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

FINAL REPORT

by

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SUMMARY

Using equipment previously constructed on the program L-M mouse cells have been grown in a 5-liter fermenter in medium 199 supplemented with peptone and serum. Changes in pH, O-R potential, dissolved oxygen and cell number and viability have been continuously monitored. Cells also have been grown under conditions of controlled pH and O-R potential and the relationship of O-R potential to growth rate has been determined.

Work has been completed on a computer program for analysis of chromosome changes in aneuploid cell populations. The method also is applicable to study of diploid cells. Automated methods combined with computer analyses permit a rapid and sensitive monitoring of chromosomal shifts in cell population.

Using the computer program, as well as more conventional methods of chromosomal analysis, data is presented on the effects of several types of environmental selection pressures on the chromosomal complement of L-M mouse cells and $BHK_{21}PD_4$ cells.

Detailed studies of the amino acid nutrition of L-M mouse cells in chemically defined medium is presented. Combining the simplicity of Eagle basal medium with the sensitivity and accuracy of an amino acid analyzer, it has been possible to obtain critical evidence concerning the rates of use of individual amino acids, the role of imbalance of amino acids in utilization patterns and the effects of supplementing nonessential amino acids.

Finally, studies of the nutrition of $BHK_{21}PD_4$ cells have led to the development of a very efficient medium (schedule 8) for this cell line. The comparative costs and yield is compared with more conventional media. Improved methods of storage and of freezing seed stocks of these cells also are presented.

FOREWORD

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

Gregory Baumann
Richard Giles
William Kerr, Jr.
Edward Lunk
Jens Pedersen
Richard Platte, Jr.
Howard Stockdale, Ph.D.
Gary Stoner

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INTRODUCTION

The pruposes of this project were (1) to determine the degree of drift in cell populations during continuous cultivation and during build-up from seed stocks to fermenter growth, (2) to determine the major causes of drift, and (3) to explore control procedures for minimizing these variables.

L-M mouse fibroblasts and $BHK_{21}PD_4$ hamster cells were used as models. The former is heteroploid, the latter is near diploid. Growth rates, nutritional requirements and metabolic behavior were examined. Markers used to follow population changes have been (1) chromosomal (number, kind, markers, etc.), (2) alkaline phosphatase activity, and (3) colony type.

The project had to be terminated prematurely when Dr. Merchant left Ann Arbor to accept the position of Director of the W. Alton Jones Cell Science Center, Lake Placid, New York.

THE POPULATION DOUBLING TIME OF STRAIN L-M MOUSE CELLS IN SUSPENSION CULTURE AS A FUNCTION OF CONSTANT, CONTROLLED REDOX POTENTIAL

Jens U. Pedersen

ABSTRACT

Strain LM mouse fibroblasts was grown in suspension culture in a modified, 5-liter New Brunswick fermenter in antibiotic free medium 199P SP FCS_5 under constant, controlled redox potential, pH = 6.80 ± .06, and temperature = 35.0 ± .5°C. Also kept constant were the agitation rate = 145 rpm, influent gas flow rate to the overlay = 300 ml/min, and carbon dioxide concentration in the influent = 5% v/v. Dissolved oxygen was monitored. The redox potential was controlled via variation of the partial pressure of oxygen in the overlay. The population doubling time during the logarithmic growth phase was determined as a function of the redox potential in two separate fermentations of 48 to 46 days' duration each comprising eight growth curves. During the first fermentation, with pH control via .32N HCL and .32N NaOH, the redox potential was varied in the range E_s = 5 - 146 mV, where E_s is the potential measured against the silver-silver chloride-saturated potassium chloride reference electrode, and the resulting population doubling time determined from each growth curve varied from 47.5 hr (from total count or 45.0 hr from viable count) at $\bar{\bar{E}}_s^*$ = 29 mV through a minimum of 29.0 hr (or 27.5 hr) at $\bar{\bar{E}}_s$ = 56 mV to 46.0 hr (or 45.3 hr) at $\bar{\bar{E}}_s$ = 119 mV. During the second fermentation, with pH control via .3 $NaHCO_3$ and gaseous carbon dioxide, the redox potential was varied in the range E_s = 29 - 211 mV, and the population doubling time varied from 66.5 hr (or 66.5 hr) at $\bar{\bar{E}}_s$ = 35 mV through a minimum of 24 hr (or 23 hr) at $\bar{\bar{E}}_s$ = 176 mV to 65.0 hr (or 70.0 hr) at $\bar{\bar{E}}_s$ = 202 mV.

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*See Definitions on page 30.

INTRODUCTION

A fermentation run (NB-15) was carried out in the 5-liter New Brunswick fermenter with the objective to confirm the results of run NB-11 reported in the previous progress report (Merchant, 1969). Due to severe toxicity problems and occurrences of oxygen probe membrane ruptures three unsuccessful runs were attempted. The toxicity occurred without any changes in procedure from previous runs, but on the basis of the observations described below (under Toxicity), it was decided to introduce some changes in the set-up used for pH control before attempting a subsequent run. The differences between NB-11 and NB-15 are described below.

MATERIALS AND METHODS

CELL STRAIN

The cells used in the fermenter experiments are strain LM mouse fibroblasts and have been carried continuously in monolayer and spinner cultures for 1-1/2 years on medium 199P SP FCS_5.

MEDIUM

The medium used in designated 199P SP FCS_5, the meaning of which appears below:

1. 199: 199 refers to the experimental medium no. 199 derived by Morgan *et al.* (1950), and modified by Salk *et al.* (1954), in both method of preparation and composition of base solution. The method of preparation from the single components is described in Merchant *et al.* (1964), and the base solution is Hanks balanced salt solution (using .05% sodium bicarbonate) instead of the original Earle balanced salt solution. The term M150 has also been used (Morgan *et al.*, 1955) to signify medium 199 with Hanks base solution. Helen J. Morton (1968) advocates the term M150. In this report the designation medium 199 is used as a continuation of usage in previous reports.

2. P (Peptone): The medium contains .5% w/v (weight/volume) Bacto Peptone (Difco), lot no. 144603.

3. SP (Special): A growth performance similar to that of the complete medium 199 was found when the following components were omitted:

D - ribose	Ascorbic acid
D - deoxyribose	Vitamine A
Glutathione	Adenosine Triphosphate
L-cysteine•HCl	Penicillin
	Streptomycin

This special medium, 199 SP, has been used throughout in the fermenter experiments. It was purchased as instant powdered medium from Grand Island Biological Company, N. Y., corresponding to their formula E12 (with Hanks base and glutamine, without sodium bicarbonate) but without the nine components listed above (lot nos. 674537, 685482, 685630, 930077, 930143).

4. $\underline{FCS_5}$ (Fetal Calf Serum, 5% v/v (volume percent)): The fetal calf serum was obtained from Hyland, Division of Travenol Laboratories, Inc., Los Angeles, California, and stored at -20°C until used. (Lot nos. 3135D061C1, -71C1, and -68C1 have been used.)

MEDIUM PREPARATION

A normal procedure for dissolving the powdered medium 199 SP and adding the bicarbonate, peptone, and fetal calf serum was followed. However, in order to eliminate or minimize variations in medium component concentration, osmolality, pH, ORP (D.O.), and other not known factors influenced by incubation and storage, the following points were observed:

1. Glass distilled water of high resistivity was used (~1 Mohm-cm).

2. To eliminate excess oxygen, the water was flushed with nitrogen or freshly boiled or autoclaved and cooled to ambient temperature before use.

3. The powder was dissolved in a partial volume of water such that the concentration was lower than 5x. Above this concentration all constituents in the medium will not dissolve.

4. The peptone was added as a freshly autoclaved and cooled 5x solution. The autoclaving of peptone was found to improve growth.

5. After adding all ingredients the pH was routinely checked before filtration and required to be 6.8 ± 0.07 as an indication of consistency in preparation.

6. Filter sterilization took place under nitrogen, again to avoid medium oxidation, at 2-5 psig through an absolute filter or at 10-15 psig through a .22 μ membrane filter.

7. Sterility check of the medium was carried out by incubation at 35-36°C routinely for 3 days and then it was stored at 4°C until used.

The medium for monolayers and spinner flasks was kept in tightly closed, screw-capped prescription bottles to avoid oxidation (increase in ORP) and increase in pH by equilibration with air.

The medium to be used in the fermenter was kept in receiving flasks (in essence, Erlenmeyer aspirator flasks with an upper hoseadapter) where the medium was allowed to equilibrate with the surrounding air through a cotton or glasswool filter. Conditioning (ORP - D.O. and pH) of the medium took place in the fermenter within 1 to 2 hr after charge.

COMMON EXPERIMENTAL CONDITIONS FOR THE CONTROLLED FERMENTER RUNS

The following conditions for a fermenter run were decided upon prior to the entire experimental series:

1. Temperature: 35°C was chosen on the basis of previous growth at that temperature in monolayers and spinners. It gives a reasonable high growth rate and diminishes the risk of impairment caused by accidental temperature fluctuations above 39°C, which is detrimental to the cells. The temperature was maintained at a constant, controlled level of 35.0 ± 0.5°C by means of an external water bath.

2. Agitation Rate: 145 rpm has proven to provide adequate but gentle agitation to keep the cells suspended and to provide for sufficient gas transport across the surface of the medium and adequate mixing for pH control.

3. Gas Flow Rate: The influent gas flow rate from the gas mixing set up to the overlay in the fermenter was chosen as 300 ml/min. This flow rate provides a fast enough renewal rate in the overlay to accomplish the desired ORP controlling action, without causing excessive medium evaporation. 300 ml/min is the lower limit for accurate flowmeter settings within the ranges used for the partial flows: 15 ml/min to 285 ml/min. It was decided not to sparge in order to avoid foaming, denaturation and unnecessary stripping of volatiles.

4. pH: 6.8 was chosen as the initial level for constant, controlled acidity under the assumption that lactate production, i.e., glycolysis, would be low (Willmer, 1966). Further, a carbon dioxide concentration of 5% v/v is in equilibrium with the bicarbonate concentration of medium 199P SP FCS_5 at pH = 6.8.

5. CO_2: Carbon dioxide is an important intermediate metabolite in the TCA cycle and has been used widely in concentrations corresponding to

a 5% v/v gas mixture.

6. ORP: The redox potential of the medium and suspension culture was measured with a platinum electrode versus a reference electrode having an $Ag/AgCl/AgCl_{SAT}$, KCl_{SAT} internal element and solutions and a palladium tip junction. The redox potential is given as E_S in millivolts at any existing physicochemical conditions in the suspension, where the subscript S refers to the silver-silver chloride (etc.) reference electrode. The reported values, E_S, are uncorrected for the normal electrode potential $E_{0,Ag/AgCl,etc.}$, which at 25°C is approximately 185 mV for the specific reference electrode used in the fermenter experiments. This correction has varied somewhat during the period reported but not more than about 15 mV. Hence, to obtain the redox potential in terms of E_h (relative to the hydrogen scale) all E_S values should be increased by 185 ± ~8 mV. A more precise correction is being calculated. During growth experiments the ORP was maintained at selected constant, controlled levels initially suggested by the results of monitored runs and ultimately by run NB-11.

7. D.O.: The dissolved oxygen was monitored during the fermenter experiments and is expressed as p_{O_2} in mm Hg, the partial pressure of oxygen in the gas phase which is in equilibrium with the suspension. The p_{O_2}, which corresponds to 100% saturation with air, is a function of the barometric pressure and the water vapor pressure above the suspension. The normal barometric pressure at the elevation where the experiments were conducted is 736 mm Hg. Under the assumption that the gas overlay in the fermenter is saturated with water at 35°C (p_{H_2O} = 42 mm Hg) the normal total pressure of atmospheric air is P_{tot} = 694 mm Hg and p_{O_2} = 0.2095 x 694 = 145 mm Hg.

The basic conditions for the controlled fermenter runs as described above were common for both runs NB-11 and NB-15. Due to toxicity problems mentioned elsewhere in this report, it was decided to change the pH control set-up. This resulted in the following differences between NB-11 and NB-15 experimental conditions (see Table IA).

PROCEDURE FOR A RUN

The platinum-reference electrodes were calibrated with a poising standard solution:

$$\frac{1}{300} \text{ M } K_3 Fe(CN)_6/K_4Fe(CN)_6 \quad \text{in } \frac{1}{10} \text{ M KCl}$$

which has a potential, E_h = 430 mV at 25°C. This was done before assembling the fermenter and again after termination of the run to observe possible drift of the reference electrode potential.

TABLE IA. Differences in Experimental Conditions and Systems in Two Fermenter Runs, NB-11 and NB-15

Condition or System	Fermenter Run	
	NB-11	NB-15
Base addition	0.32N NaOH	0.32N $NaHCO_3$
Acid addition	0.32N HCl	CO_2 added via influent gas
Buffer system	Phosphate: initial .012% Bicarbonate: .05%	Phosphate: initial .012% Bicarbonate: normal range during run: 0.05-0.16%
Fermenter content during autoclaving	1.5 l glass distilled water	1.5 l .5% peptone water

After autoclaving the D.O. probe was calibrated with nitrogen and air at 35°C with glass distilled water or .5% peptone still in the fermenter.

The fermenter content was expelled, save for 100 ml and the fermenter was charged with 199P SP FCS_5 to 1500 ml giving a .93 x strength medium. This was conditioned to the desired redox potential and pH via the control system. A grab sample measured with a laboratory pH meter established standardization of the fermenter pH.

Inoculation from a spinner flask with a culture in midlog phase (~1.5-2.0 x 10^6 cells/ml) brought the volume to about 1600 ml with an estimated population density of 2.5 x 10^5 cells/ml.

Samples were taken at varying intervals dependent upon the actual growth rate.

Sampling and Distribution of Sample Volume

A sample volume of 19-20 ml was drawn with a sterile syringe and special needle with pointed tip through the silicone rubber diaphragm sampler.

About 1 ml was discarded to avoid contamination with Dakin's solution used to sterilize the sampler before each sampling.

Two ml was delivered to each of three silicone* treated viability tubes

*Siliclad, Clay-Adams, Inc., New York.

for later cell count and viability determination.

Three ml was delivered to a narrow test tube kept in a 35°C water bath for pH measurement with a thin probe combination electrode immediately after the distribution of the sample volume.

One ml was delivered to a 1-dram vial with rubber lined screw cap kept in an ice bath. One was frozen at -20°C and stored for later glucose and lactate analyses. The other was stored at 4°C for subsequent inorganic phosphate (P_i) analysis.

The sampling took 20-30 sec and the distribution of the sample volume was carried out within 1 min. The pH measurement was done within 3-4 min from sampling time.

Cell Count

From each of the viability tubes was taken 1/2 ml aliquot for counting with a Coulter counter* in a conventional manner. Each aliquot was counted six times giving a total of eighteen determinations for the cell concentration.

Viability was determined by counting in a hemocytometer about 600 cells of the suspension mixed with Erythrosin B. Cells that appeared red were considered nonviable.

PCV

The packed cell volume was read from the thrombocytocrit tube. The packed cells were transferred to a screw capped test tube and washed with .85% saline and centrifuged for 5 min at 540 G. The supernate was discarded and .5 ml of distilled water was added to the pellet. The tube was sealed and frozen at -20°C for eventual nucleic acid, protein, and phospholipid determination. The supernate from the thrombocytocrit tube may be used for osmolality measurements.

TOXICITY

The following sources are the most likely causes of the observed growth impairment. While not immediately discernible, prolonged or repeated exposure to corrosive action by base, acid, and autoclaving gradually developed the problem.

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*Coulter Electronics, Hialeah, Florida.

1. Cu^{++}: Faulty construction material in a luer-lok needle used as inlet for acid and base to the fermenter: While ordered as 316 st. the luer-lok was delivered as a standard nickel plated brass part. The canula itself was stainless steel as ordered.

The plating was corroded by prolonged exposure to hydrochloric acid and sodium hydroxide and copperhydroxide was formed.

2. Fe^{+++}: Incompatibility of different kinds of stainless steel in the fermenter re-electrolytic corrosion and exposure to corrosive compounds (e.g., Cl^-, O_2, HCl, NaOH) and extreme low pH during autoclaving resulting in Fe^{+++} formation and precipitation of $Fe(OH)_3$.

3. Ag^+: Electrolytic corrosion of the silver anode of the dissolved oxygen probe due to probe membrane rupture and to electrical potential differences between the anode and the grounded parts in the fermenter.

RESULTS

The experimental findings of run NB-11 are compiled in Table IIA and graphically represented in Figures 1A and 2A.

The population doubling time was determined from both total and viable counts. Refer to the presentation of run NB-15 for a description of how the values and graphs were derived from the basic growth curves, measurements, and recorder traces.

The experimental findings of run NB-15 are compiled in Table IIIA and graphically represented in Figures 3A and 4A.

Below follows an outline of the construction of the graphs presented in Figures 1A-4A. As an example is used growth curve no. 1 of run NB-15. This growth curve is representative as for the precision with which the PDT and $\bar{\bar{E}}_s$ are obtained. Other growth curves yield narrower or wider ranges and spreads for PDT and $\bar{\bar{E}}_s$. It was decided to present both PDT_T and PDT_V vs. $\bar{\bar{E}}_s$ since the viability varied during some growth curves.

1. Figure 5A. From this growth curve was determined the slope of a straight line that could be drawn through the points that represent the logarithmic growth phase. When the points were few or had a deviation from a straight line a spread for the value of the slope was obtained. The population doubling times, PDT_T and PDT_V, were determined from

$$PDT = \frac{\log_{10} 2}{\text{Slope}} = \frac{0.3010}{\text{Slope}}$$

TABLE IIA. Run NB-11: Population Doubling Times Determined from Total and Viable Counts at Selected, Constant Levels of the Redox Potential for Eight Consecutive Growth Curves (Conditions Described in Section "Common Experimental Conditions" and in Table IA)

Growth Curve No.	Start/End Length of Growth Curve hr	PDT Based on (hr): Total Count, Most Prob.	Total Count, Range Lo/Hi	Viable Count, Most Prob.	Viable Count, Range Lo/Hi	Redox Potential in Terms of E_s (mV): $\bar{\bar{E}}_s$ Most Predom.	$\bar{E}_s$ Start/End	E_s Range Max/Min
1	0.0/ 131.1 131.1	42.3	42.1/42.5	42.3	42.1/42.5	82	91/ 68.5	111/56
2	131.1/ 276.1 145.0	46.0	45.8/46.2	45.3	45.1/45.5	119	137/103.5	146/95
3	276.1/ 380.0 103.9	29.5	29.0/30.0	28.5	28.5/29.0	56	61/ 52.0	80/40
4	380.0/ 539.3 159.3	35.0	34.8/35.2	35.0	34.8/35.2	61	75/ 55.0	91/42
5	539.3/ 714.4 175.1	47.5	47.5/48.0	46.0	45.8/46.2	29	42/ 15.0	50/05
6	714.4/ 858.4 144.0	31.0	29.0/31.0	30.0	29.5/30.0	50	75/ 38.0	100/30
7	858.4/1019.8 161.4	32.5	31.5/33.0	32.6	31.5/32.6	41	70/ 28.0	82/10
8	1019.8/1164.3 144.5	29.0	28.0/29.5	27.5	27.3/27.7	56	64/ 47.0	74/35

TABLE IIIA. Run NB-15: Population Doubling Times Determined from Total and Viable Counts at Selected, Constant Levels of the Redox Potential for Eight Consecutive Growth Curves (Conditions Described in Section "Common Experimental Conditions" and in Table IA). (Included are Mean Values of pH and Dissolved Oxygen.)

Growth Curve No.	Start/End Length of Growth Curve hr	PDT Based on (hr) Total Count Most Prob.	Total Count Range Lo/Hi	Viable Count Most Prob.	Viable Count Range Lo/Hi	Redox Potential in Terms of E_s (mV) $\bar{\bar{E}}_s$ Most Predom.	$\bar{E}_s$ Start/End	E_s Range Max/Min	D.O. Mean Value Start/End mm Hg	pH Mean ± Spread
1	0.0/ 164.3 164.3	34.0	33.5/35.5	34.0	33.5/35.5	53	59/ 46	68/ 35	1.5/7.5	6.81 ±.06
2	164.3/ 250.5 86.2	28.5	28.0/28.5	28.0	28.0/28.5	119	122/113	133/104	3/44	6.79 ±.02
3	250.5/ 418.4 167.9	66.5	64.0/68.5	66.5	64.0/68.5	35	41/ 33	46/ 29	.6/2.2	6.30 ±.04
4	418.4/ 514.7 96.3	29.5	28.0/29.5	29.0	~29	87	94/ 84	94/ 78	2/22	6.77 ±.03
5	514.7/ 625.5 110.8	24.0	24.0/26.0	24.0	24.0/26.0	168	177/155	182/155	21/>145	6.80 ±.05
6	625.5/ 754.3 128.8	65.0	64.0/66.0	70.0	70.0/72.0	202	208/197	211/187	197/494	6.79 ±.04
7*	754.3/ 970.8 216.5	46.0	~46	36.0	35.0/37.0	119	120/117	123/114	-	6.82 ±.04
8	970.8/1107.7 136.9	24.0	24.0/24.5	23.0	23.0/23.5	176	180/170	184/160	-	6.81 ±.02

*

Figure 1A. Run NB-11: PDT_T vs. $\bar{\bar{E}}_s$. The population doubling time of strain LM mouse fibroblasts in suspension culture based on the total count as a function of constant, controlled redox potential in medium 199 special supplemented with .5% peptone and 5% fetal calf serum (conditions described in section "Common Experimental Conditions" and in Table IA).

Legend:

#1 : Values obtained from growth curve no. 1

• : Coordinates ($\bar{\bar{E}}_s$, $PDT_{T, \text{most prob.}}$) i.e., most predominant $\bar{E}_s$ during pertinent part of growth curve, most probable PDT during log phase

▨ : Width: Range of $\bar{E}_s$ during pertinent part of growth curve

Height: Spread of PDT_T as determined from slope of log plot of growth curve during log phase

—— : Horizontal line, entire range of E_s encountered during growth curve

•—• : Line between points: Most likely relationship between PDT_T and $\bar{E}_s$

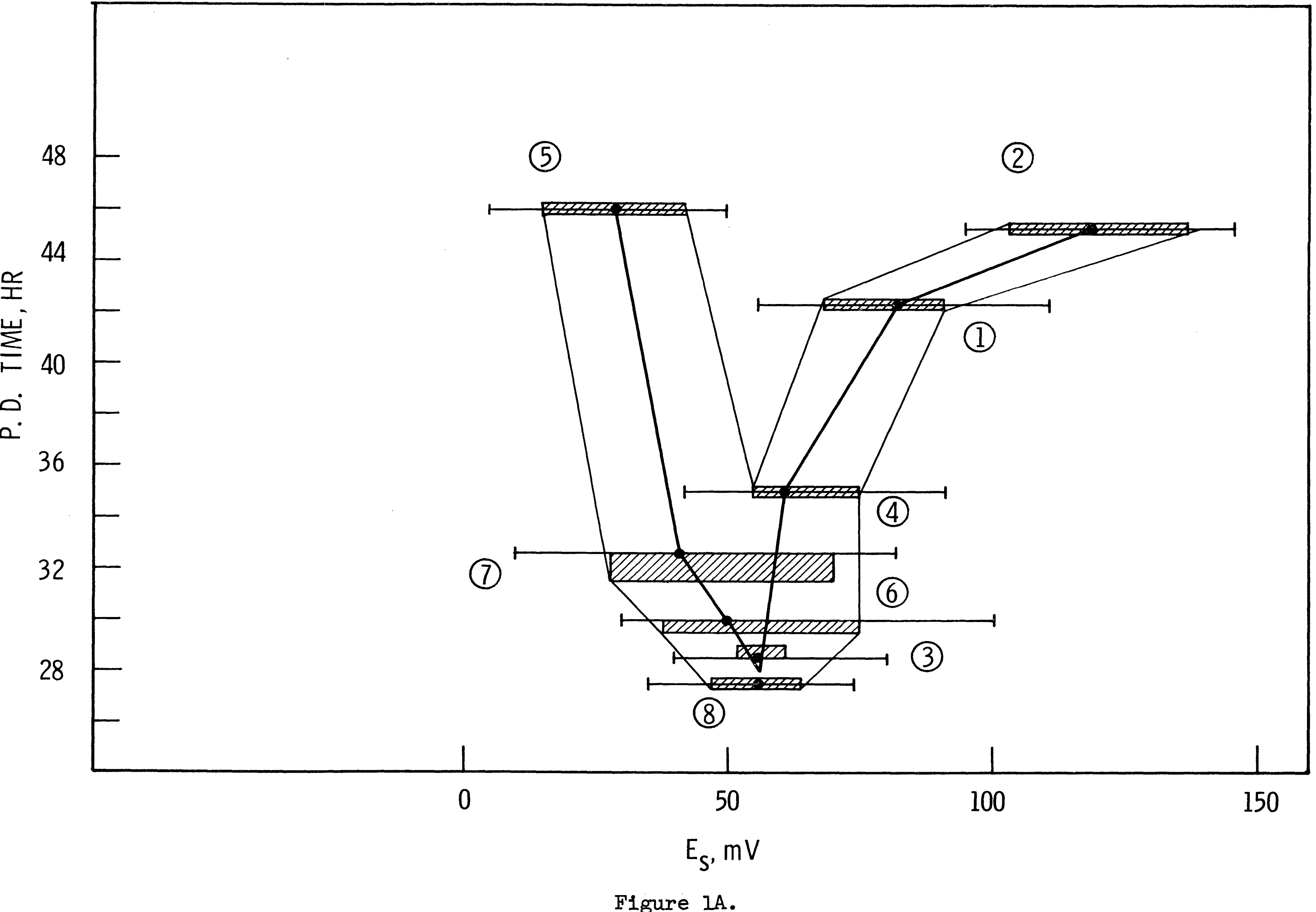

Figure 1A.

Figure 2A. Run NB-11: PDT_V vs. $\bar{\bar{E}}_s$. The population doubling time of strain LM mouse fibroblasts in suspension culture based on the viable count as a function of constant, controlled redox potential in medium 199 special supplemented with .5% peptone and 5% fetal calf serum (conditions described in section "Common Experimental Conditions" and in Table IA).

Legend:

#1 : Values obtained from growth curve no. 1

• : Coordinates ($\bar{\bar{E}}_s$, $PDT_{V,\text{ most prob.}}$) i.e., most predominant $\bar{E}$ during pertinent part of growth curve, most probable PDT during log phase

(hatched box) : Width: Range of $\bar{E}_s$ during pertinent part of growth curve

Height: Spread of PDT_V as determined from slope of log plot of growth curve during log phase

(horizontal line) : Horizontal line, entire range of E_s encountered during growth curve

(line between points) : Line between points: Most likely relationship between PDT_V and $\bar{E}_s$

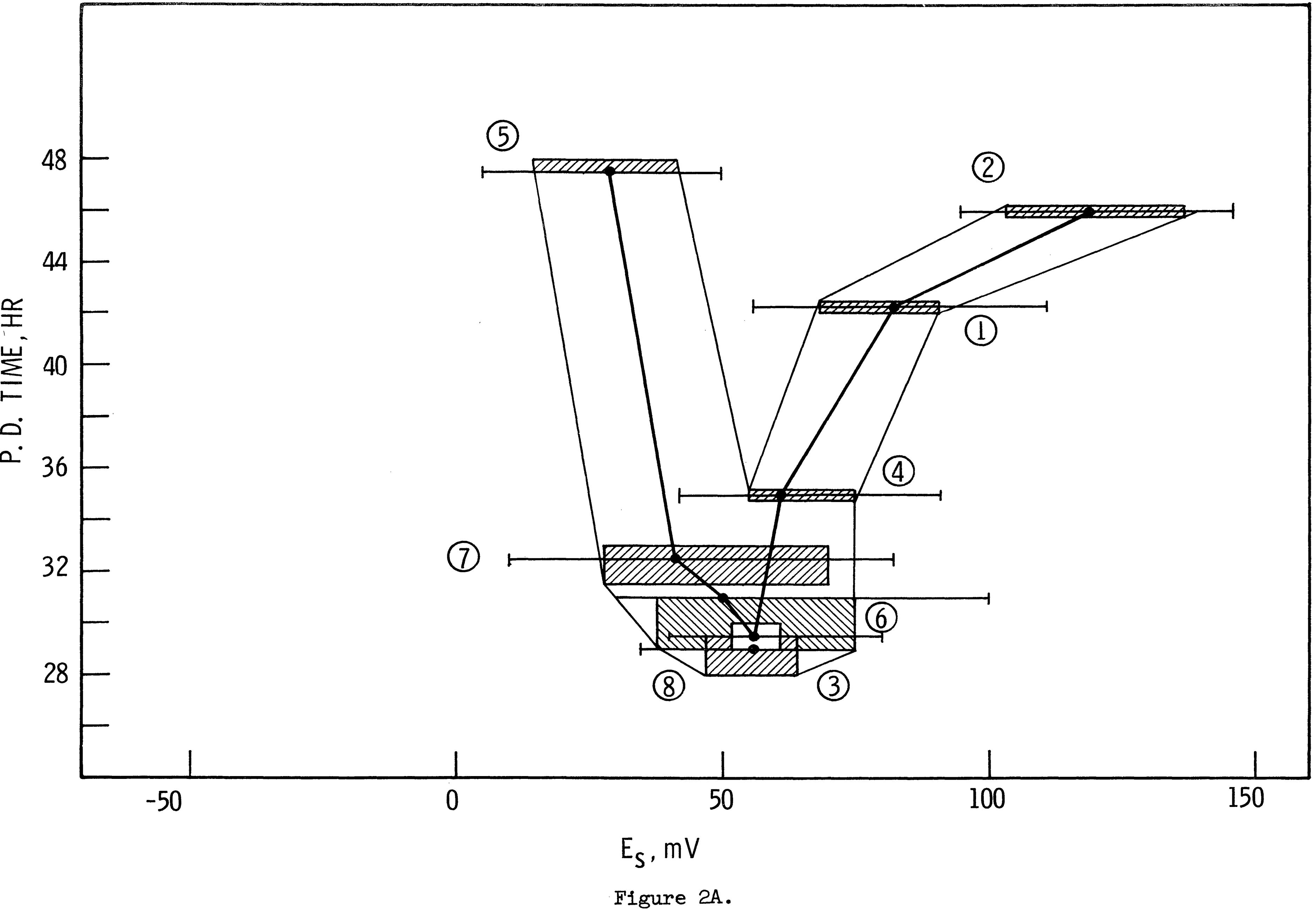

Figure 2A.

Figure 3A. Run NB-15: PDT_T vs. $\bar{\bar{E}}_s$. The population doubling time of strain LM mouse fibroblasts in suspension culture based on the total count as a function of constant, controlled redox potential in medium 199 special supplemented with .5% peptone and 5% fetal calf serum (conditions described in section "Common Experimental Conditions" and in Table IA).

Note: Point no. 7 does not belong to the family of the other points due to a higher CO_2 content in the gas phase during the growth. It is included only as a comparison. (See comment at bottom of Table IIIA.)

Legend:

#1 : Values obtained from growth curve no. 1

• : Coordinates ($\bar{\bar{E}}_s$, $PDT_{T,\text{ most prob.}}$) i.e., most predominant $\bar{E}_s$ during pertinent part of growth curve, most probable PDT during log phase

(hatched box) : Width: Range of $\bar{E}_s$ during pertinent part of growth curve

Height: Spread of PDT_T as determined from slope of log plot of growth curve during log phase

(horizontal line) : Horizontal line, entire range of E_s encountered during growth curve

(line between dots) : Line between points: Most likely relationship between PDT_T and $\bar{E}_s$

D.O. : The range of dissolved oxygen from start of growth curve till the time of the last sample used to determine the PDT, mm Hg

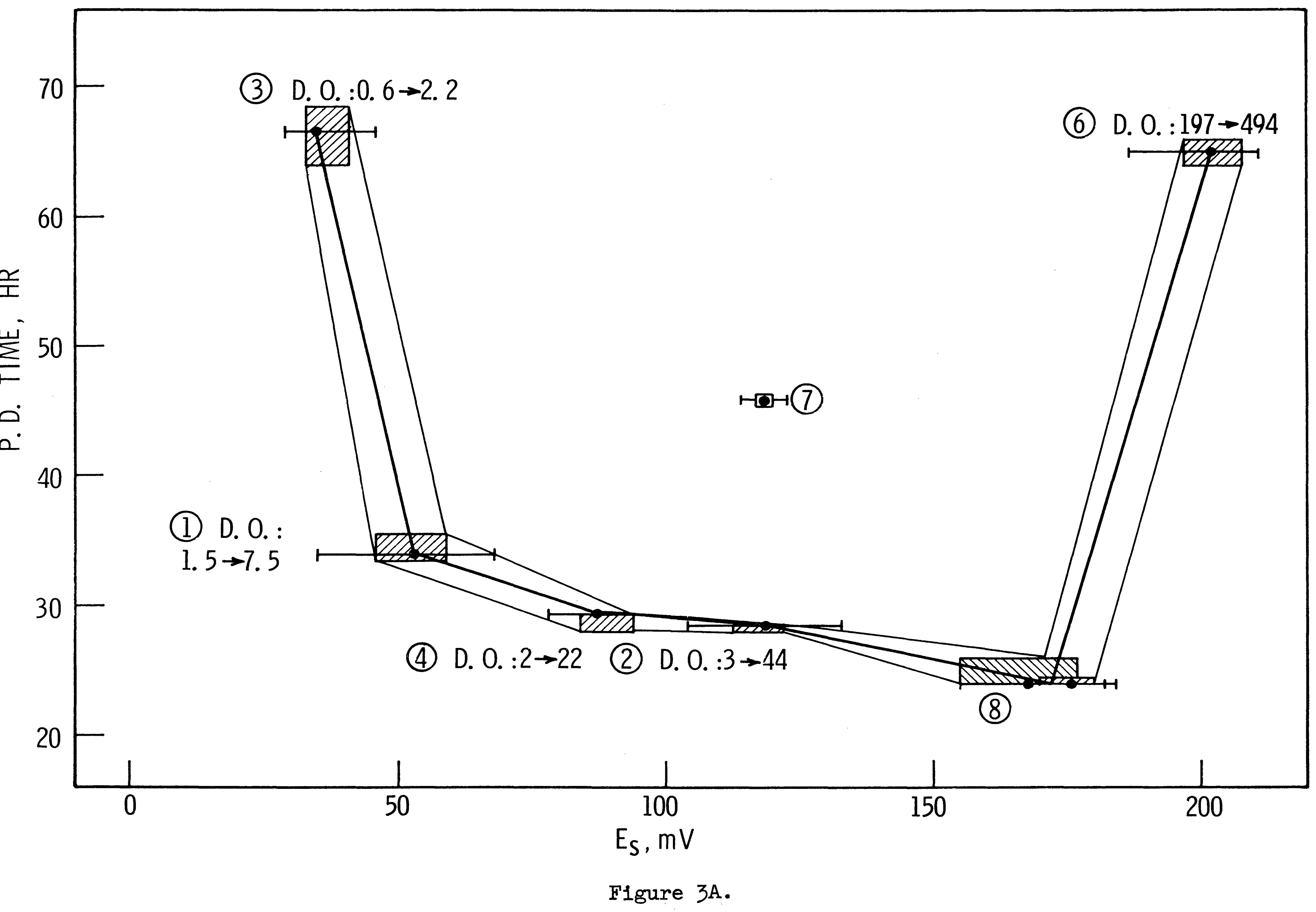

Figure 3A.

Figure 4A. Run NB-15: PDT_V vs. $\bar{\bar{E}}_s$. The population doubling time of strain LM mouse fibroblasts in suspension culture based on the total count as a function of constant, controlled redox potential in medium 199 special supplemented with .5% peptone and 5% fetal calf serum (conditions described in section "Common Experimental Conditions" and in Table IA.)

Note: Point no. 7 does not belong to the family of the other points due to a higher CO_2 content in the gas phase during the growth. It is included only as a comparison. (See comment at bottom of Table IIIA.)

Legend:

#1 : Values obtained from growth curve no. 1

• : Coordinates ($\bar{\bar{E}}_s$, PDT_V, most prob.) i.e., most predominant $\bar{E}_s$ during pertinent part of growth curve, most probable PDT during log phase

▨ : Width: Range of $\bar{E}_s$ during pertinent part of growth curve

Height: Spread of PDT_V as determined from slope of log plot of growth curve during log phase

—— : Horizontal line, entire range of E_s encountered during growth curve

•—• : Line between points: Most likely relationship between PDT_V and $\bar{E}_s$

D.O. : The range of dissolved oxygen from start of growth curve till the time of the last sample used to determine the PDT, mm Hg

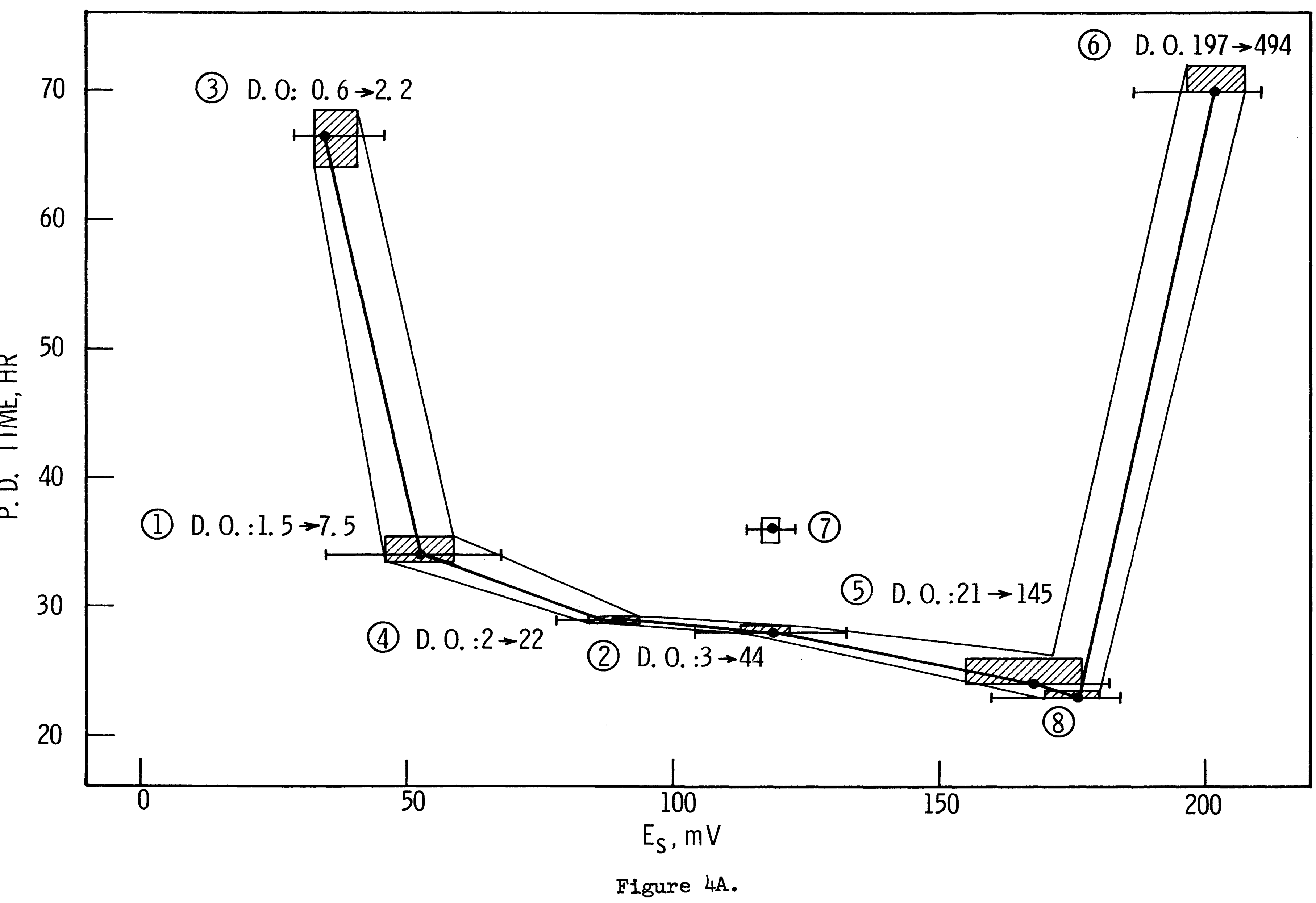

Figure 4A.

Figure 5A. Run NB-15: Growth curve no. 1: Total and viable cell count and PCV vs. time.

Legend:

● : Total cells/ml

○ : Viable cells/ml

□ : PCV, %

(hatched circle) : Estimated total cells/ml on basis of a previous count in connection with dilution of medium*

(dashed circle) : Estimated viable cells/ml on basis of a previous count in connection with dilution of medium*

(dashed square) : Estimated PCV, %, on basis of a previous count in connection with dilution of medium*

(vertical broken line) : Vertical broken line: dilution of culture. Signifies start of new growth curve or termination of run.

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*See also last entry.

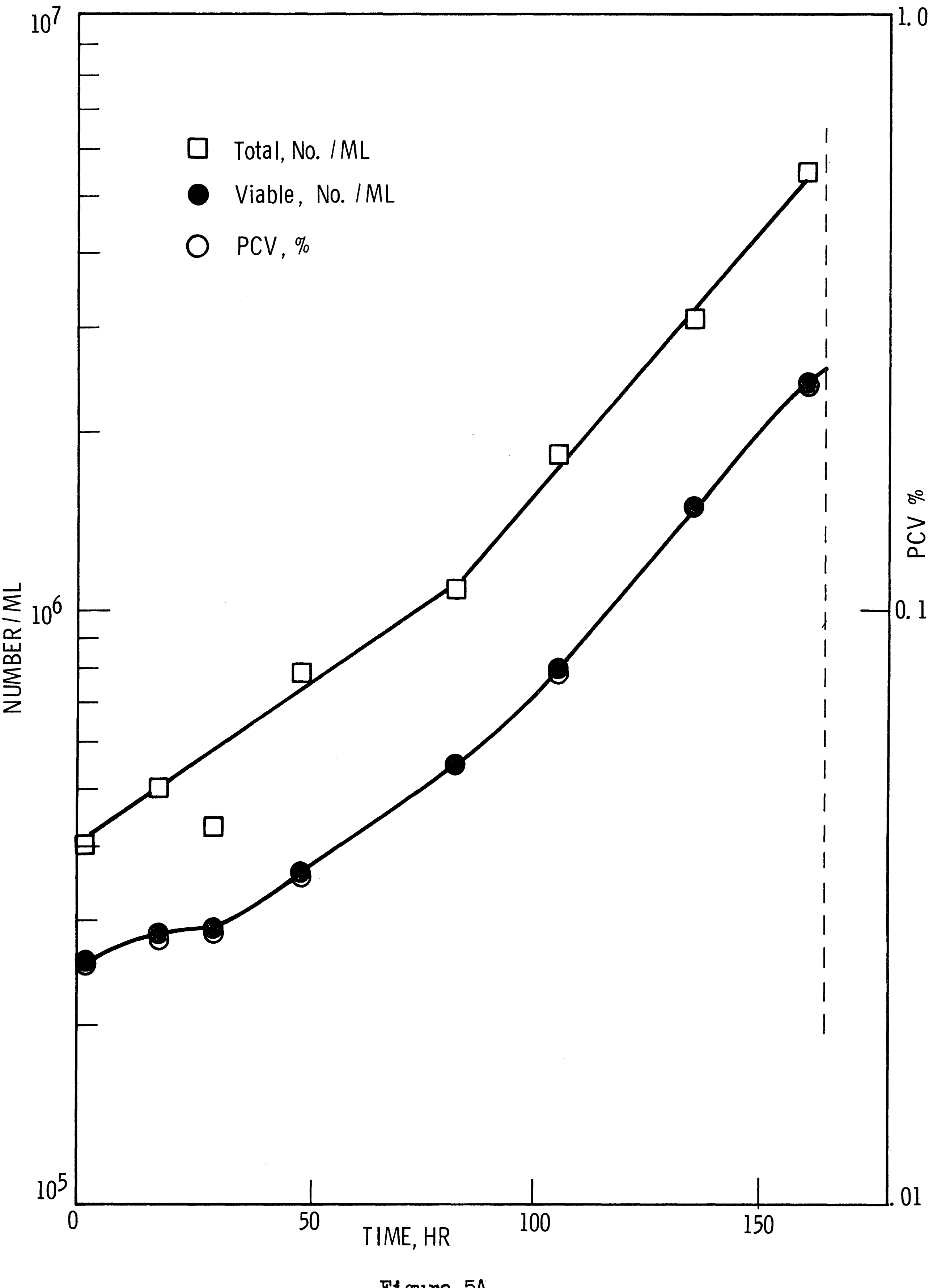

Figure 5A.

or by measuring the increment of the abscissa corresponding to a doubling of the ordinate.

Also the PCV points were plotted and the slope of the line through those points were used in deciding the logarithmic phase and hopefully supporting the PDT. (For this figure the PCVDT = 34 ± 1 hr was comparable to the PDT = 34 + 1.5/0.5 hr.) The values so obtained were tabulated in Tables IA and IIA.

2. From the continuous recordings of E_s, D.O., and pH were plotted a condensed history of the mean and range of these variables. The recorder traces were corrected for drift of analyzers, recorders and, where possible, electrodes. The pH electrode was standardized at each grab sample from the fermenter. The E_s and D.O. electrodes could be calibrated only before and after each fun.

It was found that the ORP analyzer was stable to ± 2 mV over 1-1/2 years. As mentioned under "Common Experimental Conditions," the reference electrode drift amounts to about 15 mV over 1 year despite several autoclavings. The reported values for E_s have not been corrected for electrode drift.

The D.O. electrode's service life was in one instance long enough to recheck the calibration after a run: A drift of ~ +5% was observed. The D.O. values must therefore be considered reliable.

An average mean (= $\bar{\bar{E}}_s$) was then determined from the E_s vs. time plot (Figure 6A) for the period from where the variable reached the control range until or close to the final sample used to determine PDT. This value, $\bar{\bar{E}}_s$, is considered the most predominant during the growth. However this concept is still subject to interpretation.

Likewise was done for pH.

The range of D.O. from start to final determining sample was tabulated in Table IIIA and indicated as a variable parameter on Figures 3A and 4A.

3. All the values for $\bar{\bar{E}}_s$, $\bar{E}_s$, and E_s and PDT, i.e., most predominant $\bar{E}_s$, most probable PDT including the spread and range about these values were plotted in a PDT_T vs. $\bar{\bar{E}}_s$ and PDT_V vs. $\bar{\bar{E}}_s$ graph.

4. Also P_i concentration in the cell free medium vs. time was plotted (Figure 7A). Note the constancy of (P_i) during the initial lag phase where the population density and PCV increase slightly. Further (P_i) seemed to decrease linearly between 30 and 80 hr where after exponential decrease occurred.

Figure 6A. Run NB-15. Growth Curve no. 1: pH, E_s, D.O. (mean and range) vs. time.

Legend:

: Mean trace of variable

: Upper and lower range of variable

III : Time of sample no. III: $NB\text{-}15_{III}$

: Vertical broken line: dilution of culture. Signifies start of new growth curve or termination of run

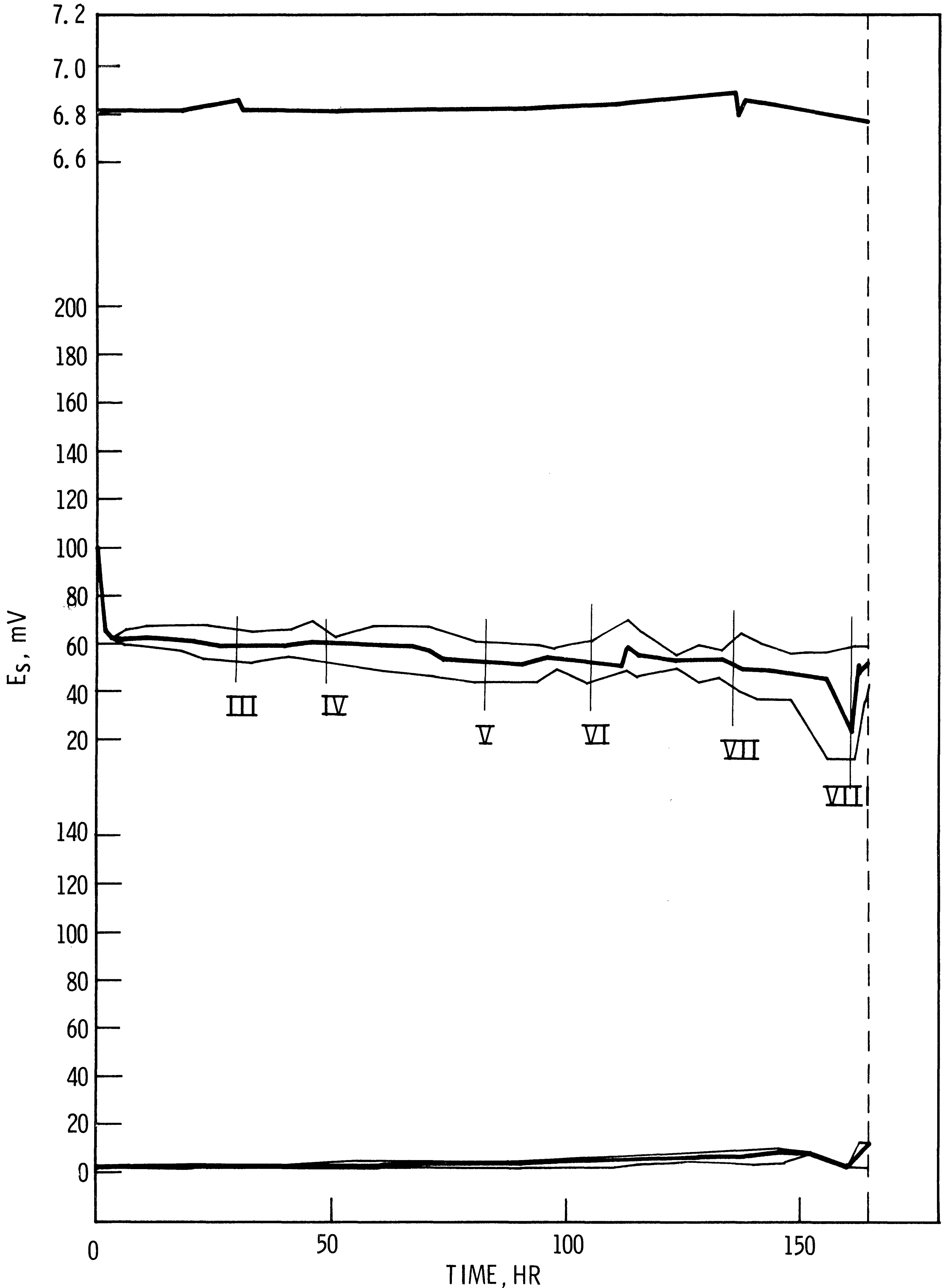

Figure 6A.

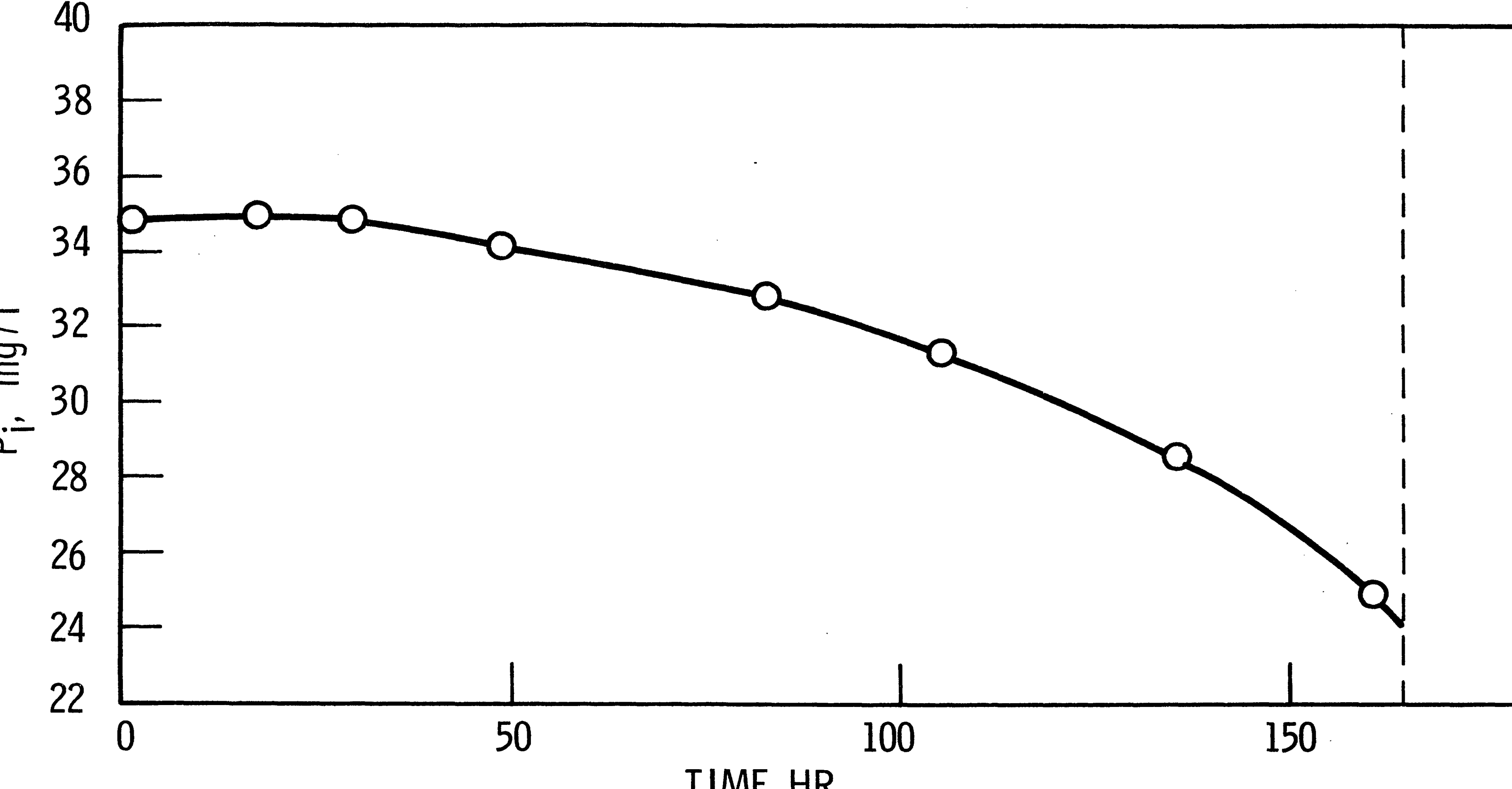

Figure 7A. Run NB-15: Growth Curve no. 1: P_i vs. time.

Legend:

○ : P_i, inorganic phosphate concentration in the cell free medium, mg/liter

DISCUSSION

There appears to be an overall qualitative similarity between the two plots of PDT vs. $\bar{\bar{E}}_S$ for the two runs NB-11 and NB-15. The curve appears concave in the approach to the indicated PTD_{min} and convex toward higher E_S values. (Note: even though NB-15 does not definitely confirm this, it certainly does not negate convexity.) The quantitative deviation is attributed to at least two factors:

1. Change in conditions are listed in Table IA.

2. Possible change in the cell population during the time elapsed between NB-11 and NB-15, despite a uniform procedure in maintaining the cells for inoculation into the fermenter.

Within one run comprising eight growth curves it was possible to establish the relationship between PDT and $\bar{\bar{E}}_S$ as shown and to essentially duplicate a point on the "curve": growth curves nos. 3 and 8 in run NB-11 and growth curves nos. 5 and 8 in run NB-15.

The exactitude of the $\bar{\bar{E}}_S$ points nos. 3 and 8 in NB-11 of course may be subject to interpretation: should they both be 56 mV? However, there is a strong indication of a minimum PDT at about 55 mV and it certainly could be anywhere from ~ 51 to 55 mV yielding maybe an even lower PDT.

For run NB-15 a minimum is indicated between 176 and somewhat below 202 mV. The data reported does not allow any conclusion whether a lower PDT_V than 23.0 hr exists in this run.

The constancy of PDT versus constant $\bar{\bar{E}}_S$ during a growth curve was maintained with an in general exponentially increasing D.O.

The concept of a constant, controlled redox potential in tissue culture as a practical and useful tool in manipulating the growth rate to obtain constancy over an extended period seems strongly indicated by the reported experiments.

I interpret the quantitative difference between the two runs to indicate the necessity of establishing the PDT vs. $\bar{\bar{E}}_S$ relationship for each particular system in question, but when this is done the data would enable a prediction of growth rates as needed.

DEFINITIONS

E_S: Redox potential as measured against the silver-silver chloride/sat. silver chloride, sat. potassium chloride/palladium junction reference electrode at any existing physico chemical conditions in the suspension, mV

$\bar{E}_S$: (also E_S mean) the mean value of E_S over a period of time where E_S varies in a cyclic manner (stemming from control action) and exhibits a reasonably constant trend. Also shorter, aperiodic fluctuations of E_S are considered valid as a basis for $\bar{E}_S$, mV

$\bar{\bar{E}}_S$: The most predominant value of the $\bar{E}_S$ during the part of the growth curve where E_S has affected the growth rate on which is based the determination of the population doubling time, mV

PDT: Population doubling time, hr

PDT_T: Population doubling time based upon total count, hr

PDT_V: Population doubling time based upon viable count, hr

P_i: Inorganic phosphate

ORP: Oxidation reduction potential. Also, redox potential, mV

199M: Short term for 199P SP FCS_5 = medium no. 199 special supplemented with .5% peptone, .05% sodium bicarbonate, and 5% fetal calf serum. (Refer to section on "Medium" for explanation of "Special" and a discussion of this medium.)

PCV: Packed cell volume, %

PCVDT: Doubling time of the culture based on PCV (- packed cell volume doubling time), hr

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CHROMOSOME MEASUREMENT:
ORGANIZATION AND APPLICATIONS OF A WORKING SYSTEM

Gregory Baumann, Richard Giles, Edward Lunk, and Richard Platte, Jr.

INTRODUCTION

A number of investigators have reported on the application of various measurements to the study of chromosome morphology, particularly with reference to the development of automated techniques for chromosome analysis of diploid cells (1-5). However, such systems are generally quite complex, require highly sophisticated computer programming, and, at this time, are extremely expensive to operate. In addition, these systems do not provide adequate descriptions of chromosomes in mixoploid cells due to the fact that any analysis of a mixoploid population must take into account the widely varying chromosome numbers and morphologies which are present in different cells within the population. Our efforts have been directed at developing an easily applied, sensitive system for providing a quantitative description of a cell population based on the measurement of chromosome length in a small sample of cells using semiautomatic measuring equipment and digital computer techniques. One goal in this system has been to reduce the importance of karyotyping and other techniques which are primarily descriptive of single cells and to stress those features which appear to be characteristic of the population as a whole. So, although this system may be used to study diploid cells (especially in following the processes of transformation which these cells undergo), it is most useful in the study of mixoploid cells where differences between single cells are of much less importance.

The specific methodology and computer programming may apply only to the exact equipment configuration being used here, but the types of equipment used are widely available, and most aspects of the logical design and methodology may be applied to any system.

Chromosome measurements are made from the projected images of 35-mm negatives using an X-Y coordinate digitizer on-line to a small computer. This equipment produces magnetic tapes containing digitized data on chromosome spreads which are further processed on a large computer system. Final output is obtained from the machine through a high speed printer, teletype terminal, and CALCOMP plotter. The entire system for preparing, digitizing, and processing chromosome data will be described.

EQUIPMENT AND METHODS

Metaphase chromosome preparations are made using a modification of the technique described by Giles, Merchant, and Masselink (6) in which cell cultures in the logarithmic growth phase are treated with colcemid to stop growth in metaphase and then are expanded in a hypotonic solution of sodium

citrate and fixed in an acetic acid-methanol mixture (see Appendix I). Microscope slides are prepared by flame drying of the fixed cell suspension and are stained with Giemsa stain.[1]

Inasmuch as the computer methods to be described are being used at this time as an adjunct to standard chromosome analysis procedures, all microscope slides are first scanned for high quality metaphase plates using a Zeiss photomicroscope.[2] Only those spreads fitting the criteria given in Table IB are used for extensive analysis although some spreads of lesser quality may be counted.

TABLE IB. Criteria used to judge a chromosome spread before using it for extensive analysis procedures.

1. Chromatid coiling, the centromeric constriction and any secondary constrictions which are present must be clearly visible.

2. If any chromosomes are found to be overlapping in the spread, the overlap must not conceal the centromere or chromatid ends of the chromosomes involved.

3. The spread must be confined to a sufficiently small area of the microscope slide for the technician to rule out the loss of chromosomes in spreading or the presence of chromosomes belonging to other spreads.

The standard procedure used to study acceptable chromosome spreads consists of making differential chromosome counts (i.e., counting separately the numbers of biarm chromosomes, telocentric chromosomes, total number of chromosomes, and total number of arms), visually identifying any marker chromosomes present, and noting the presence of abnormal chromosome morphologies. This procedure is carried out on 50-100 metaphase plates from each population examined, and those of highest quality are photographed for further study.

[1] Fisher Scientific Company, Fair Lawn, New Jersey. Code number SO-G-28.

[2] Carl Zeiss, Germany.

All photographs of mitotic figures used for measurement studies are produced using a 100 x oil immersion objective, an Optovar factor[3] of 1.25 and a projective factor[3] of 3.2. This optical configuration produces an image on the 35-mm film surface 400 times the size of the original object. Using the automatic exposure feature of the microscope, black and white negatives of consistently high quality are easily produced on Kodak high contrast film.[4] Exposed films are developed according to standard dark room techniques (see Appendix II).

Although prints are occasionally made for the production of karyotypes or visual presentation of data, all subsequent analysis is carried out using the projected images of the film negatives. Lengths are measured from these images using an X-Y coordinate digitizer. This equipment, which is used to automatically record the X and Y coordinates of any point on a planar measuring surface, was designed and built in the Department of Physics at The University of Michigan where it is used to study photographs of bubble chamber tracks (see Figure 1B). Equipment of this type enables one to easily translate analog data on a photographic image into a digital form.[5]

THE DDP-24 SYSTEM

Six X-Y coordinate digitizers are connected on-line to a 5K DDP-24 computer[6] in The University of Michigan system. A data point is recorded by manually positioning a set of movable cross hairs over the point and then depressing a foot pedal. Movement of the cross hairs is transmitted through a system of steel bands and pulleys to a rotary pulse incoder which actuates a bidirectional counter providing the digital X-Y location of the point to the DDP-24 computer through an eight-channel multiplexor.

Supervisor and control programs in the system are written in FORTRAN and assembly code and provide for interaction with the user while the measurement is being carried out by means of a small console next to each digitizer. These programs are set up to provide real-time job scheduling with five different priority levels. Of course, the system has been designed specifically

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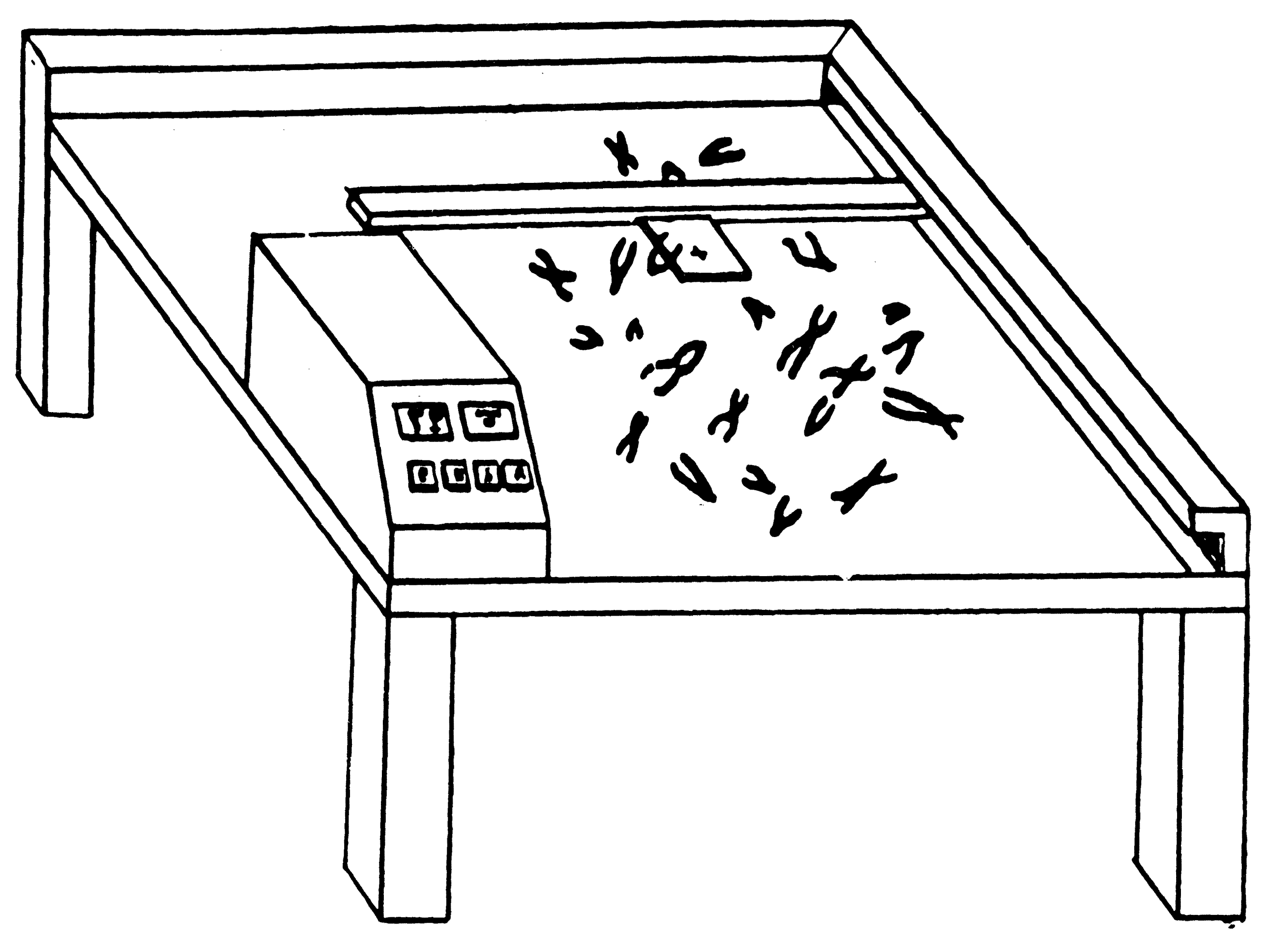
[3] The terms Optovar factor and projective factor refer to optical equipment on the Zeiss photomicroscope which provide additional magnification in the light path to the photographic film.

[4] Eastman Kodak Company, Rochester, New York. Code number M-135.

[5] Similar equipment is available commercially and can even be built with little difficulty (7).

[6] Honeywell Inc., Computer Control Co. Div., Farmington, Mass.



X-Y COORDINATE
DIGITIZER

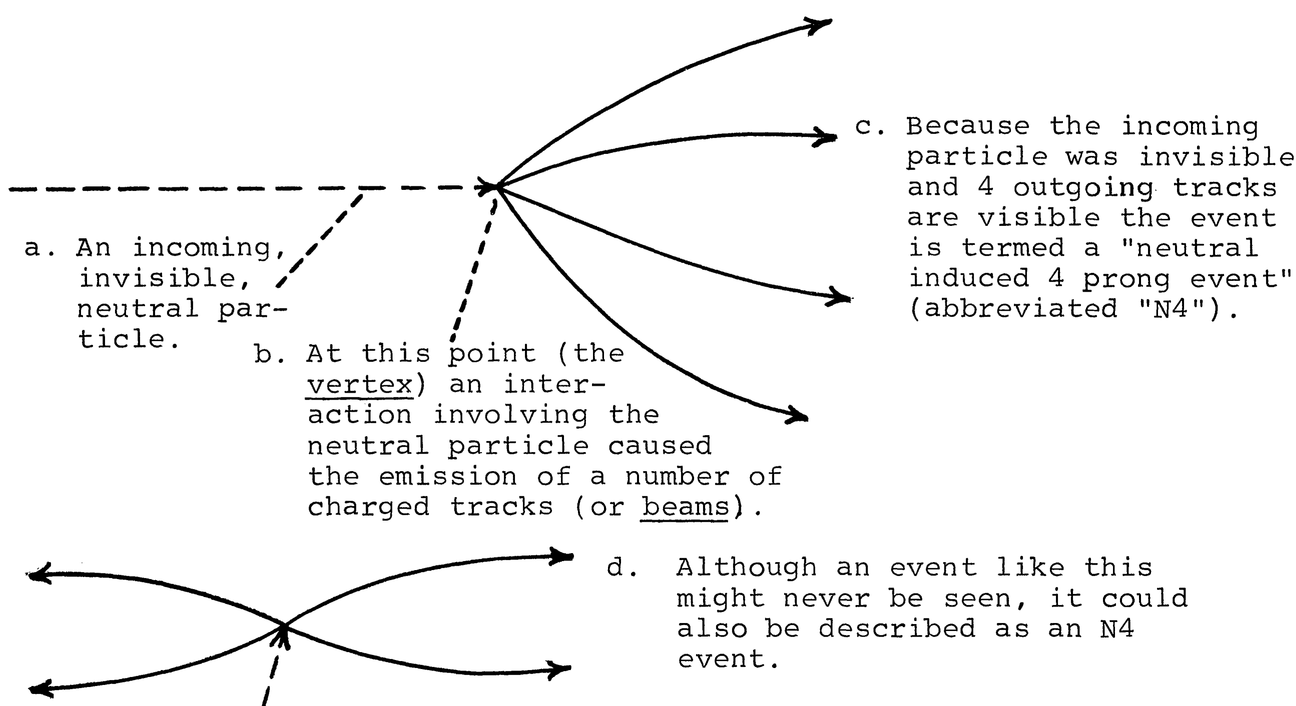
Figure 1B. A photograph of the X-Y coordinate digitizer used to make chromosome measurements.

for bubble chamber studies, and it is, therefore, necessary to describe each chromosome as if it consisted of charged tracks created by atomic particles all originating from a single point of vertex. An atomic event of this type is termed a neutral induced event and is described as having a number of prongs equal to the number of charged tracks seen to originate from the vertex (8). Figure 2B shows how chromosome structures can be redefined to fit the structure of a neutral induced event.

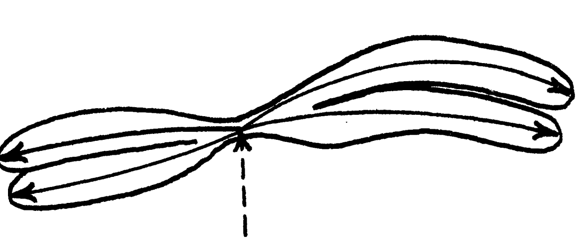
An additional problem was encountered due to the fact bubble chamber tracks are treated as three-dimensional objects and are viewed from three different views on different films. The computer is programmed to automatically switch between films during the measurement of single events. Because chromosome photographs are normally taken from only one view it was necessary to change the system to allow the user to skip the program sequence which changes views. The Department of Physics did this and modified the program to omit a number of checks normally made on bubble chamber data.

When applying this system, the user is prompted for the information needed by the computer through four display windows on the communication console (see Figure 3B). Replies to the machine are entered by depressing various combinations of the ten numerical keys and four termination keys or by depressing the foot pedal. The illumination of a large red light notifies the user of the existence of some error condition. It is possible to check and modify previously entered information through an alter sequence and to return to an earlier step in the program sequence by sending a numerical entry followed by the "C" terminator. The user signals the machine that he has completed the requested information entering sequence by depressing the "P" terminator which signals the machine to "proceed."

Although a sample session using the DDP-24 is given in Appendix III, it may be useful to describe such a session. The computer first requests a variety of items of set-up information including the user's identification number, the experiment number and roll number, each of which consists of a two-digit numerical entry followed by the "P" terminator. Then in order to alter the program sequence so that it will not switch between films, the alter sequence is used. The alter button ("A") is depressed, then the numerical "9" and, finally, the alter button is pressed again. The user is next requested to enter a frame number, event number (a number between 1 and 100, assigned to each chromosome measured in increasing order), and event type (either "2N" for a telocentric chromosome or "4N" for a biarm chromosome, as described in Figure 2B). At this point the computer will request that the user measure the vertex (centromere) of the first track followed by from two to thirteen points located along the track by locating the cross hairs and depressing the foot pedal. The idea here is to select enough points along each track so that the total length can be approximated by accumulating the distances between a series of short straight line segments (see Figure 4B). When the end of a chromatid has been reached, the "P" terminator is depressed, and the computer will request the vertex of



e. Notice the similarity between the structure in (d) and the biarm chromosome shown here. The centromere can be assumed to be the point of origin (vertex) of the chromatids which can be looked at as charged tracks. A biarm chromosome is described as an N4 event.



f. Similarly, a telocentric chromosome can be described as an N2 event.

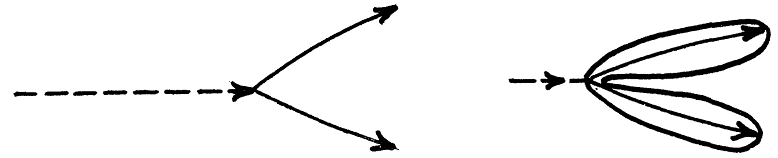


Figure 2. Pictorial development of the method used to describe chromosomes as bubble chamber events.

Figure 2B. Pictorial development of the method used to describe chromosomes as bubble chamber events.

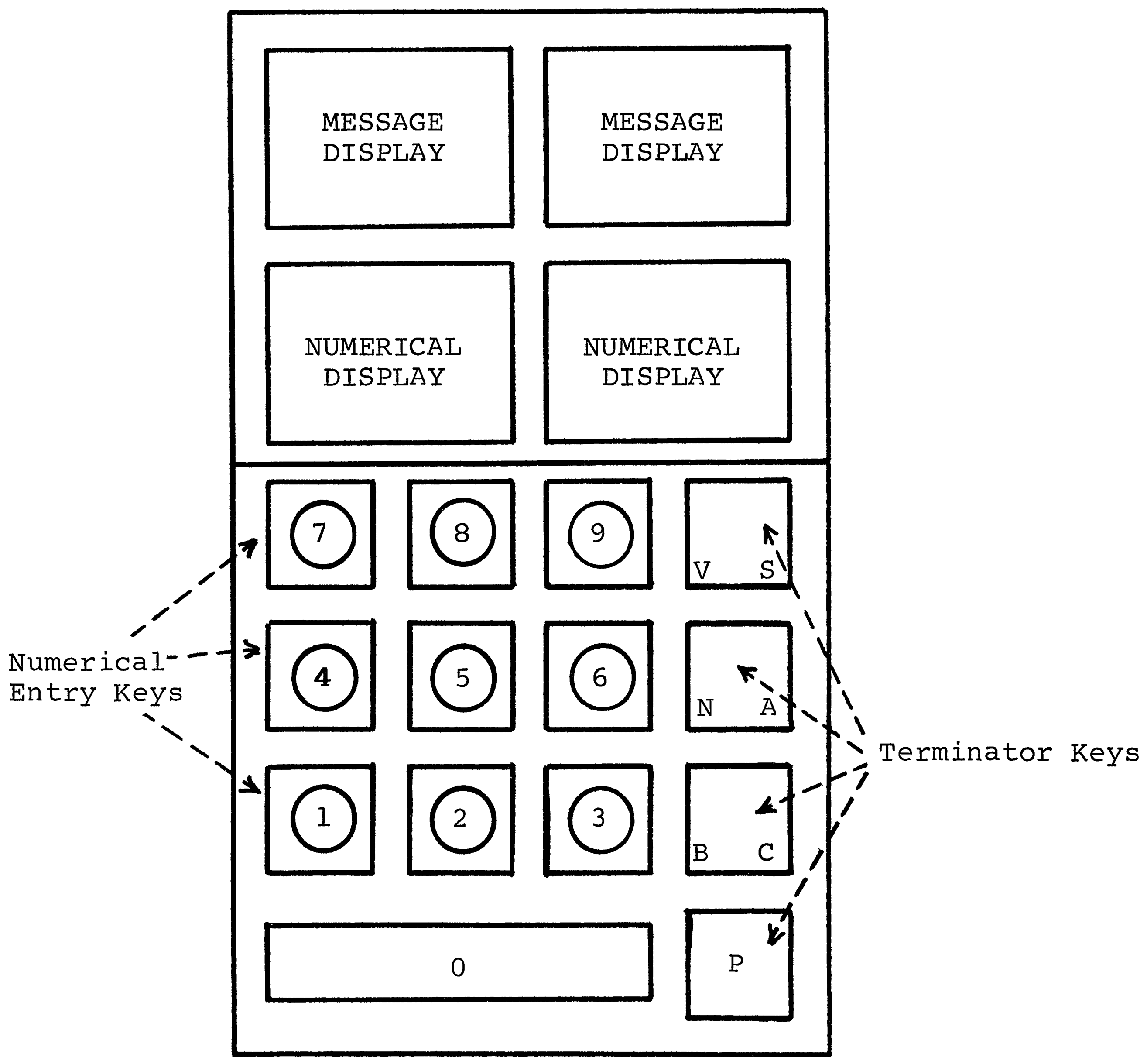


Figure 3B. Drawing of the console used for user/machine communications in the DDP-24 system.

the second track. Requests for vertices and points will continue until all the tracks for a chromosome have been measured, at which time the machine will again request a frame number. Although the system sounds complex, an experienced operator can easily measure 65 chromosomes in a single spread in 30 min, about half the time required to make the measurements by hand.

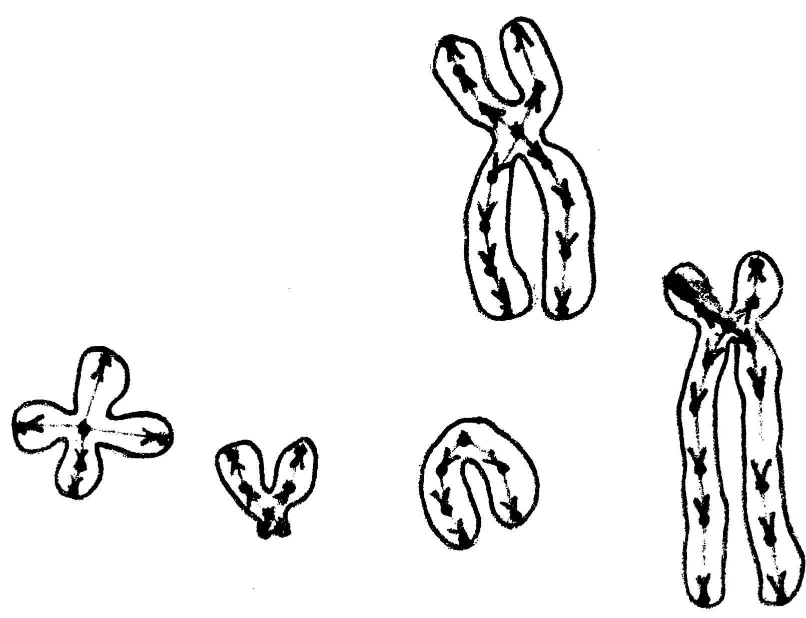


Figure 4B. Sketches of a number of chromosomes showing the locations of points which might be recorded, and the course which would be taken in locating the cross hairs on each of these points.

The projected image of the negative from which all measurements are made is ten times the size of the negative itself. In the image plane the digitizer can discriminate between two points separated by a minimum distance of 3×10^{-3} in. Therefore, if it were possible optically and photographically, the system would be capable of detecting two points separated by a distance (minimum) of 0.02 μ on the original microscope slide. This is much greater accuracy than is actually necessary for chromosome measurement studies.

Processed data from the DDP-24 computer is written on a magnetic tape through a Kennedy incremental tape drive.[7] Since output is written after each track is measured, track records from different measuring machines are completely intermixed on the tape and must be sorted out in further processing. These tapes contain 24-bit words which must be translated for processing on machines using different word sizes. The format for magnetic tape, track records is outlined in Appendix IV.

[7]Kennedy model 140 OR.

Before magnetic tapes are made generally available for further processing, they are copied. The copy tapes are written as binary tapes on a seven-track tape drive at a density of 556 bytes/in. using odd parity.

EXTRACTION AND ANALYSIS OF MEASUREMENT DATA

All further analysis of measurement data is done using the IBM 360/67 computer at The University of Michigan Computing Center. Because this computing facility allows for operation from a remote terminal or in batch mode, many of the programs being used have been written to allow man/machine interaction. Basically, two programs are used. The first, called SEARCH, scans the DDP tapes for chromosome measurement data, checks this data for errors and then rewrites the information on a second magnetic tape. CHROMOSOME, the second program, reads the material produced by SEARCH and provides the desired information on each chromosome spread examined. Both are written in FORTRAN IV (SEARCH has one subroutine in assembly code), and both include a number of features only compatible with The University of Michigan Terminal System ("MTS"), a supervisory system developed at the University.

Being a modification of the program used to sort out measurement data in the Department of Physics, SEARCH (see Figure 5B), although it has been changed slightly in our laboratory, was originally written in that department. It is the goal of this program to scan the DDP tapes with a minimum amount of data processing and extract all chromosome data. Some difficulty arises due to the fact that chromosome data is only a small fraction of the total information on a tape, and, yet, at least part of every record must be translated from the 24-bit words used by the DDP machine to IBM compatible 32-bit words. The program first translates the experiment number found in word two of the logical track record (see Appendix IV and Figure 6B). The entire record is only translated when the experiment number matches that used for all chromosome work. Next the program determines which machine was used to measure the track and transfers control of the program to a specific subroutine which handles data from that machine only. This provides the mechanism used to sort data which is completely intermixed because it was written in real-time into a meaningful form in which data is separated according to which machine it came from.

Each new track record is first checked to determine if it represents data from a new chromosome or if one or more tracks have already been read. If the current track has originated from a new chromosome, all data for the preceding event is written on the output tape. Each event is also checked to make sure it is described as coming from a structure having an N2 or N4 configuration. Thus, the program is capable of detecting a number of errors in the data as it is being sorted. These errors, which are identified by the typing of two lines of information describing the event containing the erroneous information, are listed in Table IIB.

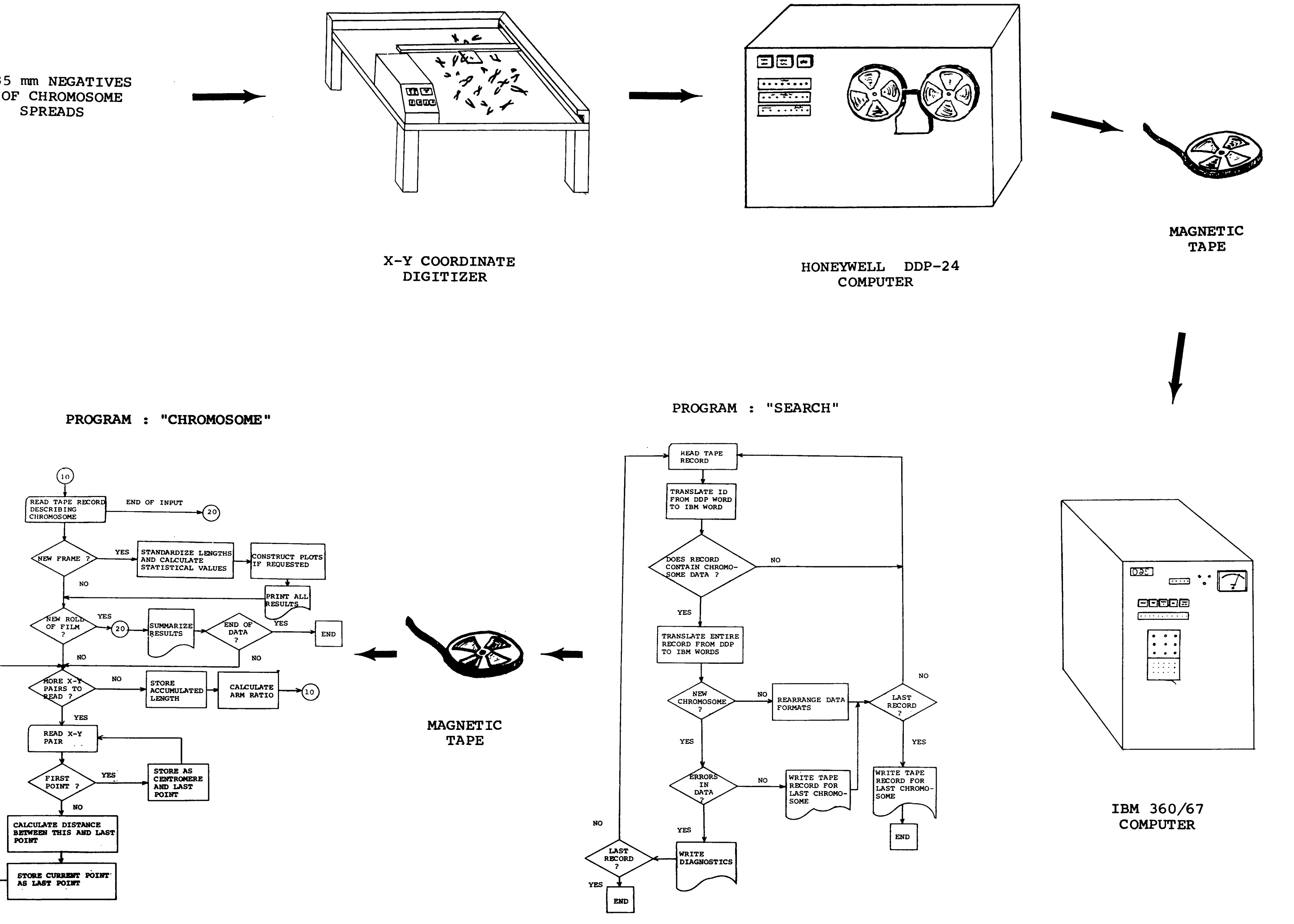


Figure 5B. Basic organization of the chromosome measurement system (from Baumann and Merchant) (11).

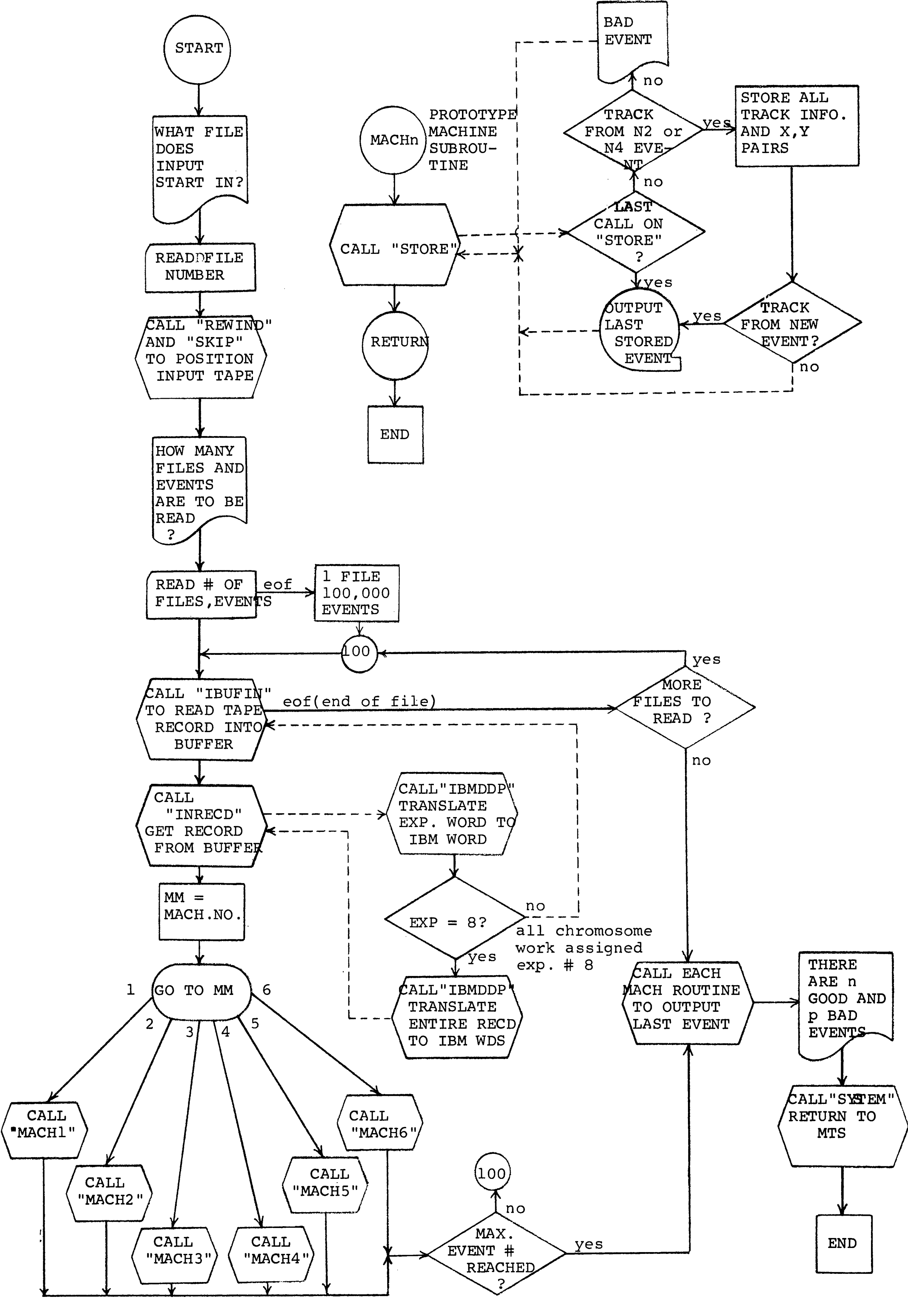


Figure 6B. Flow chart for the program "SEARCH."

TABLE IIB. The types of errors in chromosome measurement data which can be detected by SEARCH.

1. Invalid event type (every chromosome must be described as a neutral induced, two or four prong event).

2. Event does not have two or four tracks.

3. More than 16 points were recorded on a single track.

4. Not all tracks were measured.

Output from SEARCH is placed on a 9-track magnetic tape at a density of 1600 bytes/in. using odd parity. Each chromosome is described separately on the output device by a sequence of records (see Appendix V). The first record contains the roll, frame and event numbers for the chromosome in question as well as the number of coordinate pairs describing the event, the number of tracks, and the date and time of measurement. Each of the following records contains the track number, number of points on the track and a single X-Y coordinate pair.

This program makes reference to four logical input/output device numbers and is designed to be run from a remote terminal. Unit 8 is used to refer to the input magnetic tape (from the DDP system). Writing of the output tape occurs through device number 3 which must be preset to the desired starting point by the user before running the program. Units 5 and 6 are used as input and output devices, respectively, for conversational interaction with the computer. As can be seen in the sample session using SEARCH in Appendix VI, the machine requests information as to which tape file is to be scanned first and then asks how many consecutive files are to be read and how many chromosomes are present (a maximum number). By sending a logical "end of file" the user causes the program to default to scanning one file for a maximum of 100,000 chromosomes.

Application of the program is invoked by the appropriate MTS "run" commands to provide for mounting of the necessary magnetic tapes and initiation of the program itself. The use of these commands is shown in the sample session (Appendix VI). A complete listing of the source coding of this program is given in Appendix VII.

CHROMOSOME

Although it makes use of a number of MTS and IBM library subprograms, CHROMOSOME itself was written entirely in our laboratory. This program reads the magnetic tape produced by SEARCH, calculates the length of each chromosome measured, and then produces a variety of information based on the lengths of all the chromosomes in a measured spread. It may be helpful to refer to Figure 7B, which contains a similified flow chart of CHROMOSOME, while reading this description.

When the program begins execution in the computer, the subroutine CHECK is called. This program sets the input tape to the desired starting location and, if the program is being run in "batch" mode, sets up the required storage areas for constructing plots for printing on the line-printer.

The main program then begins to read input data. As the first record for each chromosome is read, it is checked to see if it represents data from a new roll or frame. Because it is possible for a new frame number to be written on the tape once accidentally, the program checks to see that all chromosomes from the preceding frame have been processed. This is done by spacing the tape ahead to see if the next event contains the same new chromosome number. If it does not, the single chromosome with an incorrect frame number is skipped, but, if it is in reality a new frame, the tape is back spaced to the beginning of the data for the new frame.

When a new frame number or end of file mark are read off the tape, all the data for the preceding frame is printed. This includes those items listed in text Table IIIB (also see Appendix IX). In addition, when an end of file mark or new roll number are encountered it is assumed that all data from an entire population sample has been examined and some summary information is printed. This summary consists of two plots. The first is a plot of arm ratio vs. relative length for each biarm chromosome. In this plot every chromosome is plotted using the same character and there is no way to differentiate data from different cells.[8] The second plot is a frequency distribution of relative chromosome and arm lengths found in all frames measured. To construct this plot each length is placed in a category with other chromosomes having similar lengths. Twenty-six different groups are used as outlined in text Table IVB.

The final plot shows the distribution of chromosome lengths (plotted with an asterisk) and arm lengths (plotted with an X) as the fraction of the total number of chromosomes or arms plotted against the length (see Appendix IX). Both plots are produced using the line printer plot subroutines which are a part of the MTS library.

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[8] A program described in Appendix XI plots this same data using a different character for each frame on the CALCOMP plotting system.

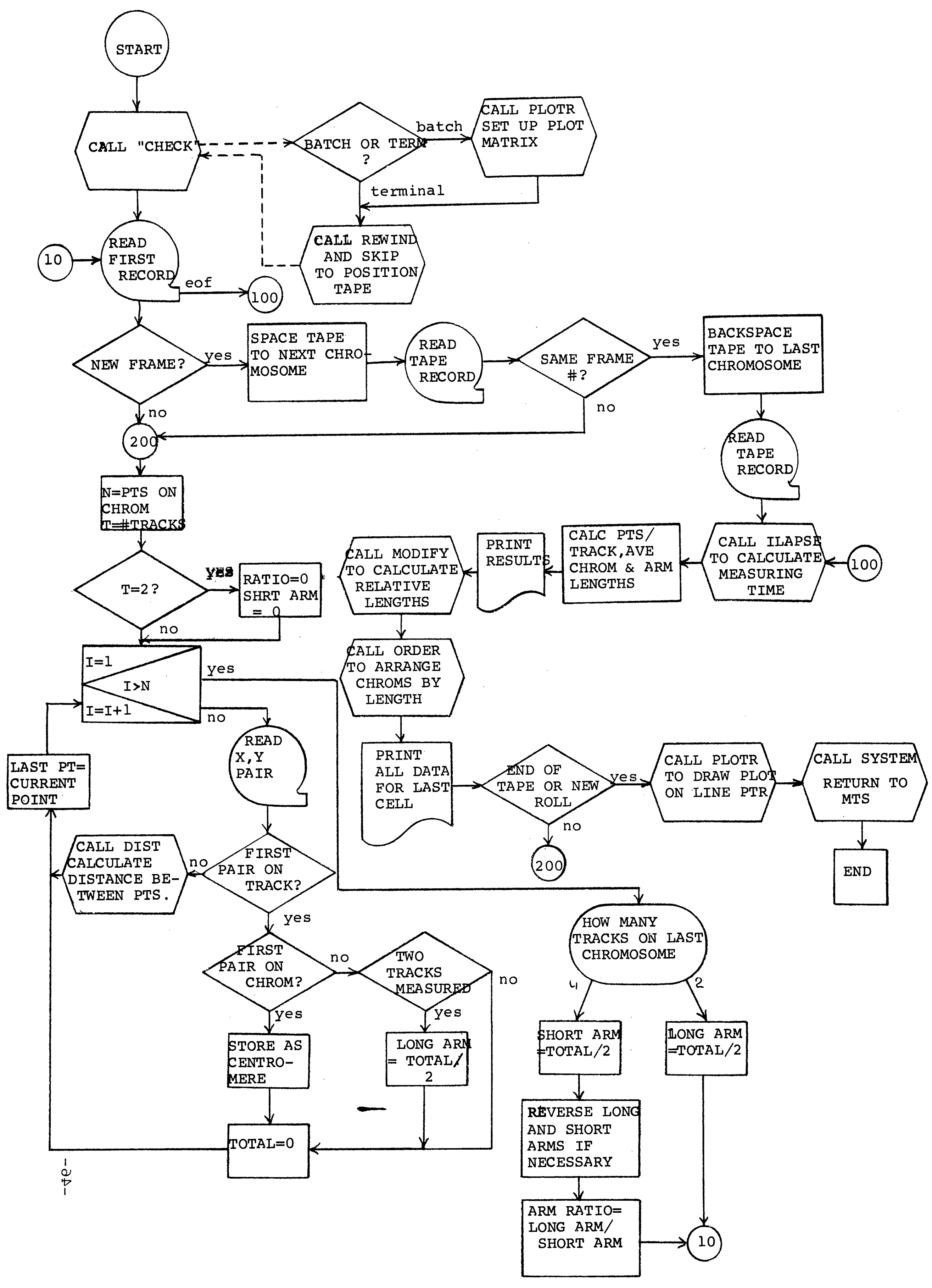

Figure 7B. Flow chart for the program "CHROMOSOME."

TABLE IIIB. A list of the information provided for each spread of chromosomes by the program CHROMOSOME.

1. Roll and frame numbers.
2. Time and date at which measuring of the spread was started and completed.
3. The elapsed measuring time.
4. Total number of chromosomes in spread.
5. Number of telocentric chromosomes present.
6. Number of biarm chromosomes present.
7. Number of tracks measured.
8. Average number of points recorded on each track.
9. Total length of all chromosomes in spread.
10. Average length of a chromosome.
11. Average arm length.
12. Separate lists of data on biarm and telocentric chromosomes (in order of decreasing total length).
    a. Location of the centromere (the first vertex recorded for the chromosome).
    b. Lengths of the long and short arms in relative length units (absolute length * 1000 / average chromosome length).
    c. Arm ratio (long arm length/short arm length).
    d. Total length of each chromosome in relative length units.
13. Considering the biarm chromosomes as one group and the telocentrics as a second, independent group, the following values are calculated for each group.
    a. Mean relative length.
    b. Variance and standard deviation of the mean.

TABLE IVB. Catagories of ranges of lengths used in the construction of arm and chromosome length frequency distribution.

GROUP	RANGE OF LENGTH
1	0 - 94
2	95 - 194
3	195 - 294
4	295 - 394
5	395 - 494
6	495 - 594
7	595 - 694
8	695 - 794
9	795 - 894
10	895 - 994
11	995 - 1094
12	1095 - 1194
13	1195 - 1294
14	1295 - 1394
15	1395 - 1494
16	1495 - 1594
17	1595 - 1694
18	1695 - 1794
19	1795 - 1894
20	1895 - 1994
21	1995 - 2094
22	2095 - 2194
23	2195 - 2294
24	2295 - 2394
25	2395 - 2494
26	2495 - ....

The number of objects falling into each of the classifications shown in Table IVB is also printed so that information on a number of plots can easily be combined manually in the event that a single plot does not contain the data for a complete population sample.

One of the procedures which must be used in preparing data for printing when an entire frame has been processed is the ordering of chromosome data according to length. This is done by the subroutine ORDER using a successor list. A successor list is an array containing the subscripts defining the order in which values should be obtained from other arrays. Once such a list has been constructed the program first looks at the "header," a variable containing the index of the first value to be printed in the final list. This index also locates the location in the successor list array containing the index value to be printed. One simply repeats this operation until all values have been printed. The advantage of this method is that all the data associated with each chromosome (centromere locations, long-arm lengths, short-arm lengths, etc.) can be ordered at the same time by merely modifying the contents of the successor list. It is unnecessary to rearrange all the data for each chromosome.

After all the summary data and lists of data described have been printed, the program procedes to process data for the next spread. All the bookkeeping information required by the machine is found on the first card image for each chromosome (see Appendix V). The event number is stored to be printed in the final print-out for the spread, and the total number of biarm or telocentric chromosomes, whichever is appropriate, is incremented. The number of tracks is used to increment the total number of tracks measured in the frame and to indicate to the machine whether or not an arm ratio is to be calculated. Using the total number of coordinate pairs for the current chromosome, the program initiates a loop to read in the appropriate number of X-Y coordinate pairs and stores this number for use in calculating the average number of points per track. The time and date are only used as they are obtained from the first and last chromosomes in a spread to calculate the elapsed measuring time (via the subroutine ILAPSE).

Within the coordinate pair reading sequence the program reads a card image and, if the data is the first record on a track, stores the length of any preceding tracks, zeros the necessary accumulator, and stores the coordinate as the last point measured. Succeeding points on a track (read from the card images which follow) are first stored as the current X-Y pair, used to calculate the distance between the current point and the last point, and then stored as the last point before the next card image is read. Distances between successive points are accumulated. When the total lengths of two tracks have been calculated in this way, they are averaged and the result is stored as the length of the long arm (in absolute units). If the program then finds points for two more tracks, their averaged length is stored as the short arm length and, after reversing these values (if necessary) an arm ratio is calculated. The first coordinate pair read for

a chromosome is stored as the centromere location.

Distances between points are calculated using the Pythagorean Theorem. To use this method, one simply assumes the two points whose coordinates are (X_1,Y_1) and $X_2,Y_2)$ to be the two vertices of the acute angles of a right triangle. A line drawn between these two points is then the hypotenuse of the triangle. The distance between the points is calculated using Equation (1).

$$C = \sqrt{(X_1-X_2)^2 + (Y_1-Y_2)^2} \tag{1}$$

Where C = the length of a line between the two points and the length of the hypotenuse of the triangle formed by the two points.

A one-line function in the main program is used to calculate C.

As was mentioned above, all chromosome lengths are stored in absolute length units. The calculation of relative lengths does not take place until the program has learned that all data for an entire spread has been read. Relative length calculation occurs in the subroutine MODIFY which places the resulting values in the storage locations originally containing the absolute lengths. Equation (2) describes the calculation used.

$$R = (L * 1000)/A \tag{2}$$

Where R = the calculated relative length.

L = the absolute length of a chromosome arm.

A = the average length of a chromosome in the current spread.

Multiplication of the absolute length by 1000 allows the computer to perform most arithemtic operations in integer mode without losing significant figures.

Any errors discovered while running the program are handled by the subroutine IFAIL which terminates execution of the program after printing the comment;

"ERROR...RETURN CODE = nnn"

where "nnn" is a numerical return code describing the error which occurred (see Appendix X).

Of course, other errors causing termination of program execution may occur. These are errors detected by the supervisory system or by the program interupt code contained in the program status word which is printed in the event of an abnormal program termination.

"CHROMOSOME" makes reference to four logical input/output device numbers. Device 5 is assigned to the magnetic tape containing chromosome data. The location at which the program is to begin reading data off the tape is set by the program after the user is interrogated via unit 6 and replies with the starting file and record number on device 3. In addition to questions to the user, all program output which requires little time to print is assigned to unit 6. Long lists of output information are assigned to logical device 7. This type of device assignment provides for the program to be easily run in either batch or terminal mode. When operating through a terminal, the program omits the printing of plots and, if unit 7 is assigned to a dummy device,[9] the printing of long output is also omitted. Terminal operation allows the user to check the operation of the program and to examine the contents of his magnetic tapes.

In batch mode it is generally sufficient to allow 3 pages of printed output and 8 sec of central processing unit time for each frame processed under the MTS system. The cost of running the program is about $.62 per frame.

USE OF COMPUTER OUTPUT

As can be seen in the preceding discussion, the system which has been developed provides a substantial quantity of information on the chromosome complement of each cell examined. However, as was mentioned, our interest is not specifically in the characteristics of single cells but in the inference which might be drawn to the makeup of the population as a whole. In examining the results of a number of population analyses made with this system some experience has been gained in determining which is the most useful data for comparing populations. This is of great significance for the reason that a variety of problems can be reduced to population comparisons. Following a population over a period of time, observing the transformation of diploid cells, comparing two apparently unrelated populations of cells, comparing a cell population to normal cells from the tissue or origin, and searching for abnormal populations of cells are all examples of comparisons between populations. The fact that chromosome measurement provides quantitative data is also helpful because such information lends itself to statistical interpretation.

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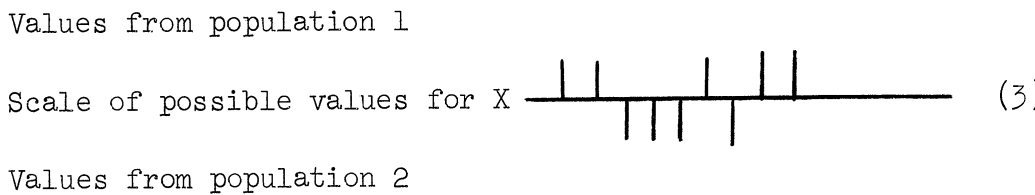
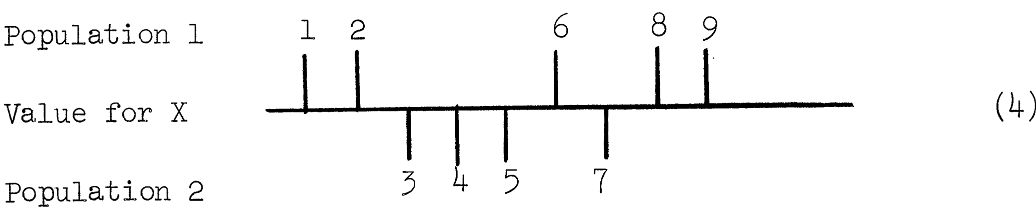
[9] In the MTS system *DUMMY* is used as such a device. Any material written on such a device is simply lost.

To date, the test which we have found most helpful for comparing characteristics is the Wilcoxon two-sample test. This is a ranking test used to determine if the values obtained for some variable in one population are lower than the values from the other population with sufficient consistency for one to say the differences between the values are probably not due to chance alone. An arbitrary value for α, the probability that the results are due only to chance, of 0.01 is currently being used as the maximum acceptable value for saying the two populations are different on the basis of the single characteristic considered in the calculation of α. This in itself is not, however, taken to mean the populations are truly different. The number of variables providing values less than 0.01 is also considered.

To apply the Wilcoxon two-sample test to the results obtained for some variable in the computer output, the values from both populations are first ranked in ascending order (Equation 3).



Then a rank is assigned to each value along the linear scale.



Next, a test statistic, W_1, is calculated as the sum of the ranks obtained from population 1.

$$W_1 = 1 + 2 + 6 + 8 + 9 = 27 \qquad (5)$$

Under the null hypothesis, H_0, all the possible combinations of rank orderings have equal probabilities of occurring, and the test statistic, W_1, is distributed according to the normal distribution with expectation $E(W_1)$ and a variance, $Var(W_1)$, calculated according to Equations (6) and (7).

$$E(W_1) = \frac{s(N + 1)}{2} \quad (6)$$

$$Var(W_1) = \frac{s(N - s)\ (N + 1)}{12} \quad (7)$$

where s = the number of values for the variable obtained from population 1.

N = the total number of values for the variable obtained from both populations.

High or low values for W_1 are considered as evidence against the null hypothesis.

The value for α is calculated using the values for W_1, $E(W_1)$, $Var(W_1)$, and the normal distribution. First z is calculated.

$$z = \frac{W_1 - E(W_1)}{\sqrt{Var(W_1)}} \quad (8)$$

Then the area outside the region between -z and +z on the normal distribution is obtained using the appropriate tables. In this way the Wilcoxon two-sample (9) is used as a two-tailed test to give the probability that one might, due to only chance, obtain any value for W_1 as extreme as or more extreme than the observed value.

The variables which have been compared thus far using this test are listed in Table VB.

It was hoped that, just as a karyotype provides a good description of a diploid cell, a list of quantitative characteristics could be provided by this analysis system which would be descriptive of the population examined. A first attempt at this consists of simply listing the mean and standard deviation of the values for each variable obtained from the entire population sample examined. Of course, these values are not as easily visualized as a karyotype.

Attempts have also been made to visually display the results obtained to aid in the interpretation of results. This is being done through the use of the two plots already mentioned, arm ratio vs. length and chromosome length frequency distributions. Execpt in the study of diploid cells, the plots of arm ratio vs. length are only of questionable value. An example of such a

Table VB. Variables being compared using the Wilcoxon two-sample test.

1. Mean length for all biarm chromosomes in each spread examined.

2. Standard deviation of (1).

3. Mean length for all telocentric chromosomes in each spread examined.

4. Standard deviation of (3).

5. (1) - (2).

6. Standard deviation of the mean length of all chromosomes in each spread.

7. Total number of arms in each spread examined.

8. Total number of chromosomes in each spread examined.

9. Values obtained by dividing the average chromosome length by the average arm length in each spread examined.

plot produced for a number of frames of data from BHK cells (BHK 21 Clone 4) on the CALCOMP plotter is shown in Figure 8B.[10]

On the other hand, chromosome length frequency distribution plots appear to be quite useful. These plots are prepared by manually combining all the plots produced by the computer for arm length and chromosome length vs. frequency constructed for a single population sample. To combine them, the numbers of items falling into each of the classifications in Table IVB are added and the totals in each group are divided by the total number of items (chromosomes or arms) being considered. A smooth curve is drawn through the resulting points.

Although it is apparent in the examples in Figure 9B that visual comparison of these plots is not difficult, the method is not very quantitative. To correct this, plans are being drawn up to write a supporting program which

[10] Supporting programs including the one used to construct these CALCOMP plots are listed in Appendix XI.

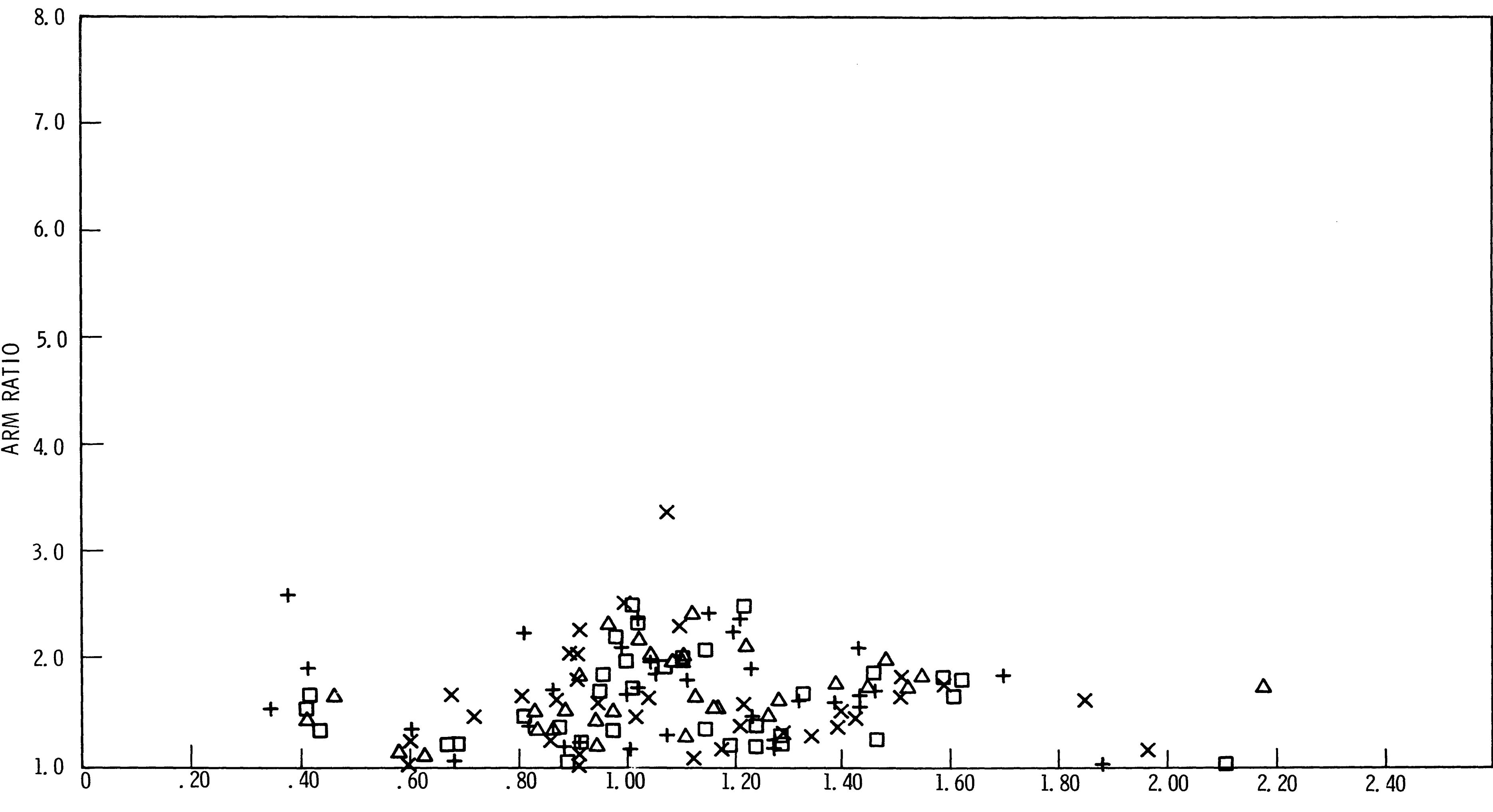
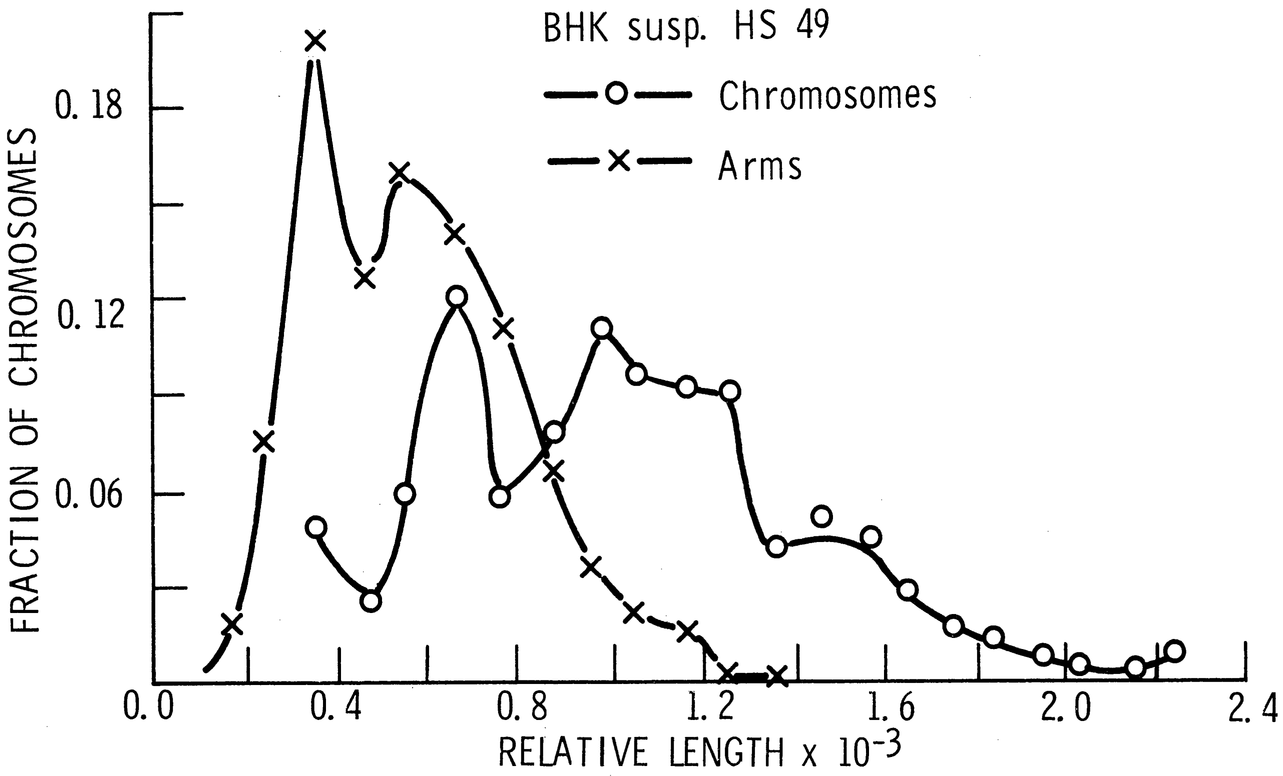


Figure 8B. CALCOMP plot of chromosome length vs. arm ratio for four BHK cells.



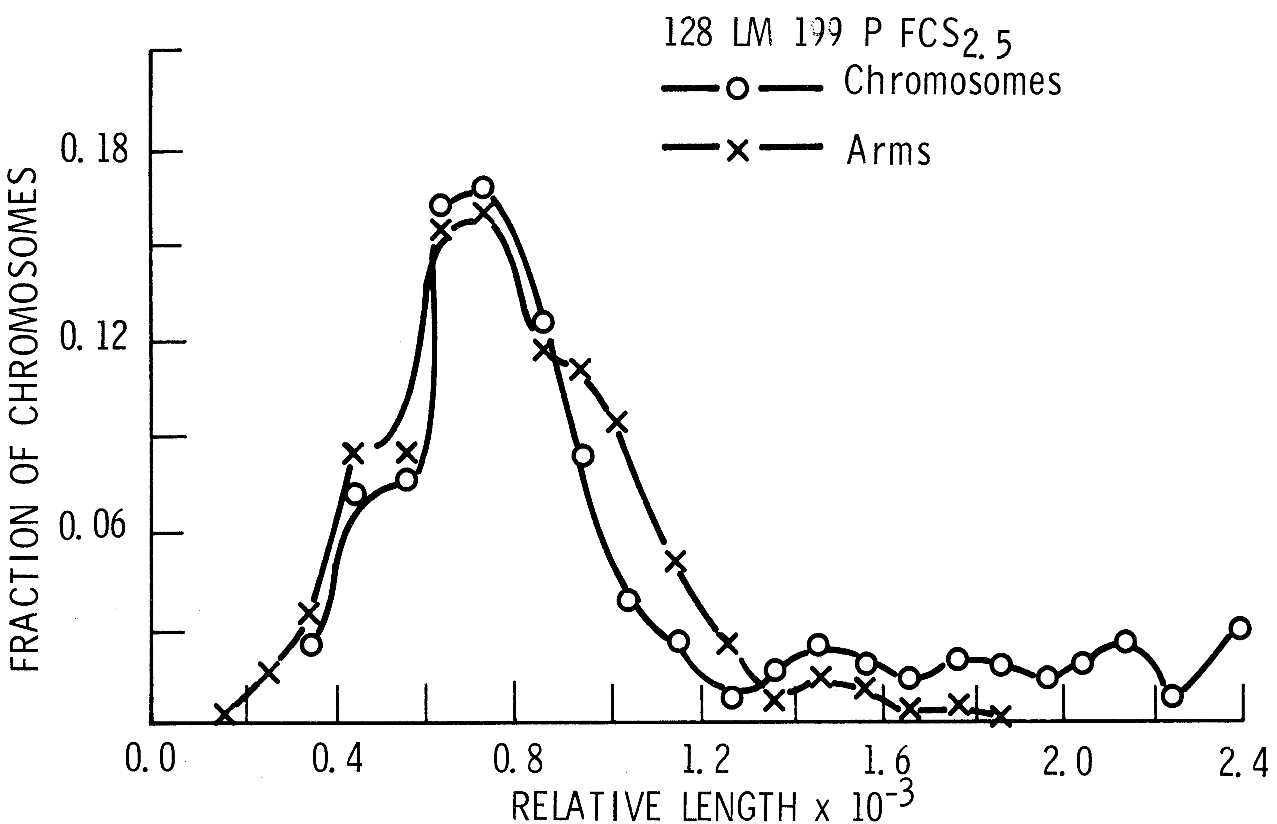


Figure 9B. Sample frequency distribution plots for chromosome and arm length in two cell lines.

will fit a line to the points using the lowest power polynomial regression resulting in a line varying no more than ± 10% from each point. The calculated formula will then be used to draw this line on a CALCOMP plot.

RESULTS

ACCURACY OF CHROMOSOME MEASUREMENT

Previous analysis of data obtained by this system (11) has shown that the results obtained are an improvement over manual measurement techniques with respect to reproducibility and the magnitude of sample variance obtained. It is felt that the errors observed are of a small enough magnitude that useful results can be obtained from chromosome measurement studies using this system.

To check the accuracy of the manual process of translating photographic images into digitized information, a FORTRAN IV program has been used to draw karyotypes using the X-Y coordinate data on the CALCOMP plotting system. An example is shown in Figure 10B. By comparing this drawing to the original photograph it is possible to determine, visually, the accuracy of the digital data. This has been done only periodically to check the operation of the system.

CHARACTERIZATION OF CELL POPULATIONS

To date, our attempts at quantitative characterization of the chromosome complements of a population sample consist simply of providing the plots which have been described and listing the means and sample standard deviations of the values being used in population comparison studies. Such a listing is shown in text Table VIB. More experience must be obtained before it will be possible to ascertain the value of having such a description.

COMPARISON OF POPULATIONS

Use of the Wilcoxon two-sample test as a two-tailed test for similarity in values from the populations used has proved very useful. Of course, more experience will be necessary before it will be possible to determine which of the nine items being compared are the most useful, but the advantages of making comparisons in multiple categories are already apparent in that they allow the introduction of a third dimension into the process. That is, the difference between populations can be described on the basis of how many categories show differences of significance as well. When more studies have been carried out it will be possible to dispose of categories which are not useful and to attach weights to the remaining ones so that a final single value can be provided on which the acceptance or rejection of the null hypothesis can be based.

TABLE VIB. Means and standard deviations for the values obtained for each characteristic examined in the cell lines listed.

CHARAC-TERISTIC	CELL LINE				
	BHK HS-49	128 L-M*	90 L-Ma*	246 L-M*	HUMAN MALE
# Chromo-somes	43.0±1.0	63.4±1.0	60.4±1.9	63.5±2.1	46.0±0.0
# Arms	76.4±1.9	76.0±1.6	74.2±1.7	76.6±2.5	87.1±3.8
$\bar{X}_{biarms}$	1117±34	1884±166	1654±65	1728±80	1056±44
σ_{biarms}	309±66	504±94	250±42	252±45	412±23
$\bar{X}_{telos}$	604±51	779±44	806±27	811±24	433±199
σ_{telos}	112±49	404±593	144±12	157±20	106±82
$\bar{X}_b - \bar{X}_t$	513±81	1115±215	851±98	927±98	619±166
$\sigma_{whole\ cell}$	369±39	526±78	381±59	429±52	
$\frac{\bar{X}_{chrom}}{\bar{X}_{arm}}$	1.78±04	1.20±0.1	1.21±.06	1.21±.06	1.91±.08

*All L-M cells are grown in medium 199 supplemented with peptone. The medium of the 128 L-M cells also contained 2.5% fetal calf serum.

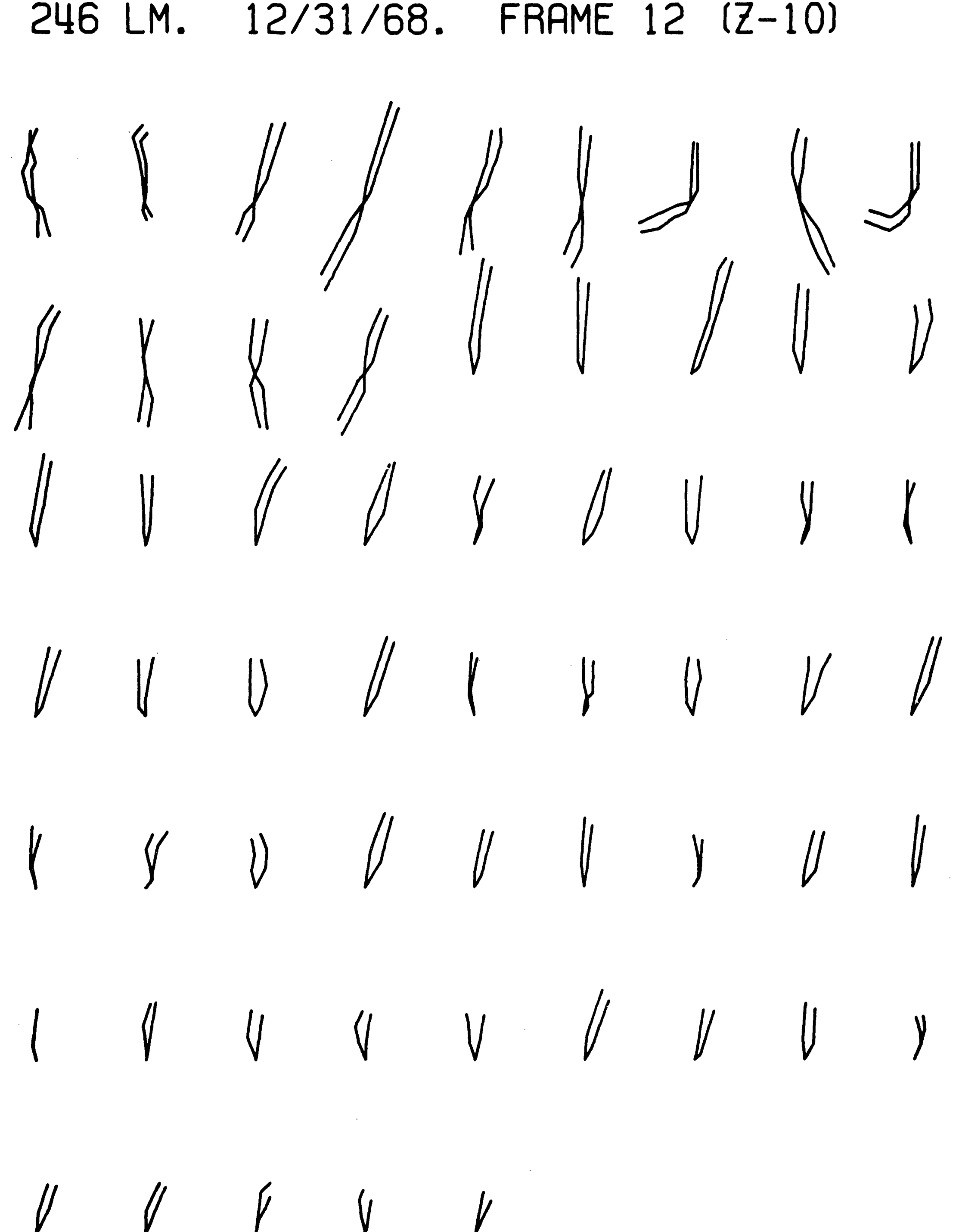


Figure 10B. Karyotype drawn by the CALCOMP plotter to check the accuracy of the measurement system (from Baumann and Merchant) (11).

Sample comparisons between a variety of populations are shown in Table VIIB.

MEASUREMENT OF HUMAN CELLS

Initial studies on the measurement of human chromosomes have shown that within a single spread all chromosomes can be paired and grouped according to the Denver classification on the basis of measurements and within a population sample of normal cells criteria can be set up for placing each chromosome in its correct group. These grouping decisions are made on the basis of relative length- and arm-ratio calculations.

It is also believed that the numerical characteristics currently being examined for cell populations may be advantageous in studies of human cells for the purpose of locating abnormalities without detailed karyotypic studies. Of course, to develop such a technique it would be necessary to use some completely automated system for collecting chromosome data. The difference between this technique and others developed recently is that it depends on numerical features of the chromosome complement other than those normally observed in systems dependent on karyotype construction.

An arm ratio-length plot showing how human chromosomes from a single spread fall into groups and pairs is shown in Figure 11B.

CONCLUSIONS

A system which has been developed for the description and comparison of the chromosome complements of cell populations has been described in detail. A semiautomatic, X-Y coordinate digitizer system is used to provide taped descriptions of chromosome spreads for analysis on the IBM 360/67 computer system at The University of Michigan. This system provides a number of numerical values which may be used as a mechanism for comparing or simply describing populations on the basis of results from a small sample of cells.

Results obtained to date have demonstrated the potential of the system, but some additional experience will be necessary to completely define the procedure for analyzing results.

TABLE VIIB. Values obtained for α in the comparison of a number of populations using the Wilcoxon two-sample test.

Cells Compared	Basis for Comparison								
	# Chroms	# arms	$\overline{X}_{biarms}$	σ_{biarms}	$\overline{X}_{telos}$	σ_{telos}	$\overline{X}_b - \overline{X}_t$	$\sigma_{whole\ cell}$	$\frac{avg.\ chrom}{avg.\ arm}$
BHK HS-49 vs. 128 LM	0.00014	0.32709*	0.00014	0.00147	0.00014	0.00014	0.00032	0.00245	0.07186
128 LM vs. 90 LMa	0.00022	0.04338*	0.00067	0.00010	0.04338	0.00010	0.00067	0.00047	0.10960
90 LMa vs. 246 LM	0.00181	0.01928*	0.00905	0.00511	0.09894	0.43539	0.14429	0.21498	0.00010
128 LM** vs. 246 LM	0.32709*	0.70394*	0.00511	0.03573	0.02321	0.00010	0.00633	0.00032	0.22628
128 LM**	0.32709*	0.70394*	0.00511	0.03573	0.02321	0.00010	0.00633	0.00032	0.22628
Human vs. BHK	0.00047	0.00047	0.00047	0.00047	0.00614	0.53526	0.25014		0.00147
Human vs. 128 LM	0.00047	0.00047	0.00047	0.01685	0.00047	0.00047	0.01314		0.00047

Complete culture designations: 128 L-M 199 P fetal calf serum 2.5%
246 L-M 199 P
90 L-Ma T_1C_3 Clone 2 199 P
BHK 21 Clone 4 suspension HS 49

*These values support other results because they indicate samples taken were not different with respect to chromosome and arm number to start with.

**These populations cannot be distinguished on the basis of enumeration alone.

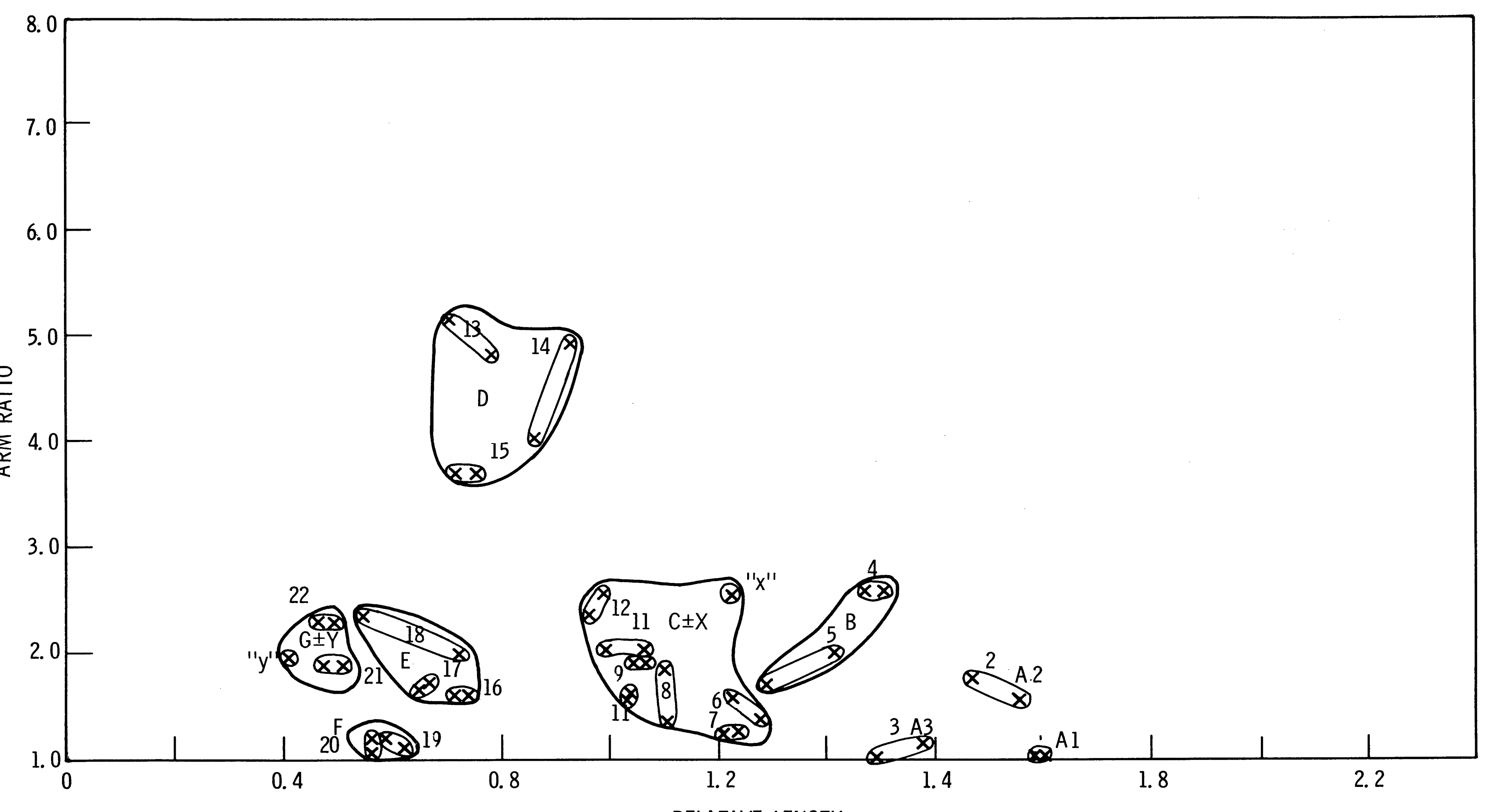


Figure 11B. Plot of arm ratio for a single human cell (male) showing how the chromosomes fall into pairs and groups.

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APPENDIX I

CHROMOSOME PROCEDURE

1. Add 0.1 ml of Colcemid solution (Grand Island Biological Co., Catalog number 521) (25 µg/ml) for every 5 ml of growth medium to a culture in middle log phase and incubate for 3-13 hours (depending on the doubling time of the cell being used).

2. Harvest cells, centrifuge at 500-800 RPM for 5 min. and discard medium. Resuspend in 3 mls of 1% sodium citrate in distilled water warmed to 37° C (prepare fresh each time). Let stand 6-8 min. at 37° C, then add one drop of fixative (1:8, acetic acid:methanol, prepared fresh each time) and centrifuge for 5 min. (at 500-800 RPM). Discard supernatent.

3. Resuspend cells in 0.5 ml fixative (for every 5 mls of original culture fluid) by adding fixative very slowly (a drop at a time). Allow to stand at room temperature for 10 min. Centrifuge, change fixative two more times allowing the suspension to stand 10 min after each change.

4. Dip a clean slide in 70% methanol, immediately drop one or two drops of cell suspension on the slide. Ignite slide by passing it through a Bunsen burner flame. Let stand 15 min. to complete drying.

5. Permanent slides are prepared as follows:
   a. Stain 40 min in dilute Giemsa solution (diluted 1:15 in distilled water before use).
   b. Rinse rapidly in distilled water (10 sec).
   c. Rinse in two changes of acetone.
   d. Rinse 3 times in acetone:xylol (2:1), 3 times in acetone: xylol (1:2), and 10 minutes in fresh xylol.
   e. Mount using a xylene soluble mounting medium.

PROBLEMS:

1. Clumping may be reduced by adding fixative more slowly or increasing the concentration of acetic acid in the fixative.
2. Cell breakage may be reduced by decreasing the concentration of acetic acid in the fixative.
3. The number of mitotic figures present may be increased by increasing the incubation time with Colcemid , but the problem may be that cells are not mitotically active.

APPENDIX II

FILM PROCESSING TECHNIQUE

All photographs are taken on Eastman Kodak high contrast copy film (HC-135) and developed as follows:

1. Develop film for the time specified by the manufacturer for the room temperature where the prepared developer has been stored. Agitate film for 10 seconds out of each minute. Eastman Kodak developer, D-19, is used.
2. Replace the developer with Eastman Kodak stop bath, SB-5a, for 30 seconds. The stop bath solution is made up in our laboratory according to the following formula[10];

Water	500 ml
28 % Acetic Acid	640 ml
Sodium sulfate (desicated)	45.0 gr
Water to make	1000 ml

3. Pour off stop bath and fix the film for 5 min in Kodak fixer (general purpose, hardening fixer).
4. Wash films for 30 min. in running water.
5. Rinse films in a solution of Kodak photo-flo.
6. Hang films to dry.

APPENDIX III

SAMPLE SESSION ON THE DDP-24

Each line in the session which follows describes the users response to the display which appears on the communications console. All of the responses described are carried out on that console.

STEP	MESSAGE DISPLAYED	KEYBOARD ENTRY	TERMINATOR	EXPLANATION
1	ENTER EXP	1	C	Returns machine to signon sequence.
2	ENTER ID	66	P	Enter user ID no.
3	ENTER EXP		A	Enter alter sequence to change program parameters so film view being used doesn't change during measurement.
4	any message	9	A	
5	any message		P	
6	ENTER EXP	8	P	Enter experiment no. assigned for chromosome measurement.
7	ENTER ROLL	40	P	Enter 4 digit (max) roll number.
8	ENTER FRAME	2	P	Enter 4 digit (max) frame number.
9	ENTER EVENT	1	P	Enter event number.
10	ENTER TYPE 1	2 or 4	N	Enter event type (2N or 4N only).
11	ENTER TYPE 2		P	Leave event type loop.
12	MEASURE VERTEX 1	none, just locate cross hairs	foot peddle	Record centromere for first track.
13	MEASURE TRACK 1 1	"	"	Locate points along track.
14	MEASURE TRACK 1 2	"	"	
⋮	⋮			
15	MEASURE TRACK 1 n		P	Signal that end of track has been reached.
16	Program automatically returns to (12) until all vertices and tracks have been measured.			
17	Program automatically returns to (8)			

PROBLEMS

1. Frame number entered in step 8 too low causing the red error indicator to light.

   a. If the entered frame number was correct (this is checked by entering the number again and pressing the "P" terminator - red light should remain on), the number as frame number for the preceding chromosome was incorrect. Enter 1-C and start over at step 1. Only the preceding chromosome must be remeasured (the one having an incorrect frame number associated with it).

   b. If the entered frame number was incorrect, enter the correct frame number followed by the "P" terminator.

2. Red light goes on after event number is entered in step 9.

   The event number entered was not greater than the one entered for the preceding event in the current frame. Enter a higher number followed by the "P" terminator.

3. Incorrect event type entered.

   Continue through the sequence until step 12 (or any other step in which "MEASURE" is displayed) is reached. Enter 6-C. This will return the program to step 10.

4. Error made in locating cross hairs, ie. a point was recorded incorrectly.

   Enter 1-C to return to the first point of the current track <u>or</u> enter 0-C to step the measurement sequence back one point at a time <u>or</u> enter 6-C to begin the event over again (this returns the program to step 10).

5. Measurement of a film completed.

   Enter 3-C to return the program to step 7 ("ENTER ROLL").

6. User desires to check previously entered information.

   a. Depress the "A" terminator.

   b. Depress the "C" terminator repeatedly to display all the values entered for the current event.

   c. Depress the "P" terminator to return to the normal program sequence

7. Measurements completed for the current session.

   a. Enter 1-C to return the program to step 1.

   b. Enter 1-P. This has the effect of signing off the current user.

APPENDIX IV

DDP MAGNETIC TAPE TRACK RECORD ORGANIZATION

The contents of each word produced by the DDP computer in writing track records are described by the following list (8).

WORD NO.	CONTENTS
1	Number of words in record
2	Experiment number
3	Roll number.
4	Frame number
5	Event number
6	Track number * 1000 + maximum track number
7	Not important
8	Scanner (user) ID * 1000 + measuring machine number
9	Date (MMDDYY)
10	Time (HHMMSS)
11	1
12	Event type word
13	Not important
14	Not important
15	Number of coordinate pairs in view
16	X coordinate of point 1
17	Y coordinate of point 1
18	X coordinate of point 2
⋮	⋮

APPENDIX V

CHROMOSOME DATA TAPE RECORDS

Each chromosome is described independently on magnetic tape by a series of tape records.

Record 1 (in format 7 I 10)

FIELD	CONTENTS
1	Roll number
2	Frame number
3	Event number
4	Total number of coordinate pairs in event (N = number of coordinate type records to follow, always greater than 2)
5	Number of tracks (2 or 4)
6	Date (MMDDYY)
7	Time (HHMMSS)

Records 2 - N+1 (in format 3 I 10)

FIELD	CONTENTS
1	Track number * 1000 + number of points on track
2	X coordinate
3	Y coordinate

While being simple to use, this type of organization is highly wasteful of magnetic tape storage space and reading time. A new, more flexible system is being worked out.

APPENDIX VI

SAMPLE SESSION USING "SEARCH"

The following is a complete teletype session showing how the program "SEARCH" is run. All lines entered by the user on the teletype keyboard are underlined, and control characters are indicated as small letters.

```
MTS (LA47-0250)
WHO ARE YOU?
UM MICBIOAAAs

#NEXT EXPECTED SYSTEM SHUT DOWN IS 5 30 PM
#$SIGNON SF40 PW=        s
#**LAST SIGNON WAS: 16:09.54   06-18-69
#  USER "SF40" SIGNED ON AT 13:26.08 ON 06-18-69
#$RUN *MOUNTs
#EXECUTION BEGINS                              *MOUNT is used to mount
 ENTER MOUNT PARAMETERS                        magnetic tapes on the
                                               tape drives at the
                                               Computing Center.
 P026 7TP,*TAPE*,MODE=50F,RING=OUT,SIZE=8000,'395L's
 DONE
 ENTER MOUNT PARAMETERS
 G113 9TP,*TAPE2*,MODE=1600,RING=IN,SIZE=80,'SF40-1's
 DONE
 ENTER MOUNT PARAMETERS
                                               Both the input and output
                                               tapes have now been mounted.

 cs
END OF FILE
#EXECUTION TERMINATED
#$COPY FROM *SOURCE* TO *TAPE2*@CCs
>REWs                                          This sequence of steps
>FSFs                                          is used to space the output
>FSFs                                          tape to the desired
>$ENDFILEs                                     starting point.
#$RUN 1SEARCH 3=*TAPE2* 8=*TAPE*s
#EXECUTION BEGINS

  UNITS 3 AND 8 MUST BE MAGNETIC TAPE
  ENTER NUMBERS IN FLOATING POINT ONLY

  INPUT STARTS IN WHAT FILE NO. (UNIT 8)??
 2.0s
```

```
 OUTPUT TAPE (UNIT 3) READY?  IF NOT, TERMINATE!
 HOW MANY FILES SHOULD BE READ?? FOR DEFAULT SEND EOF
1.0s
 HOW MANY EVENTS??
415.s

 DO  1 FILES. STOP AT EVENT      415



                              BEGINNING FILE  1

 ROLL  31,FRAME   8,EVENT 5
     ** TRACKS,FAIL CODE = 60
 ROLL  31,FRAME   8,EVENT 9
      6 TRACKS,FAIL CODE = 60
 ROLL  31,FRAME   9,EVENT 14
      6 TRACKS,FAIL CODE = 60
 ROLL  31,FRAME  12,EVENT35
      5 TRACKS,FAIL CODE = 31
 ROLL  31,FRAME  13,EVENT  6
      6 TRACKS,FAIL CODE = 60
 ROLL  31,FRAME  13,EVENT10
      6 TRACKS,FAIL CODE = 60
 ROLL  31,FRAME  13,EVENT19
      2 TRACKS,FAIL CODE = 10
 ROLL  31,FRAME  13,EVENT57
      6 TRACKS,FAIL CODE = 60
 ROLL  31,FRAME  14,EVENT 6
      6 TRACKS,FAIL CODE = 60
 ROLL  31,FRAME  14,EVENT12
      3 TRACKS,FAIL CODE = 20

 THERE ARE 271 GOOD N2 EVENTS AND 134 GOOD N4 EVENTS.

 THERE ARE  10 BAD EVENTS.
#EXECUTION TERMINATED
#$SIGNOFFs
#OFF AT 13:38.42
#ELAPSED TIME            753.676  SEC.
#CPU TIME USED            85.72   SEC.
#STORAGE USED           7266.43   PAGE-SEC.
#DRUM READS               22
#APPROX. COST OF THIS RUN         $7.26
#FILE STORAGE            589 PG-HR.    $.15
```

APPENDIX VII

SOURCE PROGRAM LISTING FOR "SEARCH"

```
      COMMON/COUNT/IEVCT,MNN,MXN,NBAD,NGOOD(2),NTAPE(2)
      DIMENSION I(2500),IA(200),MSW(7)
C     UNIT 5 IS FOR INPUT OF DATA PARAMETERS
      DO 7 K=1,7
7     MSW(K)=1
      MNN=1
      NTAPE(1)=3
      NTAPE(2)=3
900   FORMAT('0UNITS 3 AND 8 MUST BE MAGNETIC TAPE'/' ENTER NUMBERS IN F
     2LOATING POINT ONLY'/'0INPUT STARTS IN WHAT FILE NO. (UNIT 8)??')
5     WRITE (6,900)
901   FORMAT (F10.1)
      READ(5,901,END=99,ERR=99)G1
      NG1=G1-1
      REWIND 8
      IF(NG1)10,20,30
902   FORMAT ('0...NO NEGATIVE NUMBERS OR ZEROS...')
10    WRITE(6,902)
      GO TO 5
30    CALL SKIP(NG1,0,8)
903   FORMAT(' OUTPUT TAPE (UNIT 3) READY? IF NOT, TERMINATE ')
20    WRITE (6,903)
904   FORMAT(' HOW MANY FILES SHOULD BE READ?? FOR DEFAULT SEND EOF')
80    WRITE(6,904)
      READ(5,901,END=90,ERR=99) GR1
      IFCT=GR1
      IF(IFCT)70,70,81
70    WRITE(6,902)
      GO TO 80
905   FORMAT(' HOW MANY EVENTS??')
81    WRITE (6,905)
      READ (5,901,END=90,ERR=99)GR1
      MXN=GR1
      IF(MXN)85,85,98
85    WRITE(6,902)
      GO TO 81
90    IFCT=1
      MXN=1000000
      GO TO 98
906   FORMAT('0INPUT ERROR')
99    WRITE(6,906)
      CALL SYSTEM
98    NGOOD(1)=0
      NGOOD(2)=0
      NBAD=0
907   FORMAT('0DO ',I2,' FILES. STOP AT EVENT ',I7)
      WRITE (6,907)IFCT,MXN
      IEVCT=0
      DO 1000 IFC=1,IFCT
      WRITE(6,100)
100   FORMAT(1H1)
      WRITE(6,110) IFC
```

```
  110   FORMAT(1H ,30X,14HBEGINNING FILE,I3)
        NW=0
        L=IBUFIN(2500,0)
  120   IF(NW) 140,130,150
 C          READ PHYSICAL TAPE RECORD
  130   NW=IBUFIN(I,1)
        IF(NW) 140,1000,140
 C        TRANSFER LOGICAL TRACK RECORD FROM BUFFER
  140   NW=INRECD(I,IA)
        GO TO 120
  150   MM=IA(8)-(IA(8)/10)*10
        IF(LIMIT(MM,1,7)) 160,140,160
  160   ISW=MSW(MM)
        GO TO (200,210,220,230,240,250,260),MM
  200   CALL MACH1(IA,ISW)
        GO TO 300
  210   CALL MACH2(IA,ISW)
        GO TO 300
  220   CALL MACH3(IA,ISW)
        GO TO 300
  230   CALL MACH4(IA,ISW)
        GO TO 300
  240   CALL MACH5(IA,ISW)
        GO TO 300
  250   CALL MACH6(IA,ISW)
        GO TO 300
  260   CALL MACH7(IA,ISW)
  300   MSW(MM)=2
        IF(IEVCT-MXN) 140,1500,1500
  1000  CONTINUE
 C        NOW TERMINATE. MUST CALL EACH MACHINE ROUTINE TO OUTPUT
 C                LAST STORED EVENT.
  1500  ISW=3
        DO 2000 MM=1,7
        IF(MSW(MM)-2) 2000,1600,2000
  1600  GO TO (1610,1620,1630,1640,1650,1660,1670),MM
  1610  CALL MACH1(IA,ISW)
        GO TO 2000
  1620  CALL MACH2(IA,ISW)
        GO TO 2000
  1630  CALL MACH3(IA,ISW)
        GO TO 2000
  1640  CALL MACH4(IA,ISW)
        GO TO 2000
  1650  CALL MACH5(IA,ISW)
        GO TO 2000
  1660  CALL MACH6(IA,ISW)
        GO TO 2000
  1670  CALL MACH7(IA,ISW)
  2000  MSW(MM)=1
        WRITE(6,2010) (NGOOD(J),J=1,2)
  2010  FORMAT(1H0,9HTHERE ARE,I4,19H GOOD N2 EVENTS AND,I4,
      1   16H GOOD N4 EVENTS.)
        WRITE(6,2020) NBAD
  2020  FORMAT(1H0,9HTHERE ARE,I4,12H BAD EVENTS.)
        CALL SYSTEM
        END
```

```
      SUBROUTINE STORE(IA,ISW,ISET,ITST,IX,IY,LAST)
      COMMON/COUNT/ICT,LOWER,ITOP,NBAD,NGOOD(2),NTAPE(2)
      DIMENSION IX(4,16),IY(4,16),IA(200),ISET(20),ITST(4),LAST(4)
C        ISET ARRAY
C          (1)  FAIL FLAG
C                =  0   GOOD EVENT
C                  10   NOT PROPER EVENT TYPE
C                  20   NOT N2 OR N4 EVENT TYPE
C                  30   MAX TRACK G.T. 4
C                  31   TRACK # G.T. MAX TRACK #
C                  32   # POINTS ON TRACK NOT BETWEEN 1 AND 16
C                  45   ALL TRACKS NOT MEASURED
C                  60   SAME AS =20
C          (2)  EXP NO
C          (3)  ROLL NO
C          (4)  FRAME NO
C          (5)  EVENT NO
C          (6)  EVENT TYPE (2 OR 4 ONLY)
C          (7)  MACHINE NO
C          (8)  MAX NO OF TRACKS
C          (9)  DATE (MMDDYY)
C          (10)  TIME (HHMMSS)
C          (11-20)  UNIMPORTANT
      GO TO (5,20,111),ISW
 5    DO 15 K=2,5
 15   ISET(K)=IA(K)
      ISET(1)=0
      DO 17 K=8,20
 17   ISET(K)=0
 20   DO 25 K=2,5
      IF(IA(K)-ISET(K)) 111,25,111
 25   CONTINUE
      IP=IA(11)+11
      IF(IP-12) 28,29,28
 28   ISET(1)=10
      GO TO 100
 29   DO 30 K=9,IP
 30   ISET(K)=IA(K)
      ISET(7)=IA(8)-(IA(8)/10)*10
      ISET(6)=IA(12)
      IF((ISET(6)-2)*(ISET(6)-4)) 31,34,31
C        CHECK TO SEE IF EVENT TYPE IS N2 OR N4
 31   ISET(1)=20
 34   ITR=IA(6)/1000
      INTR=IA(6)-ITR*1000
      IF(LIMIT(INTR,1,4)) 40,35,40
 35   ISET(1)=30
      INTR=4
 40   IF(LIMIT(ITR,1,INTR)) 50,45,50
 45   ITR=INTR
      ISET(1)=31
 50   ISET(8)=INTR
      IP=IP+3
      LAST(ITR)=IA(IP)
      NPS=LAST(ITR)
      IF(LIMIT(NPS,1,16)) 65,60,65
 60   ISET(1)=32
      GO TO 80
```

```
   65   DO 75 NPT=1,NPS
        IX(ITR,NPT)=IA(IP+1)
        IY(ITR,NPT)=IA(IP+2)
   75   IP=IP+2
   80   DO 85 J=ITR,INTR
   85   ITST(J)=0
        ITST(ITR)=1
  100   RETURN
C          NOW OUPUT OLD EVENT--NEW EVENT IS NOW IN IA ARRAY
  111   INTR=ISET(8)
        MMN=ISET(7)
        ICT=ICT+1
        IF(LIMIT(ICT,LOWER,ITOP)) 125,350,125
  125   IF(INTR) 140,140,130
  130   DO 135 J=1,INTR
        IF(ITST(J)-1) 500,135,500
  135   CONTINUE
  140   M=ISET(6)/2
        IF(LIMIT(M,1,2)) 142,141,142
  141   ISET(1)=60
  142   IF(ISET(1)) 600,145,600
  145   NGOOD(M)=NGOOD(M)+1
        M=NTAPE(M)
  150   CALL CONVRT(ISET(9),ISET(10))
        NPS=0
        DO 160 J=1,INTR
  160   NPS=NPS+LAST(J)
C          FIRST OUTPUT RECORD IS ROLL,FRAME,EVENT #,TOTAL # COORD PAIRS IN EVENT,
C          # TRACKS, DATE, TIME
        WRITE(M,170) (ISET(J),J=3,5),NPS,ISET(6),ISET(9),ISET(10)
  170   FORMAT(7I10)
        DO 200 J=1,INTR
        ITK=J*1000+LAST(J)
        NPS=LAST(J)
        IF(LIMIT(NPS,1,16)) 175,200,175
  175   DO 190 K=1,NPS
C          TRACK RECORDS ARE (TRK #)*1000+ # POINTS ON TRACK,X COORD,
C                Y COORD
        WRITE(M,180) ITK,IX(J,K),IY(J,K)
  180   FORMAT(3I10)
  190   CONTINUE
  200   CONTINUE
  350   GO TO (5,5,100),ISW
  500   ISET(1)=45
  620   FORMAT (' ROLL',I4,',FRAME',I4,',EVENT',I2/
       2'      ',I2,' TRACKS, FAIL CODE = ',I2)
  600   WRITE(6,620)(ISET(J),J=3,6),ISET(1)
        NBAD=NBAD+1
        GO TO 350
        END
FILE
```

```
      FUNCTION IBUFIN(IA,N)
      EXTERNAL ICR
      CCMMCN /PICK/ IAT
      DIMENSION IA(1)
      INTEGER *2 NB
      IF(N) 10C, 10C, 2CC
1CC   NBR= IA(1)
      IST=1
      GC TO 400
2CC   DC 3CC K=1,NBR
3CC   IA(K)=0
      NB=NBR*4
      CALL SIGERR(ICR)
      CALL READ(IA,NB,C,LNBR,8,&5CO)
35C   IAT=0
4CC   IBUFIN=IST
      RETURN
50C   IST=0
      GC TC 40C
      END
```

```
      FUNCTION INRECD(IA,IB)
      COMMON/PICK/IAT
      DIMENSION IA(1), IB(1)
      L=IBMDDP(IA(IAT+1))
      IF(L-200) 50,1C,1CC
5C    IF(L) 100,50C,10
1CC   L=2CC
1C    KX=IAT+2
      IEXP=IBMDDP(IA(KX))
      IF(IEXP-8) 2C,3C,2C
2C    IAT=IAT+L
      INRECD=-1
      RETURN
3C    DC 3CC K=1,L
      KX=IAT+K
3CC   IB(K)=IBMDDP(IA(KX))
      IAT=IAT+L
50C   INRECD=L
      RETURN
      END
```

```
      SUBROUTINE ICR
      WRITE (6, 10)
```

```
10    FORMAT(' TAPE ERROR IGNORED')
      RETURN
      END

      SUBROUTINE MACH1(IA,ISW)
      DIMENSION IA(200)
      RETURN
      END

      SUBROUTINE MACH2(IA,ISW)
      DIMENSION IA(200)
      RETURN
      END

      SUBROUTINE MACH3(IA,ISW)
      DIMENSION IA(200)
      RETURN
      END
      SUBROUTINE MACH4(IA,ISW)
      DIMENSION IA(200)
      RETURN
      END

      SUBROUTINE MACH5(IA,ISW)
      DIMENSION IA(200),ISET(20),ITST(4),IX(4,16),IY(4,16),LAST(4)
      CALL STORE(IA,ISW,ISET,ITST,IX,IY,LAST)
      RETURN
      END

      SUBROUTINE MACH6(IA,ISW)
      DIMENSION IA(200),ISET(20),ITST(4),IX(4,16),IY(4,16),LAST(4)
      CALL STORE(IA,ISW,ISET,ITST,IX,IY,LAST)
      RETURN
      END

      SUBROUTINE MACH7(IA,ISW)
      DIMENSION IA(200)
      RETURN
      END
```

```
      SUBROUTINE CONVRT(IDAT,ITIM)
      IF(ITIM-240000) 100,100,1
 1    ITIM=ITIM-240000
C         RESET TIME AFTER MIDNITE
      LDAY=IDAT/100
      LYEAR=IDAT-LDAY*100
      LMONTH=LDAY/100
      LDAY=LDAY-LMONTH*100
      IF(LIMIT(LMONTH,1,12)) 5,100,5
C           NOW CONVERT DATE
 5    GO TO (20,40,20,60,20,60,20,20,60,20,60,80), LMONTH
 20   IF(LDAY-31) 90,87,87
 40   IF(LDAY-28) 90,87,87
 60   IF(LDAY-30) 90,87,87
 80   IF(LDAY-31) 90,85,85
 85   LYEAR=LYEAR+1
      LMONTH=1
      GO TO 88
 87   LMONTH=LMONTH+1
 88   LDAY=1
      GO TO 95
 90   LDAY=LDAY+1
 95   IDAT=(LMONTH*100+LDAY)*100+LYEAR
      IF(ITIM-240000) 100,100,1
C        REPEAT IF NECESSARY
 100  RETURN
      END
      FUNCTION LIMIT(IT,LOW,LIM)
      ITST=0
      IF(IT-LOW) 200,150,110
  110 IF(IT-LIM) 150,150,200
  150 ITST=1
  200 LIMIT=ITST
      RETURN
      END
```

```
C         SUBROUTINE "IBMDDP"

IBMDDP    CSECT
          USING   *,15
          L       1,0(0,1)
          L       1,0(0,1)
          L       0,ZERO
          SLL     1,2(0)
          SLDL    0,6(0)
          SLL     1,2(0)
          SLDL    0,6(0)
          SLL     1,2(0)
          SLDL    0,6(0)
          SLL     1,2(0)
```

```
         SLDL    0,6(0)
         C       0,NEG
         BL      KXT
         S       0,NEG
         LNR     0,0
KXT      BR      14
NEG      DC      X'00800000'
ZERO     DC      X'00000000'
         END
```

APPENDIX VIII

SOURCE PROGRAM LISTING FOR "CHROMOSOME"

```
      IMPLICIT INTEGER(A-K,M-Q,S-Z),REAL (R),LOGICAL*1(L)
      DIMENSION IDATA(7),XCOORD(100),INDEX(100),
     2YCOORD(100),S(100), HEAD(2), XBAR(2), SIGMA(2)
      REAL SIGMA
      EQUIVALENCE (TRACKS, IDATA(5)),(T,IDATA(4))
      COMMON ILONG(100), SHORT(100), RATIO(100)
      INTEGER RTOT
      IDIST (X1,Y1,X2,Y2)=SQRT (1.*((X1-X2)**2+ (Y1-Y2)**2))
      CALL CHECK (LPLOT,LSUMM)
10    LFIRST=.TRUE.
      IND=0
      LAST=.FALSE.
      LROLL=.FALSE.
      LSTOP=.FALSE.
      LWRONG=.FALSE.
900   FORMAT (7I10)
20    READ(5,900,END=9000)(IDATA(I),I=1,7)
C
C     LFIRST=TRUE FOR FIRST DATA SET IN FRAME
C     IND=DATA INDEX
C     IDATA=FIRST INPUT RECORD FOR EVENT
C     1= ROLL          2= FRAME
C     3=EVENT          4=#COORD PAIRS IN EVENT
C     5=#TRACKS (2OR4)   6=DATE (MMDDYY)
C     7=TIME (HHMMSS)
C     LSTOP=TRUE FOR END OF FILE
C     UNIT 5=INPUT DATA
      IF(LFIRST)GO TO 24
      IF(FRAME-IDATA(2)) 30,40,30
30    TSAVE=T+2
      DO 35 I=1,T
35    READ(5,902)TDAT,X,Y
      READ(5,900,END=9000)(IDATA(I),I=1,7)
      IF(FRAME-IDATA(2))31,40,31
31    LAST=.TRUE.
      DO 32 I=1,TSAVE
32    BACKSPACE 5
      READ(5,900)(IDATA(I),I=1,7)
C
C     LAST=TRUE MEANS END OF FRAME
C     LROLL=TRUE MEANS END OF ROLL AND FRAME
C
40    IF(IROLL-IDATA(1))50,60,50
50    LROLL=.TRUE.
      LAST=.TRUE.
      GO TO 60
24    LFIRST=.FALSE.
25    IROLL=IDATA(1)
      BIARM=0
      FRAME=IDATA(2)
      DATE=IDATA(6)
      TOT=0
```

```
      RTCT=C
      TIME=IDATA (7)
      PPT=0
      TR=C
C
C     RTCT=TOTAL LENGTH OF SPREAD
C     PPT=TOTAL #PAIRS IN FRAME
C
901   FORMAT ('1MEASUREMENT ANALYSIS'/' ROLL ',I3,', FRAME ',I3,
     2' STARTED AT',I7,' ON',I7)
      WRITE (6,901) IROLL,FRAME,TIME,DATE
      GO TO 100
9CCC  LSTOP=.TRUE.
      GO TO 50
C     EXPLANATION OF LSTOP ABOVE
60    IF(LAST) GO TO 7CCO
C     END OF FRAME SEQUENCE IS 7CCC
70    IF(LROLL) GO TO 8000
C     END OF SAMPLE SEQUENCE IS 800C
100   IND=IND+1
C     INCREMENT DATA INDEX
      IF(100-IND) 1C1,1C1,1C2
101   CALL IFAIL (10)
1C2   INDEX (IND) = IDATA(3)
      DATE2 = IDATA(6)
      TIME2=IDATA(7)
      PPT=PPT+T
      TR=TR+TRACKS
C
C     T=#COORD PAIRS IN EVENT
C     PPT=TOTAL PAIRS IN FRAME
C     TR=TOTAL TRACKS IN FRAME
C     BIARM=TOTAL #BIARM CHROMS
C
      IF(TRACKS-2) 11C,12C,11C
 120   RATIO(IND)=C.
      SHORT(IND)=0
      GO TO 121
 11C   BIARM=BIARM+1
 121   ACCUM=0
      CTN=0
C
C     OX,OY=OLD X AND Y
C     ACCUM=LENGTH ACCUMULATOR
C     CTN=CURRENT TRACK NO.
C     NPCT=NUMBER POINTS ON TRACK
C     CPOT=CURRENT POINT ON TRACK
C     OTN=OLD TRACK NUMBER
C     TDAT=TRACK#*1000+#PTS ON TRACK
C
      DO 15C J=1,T
902   FORMAT (3I1C)
      READ(5,902,END=14C,ERR=14C) TDAT,X,Y
      CTN=TDAT/1000
      IF(CTN-OTN) 122,123,122
122   OTN=CTN
      CPCT=1
      NPCT=MOD(TDAT,1CCC)
      OX=X
      OY=Y
```

```
      IF(CTN-1) 15C,125,15C
125   XCCCRC (INC)=X
      YCCCRC(INC)=Y
      GC TC 15C
123   CPCT=CPCT+1
      ACCUM=ACCUM+IDIST(X,Y,CX,CY)
      CX=X
      CY=Y
      IF(NPCT-CPCT) 13C,13C,15C
130   IF(CTN-2) 132,131,132
131   ACCUM=ACCUM/2
C     FINISHED WITH ONE ARM
      ILCNG(INC)=ACCUM
      RTCT=RTOT+ACCUM
      ACCUM=0
      GC TC 150
132   IF(CTN-4) 15C,133,150
133   ACCUM=ACCUM/2
      RTCT=RTOT+ACCUM
      SHCRT(IND)=MINC(ILCNG(IND),ACCUM)
      ILCNG(IND)=MAXC(ILCNG(INC),ACCUM)
      IF(SHCRT(IND))16C,160,134
 134  CONTINUE
      RATIC (INC)=(ILCNG(INC)*1.0)/SHCRT(IND)
16C   IF(J-T) 161,20,161
161   CALL IFAIL(3C)
150   CCNTINUE
      GC TC 20
 9C3   FCRMAT ('CTRACK =',I3,', PCINT =',I3,', EVENT =',I4)
 14C   WRITE(6,903) CTN,CPCT,INC
      CALL IFAIL (2C)
C
C     ENC FRAME SEQUENCE
904   FCRMAT ('OFINISHEC AT',I7,' CN',I7)
70CC  WRITE (6,9C4) TIME2,CATE2
      CALL ILAPSE(CATE,CATE2,TIME,TIME2)
C     R=PCINTS/ARM
      R=PPT/(TR*1.C)
      J=INC-BIARM
C     J=#TELOS
905   FCRMAT ('0',I3,' CHRCMCSCMES,',I3,' TELCCENTRICS,',I3,' BIARMS'/
     4' ',I5,' TRACKS MEASUREC,',F5.2,' PCINTS/TRACK')
      WRITE(6,905)INC,J,BIARM,TR,R
C
C     R=AVE. LENGTH/CHRCMCSCME
C     R1=AVE. LENGTH/ARM
C
      R1=RTCT*1.C
      R=R1/INC
      R1=R1*2/TR
906   FCRMAT ('OLENGTHS...TCTAL =',I1C,',AVE/CHRCM=',F8.3,', AVE/ARM=',
     2F8.3)
      WRITE(6,9C6)RTCT,R,R1
      CALL MCDIFY(R,R1,INC)
      CALL CRDER (HEAC,S,INC,LWRCNG)
      IF (LWRONG) CALL IFAIL(4C)
      CC 72C0 J=1,2
      XBAR(J)=0
      SIGMA(J)=0
      CTN=0
```

```
C       CTN IS LOOP CHECK
        HL=HEAD(J)
        IF(HL)7200,7200,7120
907     FORMAT ('OCODE   INDEX XCOORD YCOORD LARM   SARM   ',
       2'RATIO LENGTH'/' ')
7120    WRITE(7,907)
7160    CTN=CTN+1
        TOT=ILONG(HL)+SHORT(HL)
        XBAR(J)=XBAR(J)+TOT
        SIGMA(J)=SIGMA(J)+TOT*TOT
908     FORMAT (' ',6I6,F6.2,I6)
        WRITE(7,908)CTN,INDEX(HL),XCOORD(HL),YCOORD(HL),ILONG(HL),
       2SHORT(HL),RATIO(HL),TOT
        IF(S(HL))7200,7200,7140
7140    HL=S(HL)
        IF(INC-CTN)7150,7150,7160
7150    CALL IFAIL(60)
7200    CONTINUE
7300    CTN = INC-BIARM
        IF (BIARM.EQ.0)BIARM=1
        IF(CTN.EQ.0)CTN=1
        SIGMA(1)=(BIARM*SIGMA(1)-XBAR(1)**2)/(BIARM*BIARM)
        SIGMA(2)=(CTN*SIGMA(2)-XBAR(2)**2)/(CTN*CTN)
        R=SQRT(SIGMA(1)*1.0)
        R1=SQRT(SIGMA(2)*1.0)
        XBAR(1)=XBAR(1)/BIARM
        XBAR(2)=XBAR(2)/CTN
909     FORMAT ('OBIARMS')
910     FORMAT ('OTELOCENTRICS')
911     FORMAT(' N =',I3/' XBAR =',I5/' VAR =',F8.0/' S.D. =',F5.0)
        WRITE(6,909)
        WRITE(6,911)BIARM,XBAR(1),SIGMA(1),R
        WRITE(6,910)
        WRITE(6,911)CTN,XBAR(2),SIGMA(2),R1
        IF(LSUMM) CALL STORE2(XBAR,SIGMA,BIARM,CTN)
        IF(LPLOT) CALL PLOTR1 (INC,BIARM,LFRAM)
        LAST=.FALSE.
        INC=0
        IF(LROLL) GO TO 8000
        GO TO 25
C       END POPULATION OR END RUN SEQUENCE
8000    IF(LPLOT) CALL PLOTR2
        IF(LSUMM) CALL STORE3
        IF(LSTOP) CALL EXIT
        FRM=0
        INC=0
        LROLL=.FALSE.
        IF(LSTORE)CALL STORE1
        GO TO 25
        END
```

```
      SUBROUTINE ILAPSE (D1,D2,T1,T2)
      IMPLICIT INTEGER (A-Z)
      IF(D1-D2)10,20,10
900   FORMAT ('0MEASURING TOOK MORE THAN 1 DAY ')
10    WRITE(6,900)
      RETURN
20    HH1=T1/10000
      HH2=T2/10000
      T1=T1-HH1*10000
      T2=T2-HH2*10000
      HH1=HH2-HH1
      MM1=T1/100
      MM2=T2/100
      IF(MM1-MM2)30,30,21
21    MM2=MM2+60
      HH1=HH1-1
30    MM1=MM2-MM1
      SS1=MOD(T1,100)
      SS2=MOD(T2,100)
      IF(SS1-SS2)40,40,39
39    SS2=SS2+60
      MM1=MM1-1
40    SS1=SS2-SS1
901   FORMAT (' ELAPSED TIME=',I3,' HRS.',I3,' MIN.',I3,' SEC.')
      WRITE(6,901)HH1,MM1,SS1
      RETURN
      END



      SUBROUTINE MODIFY(R,R1,IND)
      IMPLICIT INTEGER(A-Q,S-Z), REAL(R)
C
C     RELATIVE MEAS. DETERMINED USING AVE. CHROM. LENGTH
C     RETURN VALUE IS REL. LENGTH*1000
      COMMON LONG(100), SHORT(100), RATIO(100)
      DO 10 I=1,IND
      LONG(I)=(LONG(I)*1000)/R
      IF(SHORT(I))20,10,20
20    SHORT(I)=(SHORT(I)*1000)/R
10    CONTINUE
      RETURN
      END



      SUBROUTINE IFAIL(I)
900   FORMAT ('0ERROR...RETURN CODE =',I4)
```

```
      WRITE(6,9CC)I
      CALL EXIT
      ENC
      SUBRCUTINE CRCER (HEAC,S,IND,SWRCNG)
      IMPLICIT INTEGER(A-C,S-Z), REAL (R)
      CCMMCN LARM(10C), SARM(100), RATIC(100)
      DIMENSION S(INC),HEAC(2)

      LCGICAL SWRCNG*1
      HEAC(1)=C
      HEAC(2)=C
      M=C
 1CC  M=M+1
      IF(M.GT.INC) RETURN
      S(M)=C
      J=2
      IF(RATIO(M).GT.C.C)J=1
      IF(HEAC(J).NE.C)GC TC 11C
      HEAC(J)=M
      GC TO 100
 11C  LAST=C
      I=HEAC(J)
 20C  IF((LARM(M)+SARM(M)).LT.(LARM(I)+SARM(I)))GO TO 21C
      IF(LAST.EQ.0)GC TC 205
      S(M)=I
      S(LAST)=M
      GC TO 10C
 2C5  S(M)=I
      HEAC(J)=M
      GC TO 100
 21C  IF(S(I).NE.0)GC TC 22C
      S(I)=M
      GC TC 10C
 22C  LAST=I
      I=S(I)
      IF(C.LT.I.AND.I.LT.M.ANC.LAST.NE.I)GC TO 200
      SWRCNG=.TRUE.
 9CC  FCRMAT('OORDER/LCCP ERRCR - CHRCMCSOME',I5)
      WRITE(6,9C0)M
      RETURN
      END



      SUBRCUTINE CHECK (LPLCT,LSUMM)
      IMPLICIT INTEGER (A-K,M-Y),LOGICAL*1(L),REAL(Z)
      REAL R
      CATA B,T,Y,N/'B','T','Y','N'/
      REWINC 5
 9CC  FCRMAT ('OBATCH CR TERMINAL??')
      WRITE (6,900)
 9C1  FCRMAT (A1)
 5    READ(3,9C1,END=888)A
      IF(A-B)1C,2C,1C
 1C   IF (A-T) 3C,4C,3C
 9C2  FCRMAT (' INVALIC WCRC...SENC B CR T')
 3C   WRITE(6,9C2)
      GC TC 5
 2C   LPLCT=.TRUE.
```

```
      LSUMM=.TRUE.
      CALL STORE1
      CALL PLOTR3
C     BATCH DEFAULTS TO ALL POSSIBLE PLOTS AND SUMMARIES
903   FORMAT (F10.1)
C     BATCH REQUIRES NEXT TWO CARDS HAVE FILE AND RECORD IN FLOATING POINT
904   FORMAT(' WHAT FILE DOES DATA START IN (FLOATING PT.)?')
50    WRITE (6,904)
      READ (3,903,END=888,ERR=777)R
      NF=R-1.
905   FORMAT (' WHAT RECORD?')
      WRITE (6,905)
      READ(3,903,END=888,ERR=777)R
      NR=R-1.
      CALL SKIP (NF,NR,5)
      RETURN
888      CALL IFAIL(80)
777   CALL IFAIL(50)
906   FORMAT(' WANT SUMMARY?')
40    WRITE(6,906)
C     TERMINAL DEFAULTS TO NO PLOTS
      READ(3,901,END=80,ERR=777)A
      IF(A-Y)75,60,75
60    LSUMM=.TRUE.
      CALL STORE1
70    LPLOT=.FALSE.
      GO TO 50
75    IF(A-N)90,80,90
907   FORMAT(' INVALID ANSWER...YES OR NO?')
90    WRITE(6,907)
      GO TO 40
80    LSUMM=.FALSE.
      GO TO 70
      END



      SUBROUTINE STORE1
      ENTRY STORE2
      ENTRY STORE3
      RETURN
      END



      SUBROUTINE PLOTR3
      COMMON LONG(100),SHORT(100),RATIO(100)
      INTEGER A(52),C(52),INC,BIARM, LONG,SHORT,IMAGE(2784), IDATA(6)
      DIMENSION X(52),IRAT(3)
      LOGICAL FRAME*1
      DATA IDATA /'FRAC','TION',' OF ','CHRO','MOSO','MES '/
     2,IRAT/'ARM ','RATI','C   '/
C
C     A IS STORAGE FOR ARM LENGTH DISTRIBUTION, C IS FOR
C     CHROMOSOME LENGTH DISTRIBUTION, X IS FOR VALUES OF X
C     COORDINATE
```

```
C
      LEN(JB)=(JB+5)/50+1
C     LEN COMPUTES SUBSCRIPT
      DO 1 I=1,52
1     X(I)=(I-1)*0.05
5     DO 10 I=1,52
      A(I)=0
10    C(I)=0
      CHROM=0.
      ARM=0.
C     CHROM AND ARM ACCUMULATE NUMBER OF OBJECTS BEING CONSIDERED
C     IN THEIR ASSOCIATED DISTRIBUTIONS
C
      OVER=0
      CALL PLOT2 (IMAGE,2.5,0.,4.0,1.0)
      RETURN
      ENTRY PLCTR1 (IND,BIARM,FRAME)
      DO 30 I=1,IND
      IF (SHORT(I))29,31,32
C     29 MEANS ERROR - NEGATIVE SHORT ARM LENGTH
29    CALL IFAIL(70)
31    JC=LEN(LONG(I))
      IF(JC-52)34,34,33
33    JC=52
34    A(JC)=A(JC)+1
      C(JC)=C(JC)+1
      GO TO 30
32    JC=SHORT(I)+LONG(I)
      X2=JC/1000.
      IF(4.0-RATIO(I))100,101,101
100   OVER = OVER + 1
      GO TO 102
101   CALL PLOT3('X',X2,RATIO(I),1,4)
102   CONTINUE
      JC=LEN(JC)
      IF(JC-52) 36,36,35
35    JC=52
36    C(JC)=C(JC)+1
      JC=LEN(SHORT(I))
      IF(JC-52)38,38,39
39    JC=52
38    A(JC)=A(JC)+1
      JC=LEN(LONG(I))
      IF(JC-52)41,41,40
40    JC=52
41    A(JC)=A(JC)+1
30    CONTINUE
      ARM=ARM+IND+BIARM
      CHROM=CHROM+IND
      RETURN
      ENTRY PLCTR2
903   FORMAT('1RATIOS > 4.0 ... ',I3)
      WRITE(7,903) OVER
      CALL PLOT4 (9,IRAT(1))
      WRITE(7,901)
      CALL PLOT2 (IMAGE,2.5,0.,0.3,0.)
      DO 80 I=1,51,2
      Y=A(I)+A(I+1)
      A(I)=Y
      Y=Y/ARM
```

```
      CALL PLOT3 ('X',X(I),Y,1,4)
      Y=C(I)+C(I+1)
      C(I)=Y
      Y=Y/CHROM
      CALL PLOT3('*',X(I),Y,1,4)
80    CONTINUE
900   FORMAT('1FREQUENCY DISTRIBUTION OF ARM (X) AND CHROMOSOME (*) LENG
     2THS'/' ',F4.0,' ARMS MEASURED'/' ',F4.0,' CHROMOSOMES MEASURED')
      WRITE (6,900)ARM,CHROM
      JC=A(52)+C(52)
902   FORMAT (' ',I3,' VALUES > 2.5')
      WRITE(6,902) JC
      CALL PLOT4(23,IDATA(1))
901   FORMAT ('0',20X,'RELATIVE LENGTH')
      WRITE (6,901)
904   FORMAT(' ARMS',26I3/' CHROMS',26I3)
      WRITE(7,904)(A(I),I=1,51,2),(C(J),J=1,51,2)
      GO TO 5
      END
```

APPENDIX IX

SAMPLE SESSIONS USING "CHROMOSOME"

Although "CHROMOSOME" is usually run in batch mode, it can be run from a remote teletype terminal to check data or program operation. Operation of the program depends slightly on the mode being used as can be seen in the examples which follow.

OPERATION FROM A REMOTE TERMINAL : All lines entered by the user are underlined, control characters used are in lower case type.

```
MTS (LA20-0036)
WHO ARE YOU?
UM MICBIOAAAs

#$SIGNON SF40 PW=        s
#**LAST SIGNON WAS: 09:24.40   07-15-69
#  USER "SF40" SIGNED ON AT 09:38.14 ON 07-15-69
#$RUN *MOUNT PAR=G113 9TP,*TAPE*,MODE=1600,SIZE=80,'SF40-1's
#EXECUTION BEGINS
 DONE
#EXECUTION TERMINATED
#$RUN CHROMO 5=*TAPE* 7=*DUMMY*s

  BATCH OR TERMINAL??
TERMINALs
  WANT SUMMARY?                              The summary option is
NOs                                          not operational at this
                                             time.
  WHAT FILE DOES DATA START IN (FLOATING PT.)?
1.s
  WHAT RECORD?
1.s



  MEASUREMENT ANALYSIS
  ROLL  40, FRAME  1 STARTED AT   2757  ON  70969

  FINISHED AT  12116 ON  70969
  ELAPSED TIME= 0 HRS. 53 MIN. 19 SEC.

   47 CHROMOSOMES,  1 TELOCENTRICS, 46 BIARMS
    186 TRACKS MEASURED, 5.96 POINTS/TRACK

  LENGTHS...TOTAL =     11634,AVE/CHROM= 247.532,AVE/ARM= 125.097
```

```
BIARMS
N = 46
XBAR = 1014
VAR = 148390.
S.D. = 385.

TELOCENTRICS
N = 0
XBAR =  0
VAR =    -0.
S.D. =   0.

#EXECUTION TERMINATED
#$SIGNOFF
    .
    .
    .
```

OPERATION IN BATCH MODE : Printed lines which were read off data cards are underlined in the batch session which follows.

```
$RUN *MOUNT PAR=G113 9TP,*TAPE*,MODE=1600,SIZE=80,'SF40-1'
EXECUTION BEGINS
DONE
EXECUTION TERMINATED
```

```
$RUN CHROMO 5=*TAPE*
EXECUTION BEGINS

BATCH OR TERMINAL??
WHAT FILE DOES DATA START IN (FLOATING PT.)?
WHAT RECORD?
```

Three data cards are read in at this point but are not printed. They contain the data requested.

MEASUREMENT ANALYSIS

ROLL 9, FRAME 1 STARTED AT 211202 ON 51869
31

FINISHED AT 214634 ON 51869
ELAPSED TIME= 0 HRS. 34 MIN. 32 SEC.

64 CHROMOSOMES, 50 TELOCENTRICS, 14 BIARMS
156 TRACKS MEASURED, 6.57 POINTS/TRACK

LENGTHS...TOTAL = 9317,AVE/CHROM= 145.578, AVE/ARM= 119.449

CODE	INDEX	XCOORD	YCOORD	LARM	SARM	RATIO	LENGTH
1	46	-1250	170	1449	1305	1.11	2754
2	64	-1573	62	1552	1030	1.51	2582
3	18	-1018	852	1538	989	1.56	2527
4	4	-18	446	1243	1208	1.03	2451
5	31	-637	272	1119	1016	1.10	2135
6	57	-1870	-163	1064	1050	1.01	2114
7	47	-1130	-82	1408	597	2.36	2005
8	36	-412	-294	1016	892	1.14	1908
9	22	-1227	1239	954	748	1.28	1702
10	15	-768	1198	1078	487	2.21	1565
11	56	-1711	-252	776	769	1.01	1545
12	9	-206	725	1078	288	3.74	1366
13	48	-1125	-487	789	535	1.47	1324
14	50	-1340	-593	686	350	1.96	1036

CODE	INDEX	XCOORD	YCOORD	LARM	SARM	RATIO	LENGTH
1	26	-1507	1032	1202	0	0.0	1202
2	24	-1483	1224	1160	0	0.0	1160
3	7	36	718	1133	0	0.0	1133
4	12	-587	1260	1126	0	0.0	1126
5	58	-1956	-25	954	0	0.0	954
6	1	470	26	947	0	0.0	947
7	40	-873	425	934	0	0.0	934
8	53	-1434	-739	927	0	0.0	927
9	14	-910	1487	920	0	0.0	920
10	23	-1227	989	906	0	0.0	906
11	2	53	-73	892	0	0.0	892
12	45	-1065	666	886	0	0.0	886
13	30	-566	553	886	0	0.0	886
14	16	-732	1038	872	0	0.0	872
15	29	-468	567	865	0	0.0	865
16	49	-1040	-650	831	0	0.0	831
17	41	-1052	206	831	0	0.0	831
18	42	-917	115	817	0	0.0	817
19	11	-598	1723	803	0	0.0	803
20	32	-600	144	789	0	0.0	789
21	25	-1589	1472	789	0	0.0	789
22	10	-267	1315	776	0	0.0	776
23	21	-995	1125	748	0	0.0	748
24	37	-350	-390	714	0	0.0	714
25	65	-1667	-68	700	0	0.0	700
26	51	-1375	-800	686	0	0.0	686
27	35	-730	-150	686	0	0.0	686
28	33	-513	75	680	0	0.0	680
29	54	-1569	-447	673	0	0.0	673
30	34	-642	-60	666	0	0.0	666

31	28	-1772	852	659	0	0.0	659
32	60	-1883	486	645	0	0.0	645
33	62	-1549	393	638	0	0.0	638
34	43	-869	-26	638	0	0.0	638
35	63	-1629	288	631	0	0.0	631
36	61	-1472	482	631	0	0.0	631
37	8	-130	782	631	0	0.0	631
38	52	-1490	-976	604	0	0.0	604
39	20	-1084	967	604	0	0.0	604
40	13	-685	1303	604	0	0.0	604
41	3	52	335	604	0	0.0	604
42	44	-976	-70	597	0	0.0	597
43	27	-1756	988	583	0	0.0	583
44	55	-1893	-549	549	0	0.0	549
45	39	-801	562	480	0	0.0	480
46	59	-2040	131	473	0	0.0	473
47	19	-1015	775	439	0	0.0	439
48	38	-752	-511	432	0	0.0	432
49	6	-60	509	364	0	0.0	364
50	17	-922	1082	336	0	0.0	336

BIARMS
N = 14
XBAR = 1929
VAR = 259419.
S.D. = 509.

TELOCENTRICS
N = 50
XBAR = 738
VAR = 38479.
S.D. = 196.

MEASUREMENT ANALYSIS

ROLL 9, FRAME 2 STARTED AT 214904 ON 51869
31

FINISHED AT 222316 ON 51869
ELAPSED TIME= 0 HRS. 34 MIN. 12 SEC.

64 CHROMOSOMES, 52 TELOCENTRICS, 12 BIARMS
152 TRACKS MEASURED, 7.44 POINTS/TRACK

LENGTHS...TOTAL = 11318,AVE/CHROM= 176.844, AVE/ARM= 148.921

CODE	INDEX	XCOORD	YCOORD	LARM	SARM	RATIO	LENGTH
1	13	-405	599	1752	1023	1.71	2775
2	69	-792	166	1300	1244	1.05	2544
3	93	-1412	552	1198	983	1.22	2181
4	108	-1921	-214	1130	989	1.14	2119
5	5	216	260	1057	1000	1.06	2057
6	95	-1603	1077	1543	424	3.64	1967
7	26	-885	745	1080	836	1.29	1916
8	77	-962	-9	933	769	1.21	1702
9	75	-653	-331	1159	497	2.33	1656
10	92	-1220	969	791	740	1.07	1531
11	81	-1010	-814	650	220	2.95	870
12	89	-1254	292	542	282	1.92	824

CODE	INDEX	XCOORD	YCOORD	LARM	SARM	RATIO	LENGTH
1	105	-1670	381	1453	0	0.0	1453
2	25	-697	702	1424	0	0.0	1424
3	20	-693	1413	1249	0	0.0	1249
4	83	-1285	-607	1159	0	0.0	1159
5	17	-244	1608	1142	0	0.0	1142
6	91	-1187	665	1136	0	0.0	1136
7	106	-1576	-75	1085	0	0.0	1085
8	88	-1246	125	1051	0	0.0	1051
9	107	-1592	-402	1012	0	0.0	1012
10	99	-1621	1327	1000	0	0.0	1000
11	79	-612	-994	1000	0	0.0	1000
12	73	-610	-38	995	0	0.0	995
13	87	-1521	-557	978	0	0.0	978
14	9	-110	625	978	0	0.0	978
15	23	-954	1384	972	0	0.0	972
16	18	-294	1350	955	0	0.0	955
17	78	-272	-820	916	0	0.0	916
18	24	-1026	1539	899	0	0.0	899
19	19	-528	1385	893	0	0.0	893
20	74	-610	-280	887	0	0.0	887
21	8	12	103	848	0	0.0	848
22	94	-1387	931	825	0	0.0	825
23	80	-996	-1098	825	0	0.0	825
24	7	278	21	802	0	0.0	802
25	12	-326	980	797	0	0.0	797
26	3	91	823	769	0	0.0	769
27	104	-1846	327	757	0	0.0	757
28	102	-1868	565	746	0	0.0	746
29	86	-1504	-400	723	0	0.0	723
30	1	257	559	706	0	0.0	706
31	11	-208	759	701	0	0.0	701
32	10	-147	424	695	0	0.0	695

33	4	-44	960	689	0	0.0	689
34	76	-794	-456	678	0	0.0	678
35	97	-1428	1292	672	0	0.0	672
36	98	-1467	1752	667	0	0.0	667
37	27	-837	519	667	0	0.0	667
38	22	-672	1150	667	0	0.0	667
39	2	148	722	661	0	0.0	661
40	100	-1833	845	655	0	0.0	655
41	84	-1254	-196	655	0	0.0	655
42	90	-1389	274	650	0	0.0	650
43	101	-2096	672	610	0	0.0	610
44	15	-189	1351	593	0	0.0	593
45	103	-2084	482	576	0	0.0	576
46	82	-1204	-980	531	0	0.0	531
47	16	-220	1440	514	0	0.0	514
48	96	-1537	1266	458	0	0.0	458
49	14	-338	72	424	0	0.0	424
50	28	-786	480	373	0	0.0	373
51	85	-1345	-220	361	0	0.0	361
52	21	-734	1322	339	0	0.0	339

BIARMS
N = 12
XBAR = 1845
VAR = 312844.
S.D. = 559.

TELOCENTRICS
N = 52
XBAR = 804
VAR = 61409.
S.D. = 248.

RATIOS > 6.0 ... 0

ARM RATIO

6.000
5.000
4.000
3.000
2.000
1.000

0.000 .250 .500 .750 1.000 1.250 1.500 1.750 2.000 2.250 2.500

FREQUENCY DISTRIBUTION OF ARM (X) AND CHROMOSOME (*) LENGTHS

154. ARMS MEASURED

128. CHROMOSOMES MEASURED

FRACTION OF CHROMOSOMES

.300

.240

.180

.120

.060

0.000

0.000 .250 .500 .750 1.000 1.250 1.500 1.750 2.000 2.250 2.500

RELATIVE LENGTH

APPENDIX X

ERROR RETURN CODES USED BY "SEARCH" AND "CHROMOSOME"

Error return codes from "SEARCH" (none of these are fatal)

CODE	ERROR
10	Not proper event type
20	Not an N2 or N4 event type
30	Maximum track number greater than 4
31	Track number greater than maximum track number
32	Number of points on track not between 1 and 16
45	All tracks not measured
60	Not an N2 or N4 event type

Error returns from "CHROMOSOME" (all are fatal)

CODE	ERROR
10	More than 100 chromosomes in one frame
20	End of file mark or error encountered in track record read sequence
30	Number of coordinate pairs on tape not same as declared number
40	Error (loop) encountered in ordering chromosome data
50	Error encountered in attempt to read information in subroutine "CHECK"
60	Unrecoverable loop discovered in data output sequence
70	Negative short arm length discovered
80	Logical end of file mark encountered while attempting to read control information

APPENDIX XI

SUPPORTING PROGRAMS

These programs are not yet completely documented.

THE EFFECTS OF ENVIRONMENTAL SELECTION PRESSURE ON THE CHROMOSOMAL COMPLEMENT OF L-M MOUSE CELLS AND $BHK_{21}PD_4$ CELLS

Richard Giles, Gregory Baumann, Edward Lunk, and Richard Platte, Jr.

SUMMARY

L-M cells, normally grown in modified medium 199 plus .5% Bacto peptone, in monolayer were subjected to the selective effects of supplementation of the growth medium with fetal calf serum, growth in spinner culture, and growth in a 1.5-liter fermenter (with automatic control of pH and OR potential). The chromosomes of several populations of L-M cells, which had been exposed to one or a combination of environmental changes, were analyzed to determine if any detectable alterations had occurred in the number or type of chromosomes, or the frequency distribution of characteristic marker chromosomes. Population samples were selected to investigate the stability and reversibility of detected changes. $BHK_{21}PD_4$ cells, were exposed to environmental changes similar to those which challenged the L-M cells, in order to observe the effects of various selection pressures on a near diploid permanent cell line. It was of particular interest to discover whether or not a cell population was significantly altered in chromosomal pattern during the process of going from small scale monolayer cultures to large scale fermenter cultures via spinner culture.

MATERIALS AND METHODS

Chromosome preparations were made according to the procedure reported previously (1). Colcemide exposure was varied from 4-8 hr for $BHK_{21}PD_4$ cells, to 12 hr for L-M cells. Metaphase cells were selected for analysis based on favorable fixation and staining of well spread chromosomes. Spreads were selected which appeared to be complete; i.e., were oval and/or had a visible haze of precipitated cytoplasm. Spreads with complexes of overlapping chromosomes were rejected. The number of chromosomes, number of biarmed chromosomes, number of telocentric chromosomes, and number of chromosome arms were counted.

The frequency and distribution of two marker chromosomes characteristic of L-M cells (2), and of an unusual morphology (see Figure 1C), which have been previously used to follow changes in the chromosomal pattern of L-M cells on a finer scale than chromosome number (1), were tabulated. Chromosomes, which appear quite similar to either the E or F marker (see Figures 1C-3C), were scored as E or F derivatives (E' or F'). This was done because of the fact that variations in coiling, fixation, staining, and the physical orientation of the chromosome can make the identification of all marker characteristics difficult in a particular cell. The E marker will often not show the secondary constriction nearest the centromere for the above reasons, and was scored as an E derivative in this case. F derivatives were of two major types. The first type of F derivative corresponds to the E' type of chromosome; i.e., a long subtelocentric chromosome is observed with one constriction

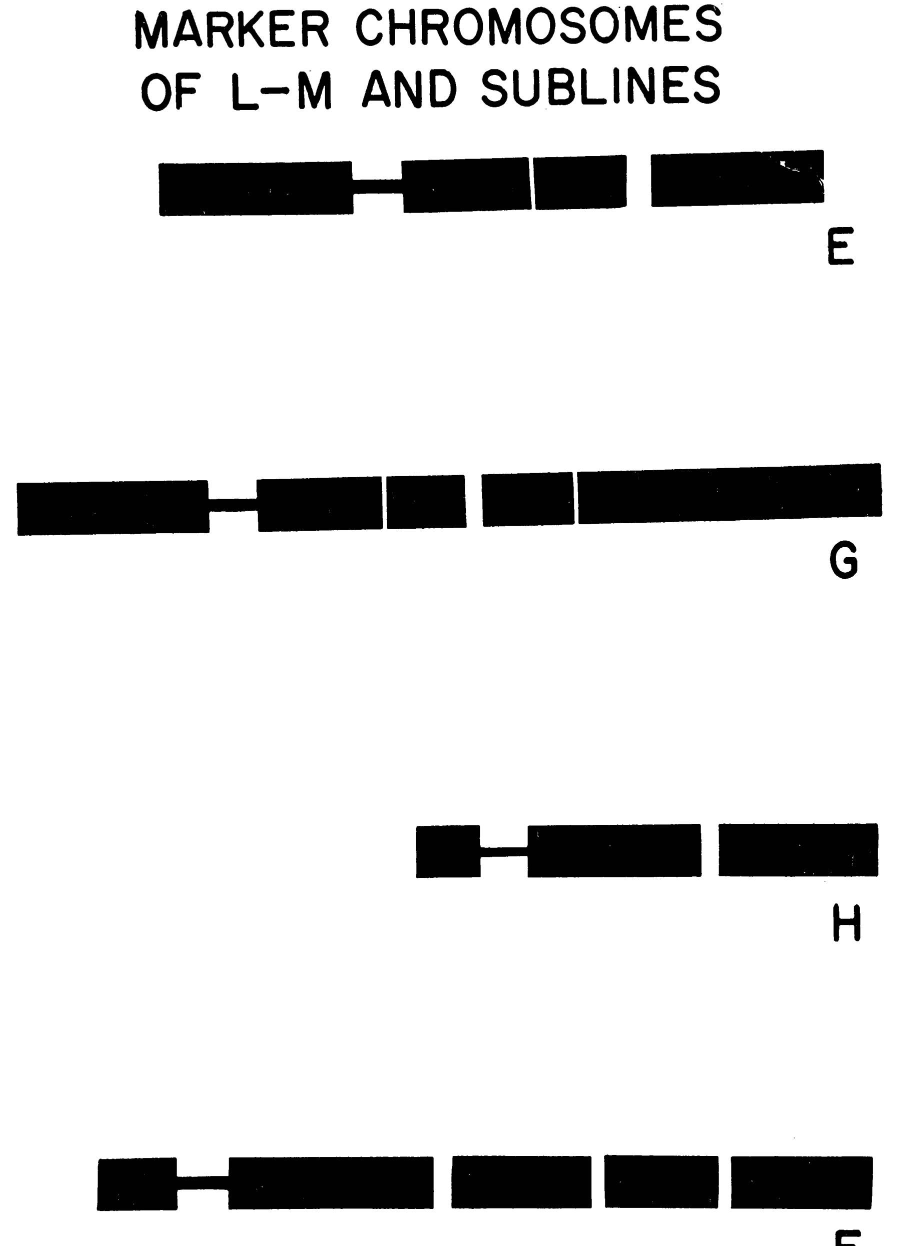

Figure 1C. Marker chromosome of the L-M strain and substrains.

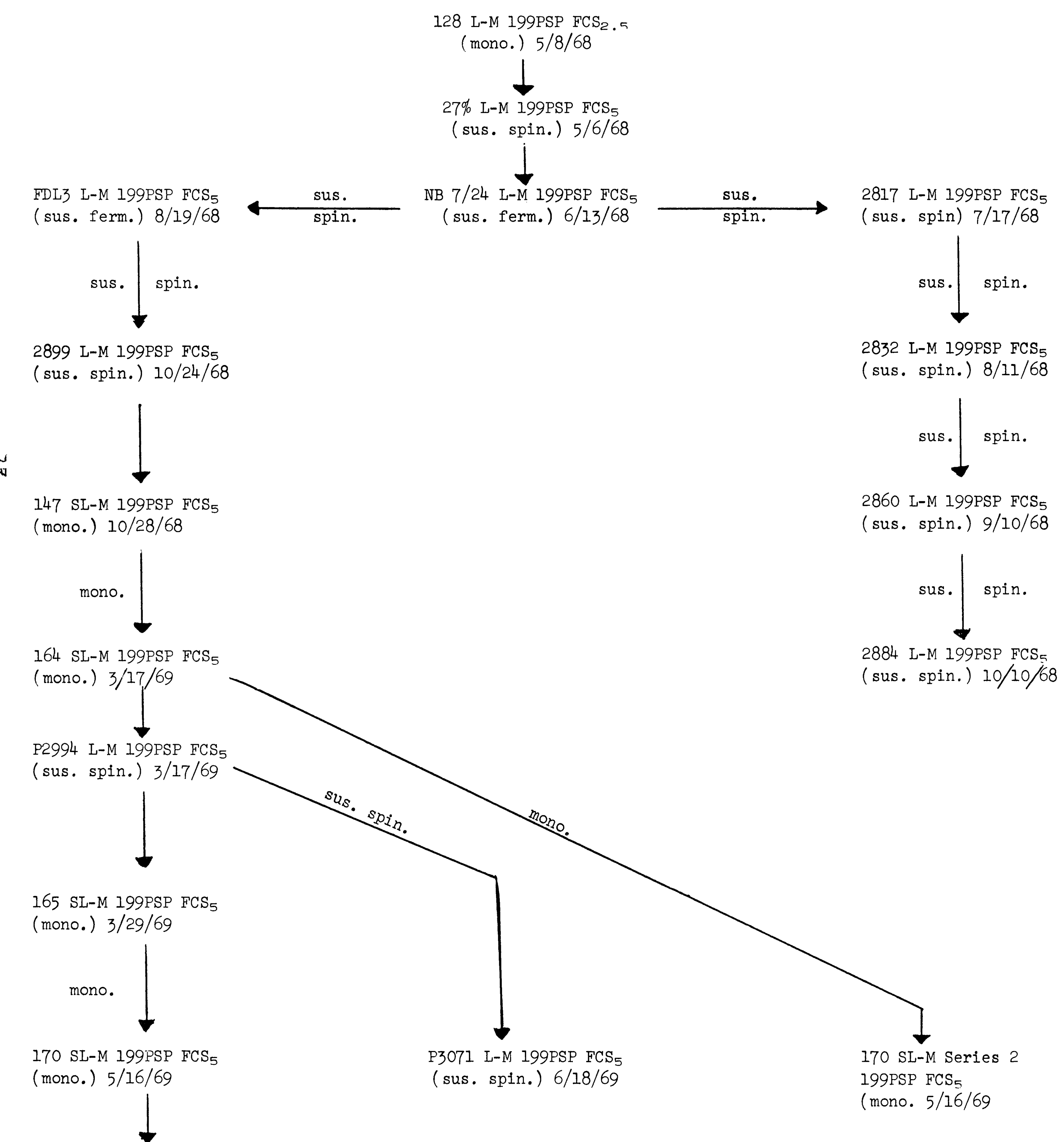

Figure 2C. Origin of L-M monolayers and suspension cultures.

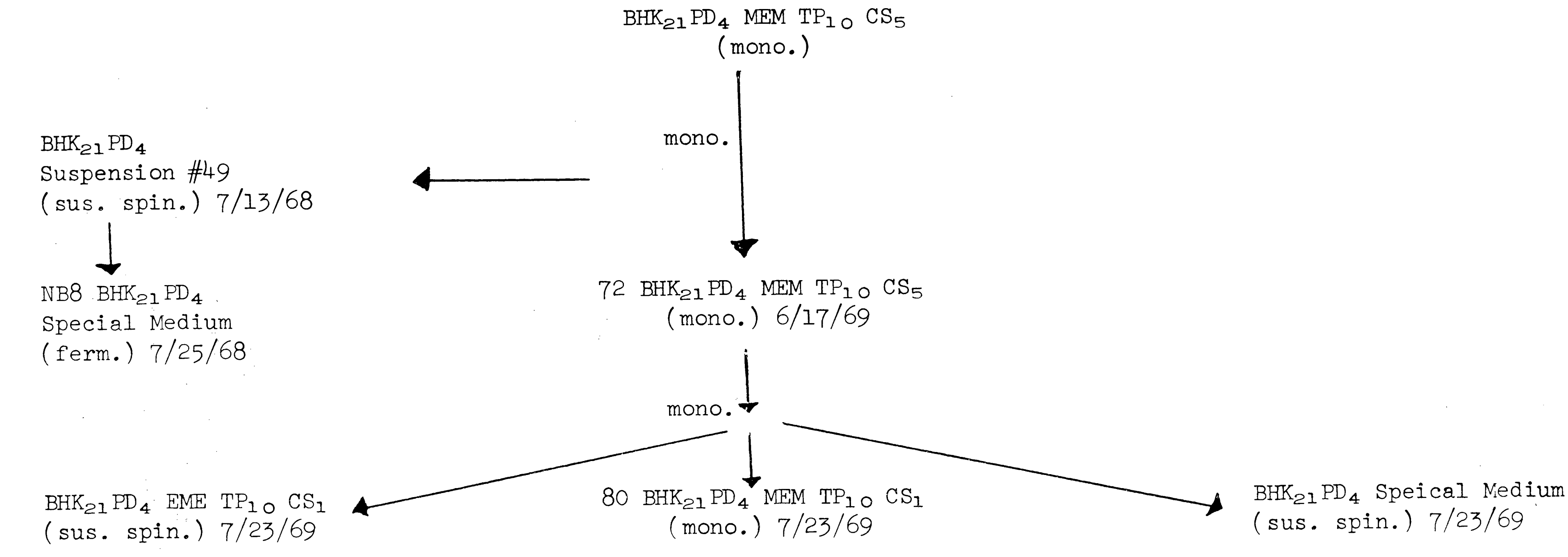

Figure 3C. Origin of the $BHK_{21} PD_4$ monolayers and suspension cultures.

more or one less than normally seen in the majority of F chromosomes. The second type of F derivatives are subtelocentric chromosomes, which appear to have resulted from a structural modification of the F marker, and always involve a more extensive difference than the presence or absence of a constriction. A marker and an apparent derivative are only observed at a low frequency (2%) in the same cell (1).

The history of the L-M cell and the medium and culturing procedures have been reported elsewhere (1,3). Figure 2C presents a diagramatic picture of the history of the L-M cell samples selected for analysis. The number immediately preceding the strain designation of monolayer cultures is the passage number, which indicates the number of subcultures. The growth medium is indicated after the strain designation. This is modified medium 199 (4) plus 0.5% peptone (199P or 199PSP) supplemented with fetal calf serum (FCS). The concentration of serum is denoted by the subscript. The date the culture was initiated is included, as is the mode of culture (sus. = suspension, ferm. = fermenter, spin. = spinner, and mono. = monolayer). Two types of fermenters were employed, with the specific fermenter used for a particular culture indicated by a code preceding the strain designation. NB 7/24 stands for the seventh experiment run in the 5-liter New Brunswick fermenter, equipped for continuous pH and oxidation-reduction potential control, while FDL3 indicates the third experiment run in the 15-liter (L) Fermentation Design fermenter. The number preceding the strain designation of a spinner suspension culture (5) is a culture identification number, not a passage number. Labels on the arrows indicate the mode of intervening culture on the same growth medium (Figure 2C).

The $BHK_{21}PD_4$ cell line was obtained from Dr. Robert Brackett, Parke-Davis and Company, Detroit, Michigan, the PD_4 signifying Parke-Davis Clone 4. A history of this line as carried in this laboratory appears in Figure 3C. The nomenclature used is basically the same as that used to designate L-M cell cultures, with the following abbreviations applicable: MEM = Eagle's minimum essential medium; CS = calf serum; Sched 8 = Schedule 8; Spinner medium = Special Medium; and TP = Tryptose Phosphate Broth.

RESULTS

L-M CELLS

A previous study on the chromosomal complement of L-M cells grown in monolayer on modified 199P showed this strain to have a modal range of 64-65 chromosomes per cell, with other chromosome numbers present at significantly lower frequencies (1). The modal number of biarmed chromosomes fell in the range of 12-14. Two marker chromosomes, the E (a submetacentric with two secondary constrictions) and the F (a subtelocentric with 3-4 secondary constrictions) were noted to occur frequently (in more than 50% of the cells),

and often in combination. The modal number of chromosomes in the L-M cell grown in modified 199P appeared quite stable in monolayer culture over a four-year period. At the end of this period the percentage of modal cells had increased significantly (from 35% to 49%), while the marker chromosome frequency remained nearly the same. Transfer to chemically defined medium (2x concentration of Eagle's basal medium in Hanks BSS) as well as animal passage in C_3H/An mice, were accompanied by shifts in the chromosomal pattern.

It was of interest to learn if population selection could be detected, via chromosomal analysis, as a result of changing the mode of culture. The possibility that selection occurs when a cell strain is transferred from a monolayer system to a suspension system, and finally to a large scale fermenter culture, could have a critical effect on the ability of a large scale culture system to produce uniform results, important for such applications as virus-vaccine production.

Baumann and Merchant (6) studied the chromosomal pattern of 128 L-M 199PSP $FCS_{2.5}$ (Figure 4C) and NB 7/24 L-M 199PSP FCS_5 (Figure 5C) to determine the effect, on the chromosomal pattern of the L-M cell, of transfer from monolayer culture to fermenter culture, via spinner culture. In addition, the effect of transferring L-$M_aT_1C_3$ Clone 2 cells from monolayer to spinner suspension culture was also studied (the L-M_a cell line was derived from in vivo passage of L-M 199P cells (3)). Because of the relevance of this data to the chromosomal analysis of subsequent populations (Figure 2C) the results of this analysis are repeated in the context of the current report. The chromosomal pattern of 128 L-M 199PSP $FCS_{2.5}$ underwent a significant change as a result of its period in spinner and fermenter culture. The number of chromosomes and number of chromosome arms showed an increase of one in modal number coupled with an approximate doubling in the frequency of modal cells (see Table IC). The telocentric chromosomes, which presented a modal range of 48-53 in 128 L-M, had altered to depict a sharp mode of 52 in the NB 7/24 L-M fermenter culture. The modal number of biarmed chromosomes remained unchanged at 13 during this selection pressure, though the frequency of modal cells of this category also roughly doubled. A shift of one chromosome in the modal number of chromosomes in a mixoploid cell line is probably not significant by itself. However, this shift, accompanied by the dramatic increase in the frequency of modal cells, indicates a definite selection in the cell population. The L-$M_aT_1C_3$ Clone 2 line showed small variations in modal number and the frequency of modal cells between monolayer culture (101 L-$M_aT_1C_3$ Clone 2) and spinner suspension culture (2827 L-$M_aT_1C_3$ Clone 2). Because of the magnitude of the variations observed in the L-M_a cell system, it appears that the selection pressure of transferring L-M_a cells from monolayer to spinner culture is small, when compared to the selection observed for the L-M cell transferred from monolayer to the fermenter system. This may be due to the continuous adjustment of the pH and oxidation-reduction potential in the fermenter system, or the fact that the L-M_a cells were only exposed to suspension culture for approximately half the time that the L-M cells were in suspension.

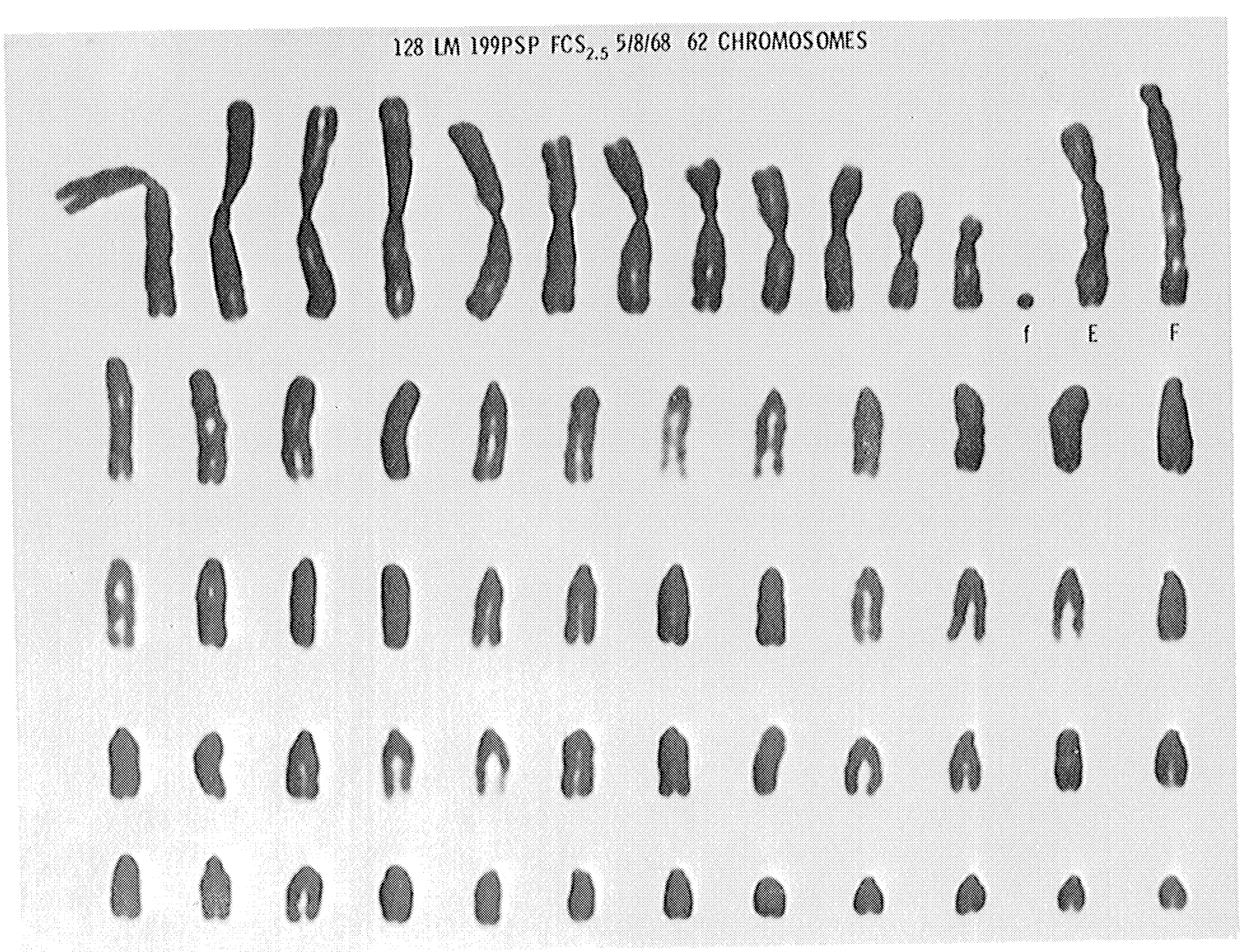

Figure 4C. Karyotype of a cell from 128 L-M grown on modified medium 199 plus 0.5% peptone and 2.5% fetal calf serum in monolayer culture.

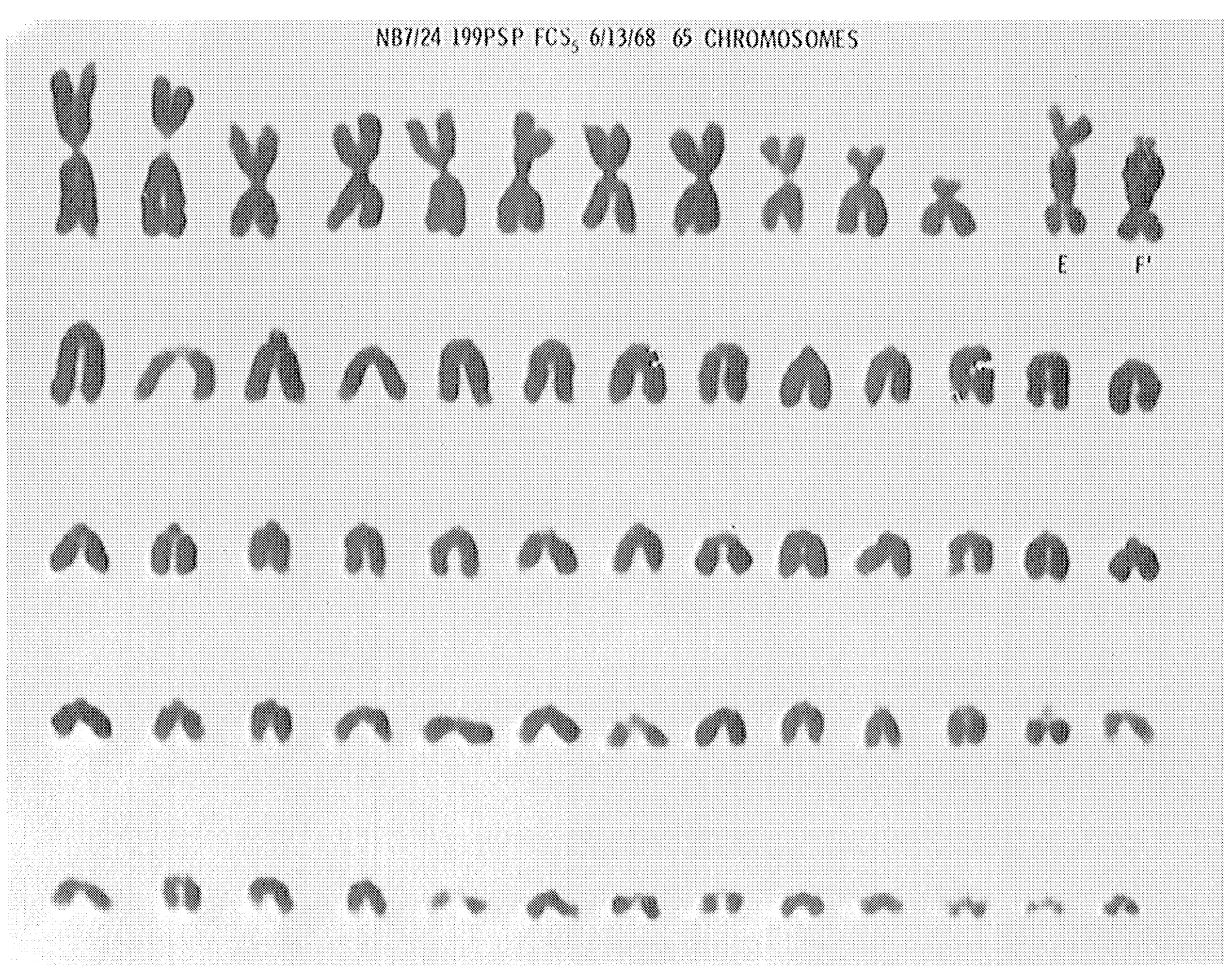

Figure 5C. Karyotype of a cell from NB 7/24 L-M grown on modified medium 199 plus 0.5% peptone and 5% fetal calf serum in fermenter culture.

TABLE IC. Frequency distribution of total chromosome numbers and numbers of biarmed chromosomes in 128 L-M, NB 7/24 L-M, 2817 L-M, 2832 L-M, 2860 L-M, 2884 L-M, P3071 L-M, 170 SL-M Series 3, 170 SL-M Series 2, 101 L-$M_aT_1C_3$ Clone 2, and 2827 L-M_a T_1C_3 Clone 2. The cells were grown in modified medium 199 supplemental with 0.5% peptone and 5% fetal calf serum, with the exception of 128 L-M, which has the fetal calf serum reduced to a concentration of 2.5%.

Cell Line and Date	Number of Chromosomes																	
	51	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69
128 L-M 199PSP FCS$_{2.5}$ 5/8/68 50 cells						1		3	6	4	5	10	12	6	2	1		
NB 7/24 L-M 199PSP FCS$_5$ 6/13/68 50 cells				1						1	4	2	14	26	1	1		
2817 L-M 199PSP FCS$_5$ 7/17/68 50 cells			1			1	2	1	1	3	1	9	8	16	5		2	
2832 L-M 199PSP FCS$_5$ 8/11/68 50 cells									1	1	7	12	18	10	1			
2860 L-M 199PSP FCS$_5$ 9/10/68 50 cells								2	2	7	6	10	15	7	1			
2884 L-M 199PSP FCS$_5$ 10/10/68 50 cells										3	4	11	11	16	5			
P3071 L-M 199PSP FCS$_5$ 6/18/69 150 cells		2		2	4	6	8	13	19	35	25	24	6		3		3	
170 SL-M Series 3 199PSP FCS$_5$ 5/16/69 50 cells				1	1		1	1	5	9	13	9	7	3				
170 SL-M Series 2 199PSP FCS$_5$ 5/16/69 50 cells				1			1	1	3	3	8	21	8	3				1
101 L-$M_aT_1C_3$ C/2 199PSP FCS$_5$ 7/9/68 50 cells	2	1	2	1	2	3	3	2	6	7	11	6	2	1	1			
2827 L-$M_aT_1C_3$ C/2 199PSP FCS$_5$ 7/26/68 50 cells				1		1	1	3	4	8	11	12	8	1				

Cell Line and Date	Number of Biarmed Chromosomes																		
	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
128 L-M 199PSP FCS$_5$ 5/8/68 50 cells		1	4	9	22	13	1												
NB 7/24 L-M 199PSP FCS$_5$ 6/13/68 50 cells				4	40	5	1												
2817 L-M 199PSP FCS$_5$ 7/17/68 50 cells	1	1	2	14	18	13													
2832 L-M 199PSP FCS$_5$ 8/11/68 50 cells		2	4	12	22	5	2	2	1										
2860 L-M 199PSP FCS$_5$ 9/10/68 50 cells		1	5	4	24	14	1	1											
2884 L-M 199PSP FCS$_5$ 10/10/68 50 cells	2		2	11	16	14	4	1											
P3071 L-M 199PSP FCS$_5$ 6/18/69 150 cells	1	1	2	11	57	29	14	7	8	5	4	8			2				1
170 SL-M Series 3 199PSP FCS$_5$ 5/16/69 50 cells				1	20	16	10	3											
170 SL-M Series 2 199PSP FCS$_5$ 5/16/69 50 cells			1	4	25	8	9	3											
101 L-$M_aT_1C_3$ C/2 199PSP FCS$_5$ 7/9/68 50 cells			2	6	20	18	3	1											
2827 L-$M_aT_1C_3$ C/2 199PSP FCS$_5$ 7/26/68 50 cells		1	3	7	15	20	3	1											

From NB 7/24 L-M, a continuous series of spinner suspension cultures was initiated, of which 2817 L-M, 2832 L-M, 2860 L-M, and 2884 L-M are representative samples taken over a three-month period. A striking observation evident on an examination of the chromosomal analysis of these samples is the constant repetition of 13 (the mode in 128 L-M and NB 7/24 L-M) as the modal number of biarmed chromosomes (Table IC). It appears that this characteristic is remarkably stable with respect to the selection pressure of suspension culture. It is also noteworthy that the high frequency of modal cells (biarmed chromosomes) observed in NB 7/24 L-M was not maintained in the spinner culture series. The modal number of chromosomes for each sample of the spinner culture series flucturated from 64-65, a range previously reported for L-M cells grown in monolayer culture on serum free medium (1). Based on changes in the modal numbers of chromosomes from this series and the cited reference, it appears that there is little selection pressure in continuous spinner suspension culture, but strong selection pressure in fermenter culture. However, an examination of the marker frequency and distribution (Table IIIC) of the E and F markers and their respective derivatives, appear to indicate that there was actually a distinct selection as a result of continuous spinner suspension culture. The great difference between the frequency of the F + F' category found in 128 L-M and NB 7/24 L-M compared to the frequency observed in spinner culture, seems to indicate selection on a finer level than chromosome number. With the decrease in frequency observed between 2860 L-M and 2884 L-M (.78 to .58), some doubt is introduced as to the long-term stability of this marker frequency change.

A second major series of cultures (see Figure 2C) was obtained through the subculture of NB 7/24 L-M in several spinner cultures, passage in a 15-liter fermenter culture (FDL3 L-M), followed by further spinner subculture, and monolayer culture (147 SL-M 199PSP FCS_5 10/28/68). The monolayer cultures were given the S prefix at this point to indicate that the cells had been previously cultured in suspension. 164 SL-M was used to continue, unbroken, the current monolayer lineage (SL-M Series 2), as well as to initiate a new suspension culture series, from which monolayers were subsequently planted (SL-M Series 3). Samples were selected from this series to investigate: (1) the reversibility of changes resulting from selection in suspension culture (170 SL-M Series 2 vs. 128 L-M); (2) the selective effect of periodic suspension culture separated by periods of monolayer growth (170 SL-M Series 2 vs. 170 SL-M Series 3); and (3) the effect of continuous suspension culture of cells previously grown in suspension and returned to monolayer culture (P3071 L-M vs. 2817 L-M through 2884 L-M).

"170 SL-M Series 2 vs. 128 L-M": In 170 SL-M Series 2 the modal number of chromosomes showed a decrease of one from the value observed for 128 L-M (64). This was accompanied by a definite rise in the fraction of cells having the modal number of chromosomes. The biarmed chromosome mode remained at 13, with a high percentage of cells in both populations possessing this mode (44% and 50%). The modal number of chromosomes shown by 170 SL-M Series 2 was two less than the strong mode of 65 observed for NB 7/24 L-M. Apparently, there

was a strong selection away from the chromosomal pattern displayed by NB 7/24 L-M in either subsequent suspension culture or monolayer culture. The frequency of the F + F' category was observed to be .48, a value noticeably higher than that detected in 128 L-M, but quite a bit lower than the frequency observed for cells carried continuously in suspension culture (2817 L-M through 2884 L-M). The overall chromosomal pattern of 170 SL-M Series 2 is different from both 128 L-M and NB 7/24 L-M, but whether this series is gradually returning to the pattern of the parent monolayer or is establishing a new chromosome pattern, is not evident.

"170 SL-M Series 2 vs. 170 SL-M Series 3": SL-M Series 3 had a modal number of chromosomes of 62 and a low frequency of modal cells compared to Series 2. Both populations had a high frequency of the biarmed chromosomes mode of 13. Interestingly, the marker frequency (Table IIC) of the F + F' category for 170 SL-M Series 3, .78, is quite consistent with the frequencies noted for 2817 L-M, 2832 L-M, and 2860 L-M, suggesting, again, that the high frequency of this category results from selection in the transfer of L-M cells from monolayer to spinner suspension culture. A further argument in support of this contention is that the comparatively low frequency of this category in Series 2, which was not selected a second time in spinner suspension culture, is due to the fact that the absence of selection pressure allowed the frequency of the F + F' category to drift toward a pattern characteristic of the parent monolayer. The frequency of the E (E') category observed in the parent monolayer (128 L-M), while the slightly higher value noted for this category in Series 3 is closer to the range seen in suspension culture (see Table IIC). We have an indication, on the basis of marker frequency, that suspension culture results in a population selection which may be reversible.

"P3071 L-M vs. 2817 L-M through 2884 L-M": P3071 L-M, which resulted from the spinner suspension culture used to initiate 170 SL-M Series 3, had a modal number of chromosomes of 61, with a significant number of cells having 62 and 63 chromosomes (see Table IC). The spinner suspension culture series 2817 L-M through 2884 L-M, characterized by modal numbers of chromosomes from 64 to 65, appears to have a significantly different population structure based on chromosome number, although all these samples had a modal number of 13 biarmed chromosomes (Figure 6C). This downward shift in the modal number of chromosomes observed for P3071 L-M may be a result of two factors: (1) the total time P3071 L-M was exposed to the selection pressure of suspension culture was roughly 7-1/2 months compared to 4 months for 2884 L-M; and (2) an intervening period of monolayer culture in the history of P3071 L-M of approximately 5 months. In any case, it is obvious that variations in culture method, such as switching between monolayer culture and suspension systems causes significant flucturations in the chromosomal pattern.

$BHK_{21}PD_4$ CELLS

The chromosomal pattern of $BHK_{21}PD_4$ cells closely resemble the normal

TABLE IIC. Frequency and distribution of the marker chromosomes in 128 L-M, NB 7/24 L-M, 2832 L-M, 2860 L-M, 2884 L-M, 1% SL-M Series 2, 1% SL-M Series 3, P3071 L-M.

Cell Line and Date	Marker Frequency and Distribution (%)				
	None	E or E'	F or F'	Expected E or E' + F or F'	Observed E or E' + F or F'
128 L-M 199PSP $FCS_{2.5}$ 5/8/68 50 cells	22	78	20	15.6	20
NB 7/24 L-M 199PSP FCS_5 6/13/68 50 cells	10	90	20	18.0	20
2817 L-M 199PSP FCS_5 7/17/68 50 cells	2	80	76	60.8	58
2832 L-M 199PSP FCS_5 8/11/68 50 cells	2	94	74	69.6	70
2860 L-M 199PSP FCS_5 9/10/68 50 cells	4	84	78	65.5	66
2884 L-M 199PSP FCS_5 10/10/68 50 cells	2	92	58	53.3	52
P3071 L-M 199PSP FCS_5 6/18/69 150 cells	23	82	88.7	73.5	72.7
170 SL-M Series 3 199PSP FCS_5 5/16/69 50 cells	2	94	78	73.3	74
170 SL-M Series 2 199PSP FCS_5 5/16/69 50 cells	14	78	48	37.4	40

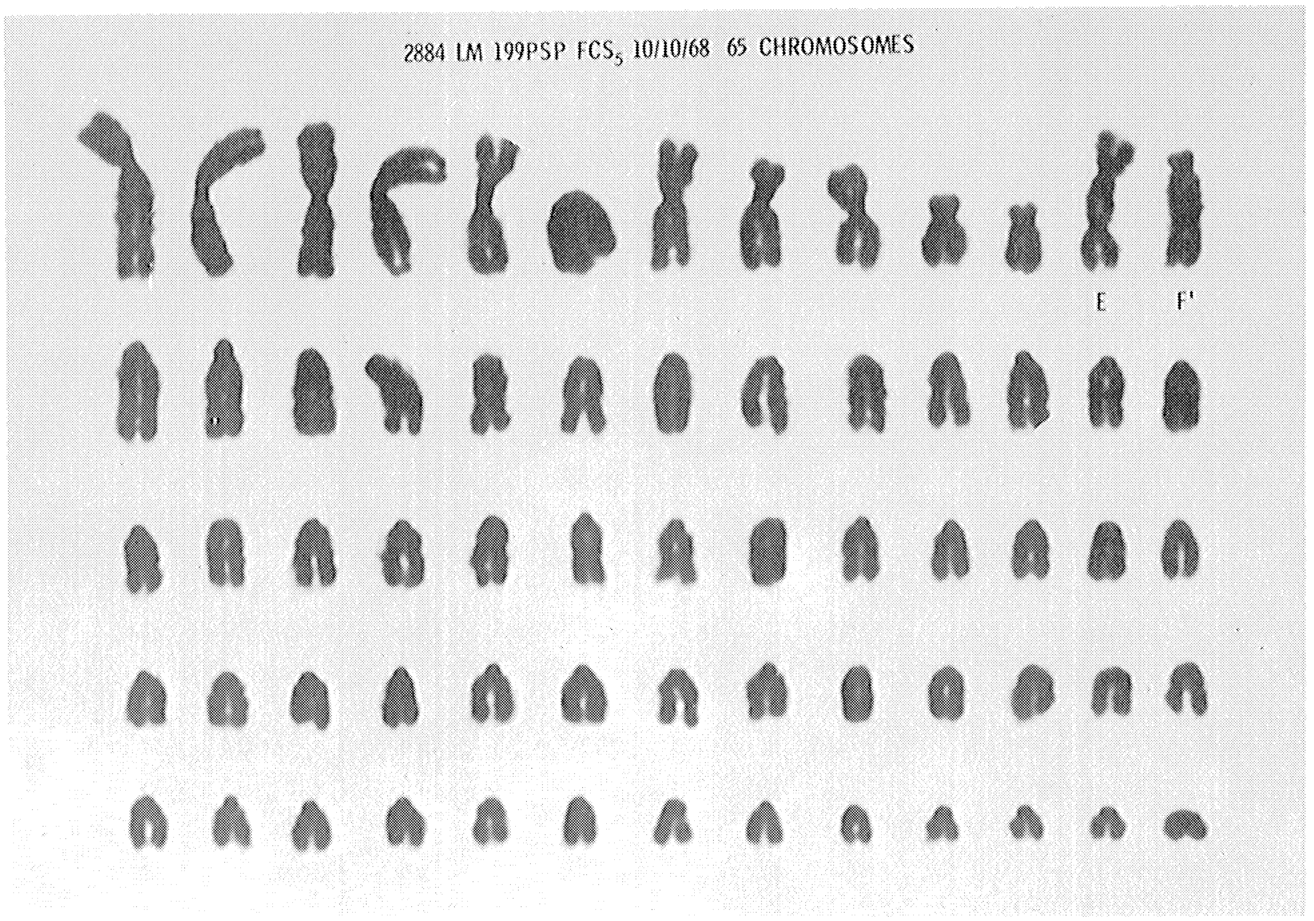

Figure 6C. Karyotype of a cell from 2884 L-M grown on modified medium 199 plus 0.5% peptone and 2.5% fetal calf serum in spinner culture.

pattern presented by the Syrian or Golden Hamster (7), which has four pairs of telocentric chromosomes, a pair of very small subtelocentrics (mT), a pair of very small metacentrics (sM), and recognizable X and Y chromosomes, with the remaining 15 pairs all biarmed chromosomes of intermediate lengths and ratios. BHK_{21} cells (Clone 13) have been reported to be uniformly male (8). The fact that there are three pairs of chromosomes and one group of four pairs, which may be readily detected and monitored to follow genotypic changes during in vitro culture, makes this near diploid continuous cell line particularly well suited to studies where it is desired to follow population selection based on karyotypic changes.

$BHK_{21}PD_4$ suspension no. 49 had a high frequency of cells with 43 and 44 chromosomes (see Table IIIC), with the diploid number predominating. The modal number of biarmed chromosomes was 36, which is equal to the number of biarmed chromosomes in the normal Syrian hamster (scoring the mT chromosomes as biarmed).

NB 8 $BHK_{21}PD_4$ showed a bimodal pattern of chromosome number, with males of 42 and 44. Figure 7C shows a pseudo diploid cell with 3 mT chromosomes, the normal number of telocentrics, and a normal pair of XY chromosomes, but the cell is missing one biarmed chromosome. The modal number of biarmed chromosomes was 34, as a result of grouping the mT chromosomes with the telocentrics, which was done in this ans subsequent analysis of $BHK_{21}PD_4$ cells. Apparently the transfer of $BHK_{21}PD_4$ cells to fermenter culture results in population selection somewhat different from that observed for the L-M cell (i.e., increased uniformity for the L-M cell system versus increased variability for the $BHK_{21}PD_4$ cell system).

72 $BHK_{21}PD_4$ also showed a bimodal pattern of chromosome number with modes of 44 and 45 (76% of the cells fell in this range), and a biarmed mode of 33. The variability in the modal number of chromosomes was due to variations in the number of telocentric chromosomes. Comparing the karyotype of a cell with 44 chromosomes (Figure 8C), to a cell with 45 chromosomes (Figure 9C), one notes several interesting differences. Figure 8C shows a cell with only slight deviations from the normal Syrian hamster karyotype, i.e., a missing biarmed chromosome and an extra mT chromosome. In Figure 9C we see a cell with the normal (hamster) number of biarmed chromosomes (excluding mT), but with an extra telocentric as well as an extra mT chromosome. In addition, the size variations in the two chromosomes labelled sM may not be entirely due to differential coiling, but may represent a structural rearrangement. Another cell with 45 chromosomes, showed an extra pair of telocentrics and a missing pair of biarmed chromosomes in addition to the typical extra mT chromosome (Table IVC). It is apparent that $BHK_{21}PD_4$ cells vary more than is shown by chromosomes' number alone.

TABLE IIIC. Frequency distribution of total chromosome numbers and numbers of biarmed chromosomes in $BHK_{21} PD_4$ suspension no. 49, NB 8 $BHK_{21} PD_4$ Special Medium, 72 $BHK_{21} PD_4$ MEM TP_{10} CS_5, 80 $BHK_{21} PD_4$ MEM TP_{10} CS_1, $BHK_{21} PD_4$ sus. spin. MEM TP_{10} CS_1, $BHK_{21} PD_4$ sus. spin. Special Medium (6).

Cell Line and Date	Number of Chromosomes													Number of Biarmed Chromosomes													
	35	36	38	39	40	41	42	43	44	45	56	57	58	22	24	25	27	28	29	30	31	32	33	34	35	36	37
$BHK_{21} PD_4$ sus. spin. #49 7/13/68						1	3	14	22	8	1												2	5	11	27	4
NB8 $BHK_{21} PD_4$ Special Medium Ferm. 7/25/68	1		1		1	6	12	6	14	5	1	3						1		1	2	1	5	17	9		
72 $BHK_{21} PD_4$ MEM TP_{10} CS_5 6/17/69							1	7	18	20	4									1	2	8	28	10	1		
80 $BHK_{21} PD_4$ MEM TP_{10} CS_1 7/23/69						3	5	9	19	11	3										4	7	13	17	9		
$BHK_{21} PD_4$ MEM TP_{10} CS_1 (sus. spin.) 7/23/69			1	1		4	10	11	11	9	0	3	1	1	1	1	1		1	4	8	9	14	2	3		
$BHK_{21} PD_4$ Special Medium (sus. spin.) 7/23/69		1			1	2	11	10	12	8	2	0	2				1				6	15	21	3	1	1	1

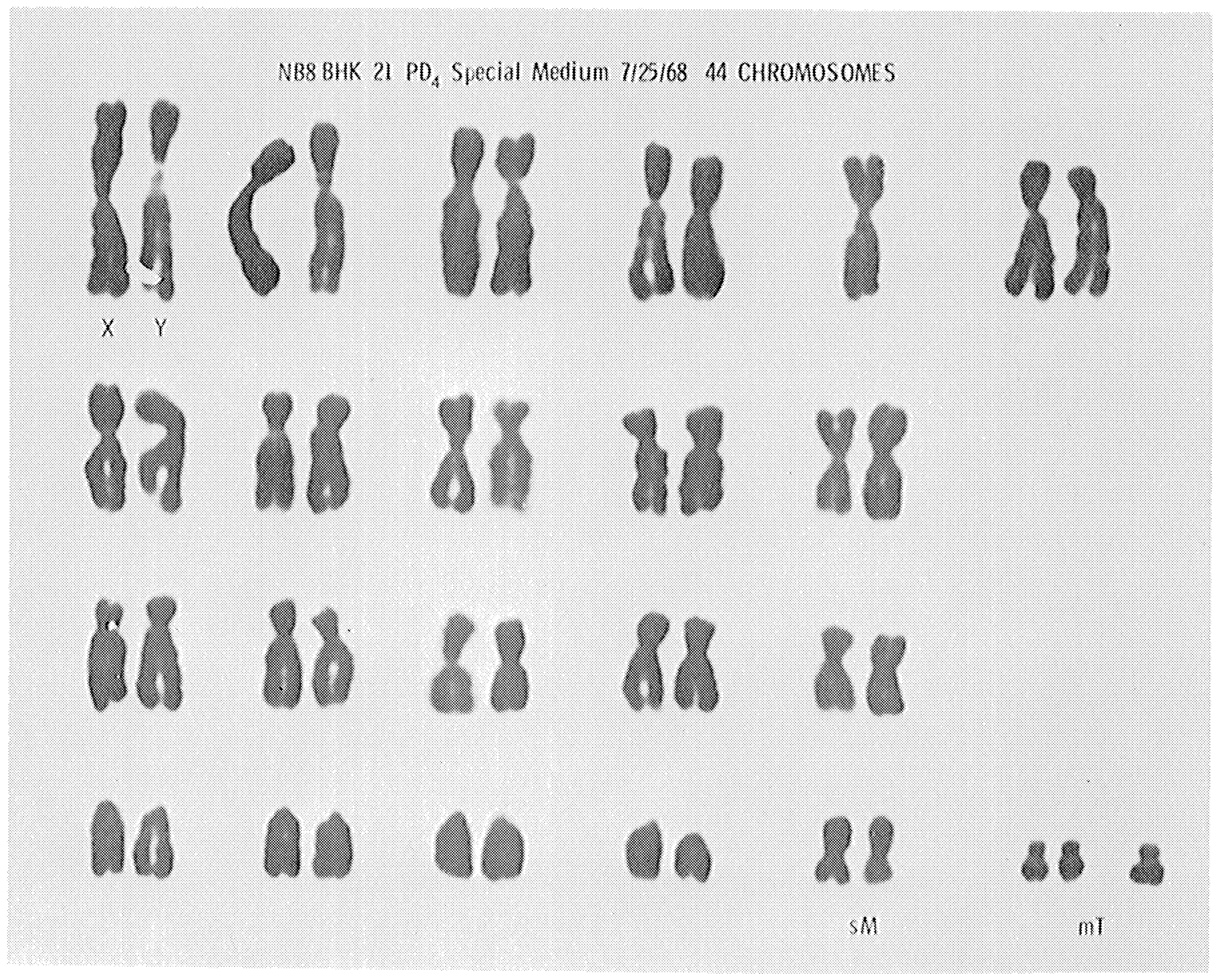

Figure 7C. Karyotype of a cell from NB 8 $BHK_{21}PD_4$ grown on Schedule 8 spinner medium in fermenter culture. Cell is pseudo-diploid.

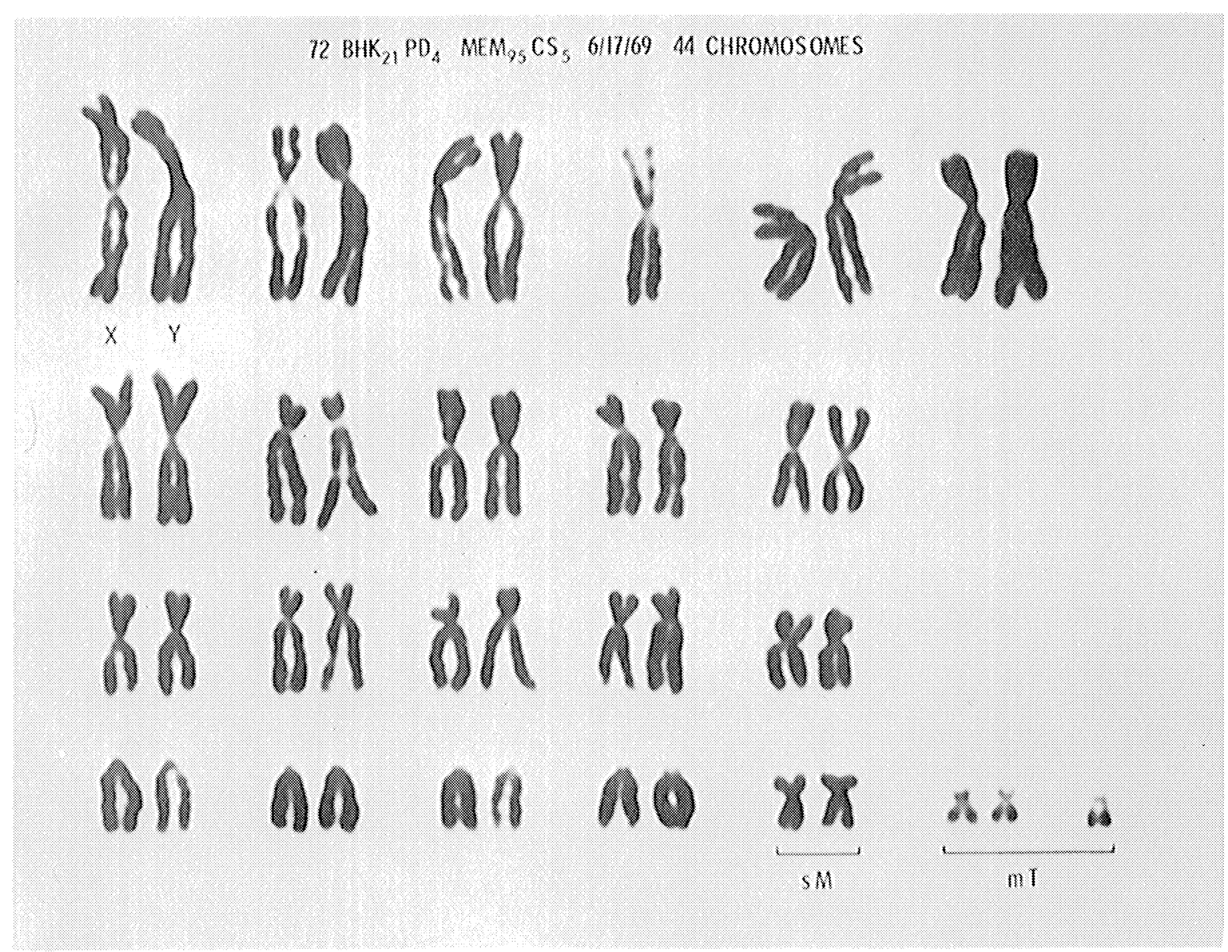

Figure 8C. Karyotype of a cell from 72 $BHK_{21}PD_4$ grown on Eagle MEM (supplemented with 10% Tryptose Phosphate Broth) plus 5% calf serum in monolayer culture. Cell is pseudo-diploid.

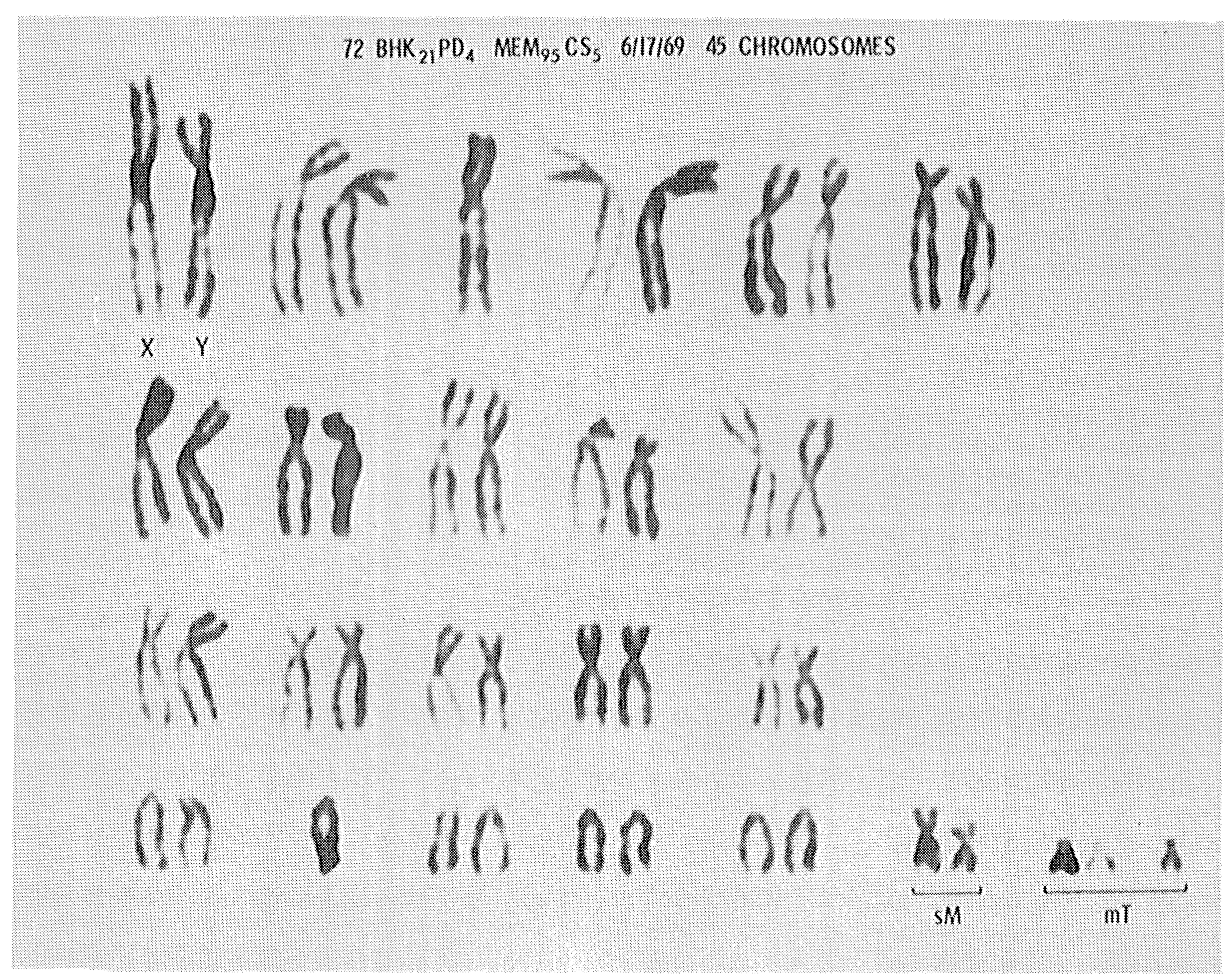

Figure 9C. Karyotype of a cell from 72 $BHK_{21}PD_4$ grown on Eagle MEM (supplemented with 10% Tryptose Phosphate Broth) plus 5% calf serum in monolayer culture. Cell is near diploid.

TABLE IVC. Frequency and distribution of mT and Q chromosomes in 72 $BHK_{21}PD_4$ (mono.), 80 $BHK_{21}PD_4$ (mono.), $BHK_{21}PD_4$ (spinner) MEM TP_{10} CS_1, $BHK_{21}PD_4$ (spinner) Special Medium.

Cell Line and Date	Marker Frequency (%)	
	3 or More mT	Q
72 $BHK_{21}PD_4$ MEM TP_{10} CS_5 6/17/69 50 cells	86	
80 $BHK_{21}PD_4$ MEM TP_{10} CS_1 7/23/69 50 cells	100	40
$BHK_{21}PD_4$ (sus. spin.) MEM[1] TP_{10} CS_1 7/23/69 50 cells	76	14
$BHK_{21}PD_4$ (sus. spin.) Special Medium 7/23/69 50 cells	70	0

[1]Methocel added.

80 $BHK_{21}PD_4$, grown on a medium with a reduced concentration of calf serum (1%), had a pronounced modal number of chromosomes of 44, with a modal number of biarmed chromosomes of 33 (mT scored as a telocentric). Three or more mT chromosomes were present in all of the cells examined, indicating that all the modal cells were pseudo-diploid. In addition, a new marker chromosome was observed, which had two secondary constrictions and a light staining (sometimes "stretched") region in the short arm adjacent to the centromere. This new marker, designated the Q marker (see Figure 10C), is quite likely a derivative of the Y chromosome, and was found in 40% of the cells examined. The appearance of the Q marker at this point in the culture history may be a consequence of the reduced calf serum concentration. The 1% level of calf serum did not produce any dramatic effect on the modal number of chromosomes, though it is noteworthy that, in comparison with 72 $BHK_{21}PD_4$, the fraction of the population having 45 chromosomes was reduced (see Table IIIC).

Two spinner suspension cultures were studied at the same time 80 $BHK_{21}PD_4$ was analyzed. One culture was grown on Eagle's MEM plus 10% TP and 1% CS (hereafter referred to as Spinner 2). Both cultures showed a biarmed chromosome mode of 33. The surprising observation was the wide range in modal number of chromosomes shown by both cultures (42-45). Of further interest was the frequency of the Q marker, which was 0 for Spinner 2 (high concentration of calf serum) and 14% for Spinner 1 (low concentration of calf serum), an additional indication that this marker may be selected by low calf serum concentration. From a comparison with $BHK_{21}PD_4$ suspension no. 49 7/13/68, it is evident that either or both of the following have occurred: (1) the stock $BHK_{21}PD_4$ cell line has changed over a one-year period to the extent that

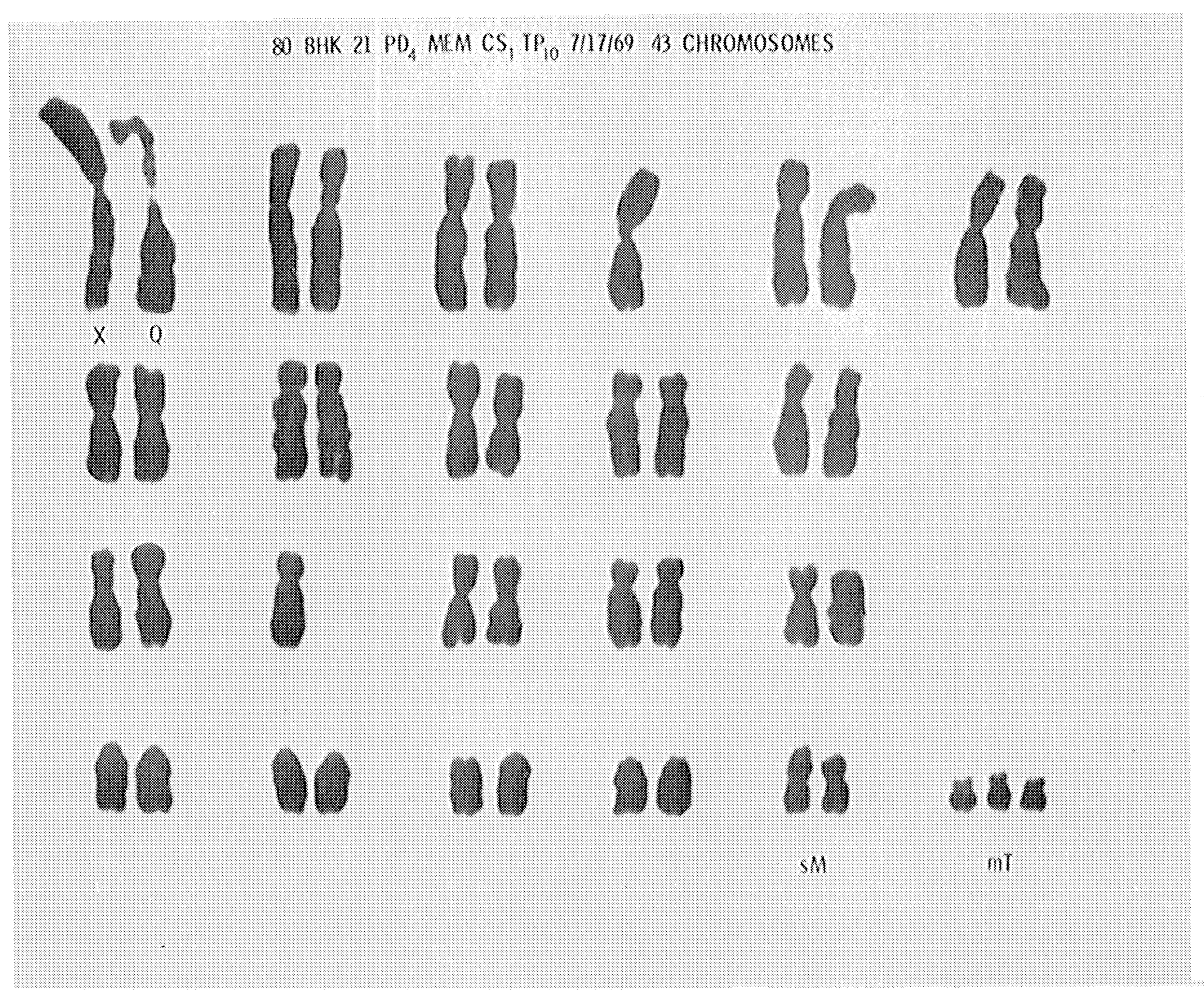

Figure 10C. Karyotype of a cell from 80 $BHK_{21}PD_4$ grown on Eagle MEM (supplemented with 10% Tryptose Phosphate Broth) plus 1% calf serum in monolayer. Cell is near diploid.

it responds differently to the selection pressure of spinner culture; (2) the spinner culture system is significantly different.

DISCUSSION

From the data presented on the L-M cell, it appears that fermenter culture exerts a strong selection pressure on the cell population. Several additional points which are evident are: (1) switching between various modes of culture produces changes in the genotypic constitution of the cell population; (2) the chromosome pattern observed in suspension culture is significantly effected by the prior modes of culture; (3) some changes resulting from population selection may be reversible over a short period of time (i.e., marker frequency and distribution), while other changes (modal number of chromosomes), may not be quickly reversible; (4) the L-M cell can be maintained in spinner suspension culture without major changes in the chromosomes pattern over a three-month period; and (5) that marker frequency appears to be a useful characteristic in evaluating the selective effects of various modes of culture, particularly spinner suspension.

It appears that a biarmed chromosome mode of 13 is an unusually stable characteristic of the L-M cell grown on 199PSP and fetal calf serum, as this value did not change under a variety of selection pressures. The consistency with which modal values fluctuated closely above the previous finding reported for the L-M cell on 199P (1) indicates that the line is probably of greater stability now than when dealt with in 1959 (9). It is certainly indicated that investigators desiring to produce large-scale cultures of consistent properties with respect to virus susceptibility, growth requirements, etc., must consider the selective effects of the sequence of different types of culture on the gernotypes and phenotypes present in any given population.

Data on the $BHK_{21}PD_4$ cell line has shown that, in comparison to the L-M cell, selection in large scale fermenter culture may not take the same direction in different cell lines (i.e., L-M cell showed increased population uniformly, while the $BHK_{21}PD_4$ population showed increased variability). Karyotypes of several $BHK_{21}PD_4$ cells indicated that the variability of this cell line is somewhat greater than is indicated by a diploid or near diploid modal number of chromosomes. The appearance of the Q marker in monolayer culture and the different frequencies observed, when this marker was scored in spinner cultures of $BHK_{21}PD_4$ cells in two different medias, may prove to be a useful system for evaluating population selection in this near diploid line.

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GROWTH OF STRAIN L-M MOUSE CELLS IN CHEMICALLY DEFINED MEDIUM

Gary Stoner

INTRODUCTION

The minimum essential amino acid requirements for maintenance of strain L mouse fibroblast cells were reported by Eagle (1). Thirteen amino acids; viz., arginine, lysine, tryptophan, histidine, leucine, isoleucine, valine, cystine, methionine, glutamine, phenylalanine, tyrosine, and threonine were found to be essential for cell maintenance. Since Eagle's original report, strain L mouse fibroblast cells have been adapted to grow in a variety of chemically defined media, and analyses of amino acid utilization by the cells in these media have been studied. However, most of these media are very complex and contain nonessential amino acids. Merchant and Hellman reported growth of strain L-M mouse fibroblast cells in Eagle's basal media at (2X) concentration containing only the thirteen essential amino acids (2). This medium is the simplest medium ever described for continuous propagation of mammalian cells. This report will contain data obtained on the kinetics of amino acid utilization and production by strain L-M mouse fibroblast cells in "(2X) Eagles" medium.

MATERIALS AND METHODS

Medium was prepared so that the only variables to be studied were the amino acids themselves. The medium was incubated for 48 hr at 37°C. Glutamine was stored separately at -20°C and added to the medium after incubation to prevent spontaneous decomposition of glutamine at 37°C. Medium was not changed throughout the growth curve.

Monolayer cultures in 30 cm^2 Falcon flasks were inoculated at an original concentration of 3.0×10^5 cells/ml. Three replicate cultures were harvested at 24-hr intervals; medium was stored at -20°C for subsequent amino acid analysis, and after washing with Hanks BSS, cells were stored at -70°C for subsequent protein analysis by the Eagle-Oyama modification of the Lowry method. A final cell count was taken at the end of the growth curve.

Amino acid analyses were performed with a Beckman Model 120C amino acid analyzer. Both short and long columns were internally standardized so that any malfunction of the machine could be detected. The short column was standardized by the addition of a standard amount of α-amino-β-guanidino-proprionic acid and the long column with a standard amount of L-norleucine. Chromatogram peak heights and widths were determined; the values were converted by a computer to the concentration of amino acids in micromoles/ml medium. The computer program accounted for the dilution of the medium by addition of the standards. It was necessary to run two long column analyses because glutamine occluded the threonine-serine peaks. Glutamine could be removed completely by hydrolysis with an equal volume of 2NHCL for 1-1/2 hr at 100°C.

RESULTS

A. GROWTH OF STRAIN L-M CELLS IN "(2X) EAGLE" BASAL MEDIUM (CONTAINING ONLY THE THIRTEEN ESSENTIAL AMINO ACIDS)

Table ID indicates the kinetics of utilization of the thirteen essential amino acids by the cells. Throughout the growth curve, it is apparent that the extent and rate of depletion of the individual amino acids varies. For instance, arginine and cystine are utilized very rapidly, whereas lysine is not utilized until the later stages of growth (perhaps along with proline for the synthesis of collagen). Isoleucine, leucine, and methionine are nearly depleted throughout growth and glutamine is entirely depleted. Figure 1D indicates that at 72 hr, ammonia production nearly parallels glutamine utilization indicating that the cells are removing the amide nitrogen of glutamine resulting in the production of ammonia. This suggests, as indicated by Eagle (3), that the amount of glutamine utilized as such for cellular biosynthetic processes is relatively small; glutamine is either converted to glutamic acid for subsequent utilization by the cells or is converted to pyrollidone carboxylic acid.

Figures 2D and 3D indicate that a number of nonessential amino acids are produced into the medium during growth. Alanine, proline, and to some extent, serine, appear to be reutilized by the cells. Glycine production occurs most significantly at the later stages of growth indicating that it may be released by plateau and decline phase cells. There is no evidence for the release of asparagine.

Figure 3D indicates the production of protein by strain L-M mouse fibroblast cells in "(2X) Eagle" medium. In this system, 130 μg protein is equivalent to 1.2×10^6 cells/ml medium. It has repeatedly been observed that protein synthesis ceases within 24 hr after glutamine is depleted, suggesting that glutamine is growth-limiting. If glutamic acid is added to the medium at a concentration of 4 mM and glutamine at 0.4 mM, growth ceases within 24 hr after glutamine is depleted, suggesting that glutamic acid will not substitute for glutamine. Also, glutamic acid does not contribute to the production of ammonia. A good correlation can be obtained when computing the conversion of essential amino acid nitrogen into protein nitrogen, nonessential amino acid nitrogen and ammonia.

B. GROWTH OF STRAIN L-M MOUSE CELLS IN "(2X) EAGLES" BASAL MEDIUM SUPPLEMENTED WITH NONESSENTIAL AMINO ACIDS

Nonessential amino acids were added to the initial medium at a concentration approximately equal to that at which they were produced at the maximum level of protein synthesis in (A) above. It can be observed in Table IID that the cells produced much smaller quantities of nonessential amino acids

TABLE ID. Micromoles of Essential Amino Acids Available/ml of Medium at Various Times Throughout Growth of Strain L-M Cells in "2X Eagle" Basal Medium

Amino Acid	Time, hr							
	0	24	48	72	96	120	144	168
	μm/ml	μm/ml	μm/ml	μm/ml	μm/ml	μm/ml	μm/ml	μm/ml
Lysine	0.310	0.310	0.310	0.300	0.300	0.290	0.260	0.200
Histidine	0.080	0.080	0.070	0.060	0.040	0.040	0.040	0.030
Arginine	0.210	0.170	0.150	0.110	0.090	0.070	0.070	0.060
Tryptophan	0.030	0.020	0.020	0.010	Trace	Trace	Trace	Trace
Threonine	0.260	0.2412	0.240	0.235	0.230	0.230	0.210	0.210
Valine	0.350	0.320	0.280	0.240	0.200	0.140	0.100	0.030
Cystine	0.190	0.130	0.090	0.070	0.060	0.055	0.050	0.050
Methionine	0.070	0.060	0.060	0.050	0.030	0.020	0.010	Trace
Isoleucine	0.350	0.260	0.220	0.190	0.160	0.090	0.050	0.010
Leucine	0.340	0.250	0.210	0.170	0.130	0.L70	0.030	0.010
Tyrosine	0.180	0.140	0.130	0.120	0.110	0.100	0.090	0.080
Qalanine	0.170	0.150	0.150	0.130	0.120	0.110	0.100	0.080
Glutamine	4.020	3.150	2.210	1.300	0.310	Trace	0	0

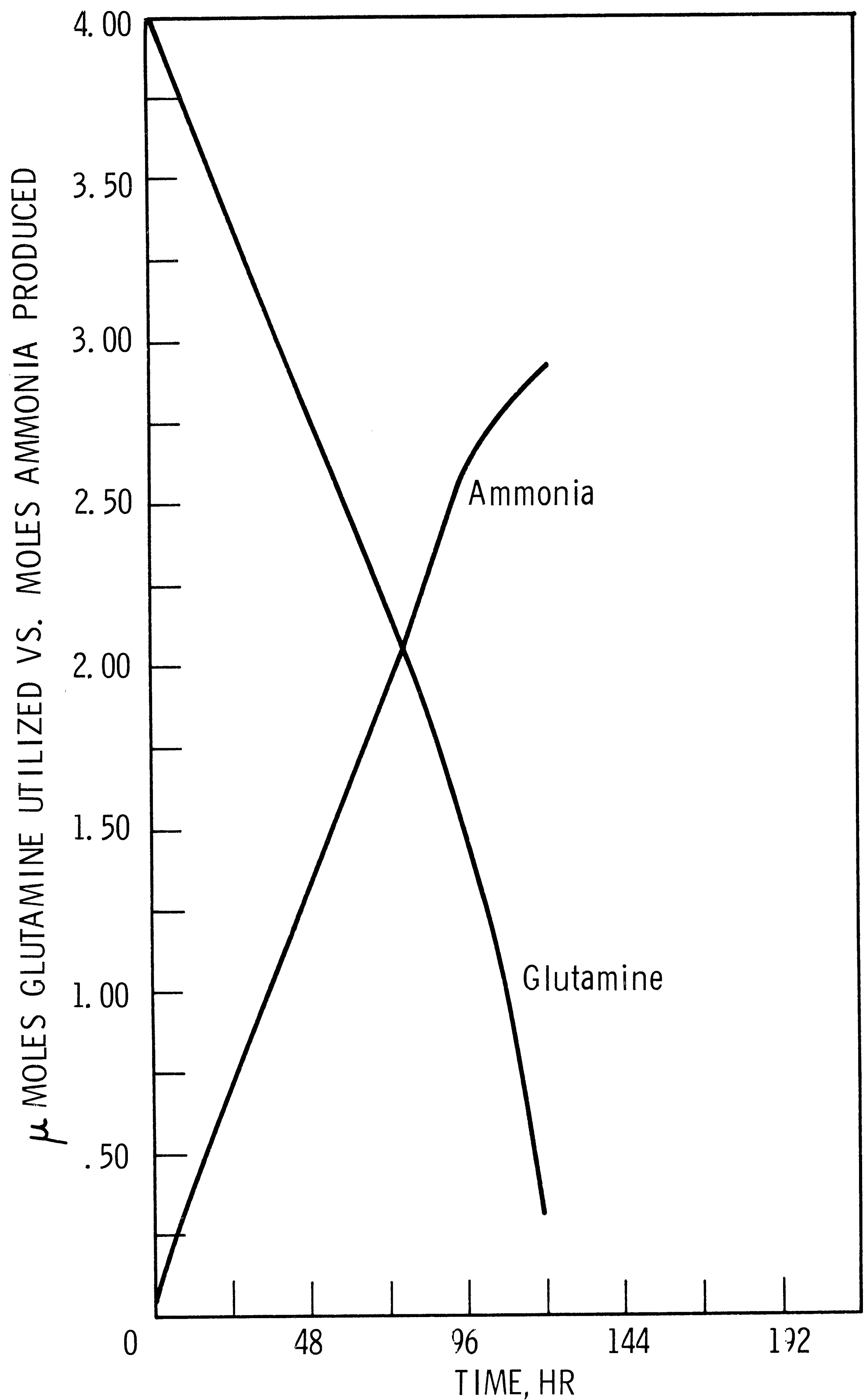

Figure 1D. Utilization of glutamine and production of ammonia by L-M cells in monolayer cultures in 2X Eagle basal medium.

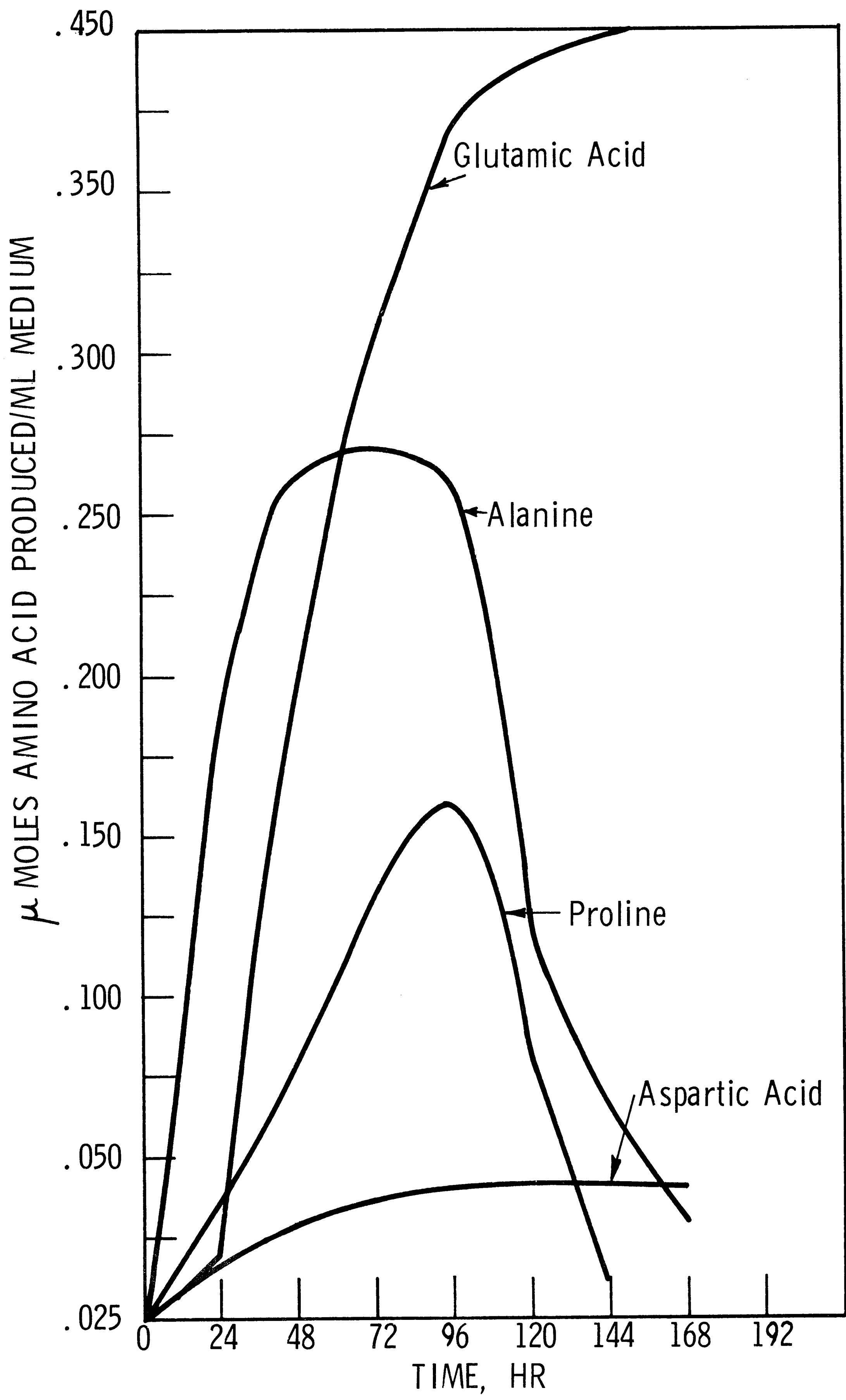

Figure 2D. Production and utilization of nonessential amino acids by L-M cells in monolayer culture in 2X Eagle basal medium.

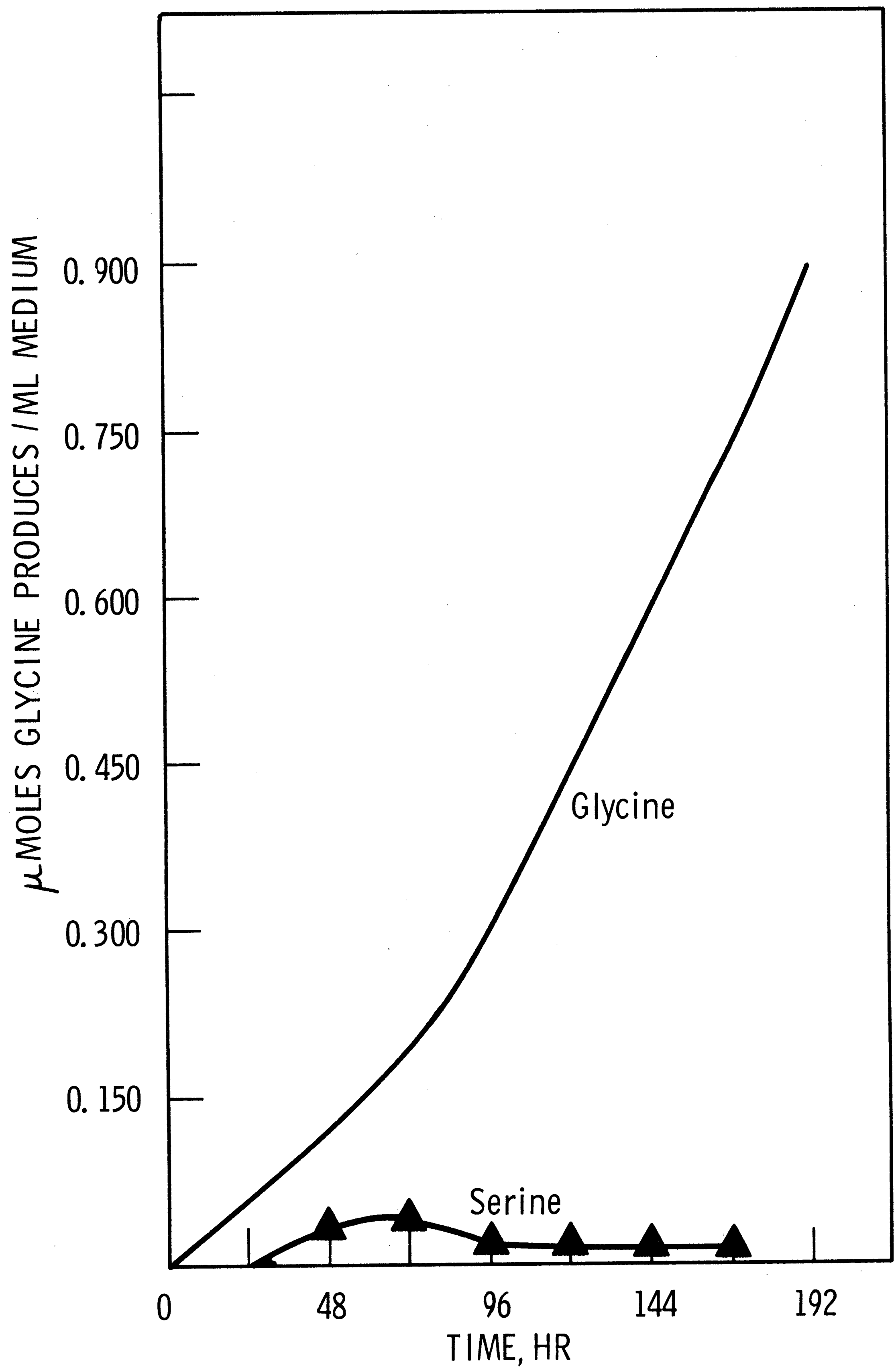

Figure 3D. Production of the nonessential amino acids glycine and serine by monolayer cultures of L-M cells in 2X Eagle basal medium.

TABLE IID. Comparison of the Production and Utilization of Nonessential Amino Acids by Strain L-M Cells in (2X) Eagle Basal Medium With and Without added Nonessential Amino Acids. Values Represent Maximum Amounts Produced and/or Utilized.

Medium	Amino Acid	μ Moles Amino Acid in Initial Medium	μ Moles Amino Acid Produced/ml Medium	μ Moles Amino Acid Utilized/ml Medium
(2X) Eagle	Glutamic acid	0	0.450	0.000
	Aspartic acid	0	0.037	0.000
	Alanine	0	0.270	0.240
	Serine	0	0.039	0.015
	Proline	0	0.160	0.150
	Glycine	0	0.900	0.000
(2X) Eagle plus added nonessential amino acids	Glutamic acid	0.450	0.130	0.050
	Aspartic acid	0.050	0.005	0.025
	Alanine	0.350	0.075	0.325
	*Serine	0.150	-	-
	Proline	0.170	0.000	0.070
	Glycine	0.450	0.450	0.000

*Serine production and utilization in medium containing nonessential amino acids has not been determined.

into the medium when they were added exogenously. In fact, with the exception of alanine and glycine, their concentration in the medium showed little variation throughout growth, suggesting that the amounts added were sufficient to maintain the necessary extra-intracellular gradient, so that excesses were not produced into the medium. Undoubtedly the cells are synthesizing nonessentials throughout growth, but are either pooling them intracellularly or synthesizing only those amounts required for cellular biosynthetic processes.

Figure 4D indicates the production of protein by strain L-M cells in "(2X) Eagel" medium containing added nonessential amino acids. It is evident that the addition of nonessential amino acids did not result in a more rapid growth rate or larger growth yield. In fact, both parameters were reduced. However, this experiment will be repeated.

C. GROWTH OF STRAIN L-M MOUSE CELLS IN "(2X) EAGLE" BASAL MEDIUM CONTAINING (4X) CYSTINE

Cystine was originally found to be growth-promoting for strain L mouse cells by Healy, Fisher, and Parker. Morgan and Morton reported a requirement for L-

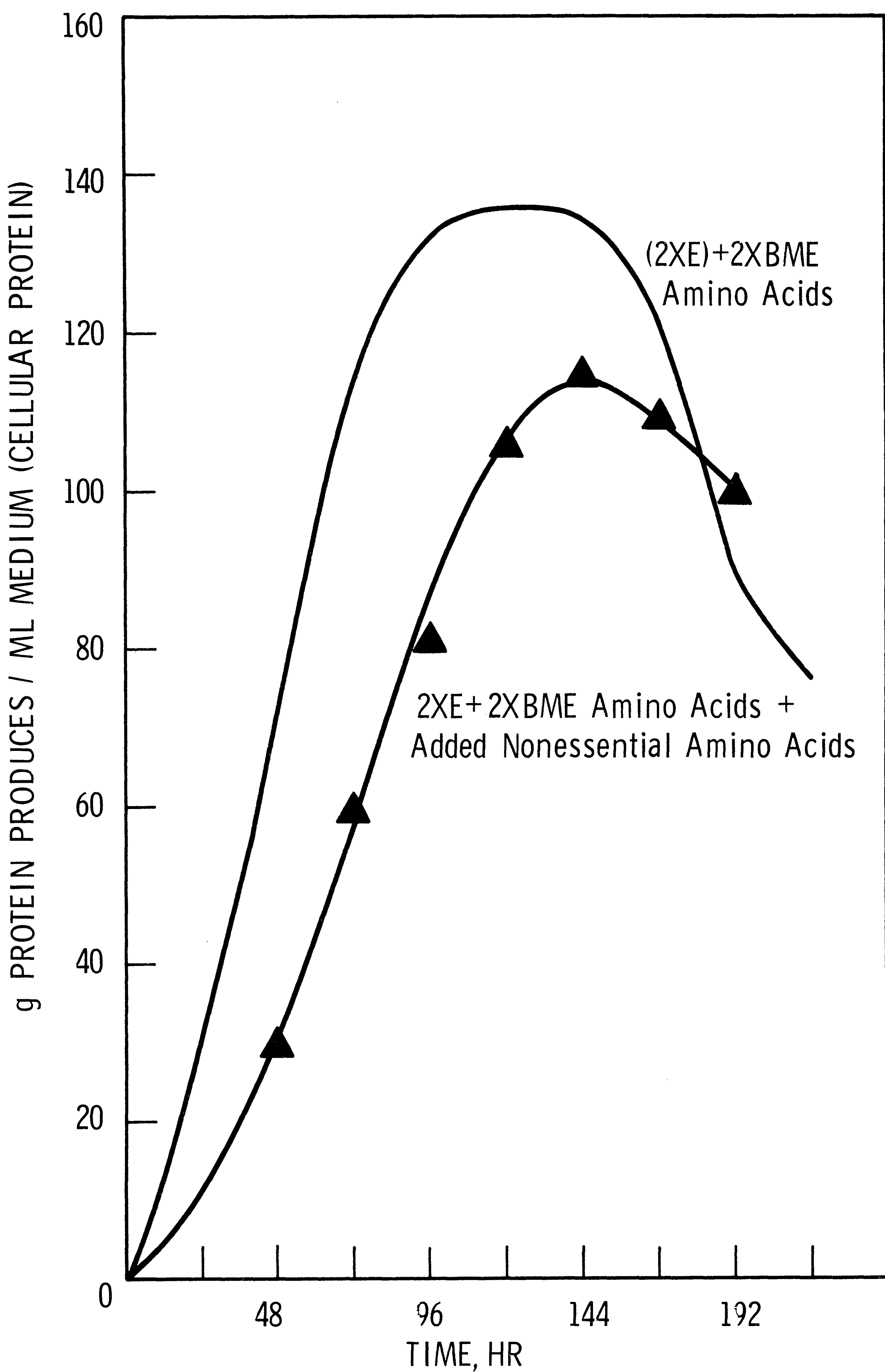

Figure 4D. Protein production by L-M cells in monolayer cultures of L-M cells in 2X Eagle basal medium with and without added nonessential amino acids.

cystine with heart muscle cells (5). As shown in Figure 5D, it is evident that addition of cystine at twice its normal concentration in "(2X) Eagles" medium results in a significant increase in growth rate although the cell yield is similar to that in "(2X) Eagle" medium. This indicates that the cells possess a larger requirement for (-SH-) groups than is provided by "(2X) Eagle" medium alone. Alternatively, cystine is converted to the reduced form, cysteine, which perhaps creates a more favorable redox potential for growth. It is felt that the cells possess a larger requirement for (-SH-) groups because in "(2X) Eagle" medium, the cells utilize cystine preferentially over the other (-SH-) donor—methionine. Yet, when cystine reaches a certain minimum level methionine utilization is markedly increased indicating that the latter now serves as the principal (-SH-) donor. It has not yet been calculated whether doubling the cystine concentration exerts a "sparing effect" on methionine utilization. It has been postulated by a number of investigators that sulfhydryl-containing compounds may provide cells with needed (-SH-) groups for the synthesis of a protein involved in the regulation of cellular division. This postulation deserves more thorough investigation.

D. SPONTANEOUS DECOMPOSITION OF GLUTAMINE IN UNINOCULATED MEDIUM INCUBATED AT 37°C FOR 250 HOURS

Many conflicting reports have been presented involving spontaneous decomposition of gluamine in medium incubated at various temperatures (6). The concentration of phosphate ions and the presence of serum appears to influence the spontaneous decomposition of glutamine to ammonia and pyrollidone carboxylic acid (7). It became necessary to determine the extent of spontaneous decomposition of glutamine in "(2X) Eagle" medium at 37°C. As can be seen in Figure 6D, throughout a time equal to a normal growth curve of strain L-M mouse fibroblast cells in "(2X) Eagle" medium, approximately 20% of the glutamine in the medium could be expected to be degraded spontaneously.

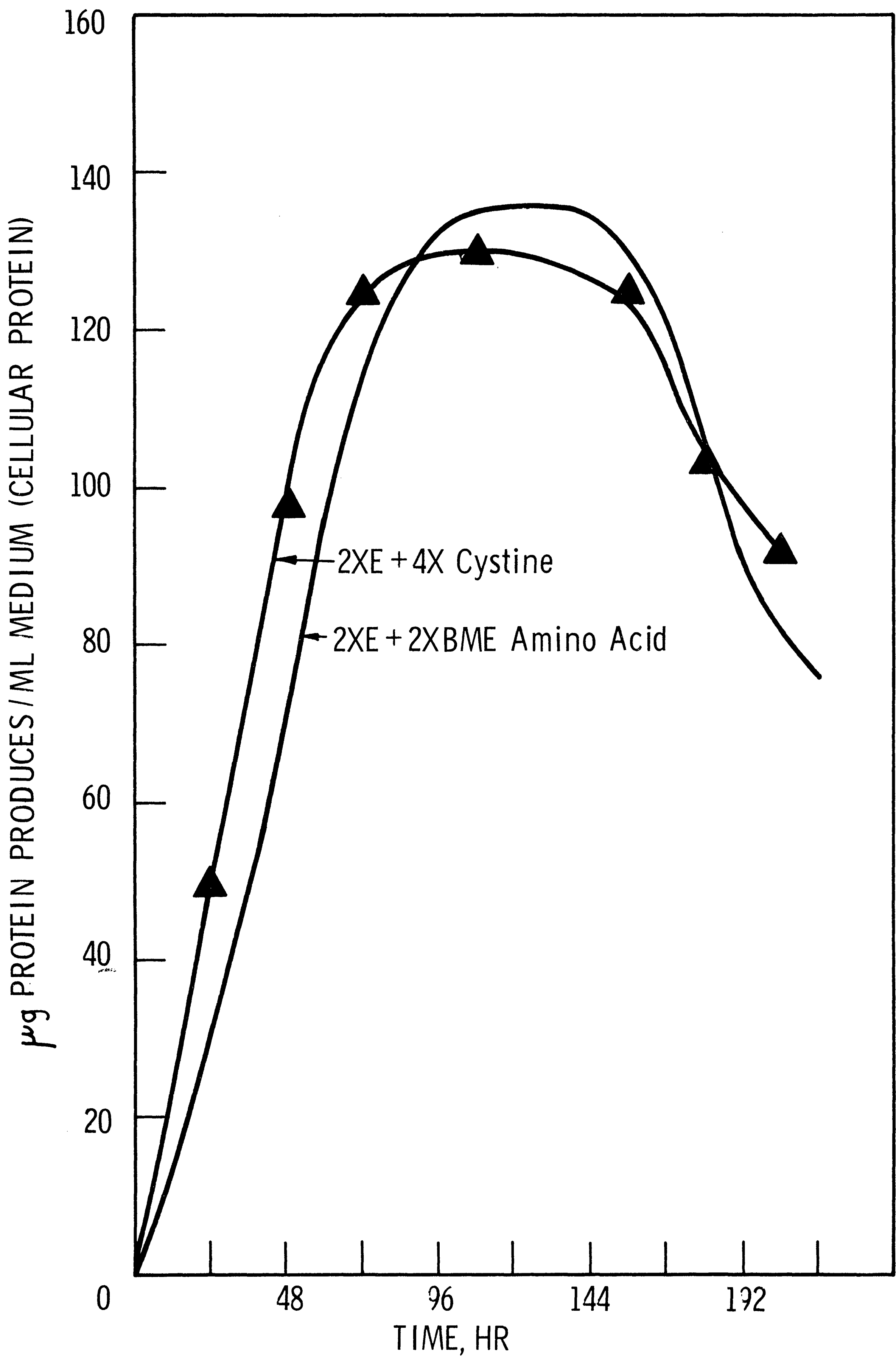

Figure 5D. Protein production by L-M cells in monolayer cultures in 2X Eagle basal medium with and without 4X cystine.

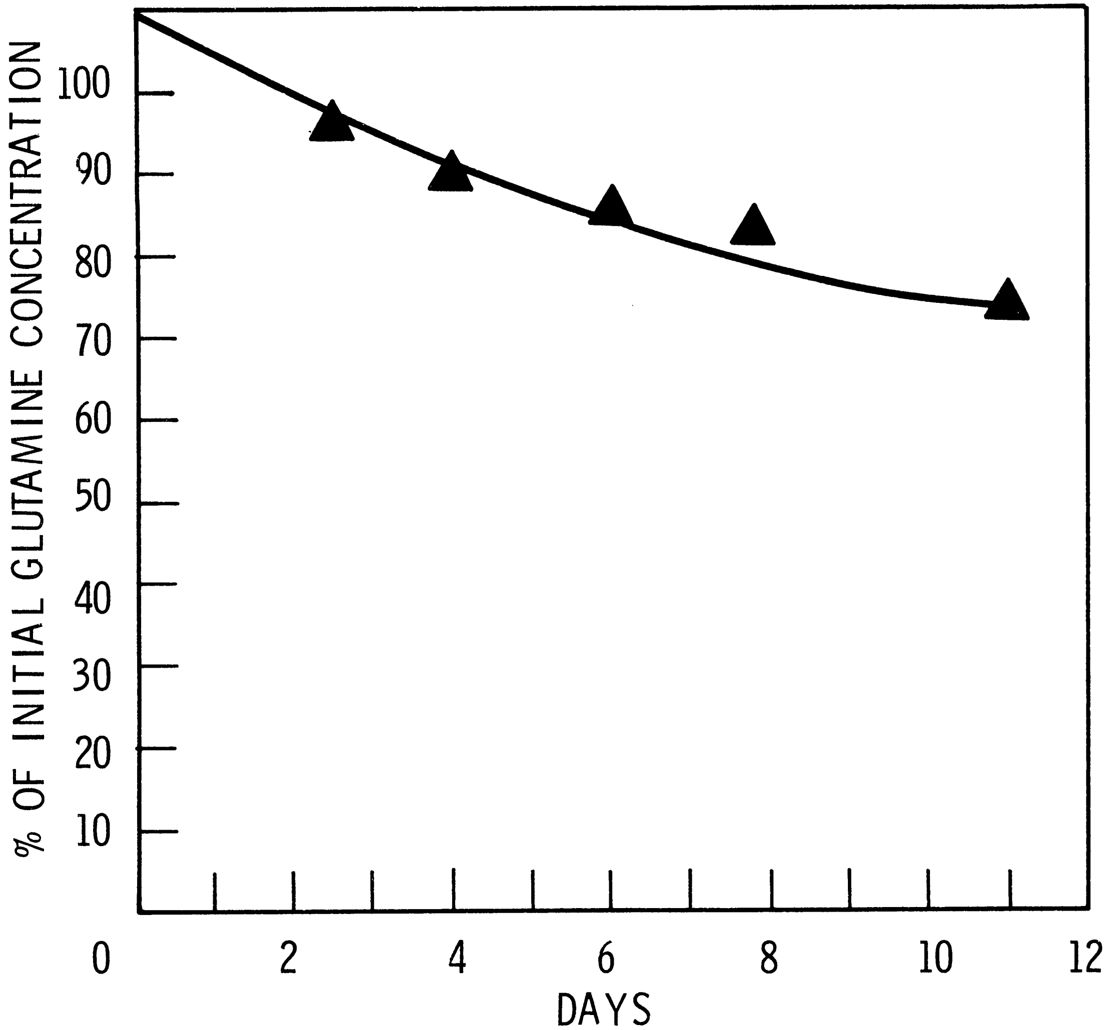

Figure 6D. Glutamine decomposition at various time intervals in uninoculated 2X Eagle basal medium incubated at 37°C.

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STUDIES OF BHK_2, PD_4 CELLS

Howard Stockdale

GROWTH OF $BHK_{21}PD_4$ CELLS IN SCHEDULE 8 AND ITS MODIFICATIONS

The standard medium for the growth of spinner cultures of BHK_{21} cells during this work was that designated "schedule 8." The formula for this is given in Table IE. A caveat is entered here that since moving to Vermont from Michigan BHK cells show increased clumping when grown in this medium. Work is currently underway to reduce the calcium level of this medium to the point where clumping is reduced without loss of cell yield.

TABLE IE

SCHEDULE 8 MEDIUM FOR SPINNER CULTURE

Calf serum	100 ml
Tryptose Phosphate	3.0 g
Glucose Anhydrous	3.0 g
Tris	3.63 g
HEPES	4.76 g
* 10 x Hanks Salts	100 ml
50 x MEM Amino Acids	30 ml
100 x Nonessential Amino Acids	30 ml
100 x BME Vitamins	30 ml
5% $NaHCO_3$ Solution	2 ml
* Calcium Chloride Solution	10 ml

Make to 1 liter final volume with water, and with pH adjusted to 7.5 with <u>IN</u> NaOH (5 ml).

*"10 x HANKS SALTS"

NaCl	56.0 g per liter
KCl	4.0 g per liter
$MgSO_4\ 7H_2O$	2.0 g per liter
$Na_2HPO_4\ 12H_2O$	2.48 g per liter
KH_2PO_4	1.20 g per liter

* Calcium Chloride Solution

18 52 g $CaCl_2\ 2H_2O$ per liter

In schedule 8 medium the limiting factor is as yet unidentified. It was considered that a vitamin, a trace element, or some undefined component might limit growth. It was observed, for instance, that during growth, acidic, ninhydrin-reacting components eluting from an ion-exchange column before aspartic acid, and contributed by tryptose, showed a great deal of depletion. Solutions containing the following compounds were made.

A. Cholesterol, calciferol, α-tocopherol and menadione.

B. Choline, pantothenate, inositol, N-acetyl glucosamine, glutathione (red), RAD, folate, ribose, deoxyribose, ascorbate, and cyanocobalamin.

C. Pyruvate, citrate, α-ketoglutarate, succinate, fumarate, malate, and oxaloacetate.

Schedule 8 medium + A, B, and C; schedule 8 +50% excess (1.5 g/l) tryptose phosphate; and schedule 8 + A, B, and C + excess TP were used for the culture of $BHK_{21}PD_4$ cells. No increase in growth rate or cell yield over that seen in schedule 8 medium was observed.

GROWTH OF $BHK_{21}PD_4$ CELLS IN LOW-SERUM MEDIA

It was felt that it might be advantageous to possess a type of BHK_{21} cell able to grow well in suspension in a low-serum medium. Three variations of schedule 8 medium, all containing 1% calf serum were made, and spinner cultures of $BHK_{21}PD_4$ cells set up after prior adaptation in monolayer culture. Because of such disadvantages as clumping, low cell yield, and slow growth, all three systems were abandoned.

However, use of MEM medium with 1% calf serum produced cells with the following properties. After two passages in monolayer, lack of cell adhesion was seen, with cells floating free or in loose clumps. Spinner culture of $BHK_{21}PD_4$ in this medium yielded 2.0×10^6 cells/ml with a doubling time of 24-25 hr. Inocula may be as low as 2.5×10^5 cells/ml. No rimming or clumping was seen in spinner culture. Cells appeared to be highly glycolytic, and death was rapid after plateau phase.

This latter medium was designated "MEM 1% C5" and its formula is given in Table IIE. Table IIIE gives a rough estimate of the comparative costs of producing a given number of $BHK_{21}PD_4$ cells with schedule 8 and MEM 1% C5 media.

TABLE IIE

MEM 1% CALF SERUM MEDIUM

MEM powder—GIBCO Cat. No. F_{16} (+ NE amino acids)	10.8 g
Tryptose phosphate	3.0 g
$NaHCO_3$	0.35 g
Calf serum	10.0 ml
Water	950.0 ml

15% (v/v) 4% 15 c.p.5. Methocell. Incorporated for spinner medium.

TABLE IIIE

COST OF MEDIA FOR $BHK_{21}PD_4$ CELLS

Item	\$ Cost per Lot at Bulk Rate		Cost per Liter Medium
	MEM 1% CS Medium		
Calf serum	18.00/500 ml		36.0 ¢
MEM powder (GIBCO Cat. No. F_{16})	37.38/50 liter lot		64.76¢
Tryptose phosphate	8.25/540 g		4.86¢
		Total	105.62¢
2 x 10^6 cells ml^{-1} formed: Cost of 1 x 10^9 cells = 52.81¢ (without tax).			
	Schedule 8 Medium		
Calf serum	18.00/500 ml		360.00¢
Tryptose phosphate	8.25/540 g		4.86¢
Tris	21.00/500 g		1.71¢
HEPES	35.00/100 g		168.00¢
Glutamic acid	7.75/1000 g		0.47¢
Dextrose	2.50/100 g		0.75¢
BME vitamins	3.60/100 ml		10.80¢
MEM amino acids	8.20/200 ml		12.30¢
NE amino acids	3.90/100 ml		11.70¢
		Total	570.59¢
5 x 10^6 cells ml^{-1} formed: Cost of 1 x 10^9 cells = 114.12 ¢.			

Note: No adjustment is made for labor as MEM 1% CS medium is virtually pre-prepared.

GROWTH RESPONSE TO SERUM CONCENTRATION

This information is incomplete, but may be of peripheral interest. During growth of $BHK_{21}PD_4$ cells in schedule 8 medium at varying serum levels, it was found that an increase of 1.5% in the serum content of the medium led to an increase of 1.0×10^6 cells/ml in the final yield of cells.

THE EFFECT OF VARYING GLUCOSE LEVELS ON GROWTH OF $BHK_{21}PD_4$ CELLS

In the previous report, it was noted that BHK_{21} cells are highly glycolytic. When cells are grown in schedule 8 medium containing 0.34% glucose, about one-third of the glucose is converted to lactic acid, a further third is not metabolized at all, and the remainder is presumably dissimilated via glycolysis and the TCA cycle. When the dry weight of cells produced is compared with the ATP yielded by glucose catabolism, an answer of 11.8 mg dry weight per μmole ATP is obtained. This is in fairly close agreement with the value obtained for bacterial systems, generally considered to be 10 μg dry weight per μmole ATP.

It therefore appears that in schedule 8 medium, glucose is well above growth-limiting levels. Better pH control, a prerequisite for vaccine production, might be achieved if glucose is lowered to the level where it is theoretically limiting (about 0.1%) hopefully reducing the amount of lactic acid formed.

Two types of experiment were attempted. (1) A series of vessels containing medium of increasing glucose content were set up to determine optimal glucose level when given as a one-step addition. (2) Alternatively it was hoped to obtain a high cell yield with minimum lactate production by adding small aliquots of glucose sequentially as required, i.e., whenever growth rate appeared to decline.

1. Schedule 8 medium was made up without added glucose dispensed into spinner flasks, glucose added to levels of 0-0.3% above the basal value. pH and cell number were followed with time. Cell yields and pH delcine are given in Figure 1E. Limitation of glucose does not provide an effective method of pH control without producing a concommitant fall in cell yield. Cell yield appears directly proportional to glucose added. There is some indication of slower growth at lower glucose levels. At a given time after attainment of maximal yield, cultures grown at lower glucose levels show a lower percentage of surviving cells. pH curves for these cultures fall into two distinct classes; cultures grown on 0.067% added glucose and below showing moderately steady pH, and cultures grown above this level showing a rapid pH decline and a later rise. Probably at a critical glucose level, enough lactate is formed to exceed the buffering capacity of the medium.

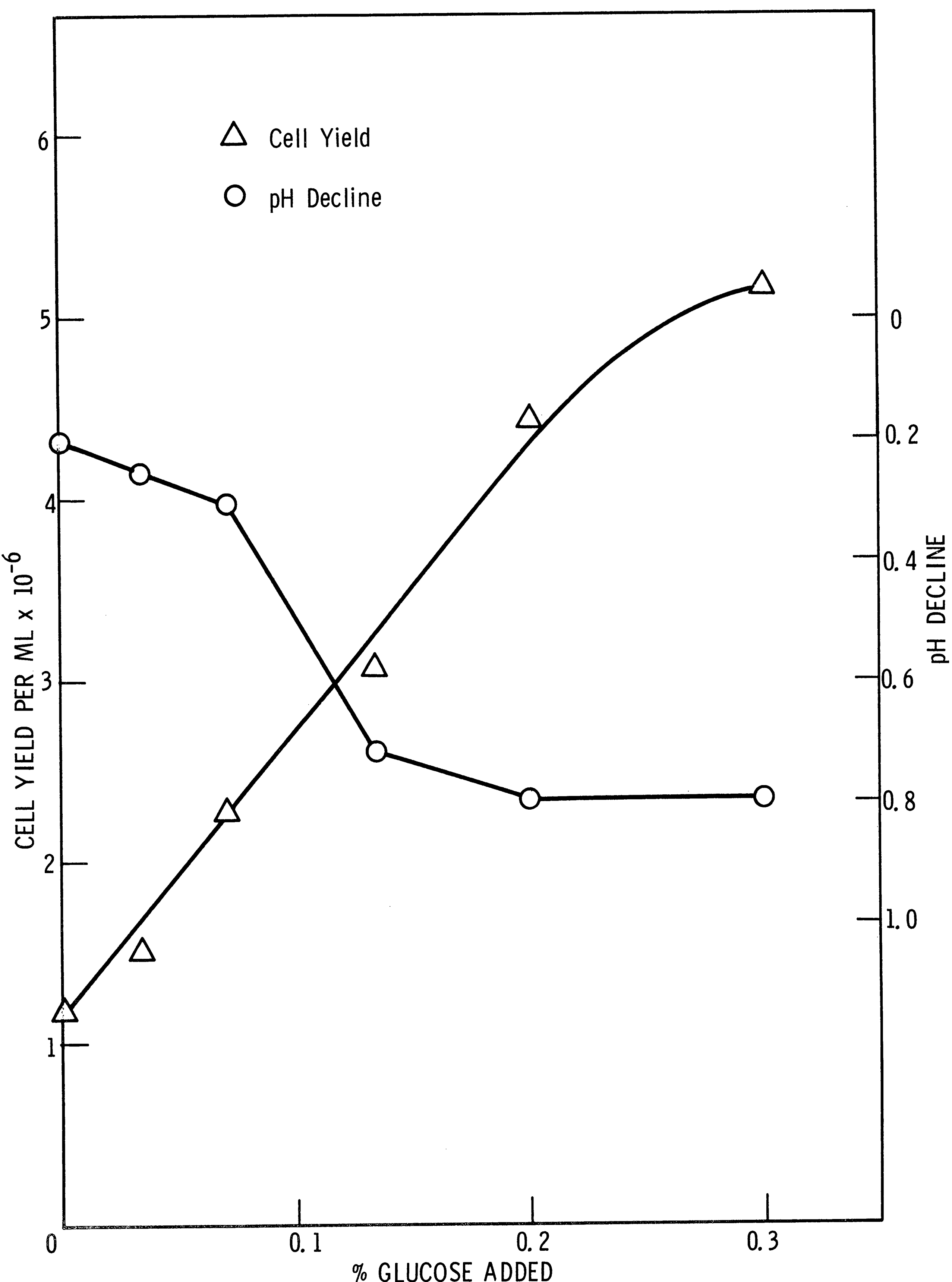

Figure 1E. Cell yield of $BHK_{21}PD_4$ and pH decline against initial glucose level.

2. For the growth curve involving sequential glucose additions, glucose was initially added at a level of over 0.02% above the basal level. Further additions were made throughout the growth period. Unfortunately, further additions of glucose were unable to stimulate rapid division once the growth rate had slowed and the cells appeared to be moving towards maintenance rather than growth. The final doubling time recorded was 129 hr. After 145 hr 0.14% glucose had been added with a yield of 28.0 x 10^5 cells/ml and a concomitant pH drop of 0.55 unit. This shows little advantage over the one-step system where 0.134% added glucose yielded 30.0 x 10^5 cells/ml with a fall of 0.52 pH unit.

It is concluded that pH control of these cultures could best be achieved by direct addition of alkali.

FREEZING OF $BHK_{21}PD_4$ CELLS FOR SUSPENSION CULTURE

An attempt was made to freeze and recover dense suspensions of $BHK_{21}PD_4$ cells for spinner to spinner transfer based on the procedure described by Walker. In Walker's paper dimethyl sulfoxide (DMSO) was used routinely as a preservative, and processing of cells at 4°C was found to be superior to that at room temperature. Paul (personal communication) states that DMSO is toxic at higher temperatures, but not at 4°C. It has recently been shown in this laboratory that BHK cells suspended in their normal growth medium with 5% DMSO at 36°C show a progressive inability to divide in fresh medium.

The present procedure involved harvesting at room temperature and cooling the cells, resuspended in fresh medium, in ice prior to addition of DMSO. Two different media, namely schedule 8, and MEM 1% CS were used in growth and processing of the cells, and in each case cells were frozen with and without DMSO by way of comparison. Media in which the cells were frozen were the same as that used for growth except that 15% (v/v) of 40 15 cps methocell was added to the freezing medium.

100 ml spinner cultures were set up and harvested in the late-log phase when the schedule 8 and MEM 1% CS-grown cultures had the following characteristics respectively: 53.4 x 10^5 cells/ml, > 99% viability; 22.0 x 10^5 cells/ml, 87% viability. Cells were harvested at RT and resuspended in freezing medium at an estimated 20-25 x 10^6 cells/ml, and cooled in ice. 5% DMSO was added to half of the samples so prepared. Samples were dispensed in 2 ml aliquots in screw cap tubes instead of sealed ampoules as it was felt that these vessels were a more likely model for the bulk handling of cells. The screw cap tubes were subjected to preliminary cooling (6-8 hr) in a styrofoam box (10 in. x 10 in. x 6 in., with 1 in. wall thickness) placed in a Revco freezer and then stored in the vapor phase of liquid nitrogen. (This is essentially the technique used in J. Pauls' laboratory.)

After six days' storage, samples were thawed rapidly, and inoculated into 100-ml spinner cultures containing the appropriate growth medium at 2.5×10^5 cells/ml. After retrieval the characteristics of the inocula were as follows.

1. Schedule 8 cells + 5% DMSO—90% viability > 99% viability after 40 hr

2. Schedule 8 cells - No DMSO—74% viability < 1% viability after 24 hr

3. MEM 1% CS cells + 5% DMSO—89% viability—always 90-97% viability

4. MEM 1% CS cells - No DMSO—0% viability

No growth was observed with inocula processed without DMSO. Cells frozen in the presence of DMSO showed apparently normal growth (see Figure 2E).

SURVIVAL OF $BHK_{21}PD_4$ CELLS AT 4°C

It is generally known that when cells of E. coli are suspended in a salt solution at 4°C at about 1×10^3 cells/ml, cell death is rapid. This effect is not shown if cells are at a density of 1×10^8 cells/ml, or if they are kept as a centrifuged pad. Mammalian cells may display similar behavior, and if so, cold storage without freezing may be advantageous if one wishes to use the same batch of cells for inocula over a period of a few days.

Two log-phase spinner cultures grown with MEM 1% CS and schedule 8 medium and containing 1.0 and 3.0×10^6 cells/ml, respectively, were stored at 4°C with stirring. At intervals cells were used as inocula for monolayer cultures (MEM 10% TP 5% CS) inoculated at 2.5×10^5 viable cells/ml. In all but the final sample (22 days) no difficulty was found in establishing monolayer cultures, and even in this case cells began to grow after a three-day lag. Death in schedule 8 medium resulted in much cellular debris. In MEM 1% CS medium it was noted that smaller cells tended to die first. In neither of these "open" cultures did the pH rise markedly from that obtaining prior to cooling.

Stationary phase cells grown on schedule 8 medium were centrifuged, the medium decanted and the cell pad stored. Monolayers were established by transferring small portions of the pad with a pasteur pipette. In this instance death was more rapid, but a viable monolayer could be obtained after 11 days storage. Results are given in Table IVE.

There is a possibility of obtaining more prolonged survival by holding cells at 1-2°C above the freezing point of the suspending medium.

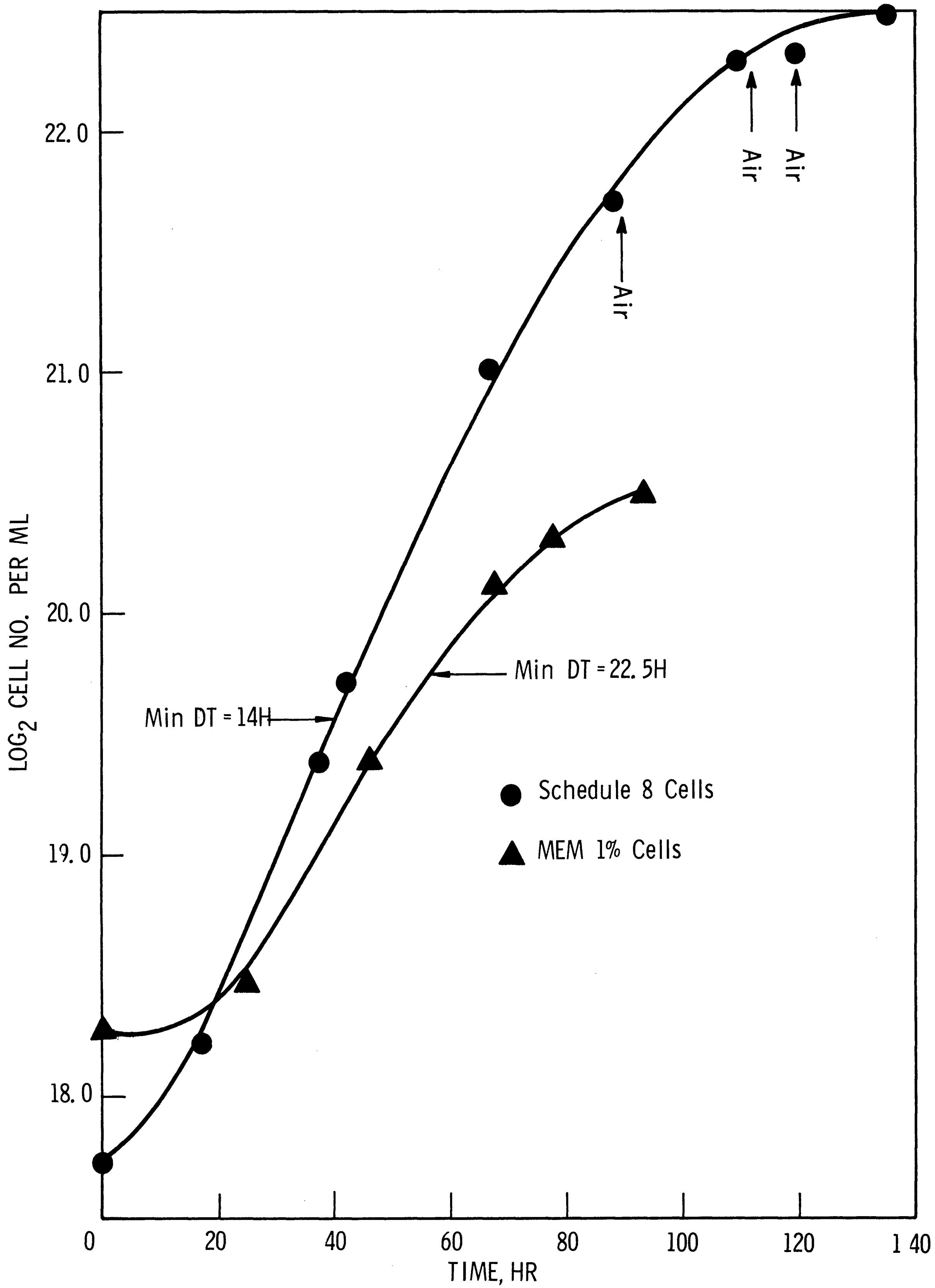

Figure 2E. Spinner growth of $BHK_{21}PD_4$ cells from frozen stock.

TABLE IVE

SURVIVAL OF $BHK_{21}PD_4$ CELLS AT 4°C

A. Spinner Cultures in Growth Medium

Time in Days	Schedule 8		MEM 1% CS		Mono Made
	% Viability	Cell No. x 10^{-5}/ml	% Viability	Cell No. x 10^{-5}/ml	
0	98	28.6	88	13.12	
1	97	30.0	87	12.2	
2	93	28.0	89	12.2	
3	83	30.1	90	12.8	
4	83	28.0	77	12.4	✓
5	86	24.0	81	9.2	
6	78	24.2	75	12.1	
11	29	10.4	68	11.0	✓
12	25	10.4	67	11.0	
15	25	8.8	59	9.0	✓
18	40.7	7.0	57	8.0	✓
22	~25	6.9	39	6.5	✓

B. Centrifuged Pad

	Time in Days					
	0	1	2	4	7	11
% Viability	86	35	41	47	36	~15
Mono Made				✓	✓	✓

UPTAKE OF AMINO ACIDS BY $BHK_{21}PD_4$ CELLS DURING GROWTH IN SCHEDULE 8 MEDIUM

All analyses were done with a Beckman 120C amino acid analyzer. Ultrafiltrates of calf serum and tryptose phosphate were examined as a preliminary to determine whether or not these materials incorporated unidentifiable components to such an extent as to render a rough estimate of amino acid uptake impossible. Although components other than the 21 common amino acids were observed, their concentration was relatively low and their elution times such that they did not interfere with the normally encountered amino acids.

Tryptose phosphate contained nine unidentified, highly acidic components, which were eluted before aspartic acid. Three unidentified components were seen among the neutral amino acids. Lysine, histidine, arginine, tryptophan aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isolencine, leucine, tyrosine, and phenylalamine were observed. The presence of cystine was suggested, but not confirmed, by a shoulder on the alanine peak. Some ammonia is present. Asparagine, and a possible small amount of glutamine, were identified tentatively. Upon autoclaving, there is some degradation of the "pre-aspartic acid" component and a loss of the presumed glutamine. Quantitatively, tryptose appears to provide the largest amount of amino acids of the medium components and is notably rich in leucine and lysine.

At the level used (10%), calf serum does not appear to contribute a major portion of free amino acids, at least in the initial stages of growth. There are at least two unidentified components, eluted before aspartic acid. A small amount of hydroxyproline is present. One double peak observed could be attributed to serine, threonine, and glutamine, or alternatively to a combination of two of these alone. The major peak in the chromatogram is evidently ammonia.

When an ultrafiltrate of whole medium was analyzed, the quantitative contribution of the unidentified components appeared to be relatively minor. There is a marked increase in ammonia levels during growth, but little loss of arginine (see Table VE). The levels of proline and glycine increase throughout growth. Aspartic and glutamic acids are diminished by more than 90%, serine shows a 75% loss, and all other amino acids are depleted to a lesser degree. None of the essential amino acids appears to be limiting. Ammonia production, and the great depletion of aspartic acid and glutamic acid suggest that these amino acids may be dissimilated via deamination, transamination, and the TCA cycle. A gross nitrogen balance of free amino acid uptake reveals that a maximum of 22% of all protein synthesized could be derived from free amino acids and the remainder is presumably obtained from the proteins and peptides present in calf serum and tryptose phosphate.

The behavior of serine, a presumably nonessential amino acid, is interesting in that it may be a possible source of the free glycine released, and that

TABLE VE

UPTAKE OF FREE AMINO ACIDS BY $BHK_{21}PD_4$ CELLS DURING GROWTH

Culture time (hr)	00.00	20.50	48.45	67.75	92.75	116.50	Change in AA Conc
Cell yield (x 10^{-5}/ml)	00.00	6.4	18.5	33.7	47.5	46.5	(μM/ml)
Amino acid (μM/ml)							
Lysine	1.160	0.995	1.080	1.120	1.16	1.08	-0.08
Histidine	0.415	0.340	0.369	0.400	0.403	0.375	-0.04
Ammonia	0.532	0.767	0.128	1.810	3.050	3.460	+2.928
Arginine	1.140	1.040	1.030	1.070	1.070	1.040	-0.10
Tryptophan	0.078	0.074	0.550	0.078	0.087	0.052	-0.026
Aspartic acid	0.475	0.305	0.136	0.085	0.048	0.036	-0.439
Threonine	0.915	0.934	0.865	1.020	0.815	0.803	-0.112
Serine	1.020	0.855	0.550	0.445	0.385	0.253	-0.769
Glutamic acid	5.350	4.970	3.070	1.520	0.069	0.070	-5.280
Proline	1.060	0.890	0.995	1.240	1.380	1.66	+0.60
Glycine	0.660	0.628	0.760	0.980	0.850	0.737	+0.077
Alanine	0.858	1.010	1.520	1.970	1.21	0.708	-0.150
Valine	1.150	1.090	1.050	1.110	1.08	0.990	0.160
Methionine	0.232	0.268	0.236	0.268	0.156	0.137	-0.095
Isolencine	0.860	0.830	0.770	0.915	0.757	0.680	-0.160
Lencine	1.400	1.170	1.150	1.280	1.040	0.915	-0.485
Tyrosine	0.320	0.345	0.322	0.465	0.285	0.265	-0.055
Phenylalanine	0.505	0.572	0.540	0.667	0.487	0.425	-0.140

the rate of uptake per cell varies markedly throughout growth (see Figures 3E and 4E). Both the rate of serine removed from the medium by a fixed number of cells present, and the amount of serine used in the production of new cells, show a rapid decline during exponential growth (both parameters are expressed in µmoles serine used per million cells over a 10-hr period). This would suggest that either a large pool of serine accumulates during growth, or that serine requirements are population-dependent, cells at high densities being better able to produce or retain serine itself, or its possible metabolite, glycine.

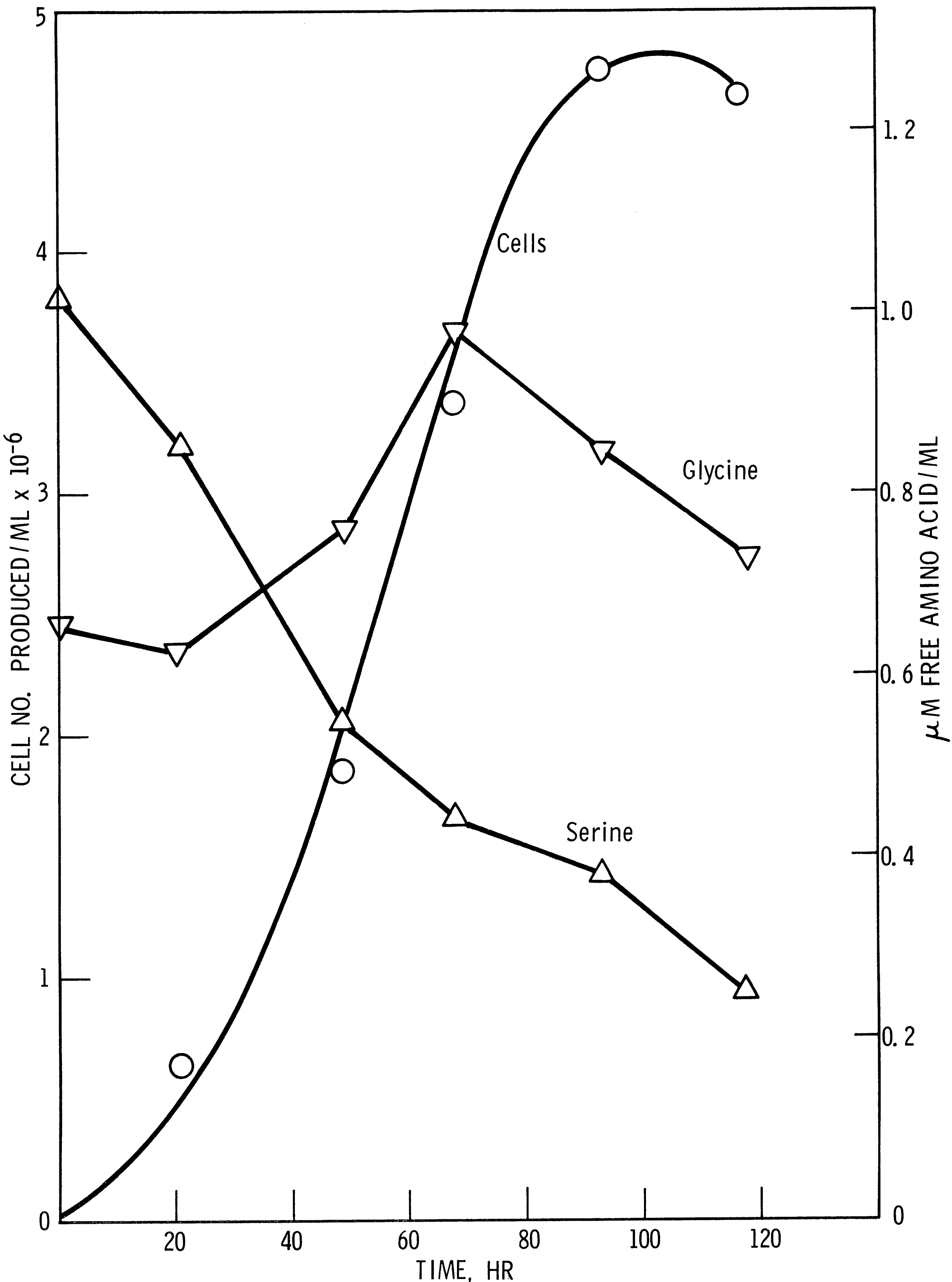

Figure 3E. Supernate levels of serine and glycine during growth of $BHK_{21}PD_4$ cells.

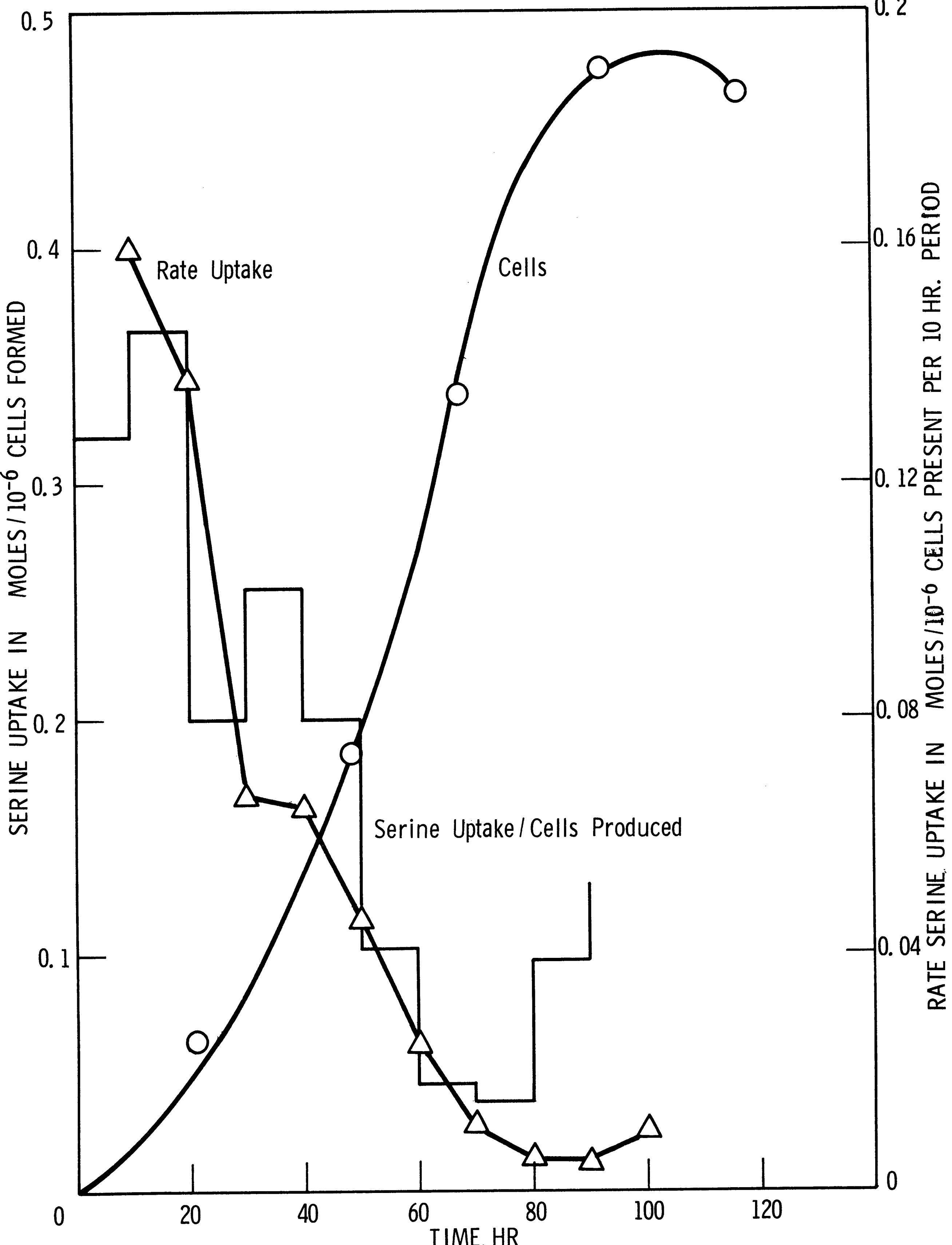

Figure 4E. Serine uptake during growth of $BHK_{21}PD_4$ cells.

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13. ABSTRACT

Using equipment previously constructed on the program L-M mouse cells have been grown in a 5-liter fermenter in medium 199 supplemented with peptone and serum. Changes in pH, O-R potential, dissolved oxygen and cell number and viability have been continuously monitored. Cells also have been grown under conditions of controlled pH and O-R potential and the relationship of O-R potential to growth rate has been determined.

Work has been completed on a computer program for analysis of chromosome changes in aneuploid cell populations. The method also is applicable to study of diploid cells. Automated methods combined with computer analyses permit a rapid and sensitive monitoring of chromosomal shifts in cell population.

Using the computer program, as well as more conventional methods of chromosomal analysis, data is presented on the effects of several types of environmental selection pressures on the chromosomal complement of L-M mouse cells and $BHK_{21}PD_4$ cells.

Detailed studies of the amino acid nutrition of L-M mouse cells in chemically defined medium is presented. Combining the simplicity of Eagle basal medium with the sensitivity and accuracy of an amino acid analyzer, it has been possible to obtain critical evidence concerning the rates of use of individual amino acids, the role of imbalance of amino acids in utilization patterns and the effects of supplementing nonessential amino acids.

Finally, studies of the nutrition of $BHK_{21}PD_4$ cells have led to the development of a very efficient medium (schedule 8) for this cell line. The comparative costs and yield is compared with more conventional media. Improved methods of storage and of freezing seed stocks of these cells also are presented.

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