Nitowsky (1962) find 10% activity in the low centrifugation fraction, 69% in the high centrifugation fraction, and 20% in the supernate.

With the L cell histochemical determination of alkaline phosphatase with β-naphthyl phosphatase shows diffuse cytoplasmic staining with moderately stained granules near the cell membrane. No staining was evident on the nucleus but was present on chromosomes during division (Cox and MacLeod, 1962). No studies to data have been reported of cytochemically determined localization of alkaline phosphatase with cell fraction from L cells.

The cytochemical determination of alkaline phosphatase levels of established cell lines show that enzyme content varies from very high constitutive to low or trace levels (Nitowsky and Herz, 1961a,b; Cox and MacLeod, 1961, 1962). Nitowsky and Herz (1961b) have detected a 500-fold difference between phosphatase activities of established cell lines; Detroit-6 and J-111 lines have the highest specific activity. Even HeLa lines maintained in different laboratories show great alkaline phosphatase variation. According to Greenstein (1954) the specific activity of cancer lines is usually less variable and shows lower values than those of normal cells from the same tissue.

Clonal lines derived from a constitutive parental line show great variation in alkaline phosphatase activity. Maio and DeCarli (1962) derived negative clones, including EUE6, from a parental line with high activity. The differences in the specific activity among the sub-lines is 1000-fold; all intermediate enzyme levels may be isolated. Nitowsky and Herz (1961b) also find the wide range of activities among clonal lines from a common parent using Chang liver, Zimmer liver, and subclones of HeLa S_3. Keefe *et al.* (1965), find similar variation in alkaline phosphatase levels in strains derived from clones of LM cells passaged through C_3H mice. And Fortelius *et al.* (1961), derived a 100% negative clonal line from a giant cell of HeLa. Such clonal variants easily lend themselves to studies of correlation between alkaline phosphatase levels and karyotype analysis.

Alkaline phosphatase is also subject to variation due to tissue of origin as reported by Nitowsky and Herz (1961a), Cox and MacLeod (1961), and others. Variations, both increases and decreases, are seen when activity levels from recent cell isolates are compared to established cell cultures. Nitowsky and Herz (1961b) find this to be true with human cell cultures; Lieberman and Ove (1958) confirm this with rabbit kidney cortex cells.

Species variation of alkaline phosphatase is studied electrophoretically by Paul and Fortrell (1961). Using liver preparations from mouse, guinea pig, rat, frog, pigeon, and perch, they see one or two bands for each species, each with a unique mobility. This indicates structural variants in alkaline phosphatase are present between species. Similar variation is seen in isozyme studies of human cell lines made by Beckman and Regan (1964). They detect different

number of bands, different staining intensities, and different mobilities with HeLa, Wish amnion, and RA lines. They feel these bands are "subject to considerable variation due to dynamic fluctuations in the population of cells." Moss (1962, 1963, 1965) and Moog et al. (1966), characterize electrophoretically the alkaline phosphatases from human and mouse intestines finding variants having different substrate preferences. Boyer (1961) demonstrates the heterogeneity of human serum and placental alkaline phosphatase, observing several new bands during pregnancy. Trubowitz and Miller (1966) describe the heterogenetic pattern of alkaline phosphatase in normal leucocytes and in leucocytes of patients with polycythemia vera, who all have increased levels of alkaline phosphatase. Even clonal derivatives of EUE with differing activity levels of alkaline phosphatase show different starch gel electrophoresis patterns (Santachiara-Benerecetti et al., 1967). Differences between the two enzyme variants are indicated by other criteria: sensitivity to inorganic phosphate, thermal stability, and time course of heat inactivation. Thus, it appears that molecular variants of the enzyme are present and can be detected by a variety of means.

As indicated previously, the amount of alkaline phosphatase detectable is partially dependent upon the growth medium, the phase of the growth curve measured, length of incubation and degree of cell crowding. Fortelius et al. (1960), observe a decrease in alkaline phosphatase after a medium change. Nitowsky and Herz (1961) observe a variation in the enzyme level when Chang liver cells are transferred from Eagles' medium containing horse serum to Puck's medium containing human serum. Keefe et al. (1965), find adaptation of LM mouse cells, normally grown in medium 199 plus 0.5% peptone, to growth in two times Eagles medium causes loss of alkaline phosphatase activity. And Melnykovych et al. (1967), demonstrate that Henle and HeLa S_3 cells have higher noninduced specific activities in Earles' rather than Hank's BSS; and claim cells harvested by scraping have a higher activity than trypsinized cells regardless of the medium.

In addition to nutritional environment, variation in alkaline phosphatase levels occur with the various stages of the population growth cycle (Nitowsky and Herz, 1961; Martin, 1964b). Cristofalo et al. (1967), using WI 38 cells and SV_{40} transformed cells find variation in alkaline phosphatase and other enzymes during a single growth cycle over a 10-day period with no medium change. Phosphatases show a decline in activity (15-25%) during days 4-6, followed by a slow increase back to the initial level. However, Regan (1966), using a synchronously dividing suspension of HeLa, finds the alkaline phosphatase level does not change significantly throughout one generation. The enzyme level is studied for the 12 hours after the synchronization is discontinued. Thus, no increase of alkaline phosphatase is measured in mitotoic or post mitotic cells.

The growth rate of cells seems to be correlated with alkaline phosphatase activity. Maio and DeCarli (1963) report that lines with very low alkaline phosphatase activity usually have a slower growth rate than the positive lines under the same growth conditions. Other workers, DeCarli et al. (1963; Earle,

(1965); Cox and MacLeod (1964) believe alkaline phosphatase levels are related to epithelial-like morphology and heteroploid karyotype.

Information is slowly accumulating about alkaline phosphatase in the cell culture system. As data from more standardized conditions become known, studies with alkaline phosphatase and other enzymes may lead to a better understanding of differentiation, dedifferentiation, and senescence.

The Relationship of Alkaline Phosphatase to Other Markers in the Cell Culture System

Studying any system from more than one point of view is usually advantageous. However, the study of genetic markers is so relatively new to the cell culture field that only a few correlation studies with two or more markers have beem made, including alkaline phosphatase and karyotype analysis. Preliminary studies of alkaline phosphatase as related to other markers have been made. Such investigations seem to hold promise for the study of hybridization and senescence.

Interest in leucocyte alkaline phosphatase levels was stimulated in 1952 by Valentine and Beck's (1952) observation that patients with chronic myelogenous leukemia had low alkaline phosphatase levels and patients with polycythemia vera had high alkaline phosphatase levels. Alter et al. (1962), and Trubowitz et al. (1962), feel this decrease in enzyme activity in leukemia is the result of a partial deletion in chromosome 21, the Philadelphia chromosome. Some ten years later King et al. (1962), and Alter et al. (1963), made the observation that individuals carrying a trisomy of chromosome 21 (mongoloids) had unusually high levels of alkaline phosphatase in the leucocytes.

Shortly thereafter DeCarli, Maio, and Nuzzo (1963) made a karyotype analysis of EUE strains (tissue culture cell lines) with high and low alkaline phosphatase specific activities. They found the low activity strains had a modal chromosome number 7-8 units less than the mode of the parental strain. In the 7-8 unit reduction, 1-2 units were in the group of long acrocentric chromosomes, with a more pronounced reduction in the short acrocentric group (Denver classification group 21-22). Later work by DeCarli et al. (1964), show that low alkaline phosphatase strains have about half the number of short acrocentric chromosomes of the high activity strains. Some reduction is also seen in the number of 13-15 group chromosomes. A definite correlation exists between the alkaline phosphatase level and the number of small acrocentric chromosomes even though differences exist in activity among lines with an equal number of small acrocentric chromosomes. Comparing strains, the increase of one chromosome (increasing from 2 chromosomes to 3) did not result in a simple additive effect in the enzyme level; such a simple additive effect in the enzyme level is seen with an increase in the number of β-galactosidase locus in E. Coli. This may be accounted for by the kinetics of enzyme formation, es-

pecially if there are two or more subunits as with alkaline phosphatase in E. Coli and S. marcesens (Rothman and Byrne, 1963) and/or changes in the regulatory gene.

Konigsberg and Nitowsky (1962) have worked with clonal sublines of Chang liver with different alkaline phosphatase levels and find differences in the modal number of chromosomes and in the number of marker chromosomes. Dodson (1966) working with RPMI 2650 developed a subline 2650-R by irradiation. The subline contained a partially deleted chromosome 21. Unexpectedly the subline showed higher alkaline phosphatase activity than the parent. The investigators tentative explanation of this unusual inverse relationship include: (1) deletion of a segment different from the Philadelphia chromosome deletion, (2) irradiation may have eliminated an inhibitory gene, and (3) an enhancer may have moved closer to the enzyme locus.

Such data indicate there is a substantial and functional correlation between alkaline phosphatase levels and chromosome group 21-22, though complexities of this relationship are, as yet, unexplored, and other chromosome loci are probably involved in alkaline phosphatase expression. To date the relationship of chromosome group 13-15 to alkaline phosphatase is undefined; Dodson's work with an irradiation mutant also needs further study.

Walker (1965) made a study of the correlation of alkaline phosphatase with another marker, colony morphology. The classification system of colony type used was devised by Murphy et al. (1962); five colony types were designated. Walker studied the alkaline phosphatase content and modal distribution of colony type of the LM cell and three of its variants. With the colony type selection data he confirmed the work of Keefe et al. (1965), that the population selection accounted for the decrease in alkaline phosphatase activity as the LM cell was adapted to growth in two times Eagles medium (LM-E) and in high density suspension culture (LM-S). He found the diffuse colonies are generally negative even in lines with alkaline phosphatase activity; and though not thoroughly substantiated, giant cell colonies usually show alkaline phosphatase activity.

Other reports of correlation of alkaline phosphatase and a second marker are almost nonexistant. Drysdale et al. (1967), studying the antigenic specificities of the H-2^k allele of LM cells found no relationship between alkaline phosphatase activity and the gain or loss of H-2 antigens. Mecs et al. (1964), found alkaline phosphatase activity increased according to the phase of the viral cycle in polio infected HeLa. However, no work has been reported concerning the relationship of cell viral susceptibility and alkaline phosphatase as markers.

Research Applications of Alkaline Phosphatase

Alkaline phosphatase as a genetic marker seems to have potential in di-

verse areas of research. As previously mentioned alkaline phosphatase levels serve as one measure of characterization of several diseases: mongoloidism, chronic myelogenouse luekemia, and polycythemia vera. Such studies coupled with karyotype analysis serve as a useful tool to study the gene dosage theory concerning the number of chromosomes and corresponding levels of enzyme production (DeCarli et al., 1964).

A problem which has plagued cell culture investigators is that cells which are differentiated in vivo usually dedifferentiate when grown in vitro; functions specific to the tissue of origin are lost in vitro. Shifts in enzyme levels are seen over a period of time as recently isolated cells become established cell lines (Lieberman and Ove, 1958). Enzyme marker studies, including alkaline phosphatase, and more work with the in vivo biochemical environment may lead to an understanding of the differentiation process.

Likewise, another problem facing cell culture investigators is the limited in vitro lifespan of diploid cells (Hayflick and Moorehead, 1961; Hayflick, 1965). Senescence, as defined for the human diploids, is little understood. Cristifalo et al. (1967), have made enzyme marker studies of aging in WI-38 strains. Over the "life span" of the diploid strains the alkaline phosphatase and LDH showed no significant variation; acid phosphatase increased with age. Though in this particular system alkaline phosphatase did not prove to be a good marker, it may be useful in related systems.

Other applications of alkaline phosphatase as a marker are in hybridization studies, and monitoring large-scale and long-term growth in vitro; further applications are unthought of as yet. Surely with time, as cell culture conditions improve and become more standardized genetic markers will become an essential and routinely used laboratory tool.

MATERIALS AND METHODS

Cell Strains

The LM strain is a subline of the L strain (clone 929) mouse cell. It has been carried in medium 199 (Parke-Davis, Detroit, Mich.) supplemented with 0.5% Bacto-peptone (199 P) (Difco Lab, Detroit, Mich.). Suspension cultures were supplemented with 0.1% methylcellulose (Dow Chemical, Midland, Mich.) to prevent clumping and to stabilize the cell membrane. Cells recovered from the Revco freezer (-70°C) were frozen down in saline (0.85%) with 20% glycerol, to minimize cell damage during freezing.

The LMa and LMf substrains are derivatives of the LM strain. LM cells were injected intramuscularly into the right foreleg of weanling, inbred C_3H mice conditioned with cortisone acetate (Keefe et al., 1965). The substrains of LM were obtained by culturing tissue from the nodules of resultant in vivo

growth of the LM cells. The Lma and LMf strains were isolated from nodules of separate mice and have been carried on 199 P.

The BHK_{21} line was derived from the kidneys of 5, one-day old Syrian hamsters. The line has been maintained on modified Eagle medium (double strength solution of amino acids, vitamins, and glutamine in Hanks balanced salt solution, 2XE) plus 10% tryptose phosphate broth and 10% fetal calf serum.

The HCAAT is a derivative of HeLa (a human carcinoma of the cervix) adapted, in this laboratory, for growth in 2XE plus 10% tryptose phosphate and 10% fetal calf serum rather than human serum.

Fractionation Procedure

Cell samples were taken from the freezer, thawed and washed twice with 15 ml of 0.85% saline. Cells were counted and then resuspended in 0.25 M sucrose so that the sample contained at least 8 ml of 5×10^6 cells/ml. A minimum total volume was required for effective sonication.

Cells were disrupted by sonication with a Biosonic II ultrasonic probe (Bronwill Scientific, Rochester, N. Y.). Complete cell disrupted was achieved with 90-120 seconds of sonication. The sample was checked for complete disruption by visual observation with a microscope. The sample was then frozen until used.

The cell fractionation procedure used was a modification of that of Hogeboom (1955). A Sorval-RC2 and Spinco preparative ultracentrifuge, with a SW 39 L swinging bucket rotor, were used for differential centrifugation. All work was done at 4°C.

Three ml of 0.34 M sucrose was overlaid with 3 ml of sample in 0.25 M sucrose. The nuclear fraction was isolated by centrifugation for ten minutes at 700 X G. A soft pellet was obtained; the supernate must be carefully removed. A micro-pipette was used for the removal of the last 1-2 ml of the supernate. Recentrifugation of the pellet in 1-2 ml of the supernate ocassionally was necessary. The mitochondrial fraction was isolated from the nuclear supernate by centrifugation for ten minutes at 500 X G. The supernate was removed and the mitochondrial fraction was washed with 5 ml of 0.25 M sucrose and centrifuged for ten minutes at 23,600 X G.

The mitochondrial supernate was centrifuged for sixty minutes at 52,100 X G to isolate the microsomal fraction. The final supernate (the supernate of the microsomal fraction) was saved. This contained the soluble ribosomes, soluble protein and cell sap. The mitochondrial supernate was not combined with the supernate from the mitochondrial wash so to prevent dilution of the final supernate. All pellets were resuspended in 1 ml of glass distilled water with very vigorous mixing; the mitochondrial fraction was hard to get into suspension

and required extended mixing. The solubility was not notably increased when resuspended in 1 N NaOH.

Lowry Protein Determination

Protein determinations were carried out according to Bessey et al. (1946), and Lowry et al. (1951), using 2% alkaline cupric carbonate in 0.1 N NaOH. This method was adequate to solubilize the protein, and thus, eliminate the step of protein precipitation with 0.5 N PCA and resolubilization in 1 N NaOH. All samples were diluted in base so as to have a final concentration of 1 N NaOH. The total volume of the reaction mixture was 2.4 ml; each point was run in triplicate. A standard curve using crystalline bovine serum albumin was prepared with each determination. OD was read at 750 mμ on a Beckman spectrophotometer; a reference blank of 1N NaOH was used.

Alkaline Phosphatase Determination—Cytochemical

The cytochemical detection of alkaline phosphatase was based on a modification of Burstone's (1961) technique. The procedure is an azo-dye coupline method; sites of enzyme activity appear as dark blue granules. The modification permits the stain to be put in solution in three separate parts, stored for 2-3 months at 4°C and mixed immediately before use.

Materials and reaction mixture:

1. Naphthyl AS-E phosphate (Nutritional Biochemical Corp., Cleveland, Ohio) in dimethyl formamide—dissolve 200 mg of naphthyl AS-E phosphate in 5 ml N, N,-dimethyl formamide (40 mg/ml DMF). Store in a brown tube, tightly stoppered at 4°C.

2. 0.1 M Tris Buffer—add 12.11 grams Sigma 7-9 biochemical buffer (Sigma Chemical Co., St. Louis, Mo.) per liter of freshly distilled water. Adjust pH to 8.8 with HCl. Store at 4°C.

3. $MgSO_4$ anhydrous—add 600 mg anhydrous $MgSO_4$ per 10 ml of Tris Buffer (0.1 M, pH 8.8). Store at 4°C.

4. Fast Blue RR—add 600 mg of Fast Blue RR to 10 ml of Tris Buffer (0.1 M, pH 8.8). Store in a brown tube, tightly stoppered, at 4°C.

To prepare the fresh staining mixture use the following ratio of components, and add in order from left to right. After preparing the mixture, centrifuge to sediment the particulate matter, use the supernate. The stain is active for 30 minutes. The cells require 20 minutes to stain.

To Prepare	Naphthyl AS-E	Tris Buffer	Anhydrous $MgSO_4$	Tris Buffer	Fast Blue RR
1 ml	0.005 ml	0.5 ml	0.01 ml	0.5 ml	0.01 ml
10 ml	0.05 ml	4.5 ml	0.1 ml	4.5 ml	0.1 ml
100 ml	0.5 ml	45.0 ml	1.0 ml	45.0 ml	1.0 ml

To stain cells attached to a surface:

Aspirate off the medium; rinse with phosphate free saline, drain; fix for five minutes with cold neutral buffered formalin, rinse twice with distilled water, drain; incubate in freshly prepared reaction mixture for 20 minutes, drain; wash twice with distilled water. Allow to dry. Falcon flask preparations may be preserved under a layer of mineral oil.

To stain cells in suspension:

Deliver cells to a small test tube, centrifuge and remove medium; incubate in freshly prepared reaction mixture for 20 minutes, mix well to disperse the cells; count the cells in a hemocytometer, count at least 100 cells.

Alkaline Phosphatase Determination—Quantitative

The quantitative determination of alkaline phosphatase was carried out according to Lowry et al. (1954), as reported in the Sigma Technical Bulletin #104 (1961). The alkaline phosphatase standard is the p-nitrophenol standard solution, Sigma #104-1. The stock solution was dispensed in 100 λ (0.1 ml) quantities into screw cap vials and frozen until ready for use. When ready for use, the stock solution was thawed and 20 ml of 0.02 N NaOH was added directly to the vial—this is the working standard. The final volume of each point on the standard curve was 2.75 ml; all points were done in triplicate. Alkaline phosphatase is reported in Sigma units. One Sigma unit of enzyme will liberate 1 μM p-nitrophenol per hour at 38°C. 1 μM is 0.1391 mg of p-nitrophenol.

The substrate in the actual alkaline phosphatase assay is Sigma 104 phosphate substrate in Sigma 104-5 alkaline buffered solution (a glycine buffer, pH 10.5). The assay may be run at any total volume, but it is critical to keep the ratio of volumes of reagents constant; 1:10:100 (sample:substrate: 0.02 N NaOH). Throughout this work a total volume of 2.22 ml was used (0.02: 0.2:2.0 ml). The OD was read at 410 mμ against a sample free blank.

Starch Gel Electrophoresis

The starch gel electrophoresis was carried out according to Smithies

(1955), 1959) with the following modifications. Starch slabs were made from hydrolyzed potato starch (Connought Lab., Toronto, Canada). The desired amount of starch was suspended in gel buffer (0.03 M boric acid, 0.012 M NaOH, pH 8.2) in a side arm erlenmeyer flask. The starch was cooked, with mixing, over an open gas burner until a viscous, translucent liquid appears with small bubbles throughout. The starch was degassed with negative pressure, and allowed to boil for about a minute. The starch was quickly poured into a clear plastic electrophoresis tray beginning at one end and pouring smoothly to the other end, being careful not to allow the starch to solidify while pouring. The trays were sealed by placing the edge of the cover down at one end of the gel and allowing it to slowly fall the length of the gel to prevent trapping air bubbles. The sample slot mold was inserted into the gel. The gel was cooled at 4°C for at least one hour before use. The gel may be stored 24 hours before use if kept tightly covered so as to prevent loss of water.

The sample was applied in one of two manners. 20-50 µl of sample was delivered to a sample well of a cold gel with a micropipette. The gel was covered with Saran wrap and electrophoresed for 30 minutes to allow the sample to enter the gel. At this point the power was disconnected and the sample wells were filled with a thick paste of starch made up in gel buffer. Excess starch and buffer were absorbed with small pieces of filter paper. Filling the sample wells permits an even current supply to reach all portions of the gel.

Or secondly the sample was introduced by wetting small strips of filter paper (2 X 20 mm) of Whatman #3 MM filter paper. The filter paper was inserted on a spatula into appropriate size slits made in the gel by a razor blade or spatula. Care was taken to prevent bubble formation at the point of insertion which may result in a distorted pattern of migration. After 30 minutes of electrophoresis the sample has entered the gel and the filter paper was removed; the slits are carefully closed back together. Either method of sample application gave satisfactory results; the filter paper technique was easier but less quantitative.

The gel was completely covered with Saran wrap throughout the run to prevent dehydration. A bridge of 4-5 layers of Whatman #1 filter paper, presoaked in bridge buffer, served as a electrical connection between the starch slab and the bridge buffer (0.3 M boric acid and 0.06 M NaOH pH 8.8). Enough bridge buffer (500 ml) was used to dissipate any heat from the electrodes. The electrodes were single strands of plantium wire, connected to a variable voltage source of direct current (Heathkit regulated power supply, model PS-4, Benton Harbor, Mich.). The voltage drop across the gel was measured with the Heathkit Vaccum Tube voltmeter, model 1 M-10 (Heathkit, Benton Harbor, Mich.).

The electrophoresis is carried out for 6 hours at room temperature with a voltage drop of 6 volts/cm of gel (180 volts across a 30 cm gel); or electrophoresis is run for 14-16 hours at 4°C at 6 volts/cm. Unfortunately with the system used only 2 volts/cm drop was obtained; the resistance across the gel

was not great enough. After completion of the electrophoresis run the gel was sliced in half in a horizontal plane, thus, all samples appear in both halves. The cutting apparatus was a plastic tray with a thin wire suspended across it serving as the cutting edge. One half was stained for alkaline phosphatase, the other half for protein. After staining the gel slabs may be preserved by wrapping in Saran wrap and storing in the cold.

The protein stain is a 0.5% solution of Amido Black in methanol, water, and glacial acetic acid (30:60:10). The stain may be stored in the cold for months; it is reuseable. The gel was exposed to the stain for one minute and destained in methanol, water, and glacial acetic (30:60:10); one hour each in the first and second rinse, overnight in the third rinse.

The alkaline phosphatase stain mixture is composed of 200 ml of 0.1 M Tris buffer pH 8.8, 100 mg β-naphthyl acid phosphate, 100 mg anhydrous $MgSO_4$, 100 mg Fast Blue RR. The mixture was centifuged before use; otherwise, small pink spots appeared on the surface of the gel due to local concentration of the Fast Blue RR. The gel slab was stained for one hour at 37°C; the gel was destained in 0.1 M Tris buffer pH 8.8. The stain must be prepared fresh daily.

Cellulose Acetate Electrophoresis

The cellulose acetate electrophoresis was run using "phoroslides" (Millipore, Bedford, Mass.) which are small rectangular slides with a flexible plastic backing coated with a layer of cellulose acetate. Samples were applied with small pieces of Whatman #3 MM filter paper; two samples per slide. Less that 1 µl of sample is necessary; it is adequate to barely dampen the strip of filter paper with sample. The "phoroslides" serves as their own bridges; the ends of the slides are dipped into the bridge buffer (0.05 M barbital buffer, pH 8.9) by bending them lengthwise, concave upwards. The samples were electrophoresed for 30-50 minutes at room temperature with 100 volts (3-4 amps).

The slides are stained for protein for 10 minutes in 0.1% naphthol blue black, in 7% acetic acid, and destained with two one-minute washes in 3% acetic acid and one wash in ethanol. Amido black stain is not recommended for staining cellulose acetate.

Slides stained for alakaline phosphatase used the same staining solution as described for the starch slabs. Cellulose acetate slides were stained for 30 minutes at 37°C. The slides were destained in two one-minute washes of 3% acetic acid and one wash with ethanol.

RESULTS

The histochemical staining results show by visual observation under a

light microscope that the LM, LMa, and LMf cells when staining positive for alkaline phosphatase stain throughout the cytoplasm and nucleus. Particularly heavy staining is evident at the cell membrane. LM cells usually stain strongly positive (taking on a deep blue color) or distinctively negative (taking on a slightly yellow tinge); ocassionally cells stain only faintly blue, the blue being detectable only at the nucleus and the membrane.

Over a six-month period the parent LM line (passage level 120-s) gave consistant staining results, staining about 30% positive with a range of 24-32% positive regardless of whether the cells were stained attached to a Falcon flask or in suspension.

The LMa clone 2 was also analyzed. Drysdale et al. (1967), report that alkaline phosphatase activity is mixed in clone 2. Passage level 89 was taken from the freezer and first analyzed two passages later. The strain shows a rise in enzyme activity with subsequent passages from the freezer.

Date	Passage Level	% Positive
4-15	91 LMa Cl 2	55
4-16	91 LMa Cl2	58
4-30	92 LMa Cl 2	78
5-20	94 LMa Cl 2	85

The BHK_{21} line and its clonal derivative, $BHK_{21}C_{13}$, were stained periodically for alkaline phosphatase. No positive cells were ever detected. No significant amount of alkaline phosphatase was determined quantitatively.

Quantitative determinations were performed on all samples of LM cells which had been frozen at -70°C for almost three years. Preliminary work was done with various types of extraction procedures: freeze and thaw, extraction with 1% and 0.5% sodium deoxycholate, extraction with Triton X-100, and sonication. The sonication procedure was chosen for all work reported here. The freeze and thaw method was very time consuming with such a dense suspension of cells, requiring more than four freezes and vigorous buzzing during thawing for complete cellular disruption. Both Triton and deoxycholate required a centrifugation step to remove cell debris; triton also has a very high absorbance at 750 mμ, interfering with protein assays.

After differential centrifugation the alkaline phosphatase activity was determined for each fraction. The data reported below is for the 2557 LM sample. The total fraction value measures the activity for the total sonicated sample; the total activity for the unsonicated sample is about 30% less than for the sonicated total. Specific activity is measured as Sigma units per mg of protein. The column labeled average specific activity is the average of

three separate experiments. Using the same fractionation procedure, a sample of HCAAT was also analyzed. These results are also reported below.

The fractionation sample were run on starch gel electrophoresis under different sets of conditions. At no time was the desired voltage of 6 volts/cm obtained. All runs were made at 2 volts/cm for 24-30 hours in the cold and for 18-24 hours at room temperature. Under these conditions the samples migrated only one inch from the origin. The following samples were used.

1. LM microsome
2. LM microsome-trypsin (10 2% trypsin/100 of sample)
3. LM microsome-triton X-100 (10 1% triton/100 of sample)
4. LM microsome and supernate combined
5. LM microsome, water layer of butanol (1:1) extract
6. LM supernate
7. LM total sonicate
8. HCAAT microsome and supernate combined
9. Human serum

When stained for alkaline phosphatase samples nos. 2,3,4,5,6, and 8 appeared to enter the gel and migrate. The human serum used never stained positive for alkaline phosphatase but stained strongly positive for protein. Alkaline phosphatase in serum is usually easily detectable on starch gel electrophoresis.

ALKALINE PHOSPHATASE ACTIVITY ON LM AND HCAAT SAMPLES

Fraction	No. of Cells Assayed	Sigma Units / 4×10^7 Cells	mg Protein / 4×10^7 Cells	Specific Activity	Average Specific Activity
2557 LM total	4.75×10^6	36.6	0.522	70.0	76
nuclear	1.9×10^8	1.22	0.027	45.0	53
mitoch.	1.9×10^8	0.50	0.088	5.7	28
micros.	8.0×10^6	20.0	0.124	161.0	118
supernate	6.0×10^6	16.0	0.313	51.0	64
		37.72			
		103% recovery			
HCAAT total	6.0×10^5	317.0	0.520	604.0	
nuclear	2.4×10^7	3.75	0.028	131.0	
mitoch.	2.4×10^7	7.25	0.016	615.0	
micros.	4.0×10^6	90.0	0.136	662.0	
supernate	1.4×10^6	187.5	0.428	438.0	
		288.50			
		91% recovery			

Cellulose acatage electrophoresis was also run with the fractionation samples. HCAAT never stained positive for protein on cellulose acetate but showed migration and strong staining for alkaline phosphatase. The LM microsomal fraction treated with trypsin showed two faint spots when stained for protein, one spot migrated very slowly, the other spot migrated very fast, much faster than the leading band of serum. The corresponding alkaline phosphatase stain showed activity only in the slow moving spot. This slow moving spot migrated at the same rate as the HCAAT alkaline phosphatase spot.

Microsomal samples extracted with triton and butanol procedures showed little if any migration, except that the butanol extract showed slight migration when stained for protein. No activity was detected when stained for alkaline phosphatase. Both 0.3 M boric acid buffer and 0.05 M barbital buffer were used as bridge buffers; the results were the same with both.

DISCUSSION

The histochemical studies of the localization of alkaline phosphatase in LM mouse cells agrees well with the findings of Cox and MacLeod (1962) and with Keefe et al. (1965). There is diffuse cytoplasmic staining with stronger staining at the cell membrane. The quantitative studies of alkaline phosphatase in the LM cell give results that might be expected based on histochemical findings. The highest specific activity for the enzyme is in the microsomal fraction, with some activity in all the other fractions of the cell. The percentage distribution of the alkaline phosphatase activity between the microsomal and the supernate fractions may depend to some extent on the amount of freezing and thawing that the samples undergo. Hugon (1967) reported that in glutaraldehyde fixed HeLa cells freezing apparently alters the distribution of the staining sites in the cell. Freezing may or may not affect the cytochemical fractionation localization results with the LM cells; as yet this is undetermined.

The quantitative determination of alkaline phosphatase in HCAAT show high enzyme activity in the mitochondrial, microsomal, and supernate fractions, but unexpectedly low activity in the nuclear fraction. Unfortunately, there is no other work in the literature with which to compare this work. Herz and Nitowsky's (1962) work with Chang liver clone BA, known to have unusually high alkaline phosphatase activity, was based on a fractionation procedure different from the one used in this work. Their fraction A, probably nuclei and mitochondria, showed only 10% of the total activity, fraction B, probably the microsomes, contained about 70% of the activity; and the supernate contained the remaining 20% activity. Different alkaline phosphatase electrophoretic variants are known (Santachiara-Benerecetti et al., 1967) and variants with different heat stabilities are known (Herz and Nitowsky, 1962). Possibly such molecular variants localize in different places in the cell; and different cells contain different proportions of the variants. Localization may possibly vary with tissue of origin and/or species of origin. As yet there is too little

data in the literature to make any generalization.

Previous attempts (Keefe et al., 1965) to show migration of LM alkaline phosphatase in starch gels have failed. Migration of the enzyme is easily demonstrated with cultured human cells in starch (Beckman and Regan, 1964). The LM alkaline phosphatase is apparently attached to some cellular components which prevent migration. Wolman and Bubis (1966) believe the alkaline phosphatase is secured to the hydrophobic layer of lipids in the membranes of the cell. Apparently in the LM cell this association or binding of the enzyme to the lipid is very strong, so strong that it requires some extraction procedure, like n-butanol (Morton, 1954) to free the enzyme. The n-butanol is a good lipid solvent. The use of a triton extraction was suggested by the work of Allen et al. (1965) and Coutinho et al. (1966). The former found increased migration in starch with the slow bands of acid phosphatase and esterases from rat liver after treatment with triton; the latter found increases in alkaline phosphatase and esterase mobility in acrylamide after triton treatment. The use of trypsin was suggested by the fact that alkaline phosphatase is very resistant to this enzyme (Pearse, 1960).

Preliminary electrophoresis studies indicate that triton, butanol and trypsin successfully release alkaline phosphatase so that the enzyme may migrate in starch; only trypsin seems to work effectively, or only the results from trypsin are interpretive in the cellulose acetate system. The slight traces of butanol left in the water layer (containing the enzyme) of the butanol extract may interfere with the cellulose acetate surface and prevent migration, and/or make detection impossible.

Other methods of electrophoresis may prove to be successful in the migration of LM alkaline phosphatase, perhaps paper or acrylamide electrophoresis. Also one method of sample application may be better than another. Smithies (1955) suggested that the pore size of the Whatman #3 MM filter paper, used for sample application, may be critical. This pore size may bind inhibitors or larger molecules which are attached to the enzymes, thus giving better separation and migration with the enzymes. He found this to be true with some serum proteins.

SUMMARY

Various cell lines have been stained histochemically for alkaline phosphatase. The results are in agreement with former reports found in the literature. Both LM and HCAAT cell samples were fractionated by differential centrifugation and each fraction was assayed for alkaline phosphatase. The LM mouse cell shows 45% of the enzyme localized in the microsomal fraction; the remaining 55% is distributed throughout the cell. Previous attempts to demonstrate migration of alkaline phosphatase in starch gels have been unsuccessful. Preliminary results indicate that various extraction procedures may free the enzyme from cellular components and allow it to migrate in starch and cellulose acetate electrophoresis.

BIBLIOGRAPHY

Agus, S. G., Cox, R. P., and Griffin, M. J. 1966. Inhibition of alkaline phosphatase by cysteine and its analogues. Biochim. Biophys. Acta 118: 363-370

Albers, H. 1953. The inhibition of phosphatase by sulfur compounds. V. Phosphatase. Ber. Deutsch. Chem. Ges. 68B:1443-1447.

Allen, S. L., Allen, J. M., and Licht, B. M. 1965. Effects of Triton X-100 used with 12 substrates and enzymes from rat liver and tetrahymena pyriformis. J. Histochem. and Cytochem. 13:434.

Alter, A. A., Lu, S. L., Pourfar, M., and Bobkin, C. 1962. Leucocyte alkaline phosphatase in mongolism: a possible chromosome marker. J. Clin. Invest. 41:1341.

Alter, A. A., Stanley, L. L., Pourfar, M., and Dobkin, K. 1963. Studies of leucocyte alkaline phosphatase in mongolism: a possible chromosome marker. Blood 22:1269-1291.

Beckman, L., and Regan, J. D. 1964. Isozyme studies of some human cell lines. Acta path. et. microbiol. scandianav. 62:567-574.

Bessey, O. H., Lowry, O. H., and Brock, M. J. 1946. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. J. Biol. Chem. 164:321-329.

Bodansky, O., and Schwartz, M. K. 1963. Comparative effects of L-histidine on the activities of 5'-nucleotidase and alkaline phosphatase. J. Biol. Chem. 238:3420.

Boyer, S. H. 1961. Alkaline phosphatase in human sera and placenta. Science 134:1002-1004.

Brunk, U., and Skold, G. 1967. Effects of DTPA on histochemically demonstrable non-specific alkaline phosphatase. Acta Histochem. 27:55-61.

Burstone, M. S. 1958. The relationship between fixation and the techniques for the histochemical localization of hydrolytic enzymes. J. Histochem. and Cytochem. 6:322.

Burstone, M. S. 1961. Histochemical demonstration of phosphatases in frozen sections with naphthol AS-phosphates. J. Histochem. and Cytochem. 9:146.

Burstone, M. S. 1962. Enzyme Histochemistry. Academic Press, New York.

Chessin, L. N., and Hirschhorn, K. 1961. Virus resustance and sensitivity in cultured human synovial cells as a possible genetic marker. Exp. Cell Res. 23:138-144.

Cox, R. P., Gilbert, P., and Griffin, M. J. 1967. Alkaline inorganic pyrophosphatase activity of mammalian cell alkaline phosphatase. Biochem. J. 105:155-161.

Cox, R. P., and MacLeod, C. M. 1961. Hormonal induction of alkaline phosphatase in human cells in tissue culture. Nature 190:85-87.

Cox, R. P., and MacLeod, C. M. 1962. Alkaline phosphatase content and the effects of prednisolone on mammalian cells in culture. J. Gen. Physiol. 45:439-485.

Cox, R. P., and MacLeod, C. M. 1963. Repression of alkaline phosphatase in human cell cultures by cystine and cysteine. PNAS 49:504-510.

Cox, R. P. and MacLeod, C. M. 1964. Repression of alkaline phosphatase in human cell cultures. CSHSOB 29:233-251.

Cox, R. P., and Pontecorvo, G. 1961. Induction of alkaline phosphatase by substrates in established cultures of cells from individuals human donors. PNAS 47:839-845.

Cristofalo, V. J., Parris, N., and Krilchevsky, D. 1967. Enzyme activity during the growth and aging of human cells in vitro. J. Cell Phys. 69:263-272.

Conyers, R.A.J., Birkett, D. J., Neale, F. C., Rosen, S., and Brundenell-Woods, J. 1967. The action of EDTA on human alkaline phosphatase. Biochem. Biophys. Acta. 139:363-371.

Coutinoo, H. B., Katehburian, E., and Pearse, A.G.E. 1966. Effects of Triton X-100 upon the mobility of esterases and alkaline phosphatase in disc electrophoresis. J. Clin. Path. 19:617-618.

Danielli, J. F. 1950. Cytological demonstration of alkaline phosphatase. Nature 165:762.

DeCarli, L., Maio, J. J., and Nuzzo, F. 1963. Alkaline phosphatase activity and chromosome variation in human cells in culture. J. Nat. Can. Inst. 31:1501-1507.

DeCarli, L., Maio, J. J., Nuzzo, F., and Benerecetti, A. S. 1964. Cytogenetic studies of alkaline phosphatase in human heteroploid cells. CSHSOB 29: 223-231.

DeLuca, J. and Nitowsky, H. M. 1964. Variations in enzyme activities during the growth of mammalian cells in vitro. Lactate and glucose-6-phosphate dehydrogenase. Biochem. Biophy. Acta 89:208-216.

Drysdale, R. G., Merchant, D. J., Shreffler, D. C. and Parker, R. R. 1967. Distribution of H-2 specificities within the LM mouse cell line and derived lines. Proc. Soc. Exp. Bio. and Med. 124:413-418.

Eagle, H. 1965. Metabolic control in cultured mammalian cells. Science 148: 45-51.

Erwin, V. G., and Whitehead, R. W. 1964. Effect of hydrocortisone on alkaline phosphatase in cultured cancer cells. Proc. Soc. Exp. Bio. and Med. 117: 754-755.

Epstein, W. 1967. An indiogenic reaction for alkaline phosphatase in disc electrophoresis. Am. J. Clin. Path. 48:530.

Fortelius, P. F., Saksela, E., and Sarin, E. 1960. A HeLa cell clonal line lacking alkaline phosphatase. Exp. Cell Res. 21:616-618.

Gartler, S. M. 1967. Genetic marker in cell culture. Nat. Can. Instit. Monograph 26:167-195.

Gartler, S. M., Gandini, E., and Appellini, R. 1962. Glucose-6-phosphate dehydrogenase deficient mutants in human cell culture. Nature 193:602-603.

Gomori, G. 1939. Microtechnical demonstration of phosphatase in tissue sections. Proc. Soc. Exp. Bio. and Med. 42:23-26.

Gomori, G. 1952. Microscopic Histochemistry. Univ. of Chicago Press, Chicago, Ill.

Greenstein, J. P. Biochemistry of Cancer. 2nd. edition, Academic Press, New York, 1954.

Griffin, M. J., and Cox, R. P. 1966. Studies on the mechanism of hormonal induction of alkaline phosphatase in human cell cultures. I. Effects of puromycin and actinomycin D. J. Cell Bio. 29:1-9.

Griffin, M. J., and Cox, R. P. 1966. Studies on the hormonal induction of alkaline phosphatase in human cell cultures. II. Rate of enzyme synthesis and properties of base levels and induced enzymes. PNAS 56:946-953.

Griffin, M. J., Cox, R. P., and Grujec, A. 1965. A chemical method for the isolation of HeLa cell nuclei and the nuclear localization of HeLa alkaline phosphatase. J. Cell Bio. 33:200-203.

Grogg, E., and Pearse, A.G.E. 1952. Coupling azo dye methods for histochemical demonstration of alkaline phosphatase. Nature 170:578.

Harris, H., Watkins, J. F., Ford, C. E., and Schoefl, G. I. 1966. Artifical heterokaryons of animal cells from different species. J. Cell Sci. 1:1-30.

Harris, M. 1967. Temperature resistant variants in clonal populations of pig kidney cells. Exp. Cell Res. 46:301-314.

Hayflick, L. 1965. The limited in vitro lifetime of human diploid cell strains. Exp. Cell Res. 37:614-636.

Hayflick, L., and Morehead, P. S. 1961. The serial cultivation of human diploid cell strains. Exp. Cell Res. 25:585-621.

Henricksen, E. 1956. Alkaline phosphatase in osteoblasts and fibroblasts cultivated in vitro. Exp. Cell Res. 11:115.

Herz, F., and Nitowsky, H. M. 1962. Alkaline phosphatase activity of human cell cultures: kinetic and physical-chemical properties. Arch. Biochem. Biophys. 96:506-515.

Hogeboom, G. M. 1955. Fractionation of cell components of animal tissues in Methods in Enzymology. Vol. 1, ed. Colowick and Kaplan, Academic Press, Inc. New York.

Hugon, J., Borgers, M. Loni, M. C. 1967. Alkaline phosphatase activity in HeLa cells. J. Histochem. and Cytochem. 15:417.

Kaplan, N. O. 1963. Symposium on multiple forms of enzymes and control mechanisms. I. Multiple forms of enzymes, Bac. Rev. 27:115-169.

Keefe, S. A., Merchant, D. J., and Kelsey, W. H. 1965. Alkaline phosphatase activity of LM mouse cells and variants. Proc. Soc. Exp. Bio. and Med. 118:1031-1037.

King, M. J., Gillis, E. M., and Baikie, A. G. 1962. Alkaline phosphatase activity in polymorphs in mongolism. Lancet ii:1302-1305.

Kongsberg, U. R., and Nitowsky, H. M. 1962. Studies of the karyotype of clonal strains of Chang liver differing in alkaline phosphatase activity. J. Nat. Can. Instit. 29:699-709.

Korngold, L. 1962. Antigens as genetic markers. Univ. of Mich. Med. Bul. 28: 337.

Krooth, R. S. 1965. Properties of diploid cell strains developed from patients with an inherited abnormality of uridine biosynthesis CSHSOB 29:189-212.

Krooth, R. S., Howell, R. R., and Hamilton, H. B. 1962. Properties of acatalasic cells growing in vitro. J. Exp. Med. 115:313-328.

Krooth, R. S., and Weinberg, A. N. 1961. Studies on cell lines developed from the tissues of patients with galactosemia. J. Exp. Med. 113:1155.

Land, D. B., and Jackim, E. 1966. A new fluorescence-yielding substrate for alkaline and acid phosphatase. Anal. Biochem. 16:481-486.

Landau, W., and Schlamowitz, M. 1961. Studies of factors related to the differentiation of alkaline phosphatase derived from several tissues. Arch. Biochem. and Biophys. 95:474-482.

Lieberman, I., and Ove, P. 1958. Enzyme levels in mammalian cell cultures. J. Biol. Chem. 233:634-637.

Loveless, A., and Danielli, J F. 1949. A dye phosphate for the histo- and cytochemical demonstration of alkaline phosphatase, with some observations on the differential behavior of nuclear and extranuclear enzymes. J. Microsc. Sci. 90:57.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the phenol reagent. J. Biol. Chem. 193:629-641.

Maio, J. J., and DeCarli, L. L. 1962. Distribution of alkaline phosphatase variants in a heteroploid strain of human cells in tissue culture. Nature 196:600-604.

Maio, J. J., and DeCarli, L. L. 1963. Induction and repression of alkaline phosphatase in human cultured cells by prednisolone, hydrocortisone and organic monophophates. Bi chem. and Biophy. Res. Comm. 11:335-342.

Maggi, V., and Riddle, P. N. 1965. Histochemistry of tissue culture cells: a study of the effects of freezing and some fixatives. J. Histochem. and Cytochem. 13:310.

Martin, G. M. 1964a. Alkaline phosphatase of human fibroblast strains under conditions or repression and induction. Abst. Fed. Pro. 23:(part i) 498 (#2382).

Martin, G. M. 1964b. Alkaline phosphatase of human skin fibroblasts cultures. I. A microassay demonstrating strain variation. Exp. and Mol. Path. 3: 622-633.

Martin, G. M. 1964c. Alkaline phosphatase of human skin fibroblast cultures. II. Kinetics of various "induction." Exp. and Mol. Path. 3:634-647.

Martin, G. M. 1964d. Variation of alkaline phosphatase activity among cells of inducible and constitutive strains of human fibroblasts. Pro. Soc. Exp. Bio. and Med. 116:490-493.

Mecs, I., Domjan, G. Y., and Szepessy, G. 1964. Alkaline phosphatase activity of normal and poliovirus infected HeLa cells. Acta Microbiologica (Budapest) 11:43-49.

Melnick, J. L. 1962. Virus susceptibility of mammalian cells. Univ. of Mich. Medical Bulletin 28:321-336.

Melnykovych, G. 1962. Effect of corticosteroids in the formation of alkaline phosphatase in HeLa cells. Biochem. Biophy. Res. Commun. 8:81-86.

Melnykovych, G. 1967. Effects of cultural conditions on alkaline phosphatase and cell culture survival in the presence of prednisolone. Exp. Cell Res. 47:167-176.

Menton, M. L., Jungle, J., and Green, M. H. 1944. A coupling histochemical azo dye test for alkaline phosphatase in the kidney. J. Biol. Chem. 153: 471-477.

Merchant, D. J., Gangal, S. G., and Holmgren, N. B. 1966. Stability of cell antigens and their value as genetic markers. In vitro II:97.

Miedema, E., and Kruse, P. F. 1967. Effect of prednisolone and contact phenomena on the alkaline phosphatase activity of HEp-2 cells. Biochem. and Biophy. Res. Commun. 26:704-711.

Moog, F., Vire, H., and Grey, R. D. 1966. The multiple forms of alkaline phosphatase in the small intestine of the young mouse. Biochem. Biophy. Acta 113:336-349.

Moorehead, P. S. 1962. Chromosome morphology as a genetic marker. Univ. of Mich. Medical Bulletin 28:294-312.

Morton, R. K. 1950. Separation and purification of enzymes associated with insoluble particles. Nature 166:1092-1095.

Morton, R. K. 1954. Purification of alkaline phosphatases of animal tissues. Biochem. J. 57:595-603.

Moss, D. W. 1965. Properties of alkaline phosphatase fractions separated by starch gel electrophoresis. Biochem. J. 94:458.

Moss, D. W. 1963. Heterogenity of human intestinal alkaline phosphatase. Nature 200:1206.

Moss, D. W., and King, E. J. 1962. Properties of alkaline phosphatase fractions separated by starch gel electrophoresis. Biochem J. 84:192.

Murphy, W. H., Bullis, C., Landau, B. J., and Acosta, R. 1962. Effects of heterologous sera on the modal distribution of variants in four strains of human epithelial cells. Cancer Res. 22:906-913.

Nitowsky, H., and Herz, F. 1961. Alkaline phosphatase in cell cultures of human origin. Nature 189:756-757.

Nitowsky, H., and Herz, F. 1961. Alkaline phosphatase activity of human cell cultures. Pro. Soc. Exp. Bio. and Med. 107:532-34.

Nitowsky, H. and Herz, F. 1963. Hormonal regulation of alkaline phosphatase in dispersed cell cultures. Biochem. Biophy. Res. Comm. 11:261.

Nitowsky, H., and Herz, F., and Geller, S. 1963. Induction of alkaline phosphatase in dispersed cell cultures by changes in osmolority. Biochem. Biophy. Res. Comm. 12:293-299.

Nitowsky, H., Herz, F., and Luka, L. 1962. Hormonal induction of alkaline phosphatase in dispersed cell cultures. Fed. Pro. 21:161.

Novick, A., and Horiuchi, T. 1961. Hyper-production of B-galactosidase by Escherichia coli bacteria. CSHSOB 26:239-249.

Paul, J., and Fortrell, P. F. 1961. Molecular variation in similar enzymes from different species. Ann. N. Y. Acad. Sci. 94:668-677.

Pearse, A.G.E. 1960. Histochemistry. 2nd. edition, Little and Brown, Boston.

Regan, J D. 1966. Alkaline phosphatase in synchronized human cells. Experientia 22:708-714.

Rothman, F., and Byrne, R. 1963. Fingerprint analysis of alkaline phosphatase of Escherichia Coli K_{12}. J. Mol. Bio. 6:330-340.

Rutenburg, A. M., Barrnett, R. J., Tsou, K. C., Monis, B., and Teague, R. 1958. Histochemical demonstration of alkaline phosphatase activity by a simultaneous coupling technique using naphthol AS phosphate. J. Histochem. and Cytochem. 6:90-91.

Santachiara-Benerecetti, S. A., Cesari, I., and DeCarli, L. 1967. Some properties of alkaline phosphatase from a human cell strain and from a clonal derivative with low activity. J. of Cell Phys. 69:169-176.

Schlamowitz, M., and Bodansky, O. 1954. Specificity of dog intestinal phosphatase antiserum. J. Biol. Chem. 206:369.

Sigma Technical Bulletin #104, 1961. Sigma Chemical Co., St. Louis, Mo.

Smithies, O. 1955. Zone electrophoresis in starch gels. Group variation in serum proteins of normal human adults. Biochem. J. 61:829-641.

Smithies, O. 1959. An improved procedure for starch gel electrophoresis; further variation in the serum protein of normal individuals. Biochem. J. 71:585-587.

Smithies, O. 1959. Zone electrophoresis in starch gels and its application to studies of serum proteins. Adv. Protein Chem. 14:65-113.

Szybalski, W. 1964. Drug resistance as a genetic marker. Krooth, R. S. (editor). Somatic Cell Genetics. Fourth Macy Conference on Genetics. Univ. of Mich. Press, Ann Arbor, Mich. p. 226-252.

Szybalski, W., and Szybalski, E. M. 1962. Drug sensitivity as a genetic marker for human cell lines. Univ. of Mich. Med. Bull. 28:277-293.

Taft, E. B., and Scott, J F. 1958. Studies on histochemistry. II. Quantitative histochemical estimation of activities of alkaline and acid phosphomonoesterases. J. Histochem. and Cytochem. 6:340.

Takamatsu, H. 1939. Soc. Path. Jap. 29:492-498.

Tierney, J. H. 1964. Fluormetric measurement of alkaline phosphatase activity in single cells of human fibroblast cultures. Abs. Fed. Pro. 23: (part 1) 489 (#2381).

Torriani, A. 1960. Influence of inorganic phosphate in the formation of phosphatase by Escherichia coli. Biochim. Biophy. Acta 38:460-479.

Trubowitz, S., Kirman, D., and Masik, B. 1962. The leucocyte alkaline phosphatase in mongolism. Lancet ii:486-487.

Trubowitz, S., and Miller, W. L. 1966. Electrophoretic heterogeneity of leucocyte alkaline phosphatase in normal man and in patients with polycythemia vera. Pro. Soc. Exp. Bio. and Med. 123:187-188.

Tsou, K. C., Su, H.C.F., and Rawnsley, H. M. 1965. Demonstration of serum alkaline phosphatase activity and isozyme on acrylamide with indoxyl phosphate. J. Histochem. and Cytochem. 13:10.

Valentine, W. N., and Beck, W. S. 1951. Biochemical studies on leucocytes and myelocytic leucemia. J. Lab. Clin. Med. 38:39-55.

Walker, J. S. 1965. M. S. Thesis, Dept. of Microbiology, Univ. of Mich., Ann Arbor, Mich.

Watkins, J F., and Grace, D. M. 1967. Studies on the surface antigens of interspecific mammalian cell heterokaryons. J. of Cell Sci. 2:193-204.

Weiss, M C., and Ephrussi, B. 1966. Studies on interspecific (rat X mouse) somatic hybrids. II. Lactate dehydrogenase and B-glucuronidase. Genetics 54:111-1122.

Weiss, M. C., and Green, H. 1967. Human-mouse cell lines containing partial complements of human chromosomes and functioning human genes. PNAS 58: 1104-1111.

Williams, H. L., and Watson, E. M. 1940. Influence of sulfhydryl compounds on the activity of bone phosphatase in vitro. J. Biol. Chem. 135:337-338.

Wolman, M., and Bubis, J J. 1966. The relation of various enzymes to cellular membranes. Histochemis 7:105-115.

E. REFINEMENT OF ANALYTIC PROCEDURES

Final touches were put on the ultra micro methods of chemical analysis and cell cloning which we reported last year. A paper based on this work was presented at the Tissue Culture Association in San Juan, Puerto Rico in June 1968 and is being prepared for publication.

F. DEVELOPMENT OF IMPROVED NUTRITIONAL SYSTEMS

1. Development of Improved Medium for Suspension Cultures

The aim of the work described was to produce an improved medium for large-scale suspension culture of mammalian cells, for the purpose of virus vaccine production. Factors that should be considered are: (1) provision of a high cell yield and support of growth at low inoculum density, thus decreasing the number of required steps in scaling-up procedures, (2) stability of medium components; (3) cost. The criterion of success would be the adequate support of a number of characterized cell lines embracing a wide viral spectrum.

It was decided to attempt to produce a rich, stable medium allowing high cell yield and to design a more economical medium by determining which nutrients could be reduced in concentration without a concomitant decrease in growth.

BHK 21 cells, clone PD 4 were chosen because of their rapid growth rate, wide virus spectrum, and their ability to grow in suspension culture. The original medium was Eagle MEM supplemented with 10% Fetal Calf Serum, and 10% Tryptose Phosphate Broth. A sodium bicarbonate buffer system was used. Unless otherwise stated, all work was done with suspension cultures.

EXPERIMENTAL RESULTS

Bicarbonate buffer is unsatisfactory in that it has a pka above physiological pH and tends towards alkalinity due to loss of carbon dioxide. Several organic buffer systems were examined, namely Tris, citrate, and HEPES, alone and in combination. The buffer finally adopted consisted of 0.02 M HEPES and 0.03 M Tris. This allowed cell growth in continuously "open" suspension culture. 0.01% $NaHCO_3$ was retained as a nutrient.

The original medium yields about 1 X 10^6 cells per ml with a doubling time of about 19 hr. Addition of a 2X concentration of MEM nonessential amino acids (NEAA) increased the yield to about 2.5 X 10-6 cells per ml with a DT of about 15 hr.

At a very cursory estimation, trace elements appeared to be nonlimiting in the standard and NEAA medium and a solution of the commonest trace elements i.e., Mn, Zn, Fe, Cu, B, Mo, and Co appears to inhibit growth.

Inorganic phosphate at the level found in standard medium (1.76 mM) does not limit growth. An increase of Pi concentration to 8.0 mM showed no stimula-

tion of yield or growth rate.

In MEM, glutamine probably serves as a source of glutamine per se and secondly as a source of carbon skeletons and amino nitrogen, via glutamate. Glutamine in stored medium has a half-life of about a week at 37°C and is converted to γ-pyrrolidone carboxylic acid. It was found that all glutamine added to the media as such could be replaced by an equal morality of glutamic acid without noticeable loss of cell yield. Presumably the glutamine requirement could be met by that present in serum.

A 3X concentration of vitamins and of essential and nonessential amino acids yielded 3 X 10^6 cells per ml. A 3X concentration of essential amino acids alone gave similar results but the other nutrients were maintained at this high concentration as it was felt that these nutrients could become limiting as yields were increased.

It was found that continuous passage in spinner medium led to a decreased cell yield due to calcium limitation. Addition of calcium to the same level as that in monolayer medium rectified this defect at the expense of increased cell clumping.

With the medium modified, as shown, 4 X 10^6 BHK cells per ml could be obtained in small (150 ml) spinner cultures. In larger vessels (500 ml medium) a count of 2 X 10^6 cells per ml was obtained. Limitation was the result, direct or indirect, of oxygen deprivation. Aerated cultures achieved a level of 5 X 10^6 cells per ml.

GROWTH MEDIUM
(Spinner)

	ml per Litre	Wt in gm Litre	Molarity
Glucose		3 g	
Tryptose phosphate		3 g	
Tris			0.03
HEPES			0.02
Calf serum	100		
MEM amino acids 50 X	30		
MEM nonessential amino acids 100 X	30		
BME vitamins	30		
$NaHCO_3$ (5% w/v soln)	2		
Glutamin Acid			0.004
10 X Hanks salts*	100		
$CaCl_2$ $2H_2O$ (1.86% w/v soln)	10		

*Hanks salts-modified to contain no glucose, phenol red, or calcium chloride. Contains twice normal phosphate concentration and 5.6 g per litre NaCl at 1 X conc.

Under these conditions (modified media with aeration) cells inoculated at 2.5 X 10^5 cells per ml regularly reach 5 X 10^6 cells per ml with a minimum doubling time of 13 hr; an overall growth period of about 100 hr. Although the data is insufficient to allow a definite statement, it appears that BHK cells can reach 4.5-5.0 X 10^6 cells per ml when inoculated at 5 X 10^4 cells.

It appears that one may eventually be able to reduce the serum component of this medium to a more economical level. Cells grown with 10% calf serum showed decreased growth yields when inoculated into media with lower serum levels, but cells grown for several passages in media containing 2.0% serum achieved a yield of 4.0-4.5 X 10^6 cells in media containing 2.5% CS.

ANALYSIS OF CELLS AND SUPERNATE

Dry weight measurements gave a value of 442 μg dry wt per million log-phase cells.

To date only one growth curve has been investigated to determine the relationship between energy production and cell yield. Glucose is removed from the supernate in an almost linear fashion, thus the rate of glucose utilization per cell present falls off rapidly, the curve of this utilization approximating to a rectangular hyperbola. The cells are highly glycoltic, but lactate accumulation in the medium decreases in the later stages of growth. During the growth of 4.75 X 10^6 cells (i.e., 2.1 μg dry wt) 1,915 μg of glucose is removed and 1,200 μg of lactic acid are produced. On the basis of ATP made available, 11.8 μg dry wt of cellular material is formed per μ mole ATP produced. This is fairly good agreement with the value of 10 μg dry wt per μ mole ATP observed in bacterial systems and suggests a similar degree of efficiency between mammalian cells and bacteria.

2. Growth in Chemically Defined Medium

The metabolism of strain LM mouse fibroblast cells adapted to growth in chemically defined medium in this laboratory is being studied. The composition of the medium is as follows:

(a) The twelve essential amino acide + glutamine at double the concentration described by Eagle as required for the maintainence of strain LM mouse fibroblast cells.

(b) Vitamins in Eagles minimal essential medium added at 2 X concentration.

(c) Glucose added at double the concentration in Hanks balanced salt

solution.

(d) Hanks balanced salt solution.

The population doubling of the LM cells in this medium is approximately 45-50 hr. The simplicity of the medium coupled with the relatively rapid growth rate of the cells renders this system optimal for basic studies of cellular metabolism and should contribute significantly to studies heretofore performed on the metabolism of the strain L mouse fibroblast cell.

RESEARCH TO DATE

Glucose Utilization

Throughout the growth curve, strain LM mouse fibroblast cells in monolayer culture utilize approximately 30% of the glucose initially present in the medium. Increasing the initial level of glucose concentration in the medium does not result in either enhanced glucose utilization or protein synthesis. Glucose uptake from the medium is determined by the glucose oxidase method.

Lactic Acid Production

When compared to the production of lactate by strain L mouse fibroblast cells in medium 199 with added peptone, strain LM mouse fibroblast cells produce considerably less lactate in the above described chemically-defined medium. Patterns of lactate production and reutilization throughout the growth curve are similar however. Lactate production is determined by the lactate dehydrogenase method.

Phosphate Utilization

Strain LM mouse fibroblast cells utilize approximately 25% of the phosphate available in the medium. Phosphate utilization is determined by reduction of ammonium molybdate according to the method of Raabe.

Amino Acid Analysis

We recently obtained through Parke-Davis Co. the use of a Beckman Spinco Model 120 C amino acid analyzer. It has been calibrated using standard amino acid mixtures and is currently being used for analysis of amino acid utilization and production by strain LM mouse fibroblast cells in the above-described medium.

DISTRIBUTION LIST

Agency	No. of Copies
Commanding General U. S. Army Medical R&D Command Attn: MEDDH-SI Washington, D.C. 20315	4
Commanding Officer U. S. Army Medical Unit Fort Detrick Frederick, Maryland 21701	5
Commanding General U. S. Army Materiel Command Attn: AMCRD-BC Washington, D.C. 20315	5
Defense Documentation Center Attn: DDCIR Cameron Station Alexandria, Virginia 22314	20
Commanding Officer U. S. Army Combat Development Command Medical Services Agency Brooke Army Medical Center Fort Sam Houston, Texas 78234	1

Unclassified

Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)

Medical School
The University of Michigan
Ann Arbor, Michigan 48104

2a. REPORT SECURITY CLASSIFICATION

Unclassified

2b. GROUP

3. REPORT TITLE

Large Scale Fermenter Growth of Animal Cells for Virus Vaccine Production: Control of Seed Stocks and Growth Conditions (U)

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Annual Progress Report (For the period 1 January 1968 to 31 December 1968)

5. AUTHOR(S) (First name, middle initial, last name)

Donald J. Merchant

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
February 1969	58	135

8a. CONTRACT OR GRANT NO.	DADA-17-67-C-7073	9a. ORIGINATOR'S REPORT NUMBER(S)	08621-2-P
b. PROJECT NO.	1B662706A096		
c. Task No.	1B662706A096-02	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)	
d. Work Unit No.	1B662706A096-02-908		

10. DISTRIBUTION STATEMENT

Each transmittal of this document outside the agencies of the U. S. Government must have prior approval of the Commanding General, U. S. Army Medical Research and Development Command, Washington D.C. 20315

11. SUPPLEMENTARY NOTES

12. SPONSORING MILITARY ACTIVITY

U. S. Army Medical R&D Command
Office of the Surgeon General, D/A
Washington, D. C. 20315

13. ABSTRACT

DD FORM 1 NOV 68 1473

Unclassified

Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT