UNIVERSITY MICROFILMS, INC., ANN ARBOR, MICH.

L. C. Card no. Mic 58-7750

KUCHLER, Robert Joseph. QUANTITATIVE ANALYSIS OF IN VITRO GROWTH CHARACTERISTICS OF MOUSE FIBROBLASTS.

University of Michigan, Ph. D., 1958
Bacteriology

Negative microfilm received from University.

# QUANTITATIVE ANALYSIS OF *IN VITRO* GROWTH CHARACTERISTICS OF MOUSE FIBROBLASTS

by

Robert J. Kuchler

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in the
University of Michigan
1958

Committee in charge:
Assistant Professor Donald J. Merchant, Chairman
Associate Professor Philipp Gerhardt
Assistant Professor Robert C. Hendrix
Assistant Professor Arthur G. Johnson
Associate Professor Alfred S. Sussman

## ABSTRACT

### QUANTITATIVE ANALYSIS OF THE IN VITRO GROWTH CHARACTERISTICS OF MOUSE FIBROBLASTS

This study was undertaken to determine the growth characteristics of a mouse fibroblast (strain L) in suspension culture. It was hoped that the information thus obtained would lead to an optimal system for the production of large cell masses that could be used in studying mammalian cell antigens. After having satisfactorily propagated these cells in suspension, it was recognized that almost any volume of cell suspension could be grown in this manner, if the speed and method of agitation were properly adjusted. It was also noted that fibroblast populations in these cultures could be characterized by a growth culture cycle which was similar to population development described for unicellular microbial cultures. The lag phase of growth was eliminated when logarithmic phase cells were used as the inoculum in media pre-adjusted for pH and temperature. This made possible the establishment of transfer methods which were highly efficient and which yielded populations with a considerable degree of uniformity.

Further application of the growth cycle attempted to define the optimal conditions for fibroblast growth. Various nutrient mixtures were studied for their ability to support and enhance growth. The minimal generation time achieved was 28 hours; the maximum number of cells was about $2 \times 10^6$ cells/ml.

Growth rate was independent of the serum concentration in the medium over a wide range. In Medium 199, maximum population density increased with the serum content to about 15%; beyond this level the extent of growth was rather constant. The rate and extent of fibroblast growth also appeared to be independent of the inoculum size.

The development of the cell population in suspension culture was also followed during the growth cycle relating the number of cells to packed cell volume. Measurements of packed cell volume showed that protoplasm increased exponentially from zero time, though a lag in the increases in number of cells was noted. The range in cell volumes of growing fibroblasts, as determined from measurements of cell diameter, was $1500u^3$ to $3000u^3$. An increase in cell size during the lag phase of growth accounted for the increase in cell mass in the absence of an increase in the number of cells. Measurements were made of DNA, RNA, total nitrogen, and dry weight during the growth cycle, and were correlated with the increases in number of cells and in packed cell volume. DNA was found to correlate directly with cell number, while RNA, total nitrogen, and dry weight increases during the lag phase were related to the increases in cell volume.

To increase the speed and precision obtained in counting fibroblasts, an electronic blood cell counter was employed. This counter, designed to count red blood cells, was found suitable for the enumeration of fibroblasts in suspension. In addition to speeding the process of cell counting by a factor of 10, the degree of variation in cell counts was markedly reduced.

## ACKNOWLEDGMENTS

The author appreciates the sincere interest of Dr. Donald J. Merchant during this work, and of his friends and associates in the laboratory who have contributed by their discussions and helpful assistance to this thesis.

TABLE OF CONTENTS


| | Page |
|---|---|
| LIST OF FIGURES | v |
| INTRODUCTION | 1 |
| HISTORICAL | 4 |
| MATERIALS AND METHODS | 18 |
| 1. Cell strains | 18 |
| 2. Culture media | 18 |
| 3. Growth of mouse fibroblasts in stationary culture | 21 |
| 4. Growth of mouse fibroblasts in fluid suspension cultures | 22 |
| 5. Mechanics involved in setting up fluid suspension cultures of mouse fibroblasts | 23 |
| 6. Procedure for setting up a replicate series of fluid suspension cultures | 23 |
| 7. Analysis of the development of a mammalian cell population | 24 |
| EXPERIMENTAL RESULTS | 33 |
| 1. The growth of mouse fibroblasts in stationary culture directly on glass | 33 |
| 2. The fluid suspension culture method | 35 |
| 3. The growth of mouse fibroblast populations in fluid suspension cultures | 37 |
| (a) The effect of age of inoculum | 37 |
| (b) The effect of serum concentration | 43 |
| (c) The effect of various media | 45 |
| (d) The effect of inoculum size | 45 |
| 4. Biosynthesis in strain L fibroblasts | 48 |
| (a) Number of cells related to packed cell volume | 48 |
| (b) Number of cells related to total cell nitrogen | 50 |
| (c) Number of cells related to cell DNA and RNA | 50 |
| (d) Number of cells related to dry weight | 53 |
| (e) Synthesis in the lag phase | 53 |
| (f) Packed cell volume at various points on the growth curve | 56 |

Page

(g) DNA, RNA and nitrogen per cell at various points on the growth curve . . . . 56
(h) Cell size distribution analysis and the growth curve. . . . . 56
5. The application of an electronic blood cell counter to define fibroblast growth . . . . . 61

DISCUSSION . . . . . 66

SUMMARY . . . . . 77

BIBLIOGRAPHY . . . . . 79

LIST OF FIGURES


| Number | | Page |
|---|---|---|
| 1. | Analysis of the development of a mammalian cell population growing in fluid suspension culture. | 25 |
| 2. | Drawing showing aperture tube, sample beaker, electrodes and flow control elements of the electronic blood cell counter . . . . . | 27 |
| 3. | Standard curve for desoxyribonucleic acid . . . . | 30 |
| 4. | Standard curve for ribonucleic acid . . . . . | 32 |
| 5. | Population growth curve of mouse fibroblasts in stationary bottle cultures. . . . . | 34 |
| 6. | Population growth curve of mouse fibroblasts in fluid suspension cultures, using plateau phase cells as the inoculum . . . . . | 38 |
| 7. | Population growth curve of mouse fibroblasts in fluid suspension cultures, using logarithmic phase cells as the inoculum in non-adjusted (pH and temperature) medium . . . . . | 40 |
| 8. | Population growth curve of mouse fibroblasts in fluid suspension cultures, using logarithmic phase cells as the inoculum in pre-adjusted (pH and temperature) medium . . . . . | 42 |
| 9. | The effect of varying the serum concentration on the growth of mouse fibroblasts in fluid suspension cultures . . . . . | 44 |
| 10. | The effect of various tissue media on the growth of mouse fibroblasts in fluid suspension cultures . . . . . | 46 |
| 11. | The effect of varying the initial cell inoculum on growth of mouse fibroblasts in fluid suspension cultures . . . . . | 47 |
| 12. | The packed cell volume related to the number of cells during the growth cycle . . . . . | 49 |

Number | Page

13. The total (TCA) precipitated cell nitrogen related to the number of cells during the growth cycle . . . . . 51

14. DNA and RNA related to the number of cells during the growth cycle . . . . . 52

15. Cell dry weight related to the number of cells during the growth cycle . . . . . 54

16. Synthesis in the lag phase . . . . . 55

17. The number of mouse fibroblasts per unit of packed cell volume during the growth cycle . . 57

18. The amount of nitrogen, DNA and RNA per cell during the growth cycle . . . . . 58

19. Cell size distribution analysis at various times during the growth cycle . . . . . 60

20. Population growth curve of mouse fibroblasts from culture analyzed for size distribution. . 62

21. The application of the electronic cell counter to analyze growth of a mouse fibroblast population . . . . . 64

22. The application of the hemocytometer to analyze the growth of a mouse fibroblast population. . 65

# INTRODUCTION

Mammalian cells were first propagated in agitated fluid suspension in 1953 by Owens, Gey and Gey (65). Prior to that time, growth had been demonstrated only in semi-solid coagulums or on a solid substrate, though it had been thought that animal cells might grow as suspended single units *in vitro*, because of the frequent occurrence of dense cell suspensions in peritoneal ascites associated with neoplasia. While culturing lymphoblastic strain MBIII in roller tubes, Owens *et al*. noted that the cells did not flatten on the glass surface but remained rounded, and while most of them adhered to the glass, others were found floating freely in the medium, giving it a turbid appearance. The free-floating cells were shown to be viable on subculturing. With slight agitation, large numbers of the remaining adherent cells could be freed from the glass. To determine whether these cells were capable of multiplying while suspended, they were inoculated into a new type of culture tube (66). The tube was fastened to the face of the roller drum, in such a way that when the drum was set in motion, the cell suspension washed over the entire surface of the tube. This new devise was called the "tumble tube." It was found that at 38 rpm none of the cells adhered to the glass but grew freely in the suspending medium as unattached, rounded cells proliferating

at rates closely analogous to populations of mammalian cells attached to a glass surface.

This new method of growing cells in suspension culture attracted the attention of Earle, who extended these studies to learn whether this phenomenon of cultivating mammalian cells in fluid suspension might be a unique characteristic of cells of the lymphoblastic series. Using a mouse fibroblast clone (strain L no. 929), Earle et al. (32) successfully grew fibroblasts in suspension in roller tubes, which were rotated around their long axis at speeds exceeding 10 rpm. This was positive evidence that a normally fixed tissue cell could be grown in fluid suspension cultures. These workers extended their studies; they subsequently showed that these cells could be grown in suspension in small erlenmeyer flasks kept in continuous motion on a rotary shaker (31).

This method of cultivation was first used in our laboratory in 1955; the immediate outlook was toward mass culture which is of necessity a basic step for the analysis of specific cell antigens, enzyme systems and hormones. With the successful propagation of mouse fibroblasts in this type of in vitro culture and with the recognition that the patterns of population development seemed to mimic those of extensively studied microbial systems, the objectives for this study became evident. They were (1) to thoroughly characterize the growth cycle of fibroblast populations in fluid suspension cultures, (2) to utilize the rate and extent of increase in numbers of fibroblasts in order to establish the optimal

conditions for growth of strain L in these cultures, and (3) to define the rate of some of the synthetic activities and to correlate these to the increase in number of fibroblasts in a growing population.

If the patterns of cell growth under defined conditions could be established, such as the rate of increase in the number of fibroblasts in a population and the rate of addition of protoplasm, then it should be possible to apply this method of analysis to changes which occur when substances which alter cellular behavior are added to the cellular environment. The cell activity may be enhanced by the addition of growth factors or impeded either partially or completely by the addition of antibodies, viruses, or chemicals; the effect, either beneficial or deleterious, would be manifested in the growth behavior. It is believed that the results of this study may provide the basis for such quantitative studies.

# HISTORICAL

Quantitative analysis of growth in the tissue culture system has always been a perplexing problem. Numerous methods have been used to measure animal cell activity in vitro, the desired endpoint being the measurement of some cellular event which occurs uniformily for a designated time interval in a constant environment. This quantitative cellular event could be an increase in cell numbers, an increase in protoplasm, disappearance of a metabolite in the growth medium, or accumulation of a by-product of cell metabolism. Other measurable criteria could be used; these are limited only by the present knowledge of cell synthesis and cell metabolism.

Many investigators before 1900 had thought of the possibility of growing tissues outside the body, but their unsuccessful attempts were generally not recorded. The cultivation of animal tissue in vitro was accomplished for the first time by Harrison in 1907 (42). He demonstrated that the neural crest of the embryonic frog, transplanted into coagulable lymph, would develop normally. Burrows (8), working with Harrison, improved the technique by using plasma instead of lymph, and succeeded in adapting the method to the cultivation of chick embryonic tissue. Carrel and Burrows (10) found that almost all adult and embryonic tissues could easily be cultivated in vitro for at least brief periods. The ex-

planted tissues showed outgrowth of connective tissue or epithelial cells, which grew in the plasma-clotted medium as a loose network of cells or as continuous radiating sheets. These workers noted nuclear divisions and stated that the explanted cells multiplied. In 1912 Carrel (9) succeeded in isolating a strain of chick-embryo heart cells which were maintained for over thirty-four years by serial subculture.

With the successful demonstration of cell growth from explanted tissue in a plasma coagulum, methods for quantitating the new cell growth were examined. The earliest means for measuring growth of explanted tissue fragments was a periodic measure of the new surface area. Such a measure would encompass increases in cell size and increases in cell number plus cell migration. Ebeling (33) showed that the surface area increased ten- to twentyfold in forty-eight hours. This method of growth measurement has many possible sources of error, because the peripheral areas of the culture may vary greatly in the density of the cell population, or the explant may contain a variety of cell types. It would follow, therefore, that interpretations based upon surface area measurements must be made with extreme caution (67).

"Mitotic coefficients" were used by Champy (13) to express the stage of culture development. The ratio of dividing to resting cells at any time would indicate the activity of the culture, and presumably its viability. Determining the ratio, however, is a tedious task because of the large number of cells that must be counted to obtain statisti-

cal reliability. In explanted cultures, the ability to divide is limited to the cells in the peripheral zone of the explant. The mitotic coefficient would not be a true value for explanted tissue as a unit. Another method has been employed by Willmer and Jacoby (84), whereby only a defined area at the edge of the colony was observed, and counts were made of the mitotic figures in that area. This method, like the one described by Champy, also appears to have quantitative pitfalls, because cell division always is limited to the periphery.

The 1930's brought the introduction of more refined techniques. The clotted plasma had always proved a barrier to the direct measurement of weights of cultures. However, Meier (62) introduced a method to dissolve the coagulated plasma leaving only the intact tissue colony. The technique used 98% alcohol to bring the concentration of alcohol in the growth medium to 10%. The plasma coagulum was dissolved; the intact colony was then placed on a weighed glass coverslip and dried. The dry mass increased tenfold in a six day period. Laser (55) varied this method, by using pepsin to dissolve the plasma clot. Meier and Laser have followed the increases in dry weight of cultures and correlated their data with increases in surface area. Their conclusion was that increases in surface area were mostly due to cell migration and not to growth and multiplication in primary explants.

Various chemical measurements have been applied in an attempt to define growth as an expression of metabolism of explanted tissue. Krontowski (52) showed that tissues could

use an amount of glucose greater than their own dry weight in forty-eight hours; the amount was found to decrease with the age of the tissue (56). Demuth (25) stated that growth in cultures increased with increasing glucose concentration. Ebeling (34) observed increased growth in cultures of fibroblasts when the glucose content was raised from 200 to 1,150 mg per cent; at higher levels glucose appeared to inhibit growth and to become toxic. Willmer (83) found the total amount of cell migration was directly proportional to glucose concentration up to 1,000 mg per cent.

Wilson, Jackson and Brues (85) have thoroughly examined glucose metabolism in tissue cultures. They explanted chick embryonic tissue in roller tubes in plasma clots. Changes in composition of the medium resulting from metabolism of the explanted tissue were then followed by analytical methods. They found that the initial rate of glucose utilization was increased when the glucose in the medium was increased from 100 to 500 mg per cent, and that glucose was used in greatest amounts when the medium containing 100 mg per cent was replaced daily. Although glucose consumption appeared necessary for survival of cultures, the rate of consumption was far in excess of that required for life and maximal growth. Complete blocking of mitosis by colchicine did not alter the rate of glucose utilization. The authors concluded that the energy requirements for life and growth are easily supplied, and that higher amounts of glucose do not increase the growth rate because it is already limited by factors such as permea-

bility, the rate of intracellular synthetic reactions, and the availability of essential foodstuffs. Further, lactic acid was formed in both the presence and absence of glucose. Its formation increased with increased glucose utilization. Evidence was also presented that tissues would utilize lactate after their supply of glucose had been exhausted. Pyruvate, too, was rapidly metabolized by the tissues; it favored growth slightly in the absence of glucose.

The lack of a quantitative measure to indicate cell growth in the tissue culture system led Willmer (83) to employ measurements of nucleoprotein phosphorous (NPP) as described by Berenblum, Chain, and Heatly (2). Willmer believed that nucleoprotein was a characteristic constituent of the nucleus and indicated increases in cell numbers. Cunningham and Kirk (19) suggested the measurement of nucleoprotein in tissue culture at about the same time but they did not then make the application.

Davidson and Waymouth (21, 22, 23) used the technique of Willmer for extracting NPP, and extended their work to include techniques for separating other phosphorous-containing compounds such as phospholipid and acid-soluble phosphorous components. A series of chick-heart explant cultures were set up in plasma clots in roller tubes and replicate tubes were harvested at intervals and analyzed. The results showed that this method provided a satisfactory index of growth.

By 1930 there was clear evidence of the existence of two definite types of nucleic acids (14). One of these was

the nucleic acid of yeast which on hydrolysis yielded a pentose identified by Levene and Jacobs (14) as ribose. The other nucleic acid, from thymus (57), yielded another pentose identified by Levene et al. (58) as desoxyribose. The assumption was made that pentose nucleic acids (RNA) were characteristic of plants, and desoxypentose nucleic acids (DNA) of animal tissues. There was always objection to this classification, because it had been known since the 19th century that the pentose derivatives were present in animal tissues. About 1924 the idea began to develop that RNA in animal tissues was more widespread than had previously been supposed. Caspersson in 1940 (11), using a quantitative ultraviolet spectophotometric technique, showed the presence of high concentrations of RNA in rapidly proliferating cells in both plants and animals. From his results Caspersson (12) concluded that a high concentration of RNA was characteristic of cells in which rapid protein synthesis was taking place. Meanwhile, the relationship of DNA to the nucleus of the cell was demonstrated histochemically by the use of the Feulgen reaction (38), which is specific for this type of nucleic acid. The general conclusions from histochemical tests have been confirmed by the procedure of cell disruption (26), with separation of the morphological components of the cells. The cytoplasmic components contain RNA while the nuclei contain DNA and small amounts of RNA.

In a later study, Davidson and Waymouth (1949) (24), using the technique of Schmidt and Thannhauser (1945) (79)

for determining the amounts of ribonucleic acid phosphorous (RNAP) and desoxyribonucleic acid phosphorous (DNAP) in tissue, adapted the method for estimating the phosphorous content of each acid after chemical separation. It was possible to follow changes in these two components in chick-heart fibroblast cultures growing in roller tubes in vitro for seven days, by renewing the fluid phase every 24 hours. Interpretation of the results was especially difficult when large explants were used because central necrosis of the culture paralleled or exceeded the production of new cells. From small explants, appreciable and sustained increases in both RNAP and DNAP were obtained when 24 explants were used which had a total weight of about 2 mg. In tests of short duration, a rise in RNAP always preceded a rise in DNAP. The final RNAP/DNAP ratio in all tests of long duration was between 5 and 6. This marked rise during active growth supported the view that protein synthesis was accompanied by increases in RNA concentration in cells.

Hull and Kirk (50) extended the work of Davidson and Waymouth by studying the uptake of $P^{32}$ as well as the synthesis of nucleic acids. When the growth medium was adequate, DNAP and RNAP increased uniformly and paralleled each other closely after an initial drop. Since it was believed that phosphate entered DNA at the time of cell division, $P^{32}$ incorporation into DNA was suggested as an index of growth in cell numbers.

Prior to 1945, plasma or fibrin clots had proved to be the most efficient substrates for routine maintenance of tissue cultures. The analyses of growth thus far presented

have all used cultures of cells embedded in clots. However, there were many objections to the use of these clots (35). Some disadvantages have been pointed out by Evans and Earle: 1. The preparation of the plasma or fibrinogen involved technical difficulties; 2. under many conditions, the clot became clouded and obscured cell detail or was liquefied and the culture was destroyed; 3. the fact that the culture was embedded within the clot and could not easily be removed interfered with determinations of growth, weight, or chemical changes of the culture, as well as the study of fixed and stained preparations; 4. the chemically undefined nature of the clot interfered with studies of the metabolism of cells in tissue cultures.

It had been known that a glass surface would support growth from primary explant material when tissue fragments were first allowed to dry on the glass in roller tubes (67). Nutrient fluid could then be added and all of the tube that was bathed with the medium would become lined with a dense growth. As growth proceeded, local areas of stress developed and whole sheets of cells became detached from the glass (67). This method eliminated the clot, but still retained the obstacle of quantitative analysis, since attempts at subculturing cells directly on glass were not successful, and the technique of transplantation as employed with plasma-clot cultures could not be applied, because the true quantity still could not be assessed. In 1947 Evans and Earle (35) introduced the tech-

nique of growing cells on glass under perforated cellophane in Carrel flasks. Excellent growth was obtained from primary explants of embryonic chick-heart and subcultures of the chick-heart and strain L cells, previously grown in a plasma matrix. Control cultures of strain L cells grown directly on glass were so poor that these workers suggested such cultures should not be relied on in studies of cell nutrition.

At about this time, there was a rapid improvement in tissue culture methods and suitable media became available. Cell growth could be obtained easily from almost any tissue on primary explantation, and many new cell strains were developed. An increasing number of studies made use of established cell lines rather than explanted tissue.

Shannon and Earle (77) reinvestigated the growth behavior of strain L fibroblasts when planted as cell suspensions from cellophane cultures on a pyrex glass substrate, and compared the growth to that obtained under perforated cellophane. The growth was luxuriant and comparable in both instances; this finding was directly opposite to the earlier study. These workers attributed the newer observation to the fact that a greater density of cells in the inoculum was required to initiate growth directly on the glass floor of the flask. The cell sheets were so friable that quite uniform suspensions could be prepared. The luxuriance of growth and ease of handling of strain L on glass indicated that a plain glass substrate was satisfactory for the routine growth of this cell type and that such a culture method should be

further studied for possible use in quantitative work with replicate cultures.

With this new procedure, whereby cells were grown directly on glass, enumeration of cells or cell nuclei became practical. Direct counting of cells was found not to be the best method, because it was difficult to remove all of the cells by scraping and it was difficult to break up small clumps. A procedure was developed by Sanford et al. (74) in which 0.1 M citric acid plus crystal violet in a hypotonic solution was used to free nuclei from the cytoplasmic material and stain them at the same time. This method was employed to improve quantitation, because with such treatment excellent suspensions of nuclei could be obtained. This technique of freeing nuclei from cells was first reported by Dounce (27). The nuclei were enumerated in a hemocytometer, to give an accurate measure of the cell population. By these methods large numbers of accurately replicated cultures could be planted from a cell suspension and followed periodically for their developmental expression (31).

As previously indicated, mammalian cells were first successfully grown in suspension cultures in 1953, and shortly afterward Earl et al. (32) showed that fibroblasts would proliferate in this type of shaken culture. The suspension culture provided a classical model for the analysis of growth of mammalian cell populations in vitro.

Various devices have been used since 1953 to maintain animal cells in suspension. Earle et al. (31) placed erlen-

meyer or boiling flasks containing cell suspensions on a New Brunswick rotary shaker. Owens et al. (66) and Graham and Siminovitch (39) employed the "tumble tube" previously described. Cherry and Hull (15) adapted a magnetic stirring apparatus, and Danes (20) sustained the cells in suspension by means of a glass stirring device driven by a motor; the stirrer was introduced from the top of the culture vessel.

Terminology analogous to that used to describe cell-number increases in microbial cultures entered this field of study about 1954. The "growth cycle" is now commonly used to describe mammalian cell growth in vitro. This cyclical development of microbial cultures can be separated into a series of "connected and consecutive phases characterized by variations in growth rate" (54). When plotting the number of individuals with time a sigmoid curve results. The sigmoid curve can be separated into four major parts at points of change in growth rate. These phases are designated as (1) lag phase, (2) logarithmic phase, (3) plateau phase, (4) log death phase (7, 54, 63). Rinaldini (72) first used the growth cycle terminology in his study of cell populations of chick embryonic heart. Graham and Siminovitch (39) and Puck et al. (71) have also suggested the use of these terms. Earle's early work on suspension cultures showed typical growth curves by nuclei enumeration; however, he did not use Rinaldini's terms (72).

Siminovitch et al. (1957) (78) extended their studies on growth to include studies of the biosynthetic activities of strain L in suspension cultures. Their cultures contained

15 to 20 ml of cell suspension in a tumbling type tube. They showed that DNA, RNA, and protein content per cell remained constant during the logarithmic phase of growth and that the L cells could be kept indefinitely in the logarithmic phase by subculturing from log phase populations. They were interested in virus replication in animal cells and proposed the theory that this system of growing the strain L fibroblasts in suspension provided a virus-host system that is analogous to the virulent bacteriophage-bacterium system.

It was early recognized that growth of unicellular populations was not a simple process of multiplying cells, but a complex one including the total synthetic activities of the cell. Since the major understanding of this entire process was first gained through study of microbial systems, it seems advantageous to review the events that led to a more thorough understanding of our present knowledge regarding the development of microbial populations.

Henrici in 1928 (43), studying micro-colonies of bacteria starting with a single cell, noted that cells grew first in length and over-all size, then divided. This is the basic law of population development. It must be thoroughly understood that every biological plot is a randomized function of all the individuals in the population. Two fundamental plots can be made as functions of time; the first, previously described, is the increase in cell numbers, and the second and more informative is the increase in cell size, which can be subdivided into many biosynthetic processes. Protoplasmic

increase precedes the first cell division, and a plot of cell size increases with time would indicate cell growth from the time a cell is placed in a favorable environment, where it can initiate positive growth. Or, in a more technical sense, a cell begins to synthesize protoplasm when it is placed in an environment of high free energy. In order to synthesize materials the laws of chemical kinetics are obeyed. As Lamanna and Mallette state (53), "Organisms function at the expense of their environment, the latter being finally degraded to a state of low free energy in which the organism can no longer function."

When a cell reaches a certain size in its internal development it divides into two new individuals. The process of division is not thought to be a limitation of environment, but rather is predetermined by internal hereditary limitations. Although increases in cell numbers was the criterion originally used to study populations of unicellular organisms, this method of population study is now considered to be a primitive yardstick, because the curve obtained for increases in cell numbers is only a composite picture of the biosynthetic development of every individual in the culture. Thus, cell division is only an effect of a complex biochemical factory of enzymatic reactions.

The studies to be reported here have attempted to investigate the phenomenon of mammalian cell growth in suspension culture. The experiments have been designed not only to analyze the population, but, more important, to characterize

the individuals in the population. It is believed that this work has provided a means for a better understanding of fibroblast growth *in vitro*. It also serves as a model system which permits an experimental approach to the study of the biochemistry of animal cells.

## MATERIALS AND METHODS

1. Cell Strains

(a) Strain L 929 Mouse Fibroblasts - This cell line was derived from normal subcutaneous tissue of an adult $C_3H$ mouse in 1940 (30) and was isolated as a clone in 1948 (75). A subculture of the clone isolate was received from Dr. Earle in 1952. It has been propagated in stationary culture since 1952 and in agitated fluid suspension culture since 1955.

(b) LLC-$M_1$ Strain Mouse Fibroblasts - This mouse fibroblast strain was isolated from a hypertrophic lymph note of a Swiss mouse by Hull in 1952 (49). This cell line has been grown in stationary culture since it was obtained in 1953 and in fluid suspension culture since 1955. The LLC-$M_1$ strain appeared to grow and synthesize cellular materials at rates which were analogous to strain L.

2. Culture Media

(a) Synthetic medium 199 - Medium 199 of Morgan, Morton and Parker contains a complete complement of amino acids and vitamins as well as accessory growth factors (64). Nucleic acid hydrolytic constituents are included as purines, pyrimidines and pentose sugars. Glutamine,

found by Fisher et al. (37) to be of great importance in cell growth, is also added. The mixture was used as a basal solution, since a completely adequate synthetic medium for animal cells in tissue culture has not been devised. During the early studies, synthetic medium 199 was prepared in this laboratory and sterilized by autoclaving at 121.6° C for fifteen minutes. Later, this medium was purchased as a 10x concentrate.*

(1) Synthetic medium 199 (60%) + Horse Serum (20%) + Hank's Balanced Salt Solution (20%) - ($199_{60}HS_{20}BSS_{20}$) - As a growth medium for fibroblasts in suspension cultures, synthetic mixture 199 was supplemented with 20% horse serum and 20% balanced salt solution. This medium was found satisfactory for the maintenance and propagation of cells through continuous subcultivation in this type of in vitro culture.

(2) Synthetic Medium 199 with 0.5% Bacto-Peptone - (199P) - Peptones have long been used to grow fibroblasts in vitro (1). Synthetic medium 199 supplemented with peptone was found to be adequate for growing fibroblasts on a glass surface and was thus used to carry stock cultures. Uniform suspensions could not be obtained in agitated cultures, however, unless this medium was supplemented with 5% horse serum ($199P_{95}HS_5$).

---

*Microbiological Associates Inc., Washington, D.C.

(b) Eagle's Synthetic Medium - This is a basal medium; it contains twelve essential amino acids, eight vitamins, and glutamine (28, 29). These ingredients are necessary for cell growth and the medium is considered to be optimal when supplemented with a dialyzed serum fraction.

(1) Eagle's Synthetic Medium (80%) + Horse Serum (20%) - (Eagle's$_{80}$HS$_{20}$) - Cell growth in this medium was compared with that in medium 199. As a growth medium for fibroblasts, it was supplemented with 20% horse serum.

(c) Lactalbumin - Yeast Extract - Eagle's - Peptone Medium - (YELP) - Cooper (18) introduced the combined use of lactalbumin and yeast extract in tissue culture. In this laboratory it had not been found satisfactory as a growth medium unless it was supplemented. Eagle's synthetic mixture and peptone were added and the result provided an adequate growth medium. The composition of the medium is as follows.

| | |
|---|---|
| Hank's balanced salt solution (10x) | 100 ml |
| Lactalbumin Hydrolysate (enzymatic) (Nutritional Biochemical) | 2.5 g. |
| Yeast Extract (Difco) | 0.5 g. |
| Bacto-Peptone (Difco) | 5.0 g. |
| Eagle's amino acid concentrate (100x) | 5 ml |
| Eagle's vitamin concentrate (100x) | 5 ml |
| Eagle's glutamine (200 millimolar) | 0.5 ml |

| | |
|---|---|
| Sodium bicarbonate | 1.0 g. |
| Distilled water | to 1000 ml |

(1) Lactalbumin - Yeast Extract - Eagle's - Peptone Medium (95%) + Horse Serum (5%) - ($YELP_{95}HS_5$) - As a growth medium, 95 parts of the YELP mixture was mixed with 5 parts horse serum. This medium was found to be more economical for large-scale experiments, since it was as satisfactory as any other medium studied and was less expensive than 199.

(d) Penicillin and Streptomycin - When a culture was set up, penicillin and streptomycin were added from a stock solution to bring the concentration of each to 100 units per ml. in the growing culture.

3. Growth of Mouse Fibroblasts in Stationary Cultures

Populations of mouse fibroblasts can be grown as a monolayer on a glass surface (77). The strain L fibroblast was grown throughout this study on one of the inner surfaces of neutraglas bottles (250-ml capacity, with a surface area on one of the sides of about 56 $cm^2$). Ten ml of growth medium containing approximately 2 x $10^5$ cells/ml was used as the inoculum. The cells settled on the surface of the glass and were bound there. As growth proceeded, increases occurred in cell numbers, until the entire glass surface was covered with cells all adherent to the glass and to each other. When a population was fully grown, the cells could be scraped off the

glass, dispersed, diluted, and replanted in fresh bottles. In early studies on growth of these fibroblasts, the procedure used by Rinaldini (72) for chick-embryo heart cells was followed. A replicate series of stationary bottle cultures was set up and cells were harvested at various time intervals to obtain a growth-response curve.

4. Growth of Mouse Fibroblasts in Fluid Suspension Cultures.

Mouse fibroblasts were propagated in agitated fluid suspension cultures in erlenmeyer flasks, closed with rubber stoppers. Flasks of 250-ml and 500-ml capacity were used; the volume of the cell suspension in each flask was about 80 or 100 ml respectively. The flasks containing the cell suspension were placed on a Eberbach rotary shaker* having a one-inch radial stroke. The speed of the shaker was adjusted to 100 rpm. Various ratios of liquid volume to size and shape of container, as well as the speed and method of agitation, have been analyzed in this study. After successfully propagating these cells in fluid suspension, it became apparent that the system had shortcomings which limited its use as a quantitative tool. The limitations, mostly mechanical, were studied first and after their satisfactory characterization, the investigation was extended.

---

*Eberbach and Son Company, Ann Arbor, Michigan.

5. Mechanics Involved in Setting Up Fluid Suspension Cultures of Mouse Fibroblasts

Fluid suspension cultures can be set up from stationary cell cultures grown on a glass surface, or directly from previous suspension cultures. The cells may be transplanted from growing (log phase) or mature (plateau phase) cultures.

In harvesting bottle cultures, the growth medium was decanted and 2 or 3 ml of fresh medium was added. The cells were scraped off the glass surface and pipetted vigorously to obtain a dense suspension of evenly dispersed cells. In harvesting from suspension cultures, the cells were sedimented at 1,000 to 1,500 rpm. The used medium was decanted and about 10 ml of fresh medium was added; the cells were dispersed by pipetting.

A 0.1-ml sample of the suspension was diluted 1:10 or 1:100 in 0.25% trypsin, in citric acid-crystal violet, or in Hank's balanced salt solution, so as to obtain a countable sample; a drop of the suspension was then placed in the hemocytometer. After the count was made, the number was multiplied by the dilution factor to express the number of cells per ml.

The volume of suspension needed to set up suspension cultures was obtained by diluting with fresh medium, to a concentration of 1 to 3 x $10^5$ cells/ml.

6. Procedure for Setting Up a Replicate Series of Fluid Suspension Cultures

A 2-liter side arm flask fitted with a bell delivery

bottom outlet was filled with 1,000 to 1,500 ml of growth medium depending on the number of replicate cultures needed in the experiment (36). A heavy cell suspension was inoculated, so as to obtain the number of cells indicated above. About 102 ml of the suspension was delivered into each of a series of 500-ml erlenmeyer flasks. When 250-ml flasks were used, approximately 80 ml of cell suspension was the inoculum. All analytical work was done on cultures grown in 500-ml flasks. One replicate culture was harvested at zero time and one to three cultures were harvested each day during the experiment.

7. Analysis of the Development of a Mammalian Cell Population

When a culture was harvested, it was split into two parts; a 1-ml and a 100-ml portion (figure 1); the remainder was discarded.

(a) Analysis of the 1-ml portion

(1) Cell counting with the hemocytometer, and the measurement of cell diameter

A. A cell count was made in a hemocytometer as previously described.

B. Gross morphological observation of the rounded fibroblasts, growing in suspension cultures, indicated cells of various sizes. Upon measurement of cell sizes with a calibrated ocular micrometer, it was found convenient to place a cell into one of four categories according to

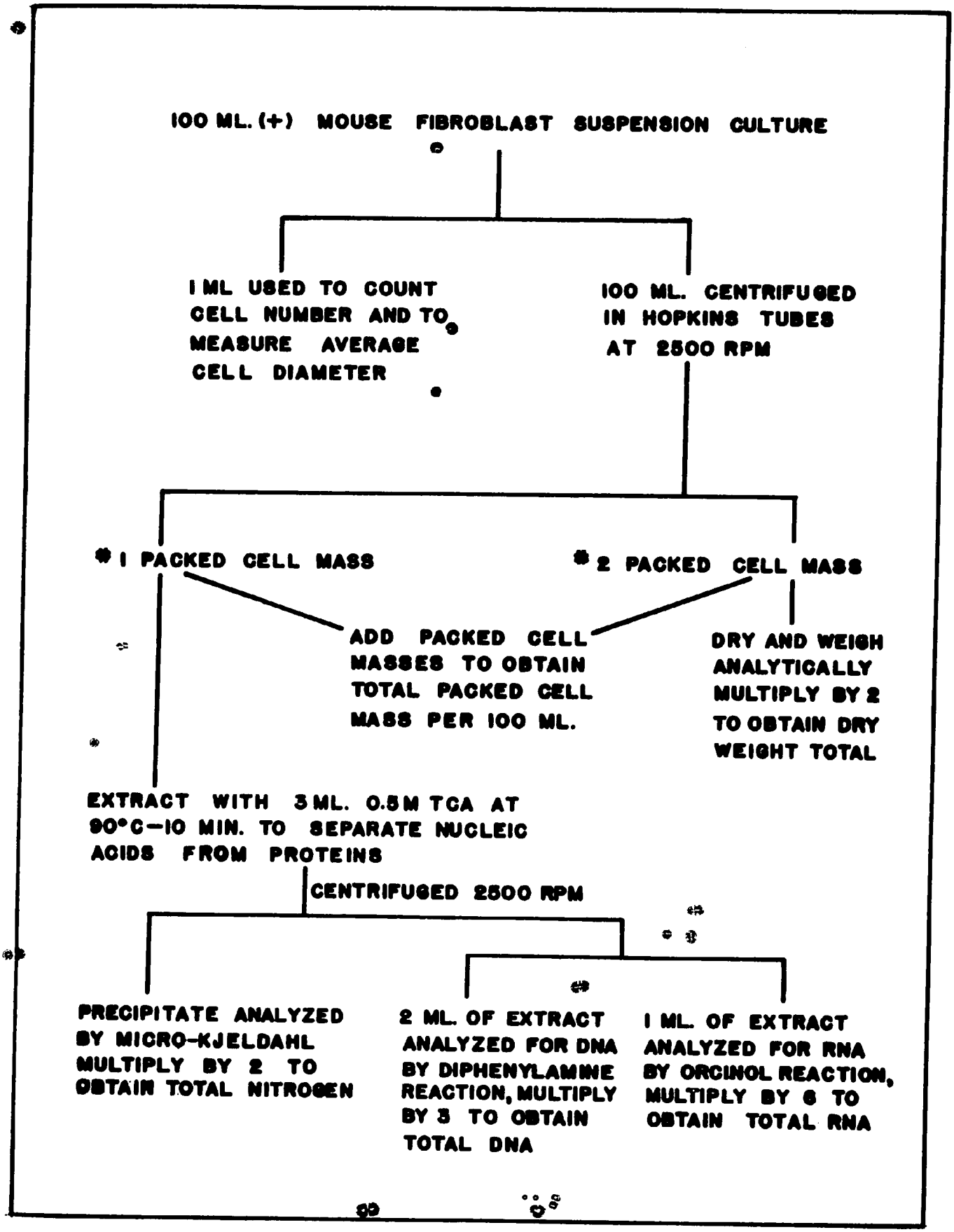



FIGURE 1. ANALYSIS OF THE DEVELOPMENT OF A MAMMALIAN CELL POPULATION GROWING IN FLUID SUSPENSION CULTURE.

size. The size of a cell could easily be determined when observed at 450x, since the cell would fit between definite lines on the ocular micrometer. The cell volume could immediately be judged, because the volume had been precalculated for each category.

(2) Cell counting with the electronic blood cell counter*

The electronic counter used in this study was based on the principle that cells are poor electrical conductors, as compared with a saline solution. In operation, a dilute suspension of cells in NaCl was drawn through a minute aperture conducting an electrical current between platinum electrodes. Each cell passing through the aperture displaced an equal volume of solution and thereby modulated the electric current. The resulting pulses were amplified and counted (6). Five-tenths ml of the 1.0 ml portion was diluted to 100 ml with physiological saline in a siliconized beaker; this gave a 1:200 dilution. Figure 2 illustrates the counting system. The tube B contained the aperture A through which the sample was drawn; C and D represent the electrodes. When stopcock F was opened an external vacuum source P initiated flow through the orifice and caused the mercury J in the manometer to assume the position shown, with the mercury in the open arm of the manometer drawn slightly below the

*Coulter Electronic, Chicago, Illinois.

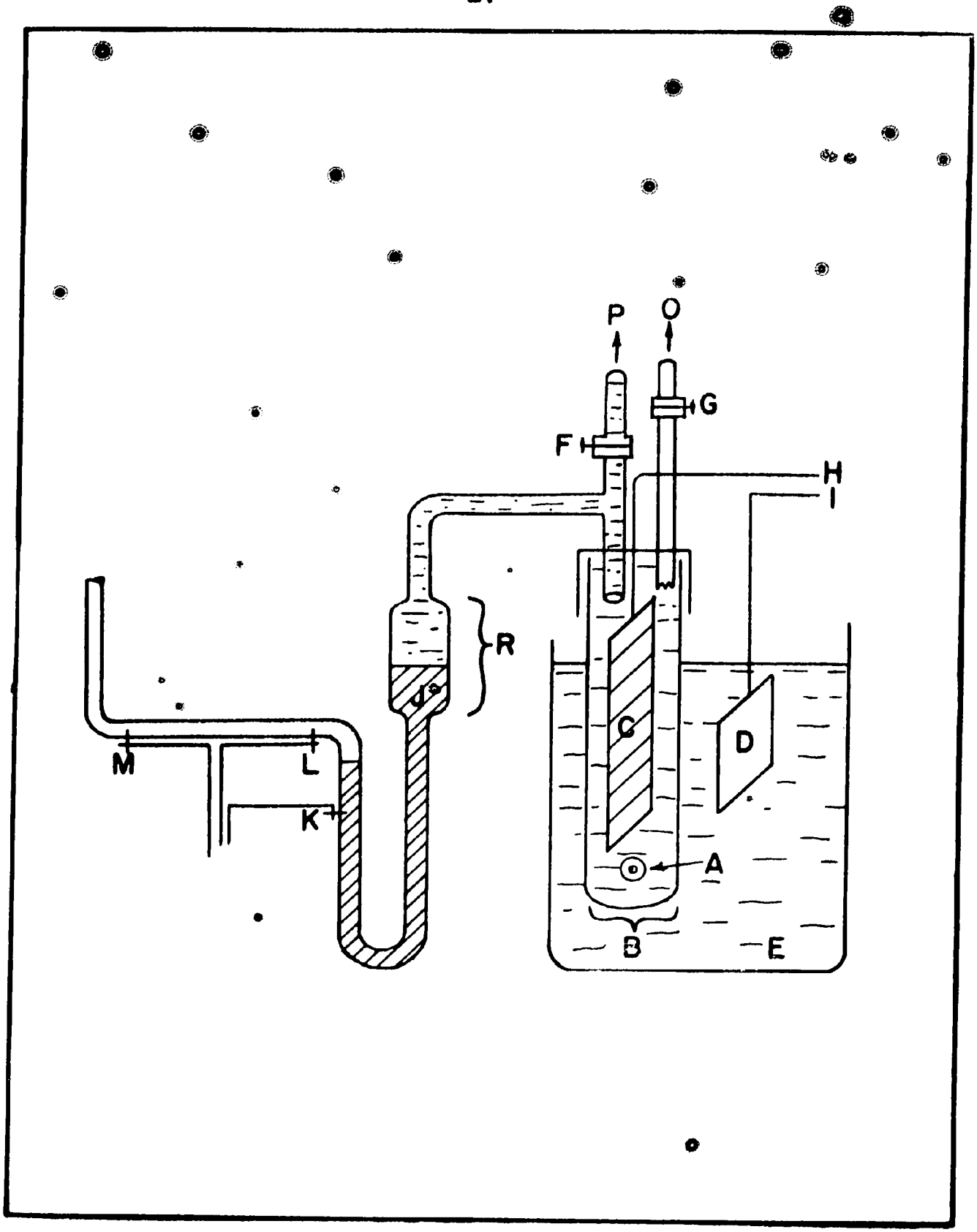



FIGURE 2. DRAWING SHOWING APERTURE TUBE, SAMPLE BEAKER, ELECTRODES AND FLOW CONTROL ELEMENTS OF THE ELECTRONIC BLOOD CELL COUNTER.

horizontal branch. When stopcock F was closed, the unbalanced manometer functioned as a siphon to continue the flow of the sample through the orifice. As the mercury in the open arm rose into the horizontal branch, it made contact with a wire electrode L sealed in the manometer wall and set in motion a high speed decade counter, which began counting the impulses that reached or exceeded the threshold level. The counter was set to include the smallest cells in these fibroblast populations. A few seconds later the mercury column made contact with a second wire electrode, M, which stopped the counter. By multiplying this count by the dilution factor, the cell count could be obtained.

(b) Analysis of the 100-ml portion

(1) Packed Cell Volume - The 100-ml cell suspension split into two equal parts (figure 1) and centrifuged 10 ml at a time in two or four Hopkins centrifuge tubes which were graduated to a total volume of 0.050 ml (50 µl), in 0.002-ml divisions. Readings of the packed cell volumes were made independently on the samples. The packed cell masses were split into two samples #1 and #2. Sample #1 was extracted with trichloroacetic acid (TCA) and sample #2 was dried to determine the weight.

(#1) Trichloracetic acid extraction - The packed

cell sample marked #1 was treated with 3 ml of 0.5 M TCA and heated at 90° C for 10 minutes to extract the nucleic acids (17). After centrifugation to pack the residue, the supernatant fluid was removed and used for nucleic acid determinations. The precipitate was used to determine the total cell nitrogen.

a. Desoxyribonucleic acid - Two ml of the nucleic acid extract was used to analyze for DNA by the Dische diphenylamine reaction (17). Four ml of reagent (100 ml of glacial acetic acid, 2.75 ml. concentrated $H_2SO_4$, 1 g diphenylamine) was added to the samples and heated in a boiling water bath for ten minutes. After cooling, the blue color was read in the Klett-Summerson colormeter at 600 mμ along with DNA standards of from 50 to 500 μg. The standard DNA curve is shown in figure 3.

b. Ribonucleic acid - Five-tenths ml of the remaining extract material was used to analyze for RNA by the Mejbaum orcinol method (17,60). Two and one-half ml of water was added to bring the volume to 3 ml. Three ml of reagent (0.33% $FeCl_3.6H_2O$ in 12 N HCl) was added to the sample. Three-tenths ml of fresh orcinol was added to the

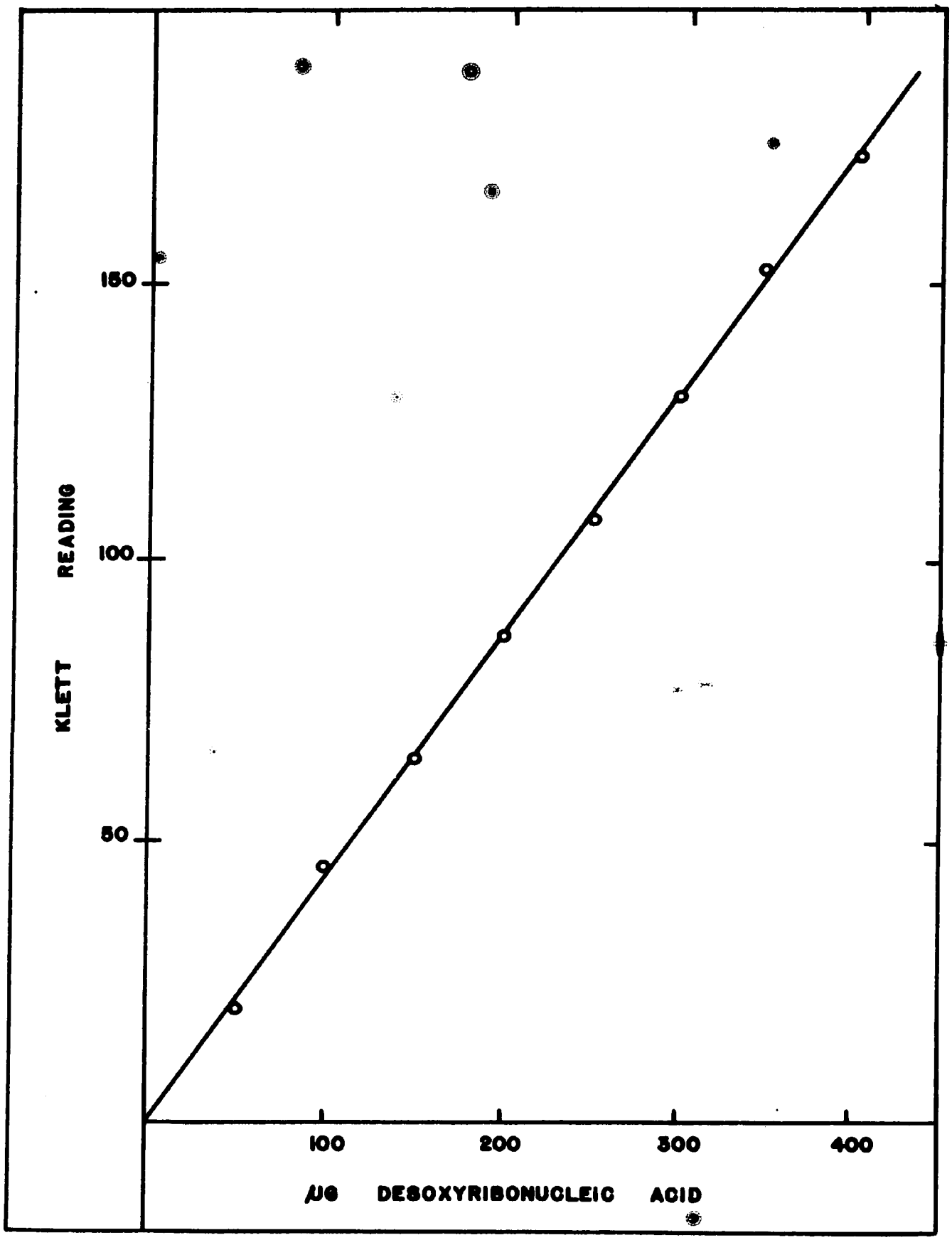



FIGURE 3. STANDARD CURVE FOR DESOXYRIBONUCLEIC ACID.

mixture and the samples were heated in a boiling water bath for 20 minutes to develop the green color. The color intensity was read at 660 mμ in the Klett-Summerson colormeter along with RNA standards from 25 to 150 μg. The standard curve is shown in figure 4.

c. Total TCA precipitable nitrogen - The residue, after removal of the nucleic acids by trichloroacetic acid extraction, was analyzed for total nitrogen by the micro-Kjeldahl technique.

(#2) Dry Weight Determination - The sample indicated #2 was resuspended in 5 ml of complete growth medium and immediately dried to constant weight in a previously weighed aluminum cup. The sample was weighed after drying and weight increase was determined by subtracting the weight of a 5-ml dried sample of medium.

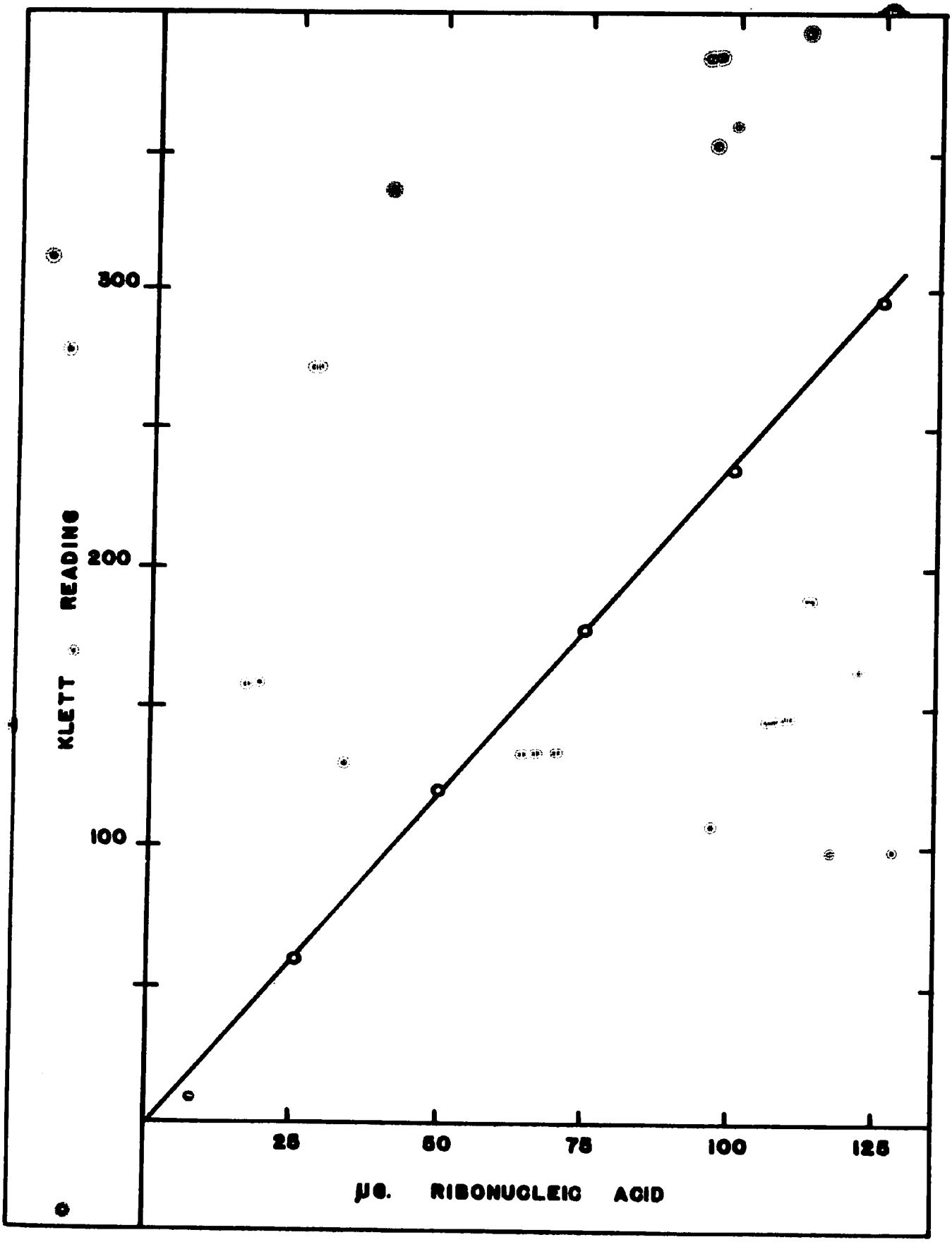



FIGURE 4. STANDARD CURVE FOR RIBONUCLEIC ACID.

## EXPERIMENTAL RESULTS

When living organisms are placed in a favorable environment, they grow and multiply; plotting the changes in the number of units with time gives the growth curve. Various cell populations have been analyzed by means of growth curves, including cells of plants, animal embryos, solid tumors, and microbes. The application of the growth-curve principle to the study of strain L mouse fibroblasts was therefore an obvious approach, and was considered necessary to further study of the cell.

### 1. The Growth of Mouse Fibroblasts in Stationary Cultures Directly on Glass

A replicate series of stationary cultures of strain L cells was prepared as follows. Ten ml of a cell suspension containing $1.8 \times 10^5$ cells/ml in a medium $199_{60}HS_{20}BSS_{20}$ was pipetted into each of a series of bottles. Duplicate bottles were harvested from the series at various time intervals during the growth of the population, and cell counts were made following resuspension of the cells. The results, when plotted as the number of cells expressed as $\log_{10}$ with time, showed that a sigmoid curve described the increases in fibroblast populations (figure 5). Following inoculation, there was approximately a twenty-four period during which the number

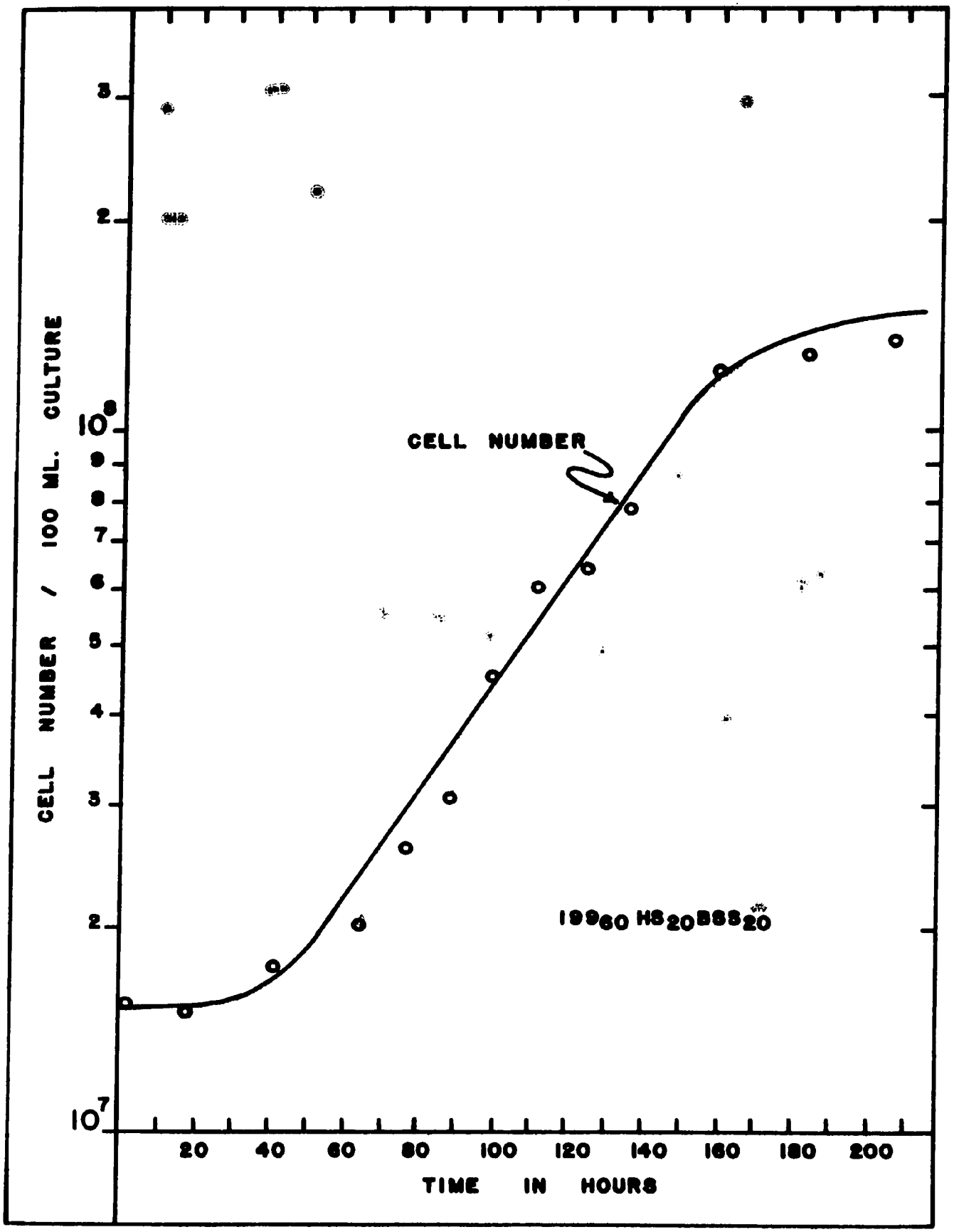



FIGURE 8. POPULATION GROWTH CURVE OF MOUSE FIBROBLASTS IN STATIONARY BOTTLE CULTURES.

of cells remained constant or, as in many experiments, decreased. After this period, cell multiplication began and soon attained a rate which remained constant for three to four days.

Thus it was realized that mouse fibroblast growth could be described in this manner and given a mathematical interpretation. The growth cycle principle has since become an important method in studies carried out in this laboratory.

2. The Fluid Suspension Culture Method

The need for large numbers of animal cells and the difficulties of quantitating replicate cultures led to an investigation of fluid suspension cultures as described by Earle et al. (32).

Many of the early studies were concerned with assessing technical details relating to the growth of animal cells in suspension cultures. Three properties of these cells were found to have a direct bearing on the technological aspects of the problem.

1. Cell Fragility - This system of cell cultivation could be utilized as an analytical tool only if cell breakage was prevented. The speed and method of agitation were found primarily important in determining the breakage factor. If the speed of the shaker was adjusted to 100 rpm. through a one-inch radial stroke, cell breakage could be completely eliminated within the limits of microscopic observation. The amount of suspension in the

erlenmeyer flask was not too critical in determining cell breakage, because comparable results could be obtained throughout a suspension-to-flask-size ratio of approximately 1:2.5 to 1:5 (e.g. the amount of suspension in a 500-ml erlenmeyer flask could vary between 100 and 200 ml).

2. Cell Size - The average volume of a fibroblast was found to be 2,200 $\mu^3$. Cells in this size range have a marked tendency to sediment and they rapidly settle from suspension under stationary conditions. Hence, the purpose of agitating cultures was to maintain the cells in suspension. It was found that the nearer the ratio of suspension to flask size approached 1:5, the more likely it would be to keep cells in suspension.

3. Cell Adhesion - Mammalian cells have a marked tendency to stick to glass and to each other. This adhesion of cells to the glass was particularly troublesome at the meniscus, and this problem has not been completely eliminated. However, it was found that the problem of adhesion was less troublesome when cells were growing in the logarithmic phase, because cells undergoing division become rounded and therefore more discrete. Binding between cells was also reduced as the 1:5 ratio of suspension to flask size was reached.

Thus problems associated with the fluid suspension

culture system were investigated and solved so that a quantitative approach to the study of growth could be made. The results presented below were all obtained in cultures of either 80-ml or 100-ml suspensions in 250- and 500-ml erlenmeyer flasks respectively. The erlenmeyer flasks were placed on a rotary shaker moving through a one-inch radial stroke at 100 rpm.

3. The Growth of Mouse Fibroblast Populations in Fluid Suspension Cultures

Having established a usable method for the growth of fibroblasts in suspension cultures, an effort was made to determine the optimal growing system and to study the cell's biosynthetic activities.

(a) The Effect of Age of Inoculum

(1) The Use of Plateau Phase Cells as the Inoculum

A typical growth curve for strain L mouse fibroblasts growing in agitated suspension culture in medium $199_{60}HS_{20}BSS_{20}$ is shown in figure 6. The population was started with $1.6 \times 10^5$ cells/ml; increases in cell numbers occurred until the characteristic maximum population was reached that could be supported by this medium. The maximum level in this culture was $1.3 \times 10^6$ cells/ml. A lag period of approximately twenty-four hours could be noted, during which time the pH of the suspending medium dropped from 7.4 to about 7.0. Exponential growth began at about the twenty-fourth hour and continued for approximately one hundred fifteen hours. The generation time in this medium was about forty hours. Two and one-half to

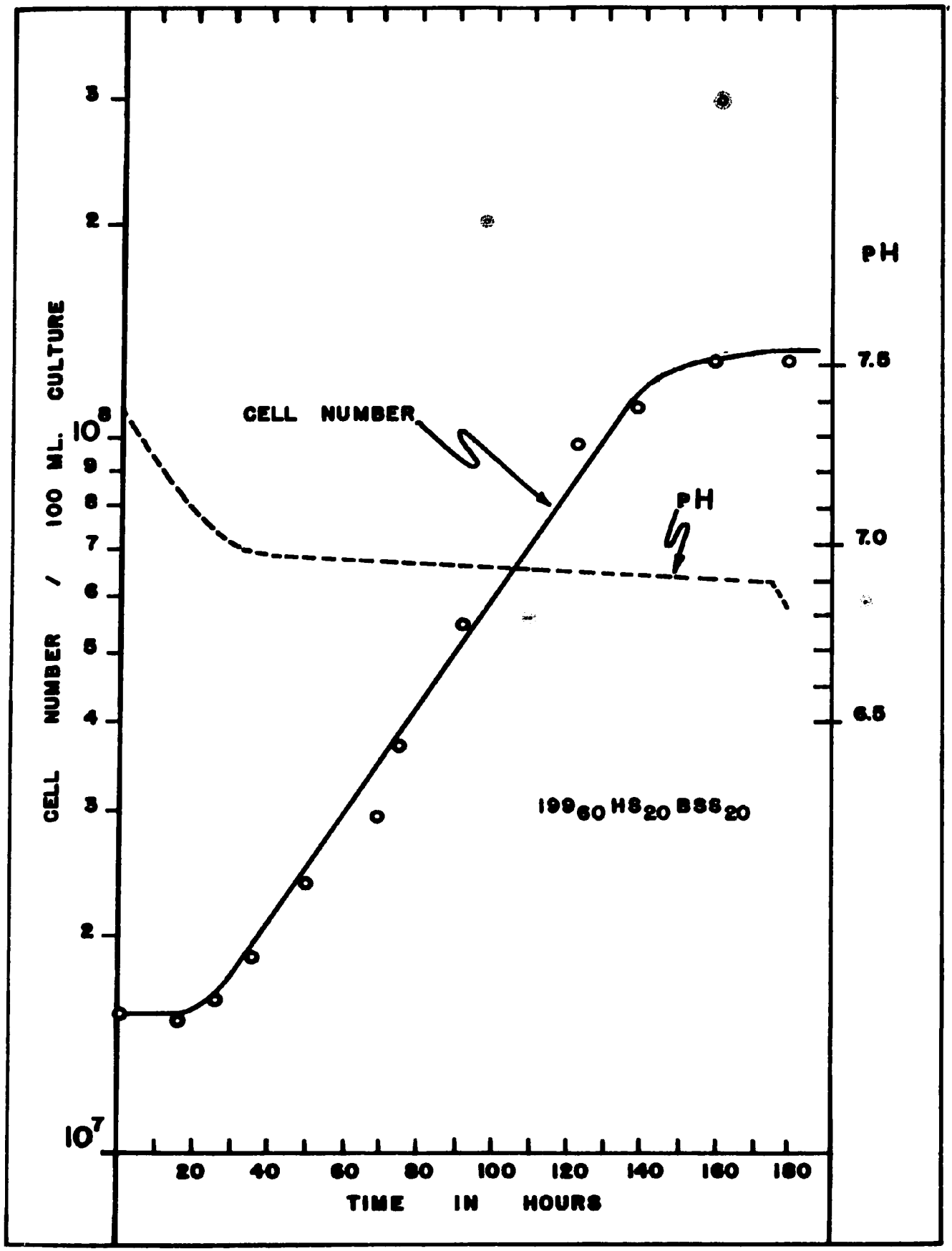



FIGURE 6. POPULATION GROWTH CURVE OF MOUSE FIBROBLASTS IN FLUID SUSPENSION CULTURES, USING PLATEAU PHASE CELLS AS THE INOCULUM.

three generations were represented by the logarithmic growth phase. The plateau phase became evident between the sixth and the eighth day; beyond this point the culture lost its viability rapidly, and indications were that the pH dropped rapidly after the plateau level was reached. It was interesting to note that the growth curve in stationary cultures was comparable to those in suspension cultures. Although a comprehensive study was not made in stationary cultures, it was concluded that the starting population in figure 5 must have been taken from plateau phase cells.

(2) The Use of Logarithmic Phase Cells as the Inoculum in Non-Adjusted (pH and Temperature) Growth Medium

Figure 7 depicts the increase in numbers of fibroblast cells in suspension cultures in $199_{60}HS_{20}BSS_{20}$. The cell inoculum was taken from a population growing in the logarithmic phase and placed in an environment where pH and temperature of the medium were not especially adjusted. The growth pattern was essentially comparable to that in figure 6, except that there was no true initial lag period (48) where complete stoppage of division would be evident. This culture cycle showed that only a decrease in the rate of multiplication occurred. Penfold (69) has emphasized the step-like character of cell processes, and the necessity for building up the right intermediates, so it would follow that logarithmic phase cells contain a steady concentration of intermediates. Theoretically, then, this population contained all the prerequisites for continued exponential multiplication. It was believed

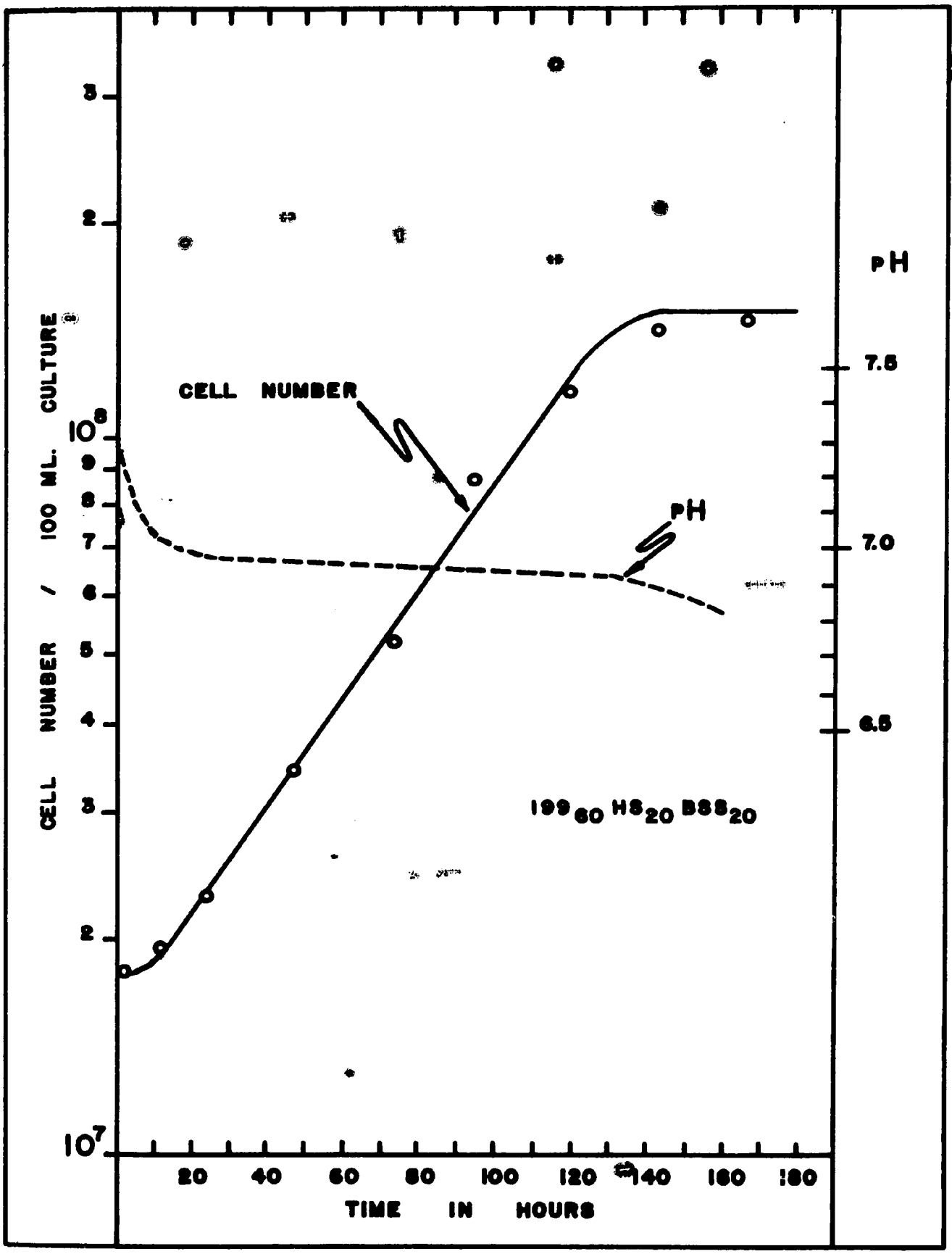



FIGURE 7. POPULATION GROWTH CURVE OF MOUSE FIBROBLASTS IN FLUID SUSPENSION CULTURES, USING LOGARITHMIC PHASE CELLS AS THE INOCULUM IN NON-ADJUSTED (pH AND TEMPERATURE) MEDIUM.

that the pH was not the rate-limiting factor, because experiments to be cited later showed that cells could grow and multiply through a fairly wide pH range. Rather, it was postulated that this was a kinetic energy imbalance which retarded biosynthetic reactions. The rate of cell division progressively increased from zero time at each lag increment until the maximum division rate was again reached, as depicted by the exponential slope.

(3) The Use of Logarithmic Phase Cells as the Inoculum in Medium Pre-Adjusted (pH and Temperature)

An exponentially dividing population introduced into medium 199$_{60}$HS$_{20}$BSS$_{20}$, pre-adjusted to 37° C and equilibrated at pH 7.0, showed no evidence of a lag phase (figure 8). It was felt that maintaining the temperature at 37° C was the important factor, because a slight shift would affect the rate of biosynthetic reactions. A single alteration in a consecutive series of enzymatic reactions would suffice to slow down the entire biological process and create a lag phase. The medium was adjusted to pH 7.0 because this appeared to be the optimal pH for growth when 20% serum was added to the medium. Lamanna and Mallette (53) have stated that variations in pH of media have less effect on the growth rate than on the total yield. Further clarification of the effect of pH on this system should be made, but the experiments were limited by the tedious process of adjustment of the bicarbonate buffer system. Figure 8 also shows that the presence of a lag phase does not affect the rate of logarithmic growth; the generation

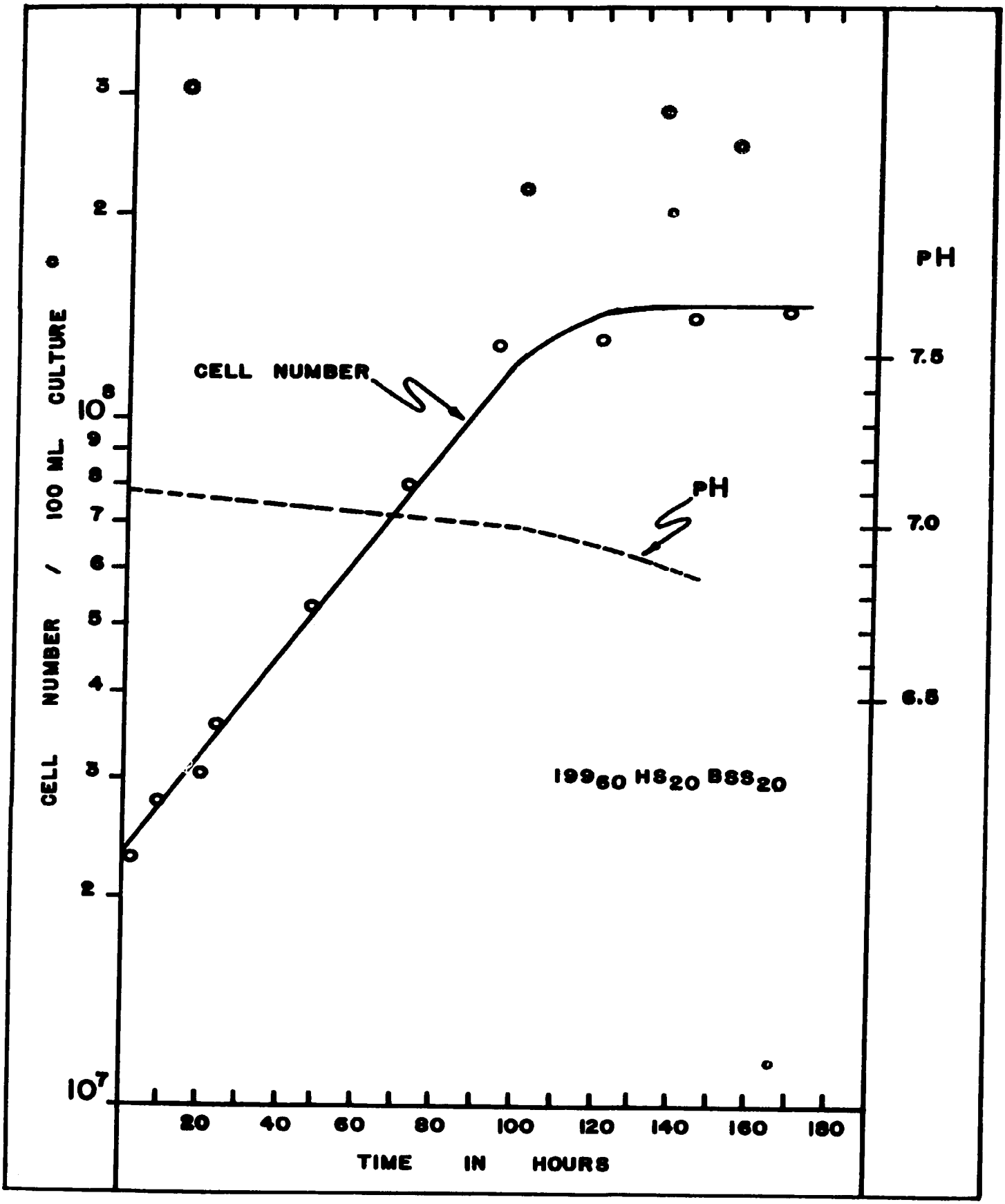



FIGURE 8. POPULATION GROWTH CURVE OF MOUSE FIBROBLASTS IN FLUID SUSPENSION CULTURES, USING LOGARITHMIC PHASE CELLS AS THE INOCULUM IN PRE-ADJUSTED (pH AND TEMPERATURE) MEDIUM.

time, the number of generations in the log phase, and the maximum cell level were about the same.

(b) The Effect of Serum Concentration

Since basic information was now available with reference to the growth cycle of mouse fibroblasts in suspension, application of the rate of fibroblast growth could be made, so as to compare growth under various conditions.

Serum had always been found essential for the propagation of animal cells in vitro. It was felt that if horse serum exerted a beneficial metabolic effect on fibroblast growth, then increased serum concentrations should increase the rate of growth. With variations of serum concentration over the range of 5 to 40% in a complete growth medium, it could be seen (figure 9) that the rate of growth in the logarithmic phase was essentially independent of the serum concentration; the rate was calculated with the formula R = 1/generation time, and the rates vary slightly between 1.8 and 2.0 x $10^{-2}$ (63). The starting inoculum for this experiment contained approximately the same number of cells in each culture; they were taken from a culture in the logarithmic phase growing in a medium composed of $199_{60}HS_{20}BSS_{20}$. The fresh medium was not preadjusted for temperature or pH. It was noted that the apparent optimum pH for growth during the logarithmic phase varied with the concentration of serum in the medium; this variability accounted for the observation that logarithmic growth could occur over a wide pH range. There appeared to be a lower than maximum cell density in the cultures containing

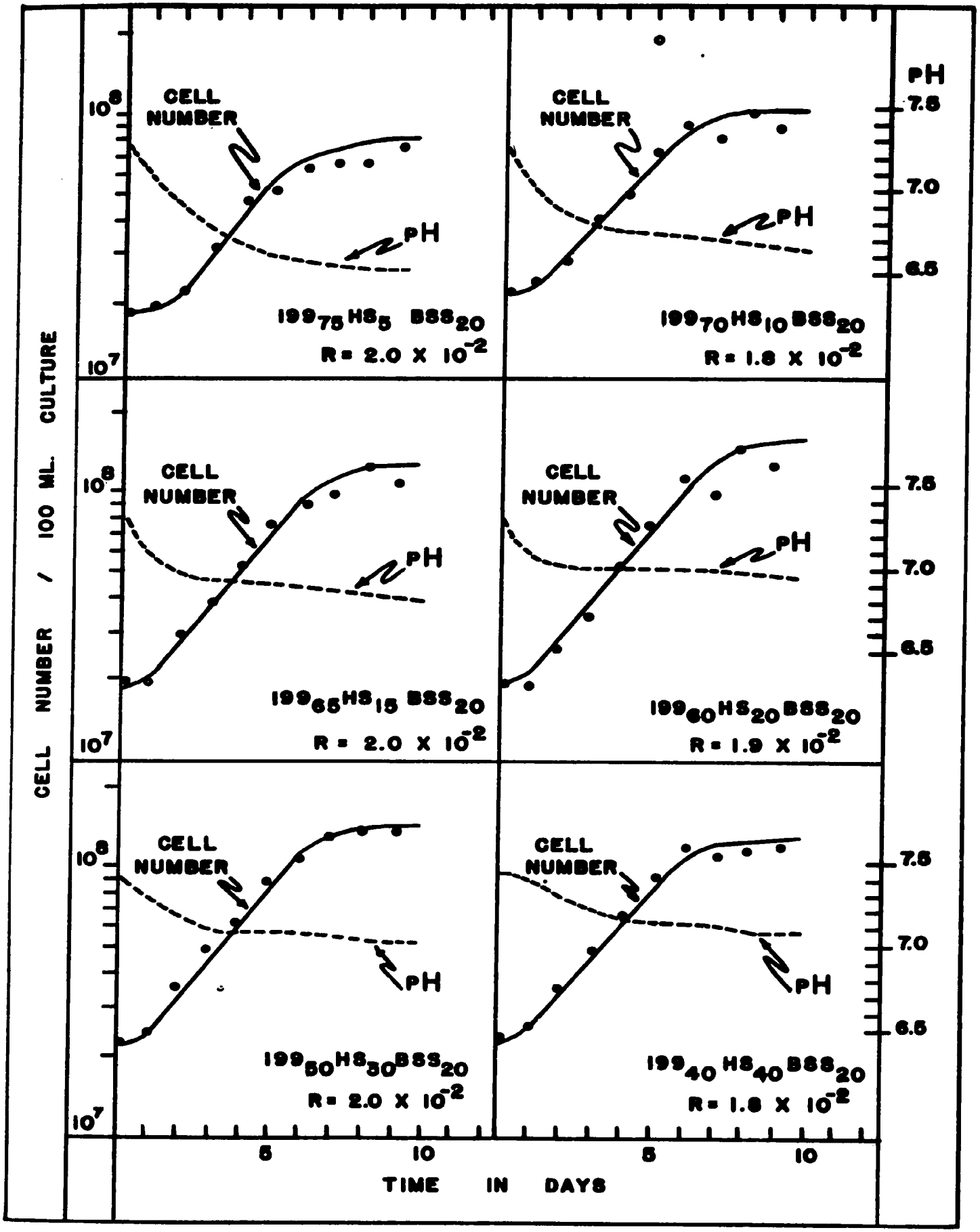



FIGURE 9. THE EFFECT OF VARYING THE SERUM CONCENTRATION ON THE GROWTH OF MOUSE FIBROBLASTS IN FLUID SUSPENSION CULTURES.

5 to 10% horse serum; this was evidenced by the plateau phase levels. However, when the serum concentration was increased to 15% or more, the maximum cell density in all cultures was essentially the same. From these results it was concluded that horse serum was essentially a buffer, and in its absence cells could not grow because of rapid pH shifts.

(c) The Effect of Various Media

Various tissue culture media do not possess analogous abilities to support growth of mammalian cells. Studies were conducted to determine which of several media could best serve the needs of further proposed work. A representative curve for each medium studied is shown in figure 10. The slope of the exponential line indicates the rate at which cell growth has occurred. The steepest slopes indicate better balanced nutritive media, for in such media substrate competition would not occur until the plateau phase was approached. In mass cultures, however, the most economical as well as the most biological informative medium would be used. It appeared that $YELP_{95}HS_5$ was the most satisfactory combination for fibroblast growth, and this medium was used in the subsequent experiments in this investigation.

(d) The Effect of Inoculum Size

Clarification was needed with regard to the interdependence of cells in shaken cultures. An experiment was devised to determine whether the size of the initial cell inoculum affected the growing system. Figure 11 shows that inoculums were varied from 1 to 3 x $10^5$ cells/ml, the rate of

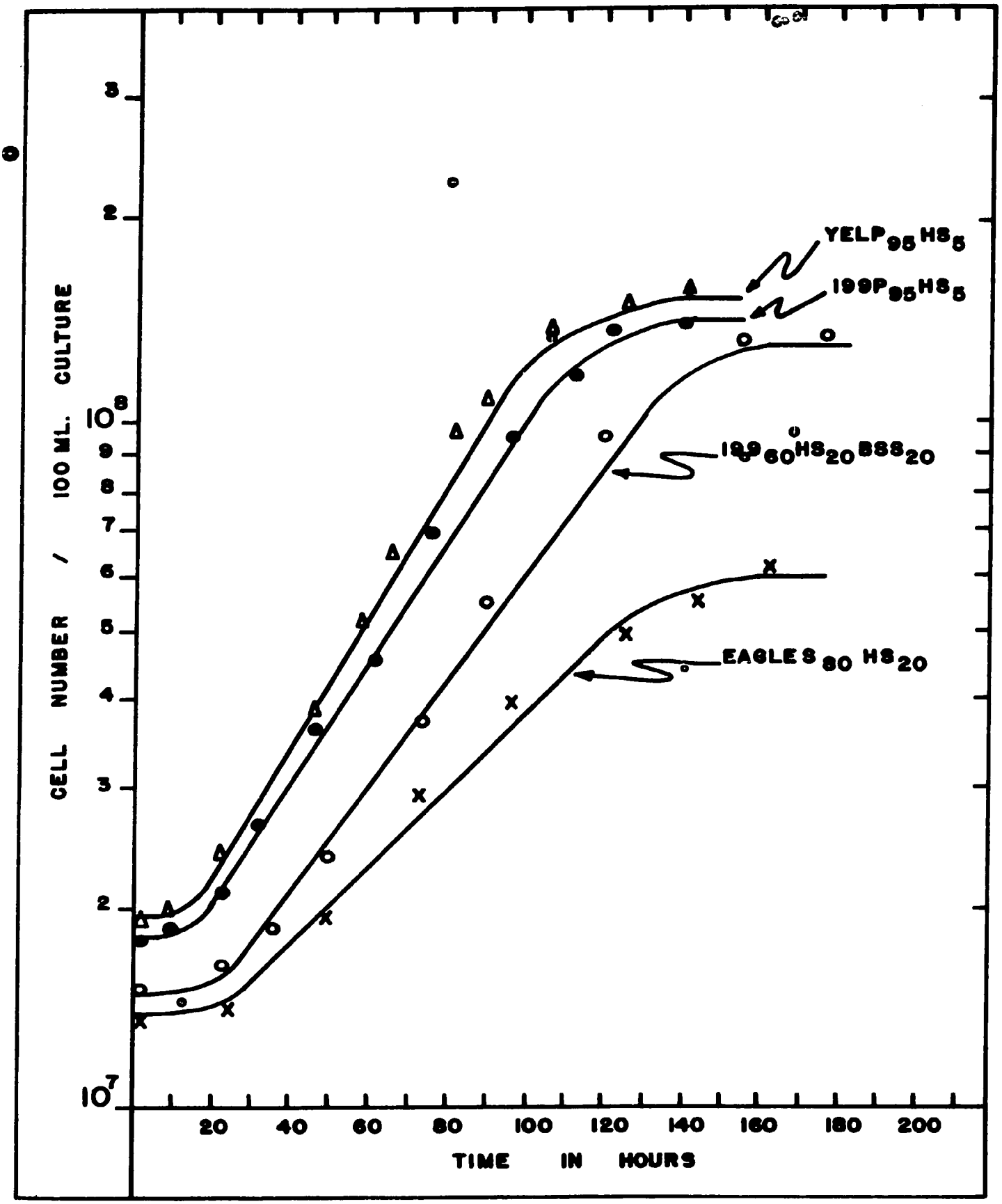



FIGURE 10. THE EFFECT OF VARIOUS TISSUE CULTURE MEDIA ON THE GROWTH OF MOUSE FIBROBLASTS IN FLUID SUSPENSION CULTURES.

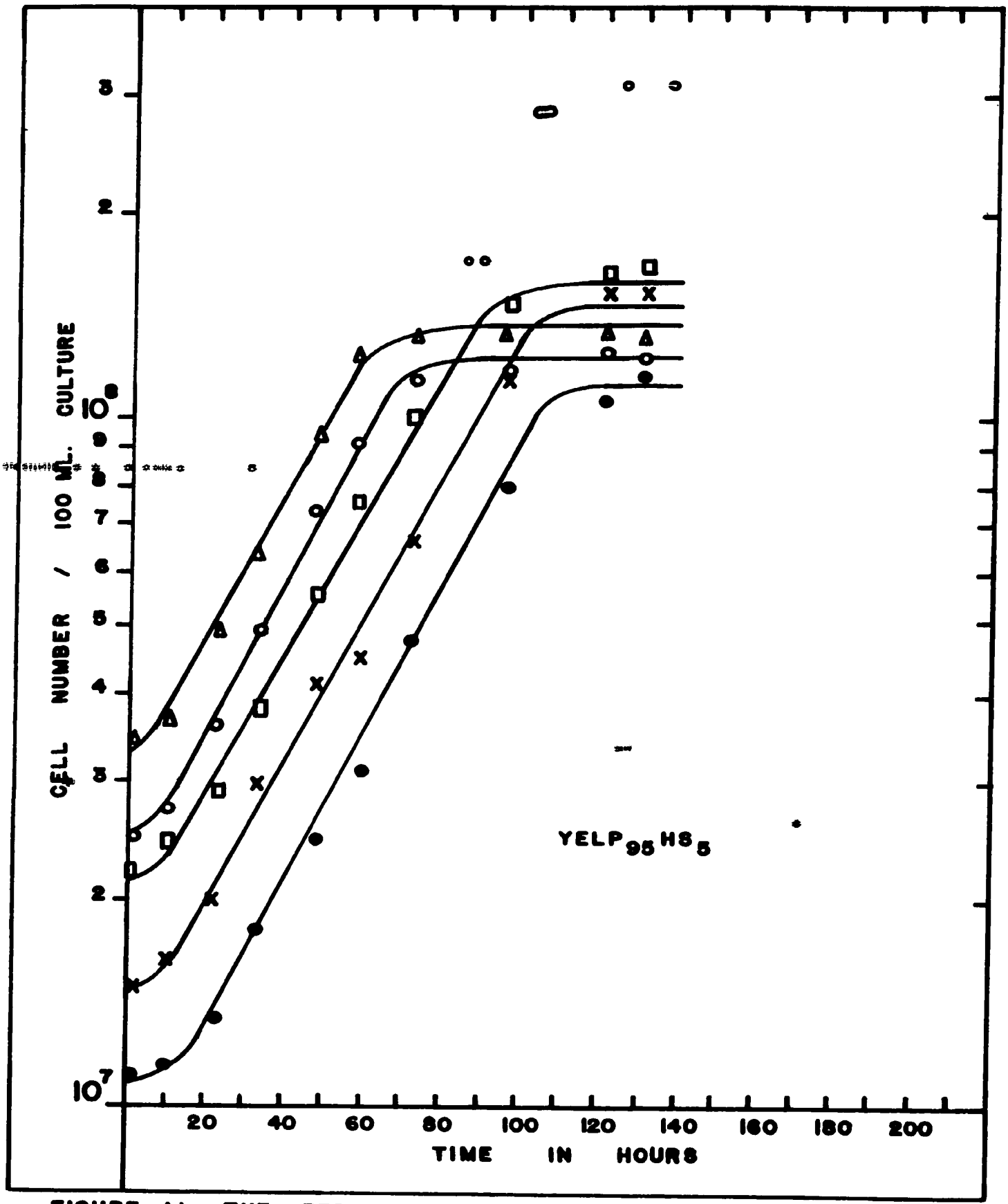



FIGURE 11. THE EFFECT OF VARYING THE INITIAL CELL INOCULUM ON THE GROWTH OF MOUSE FIBROBLASTS IN FLUID SUSPENSION CULTURES.

growth in the logarithmic phase was essentially independent of the number of cells present in the original cell population. This figure also indicates other points related to variations in the starting cell inoculum. Although the length of the lag phase varied, it was impossible to establish any significant mathematical relationship between inoculum size and the length of the lag phase, such as Hinshelwood reported (48). The maximum cell density, however, appeared to be approximately the same for all inoculums tested. These results have been verified in numerous experiments.

4. Biosynthesis in Strain L Fibroblasts

(a) Number of Cells Related to Packed Cell Volume

The previous work defined some of the conditions for the growth of strain L fibroblasts related to the increase in number of cells and the prerequisites which would result in their optimal multiplication rate. The study at this point turned to the nature of the biosynthetic capacities of these cells and the relationship of these syntheses to the growth cycle.

Figure 12 shows that growth took place by two measurable mechanisms, increase in cell numbers and increase in cell size (43). This point was substantiated in the late lag phase, where the cells continued to form new protoplasm, as evidenced by a continued increase in packed cell mass (44, 45, 46, 47, 53,63), while no increase in cell number was evident. A 100-ml culture containing approximately $2.0 \times 10^7$ lag-phase

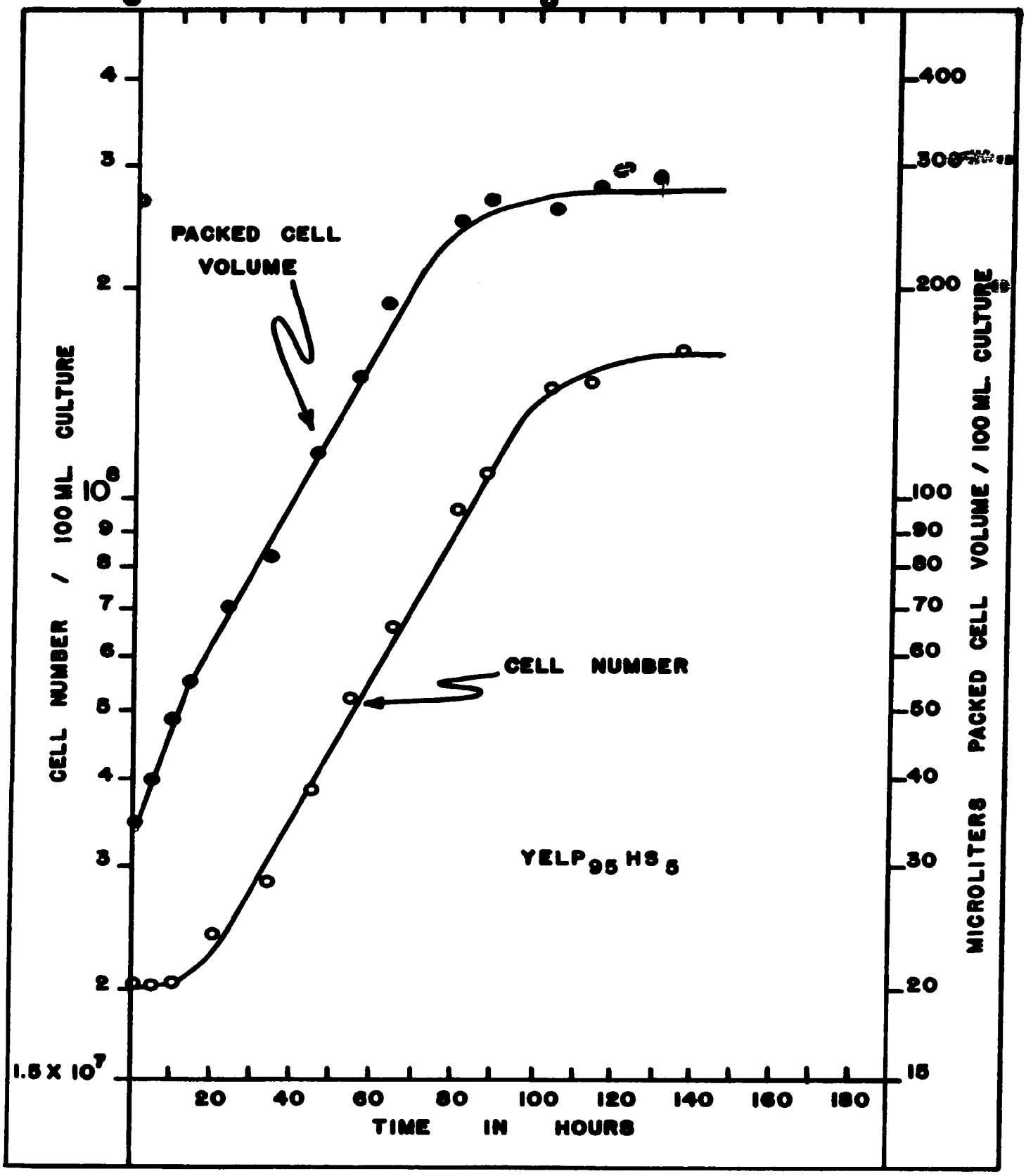



FIGURE 12. THE PACKED CELL VOLUME RELATED TO THE NUMBER OF CELLS DURING THE GROWTH CYCLE.

cells represents a packed cell volume of about 35 µl; when the number of cells had increased to $1.6 \times 10^8$, the packed cell mass was about 310 µl. At the plateau levels, packed cell mass and cell numbers had increased approximately ninefold. Two further points of interest were noted (1) the packed cell mass increased rapidly from zero time and in the late lag phase, the rate of packed cell mass increases changed to a new value and (2) the packed cell volume increases stopped when the number of cells reached about $9 \times 10^5$ cells/ml. However, the cell number continued to increase for about 36 more hours, indicating that cell division occurred in the absence of protoplasmic synthesis.

(b) Number of Cells Related to Total Cell Nitrogen

The total nitrogen as measured by the micro-Kjeldahl technique from trichloroacetic acid-precipitated material increased exponentially from zero time (figure 13). The precipitate at zero time contained $8.0 \times 10^2$ µg. nitrogen; when the population was fully grown the $1.6 \times 10^8$ cells contained $5.6 \times 10^3$ µg. nitrogen. This method serves very well in estimating the amount of protoplasmic growth, since TCA-precipitated material contains the cell protein plus other small amounts of contaminating nitrogen.

(c) Number of Cells Related to Cell DNA and RNA

Figure 14 shows that desoxyribonucleic acid synthesis paralleled increases in cell numbers. The population illustrated here contained 540 µg. DNA at zero time and increased with the culture cycle to 4,100 µg. From a gross microscopic

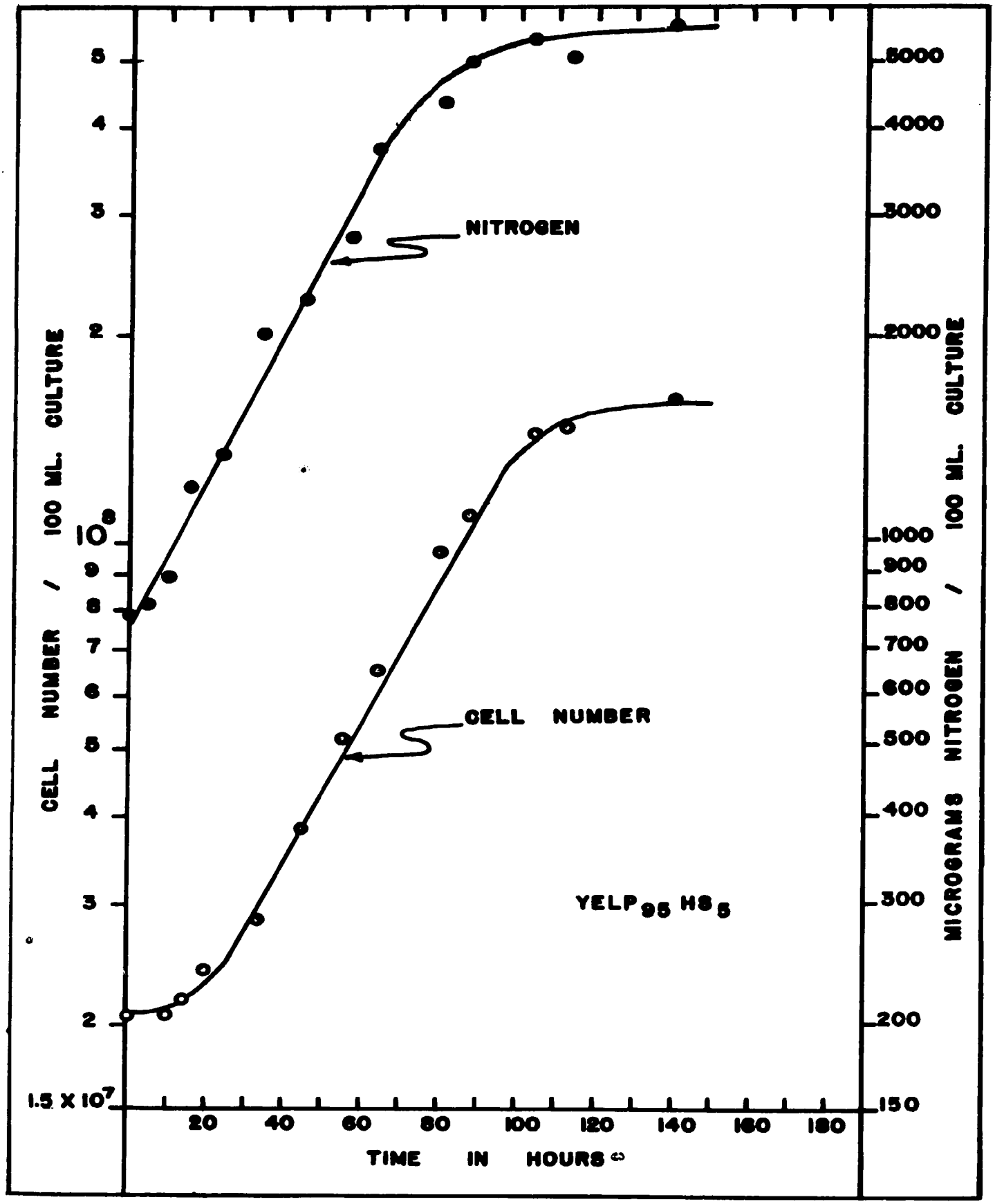



FIGURE 13. THE TOTAL (TCA) PRECIPITATED CELL NITROGEN RELATED TO THE NUMBER OF CELLS DURING THE GROWTH CYCLE.

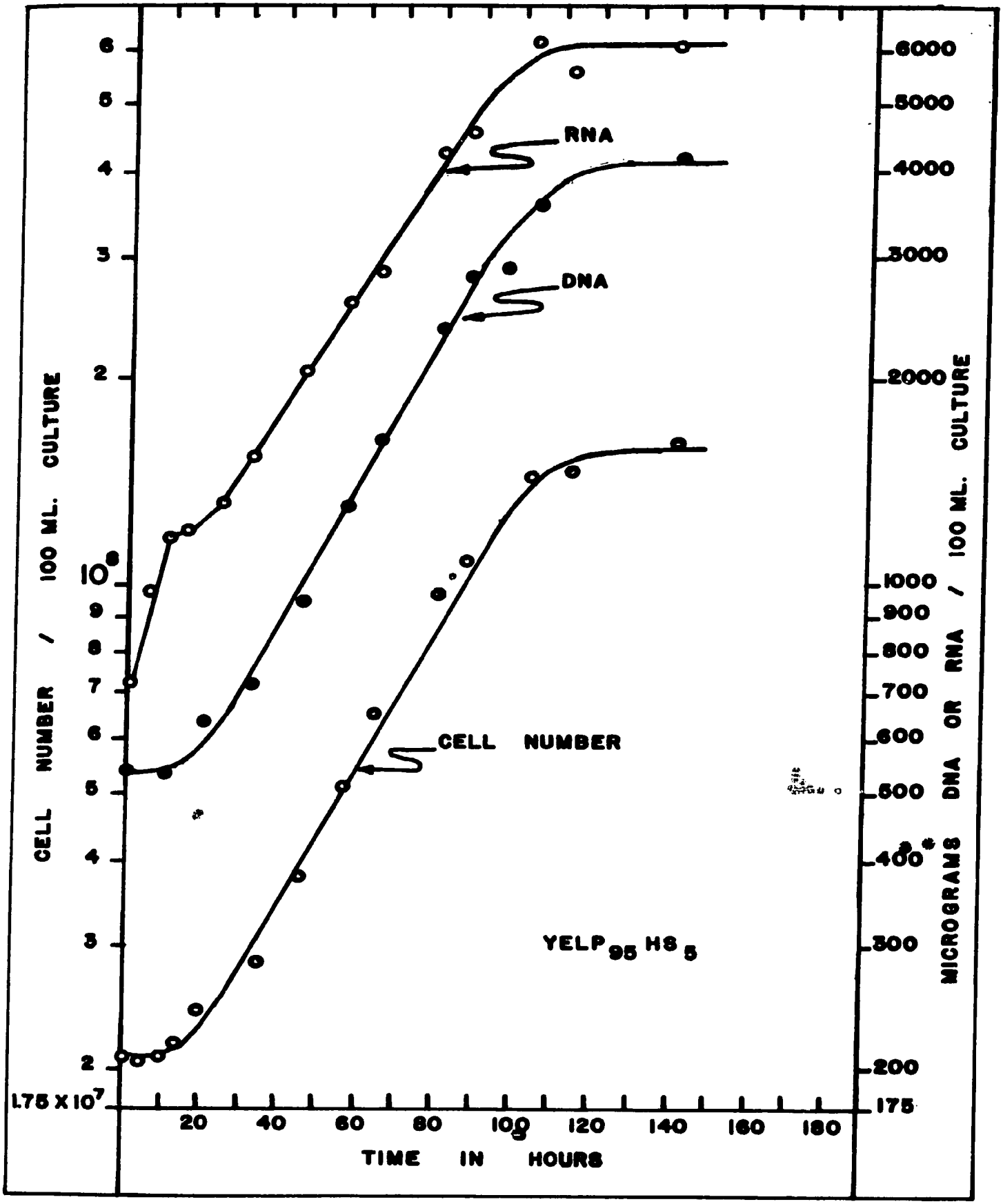



FIGURE 14. DNA AND RNA RELATED TO THE NUMBER OF CELLS DURING THE GROWTH CYCLE.

observation after the addition of a drop of crystal violet, it did not appear that many cells in fluid suspension were multinucleated, so that DNA content at any time during the culture cycle would be indicative of cell number (81).

RNA was also a measure of cell growth, but did not parallel increases in cell number; RNA increased with protoplasmic synthesis. Evidence indicated that RNA preceded and participated in protein synthesis. A rapid increase in RNA occurred immediately after cells were put into fresh medium (figure 14). The content at zero time was 720 µg. As the end of the lag phase approached, the rate of RNA synthesis changed to a new value and remained constant until the onset of the plateau phase. The final yield of RNA was 6,100 µg, an increase of 5,400 µg.

(d) Number of Cells Related to Dry Weight

Figure 15 shows the relationship between the number of cells and the dry weight. This chart shows that from a full grown culture of 100-ml volume, it was possible to obtain 60 to 70 mg. of dried cell material. It appears from the data (figures 12 and 15) that the dry weight was a constant ratio of about 16 parts for every 100 parts of the packed wet-cell volume.

(e) Synthesis in the Lag Phase

Figure 16 presents a closer view of the lag phase of growth. The results here indicate that DNA synthesis began after fifteen hours or at the same time that cell number increases were first observed. RNA and protoplasmic increases started almost immediately, when the cells were placed in the

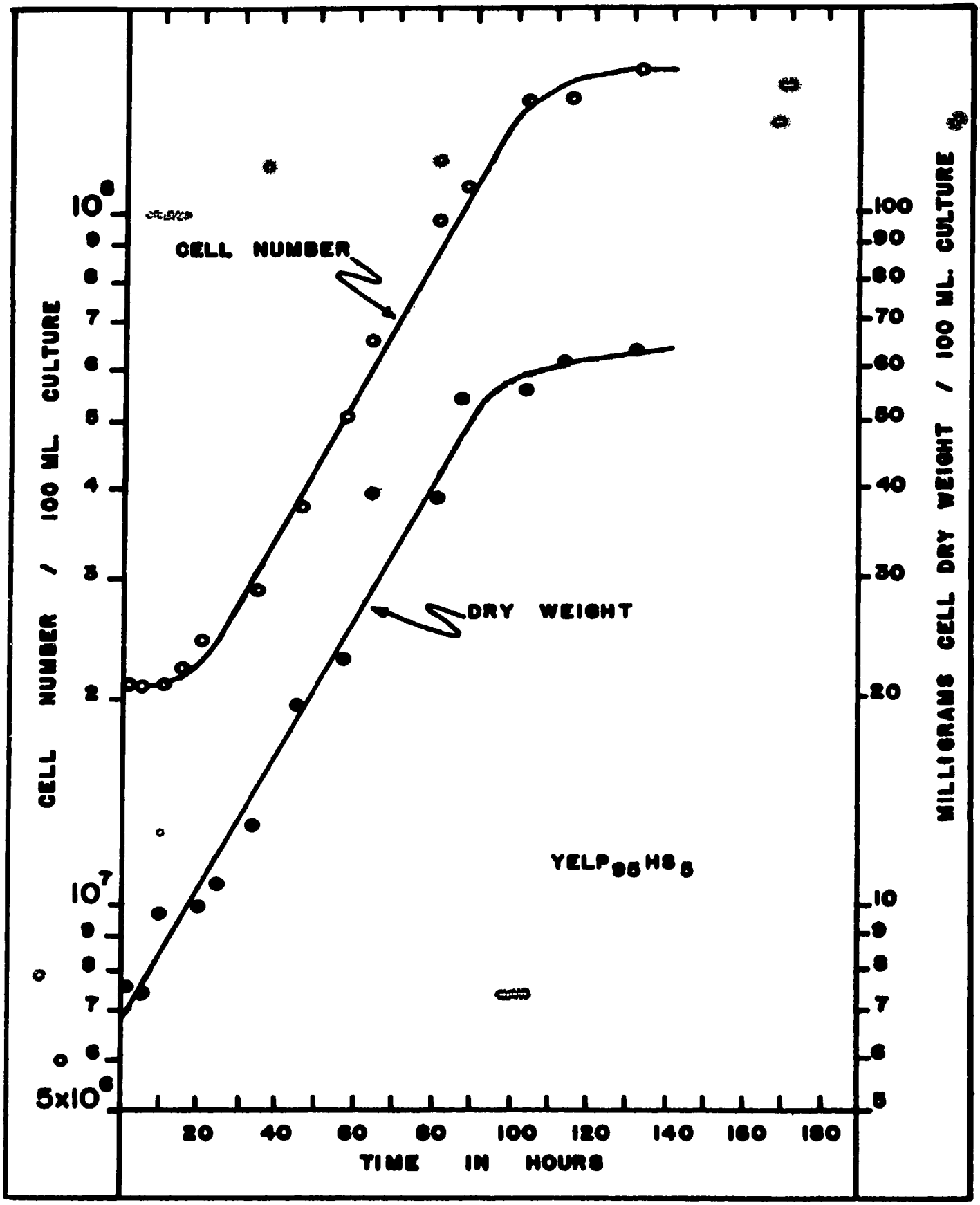



FIGURE 15. CELL DRY WEIGHT RELATED TO THE NUMBER OF CELLS DURING THE GROWTH CYCLE.

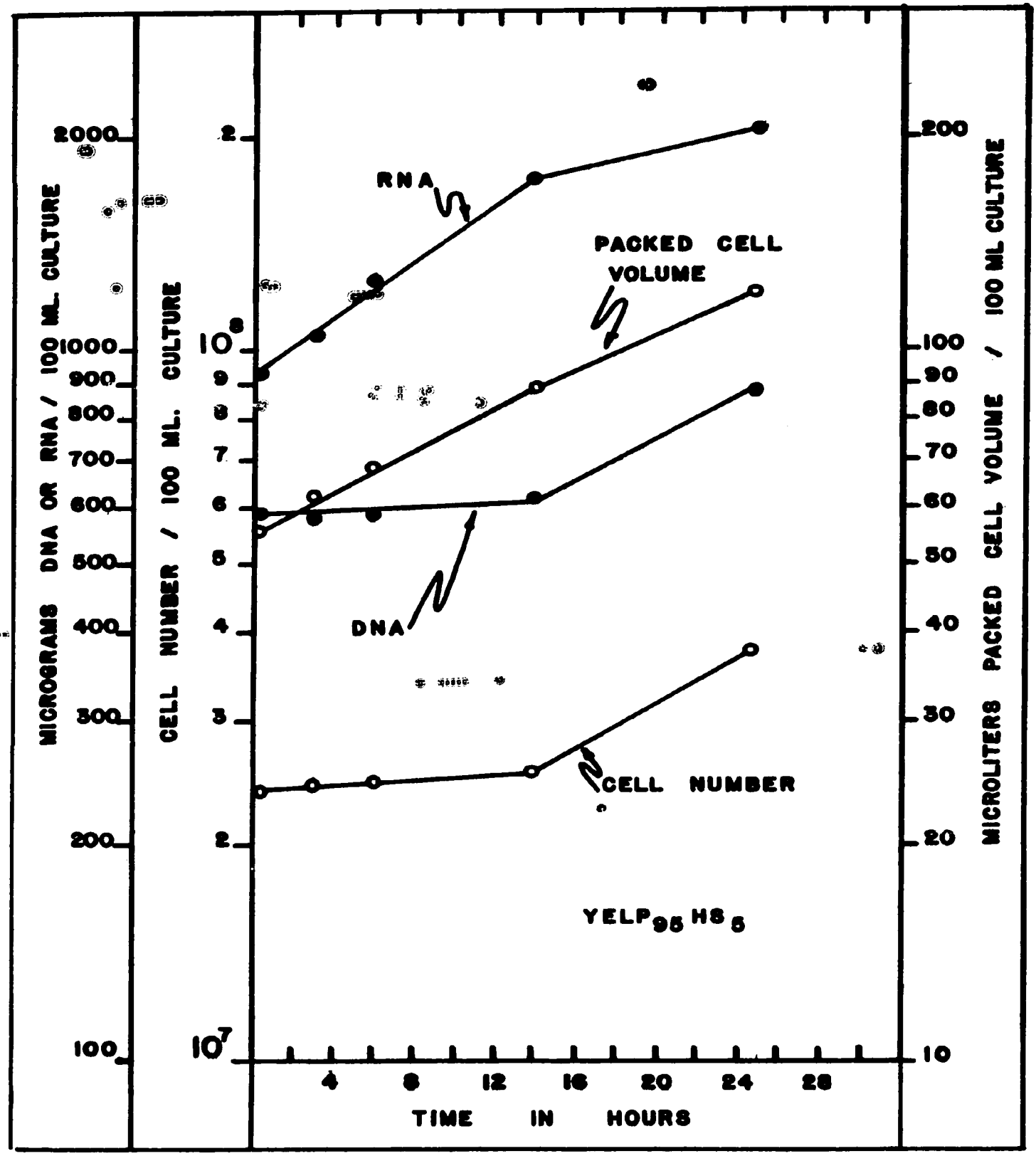



FIGURE 16. SYNTHESIS IN THE LAG PHASE.

fresh medium.

(f) Packed Cell Volume at Various Points on the Growth Curve

Figure 17 depicts the packed cell volume at various times during the growth cycle. A cell count was made when cells were packed. It can be shown that during the lag phase cells increased in size, since there was a change in number per µl of packed cell mass. In the late lag phase, the cell number per µl was reduced, because of the increased number of larger cells. The figures during the logarithmic phase indicated that the cell volume had become random, since there were approximately the same number of cells per µl throughout this period. As the plateau level was approached, the packed cell volumes represented more cells per µl, indicating the presence of cells of smaller volume.

(g) DNA, RNA and Nitrogen per Cell at Various Points on the Growth Curve

Figure 18 is a summation of the content of DNA, RNA, and total nitrogen at various time intervals during the growth cycle. It can be seen that the level of DNA was constant throughout the growth cycle indicating fixed DNAamounts per cell. RNA and total nitrogen increased during the late lag phase, but leveled off because of the random nature of the population during the logarithmic phase.

(h) Cell Size Distribution Analysis and the Growth Curve

For the purpose of quantitating the information, it

| CULTURE PHASE | HOUR OF SAMPLE | µL. PACKED CELL VOLUME PER 100 ML. CULTURE | CELL NUMBER PER 100 ML. CULTURE | CELL NUMBER PER µL. PACKED CELLS |
|---|---|---|---|---|
| LAG PHASE | 0 | 35 | $20.6 \times 10^6$ | 580,000 |
| | 5 | 40 | $20.6 \times 10^6$ | 515,000 |
| | 10 | 48 | $20.6 \times 10^6$ | 430,000 |
| LATE LAG PHASE | 15 | 55 | $21.0 \times 10^6$ | 382,000 |
| | 24 | 70 | $22.0 \times 10^6$ | 315,000 |
| LOG PHASE | 34 | 84 | $28.5 \times 10^6$ | 340,000 |
| | 45 | 116 | $37.8 \times 10^6$ | 326,000 |
| | 56 | 150 | $51.3 \times 10^6$ | 342,000 |
| | 64 | 190 | $65.7 \times 10^6$ | 346,000 |
| | 80 | 270 | $95.0 \times 10^6$ | 352,000 |
| | 87 | 275 | $105.0 \times 10^6$ | 380,000 |
| PLATEAU PHASE | 104 | 300 | $144.0 \times 10^6$ | 480,000 |
| | 113 | 310 | $145.0 \times 10^6$ | 470,000 |
| | 130 | 310 | $160.0 \times 10^6$ | 510,000 |

FIGURE 17. THE NUMBER OF MOUSE FIBROBLASTS PER UNIT OF PACKED CELL VOLUME DURING THE GROWTH CYCLE.

| CULTURE PHASE | HOUR OF SAMPLE | PICOGRAMS NITROGEN PER CELL | PICOGRAMS DNA PER CELL | PICOGRAMS RNA PER CELL |
|---|---|---|---|---|
| LAG PHASE | 0 | 37.2 | 26.2 | 35.2 |
| | 5 | 39.4 | 25.9 | 47.8 |
| | 10 | 43.3 | 25.9 | 56.4 |
| LATE LAG PHASE | 15 | 57.3 | 27.6 | 57.3 |
| | 24 | 62.9 | 29.8 | 64.5 |
| LOG PHASE | 34 | 70.2 | 25.2 | 56.2 |
| | 45 | 59.6 | 25.4 | 54.5 |
| | 56 | 54.6 | 25.6 | 50.8 |
| | 64 | 57.2 | 25.1 | 43.8 |
| | 80 | 45.2 | 24.2 | 44.1 |
| | 87 | 45.6 | 25.8 | 41.5 |
| PLATEAU PHASE | 104 | 37.2 | 25.0 | 42.8 |
| | 113 | 35.7 | 27.6 | 38.6 |
| | 140 | 35.4 | 26.0 | 37.8 |

FIGURE 18. THE AMOUNT OF NITROGEN, DNA AND RNA PER CELL DURING THE GROWTH CYCLE.

was convenient to classify the cells in four sizes on the basis of cell diameter. The four sizes were 13.0μ, 14.2μ, 16.0μ, and 17.8μ. This type of analysis was carried out because logically cell growth must be related to cell size. The cell with the greatest volume must be a mature unit, ready to divide if normal cell mitosis occurs. It is assumed, then, that a cell one-half this volume must be a product of division or it would be the smallest sized cell observed during the logarithmic phase. Smaller cells were noted in the early lag phase and in the plateau phase (the 13.0μ variety), but these were not considered in the foregoing assumption.

Figure 19 summarizes the results of microscopic measurements of cell diameter and cell volume and correlates these with the growth phase. A population started from a culture in the plateau phase contained 6% very small cells (13.0μ mean diameter). If the cell is a sphere, as observed in agitated culture, the volume can be calculated with the formula $4/3\ r^3$ and is shown to be $1150\mu^3$. Fifty-two percent of the cells were of the small logarithmic variety (14.2μ mean diameter or a volume of $1500\mu^3$); 32% of the cells were of the medium logarithmic variety (16.0μ mean diameter or a volume of $2150\mu^3$) and the remaining 10% of the cells were large (17.8μ mean diameter or a volume of $2950\mu^3$). As the population became metabolically active, the cells increased in size. The population shifted in the late lag phase to larger cells. After the second division in the logarithmic phase the population became randomly distributed, containing cells of all sizes

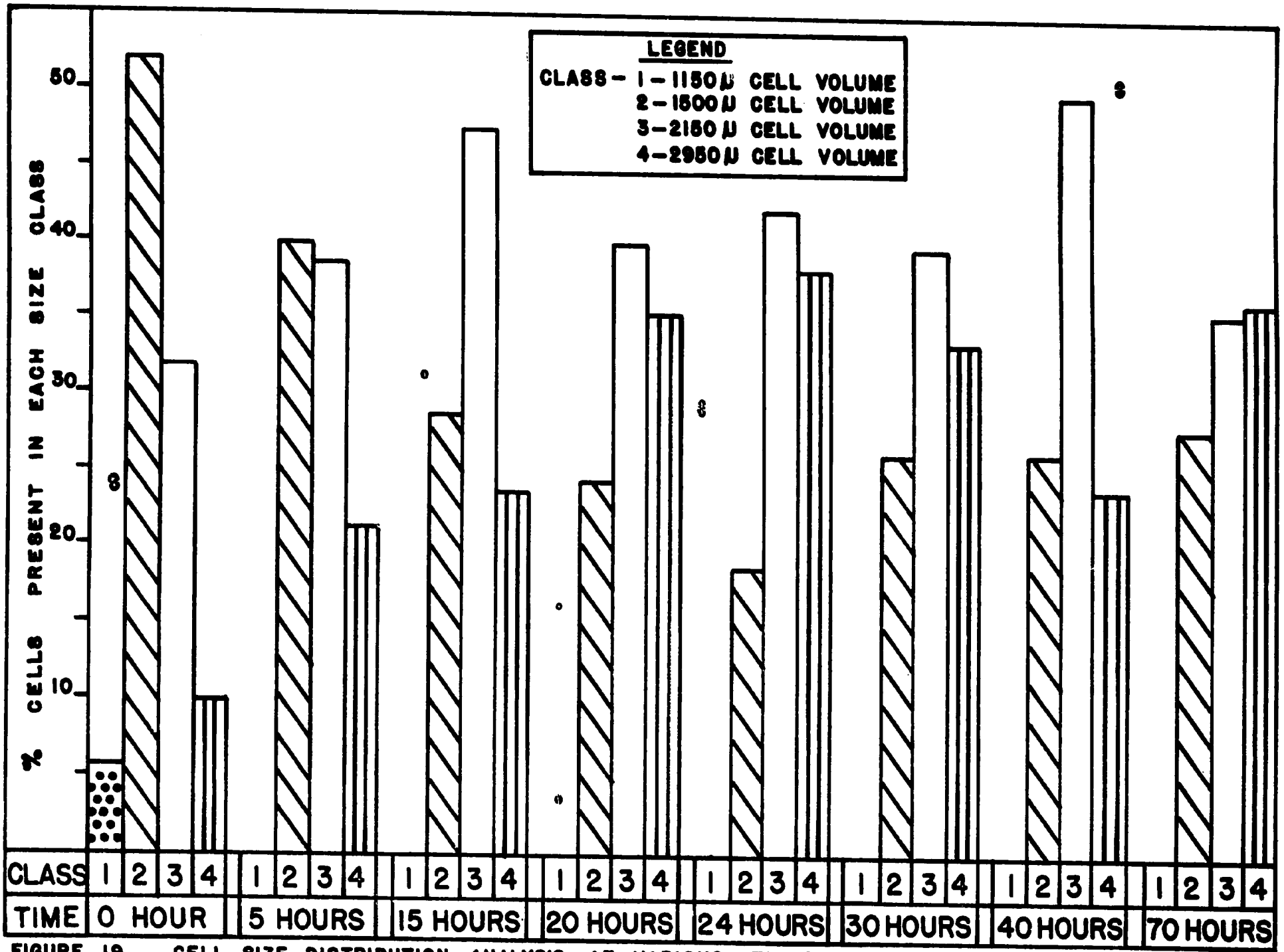



FIGURE 19. CELL SIZE DISTRIBUTION ANALYSIS AT VARIOUS TIMES DURING THE GROWTH CYCLE.

ranging from 14.2μ to 17.8μ in diameter. It can also be noted that cells of the very small variety (13.0μ diameter) were eliminated from the population at the end of five hours. These cells may have degenerated, or they may have been incorporated into the general cell population after positive growth.

These results evidently indicate that the population attained a size uniformity in the plateau phase. Figure 20 is a plot of the cell number increases in the culture cited in figure 19 and shows that population synchronization has been approached by merely allowing cells to enter the plateau phase.

5. The Application of an Electronic Blood Cell Counter to Define Fibroblast Growth

Most animal cell counting is done with blood-cell hemocytometers; this method was used in most of the work reported here. The errors inherent to this method are well known (6). Recently an electronic blood-cell counter has been developed for use in hematology, and near the end of this study its use was made available to us. It was of interest to determine the degree of precision and reliability of this instrument for counting cells grown in suspension cultures. Since a count could be made in six seconds, many more counts could be made on a given sample, and thus provided a better statistical analysis. Many more points could also be analyzed on the growth curve and this would give a more valid picture of the growth curve for these fibroblast populations.

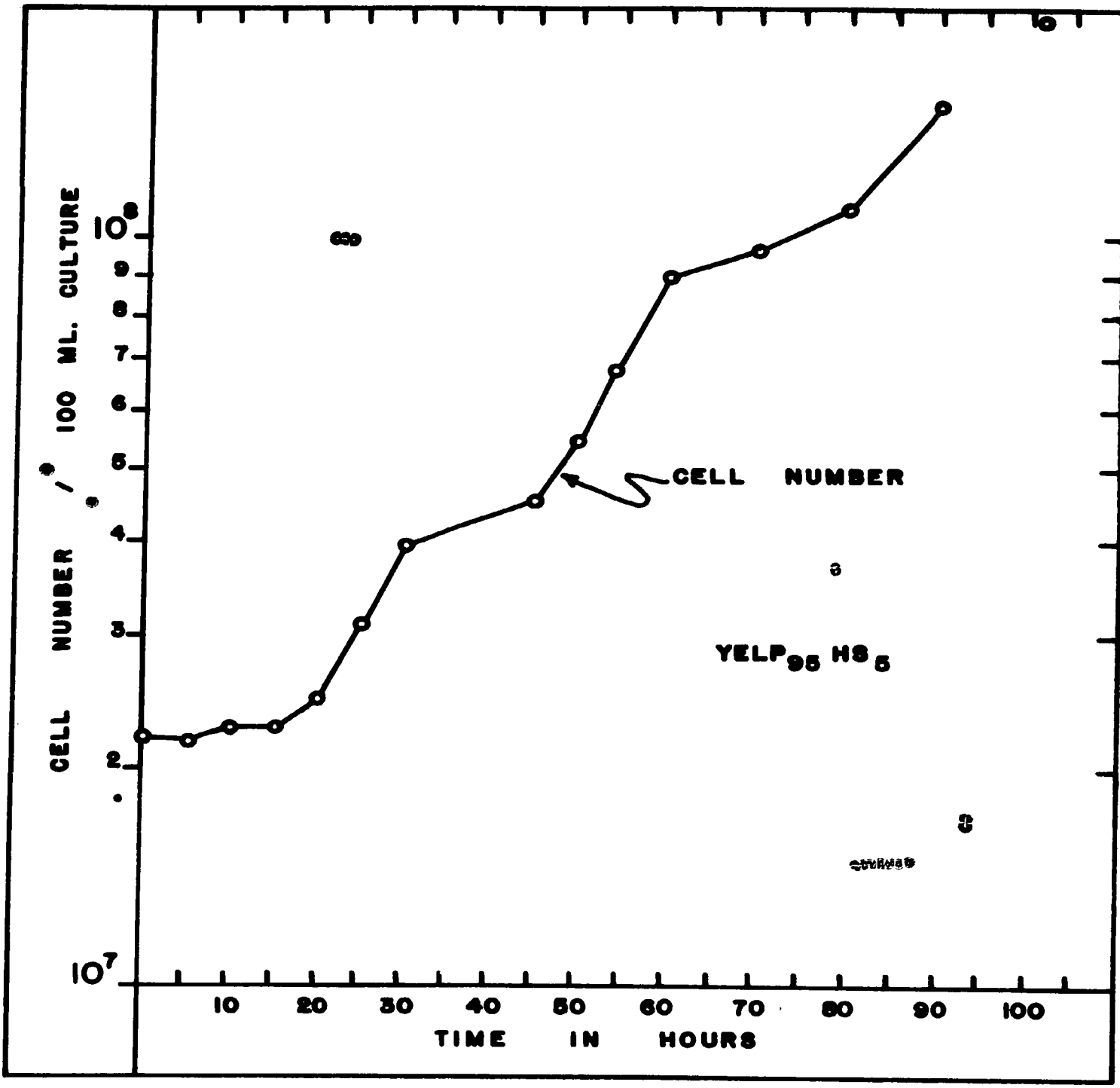



FIGURE 20. POPULATION GROWTH CURVE OF MOUSE FIBROBLASTS FROM CULTURE ANALYZED FOR SIZE DISTRIBTION.

Cultures of strain L were set up from plateau phase cells; the starting inoculum was about $1.6 \times 10^5$ cells/ml. The open circles on the growth curve shown in figure 21 represent the average of 10 to 50 cell counts made on the electronic cell counter. The length of the vertical black line above and below the open circle represents the range in cell numbers obtained in determining the average number. The standard deviation was calculated for each point and it varied between $8 \times 10^3$ and $1.6 \times 10^4$ cells/ml.

Figure 22 is a similar study using the hemocytometer counting method. This experiment was not carried out concurrently with the one cited above, but is representative of populations which have been analyzed by the microscopic procedure. Each vertical line represents the range of 10 to 20 cell counts. The standard deviation was calculated for each point and it varied between $2.5 \times 10^4$ and $3.5 \times 10^4$ cells/ml. In certain instances counts were so far off that they were eliminated, whereas in the electronic counter experiment no count was of sufficient divergence to be eliminated. Since the magnitude of the standard deviation reflects the variability within the samples, it may be concluded that the electronic counter is superior in deriving the true average cell number at points on the fibroblast growth curve.

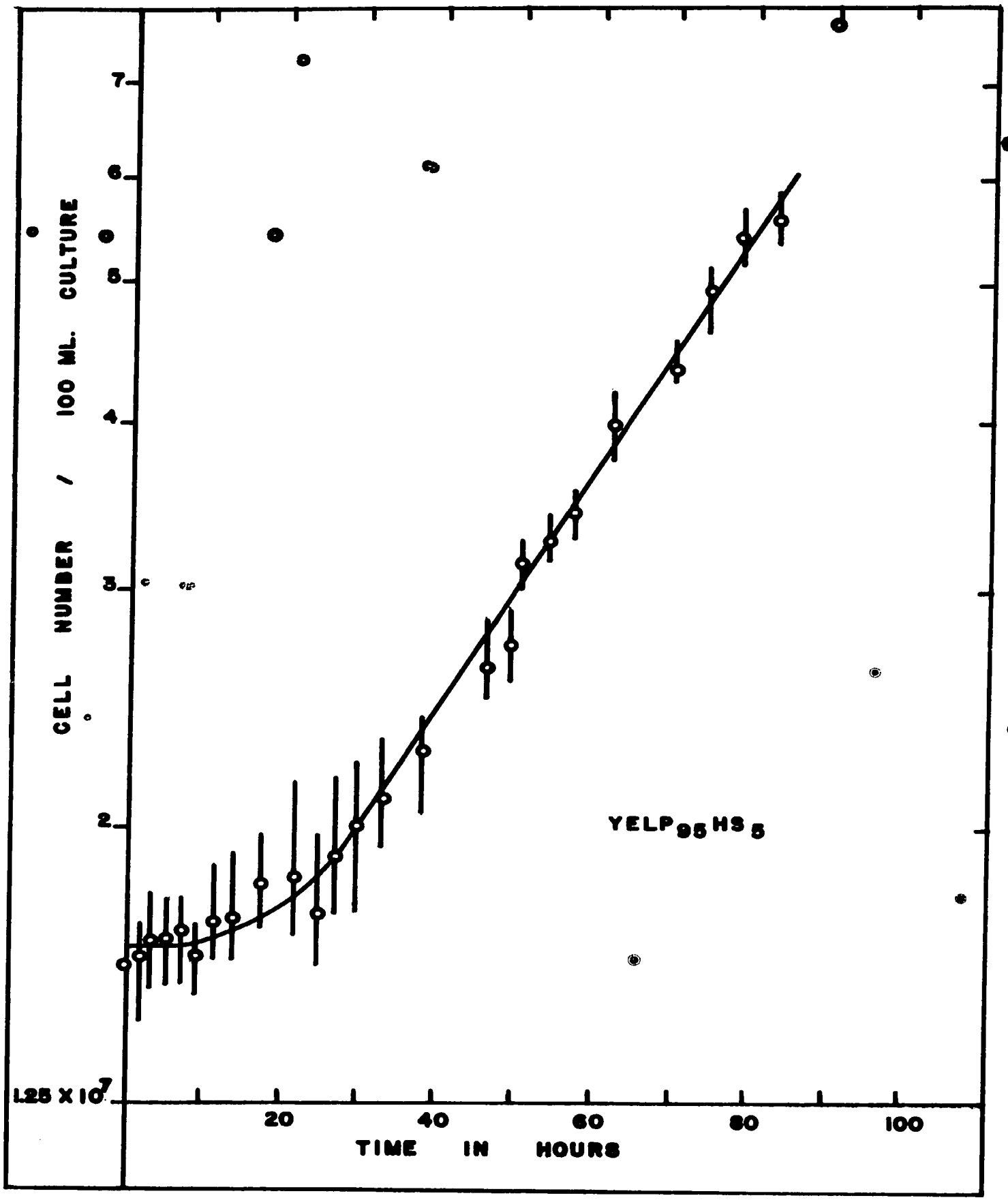



FIGURE 21. THE APPLICATION OF THE ELECTRONIC CELL COUNTER TO ANALYZE THE GROWTH OF A MOUSE FIBROBLAST POPULATION.

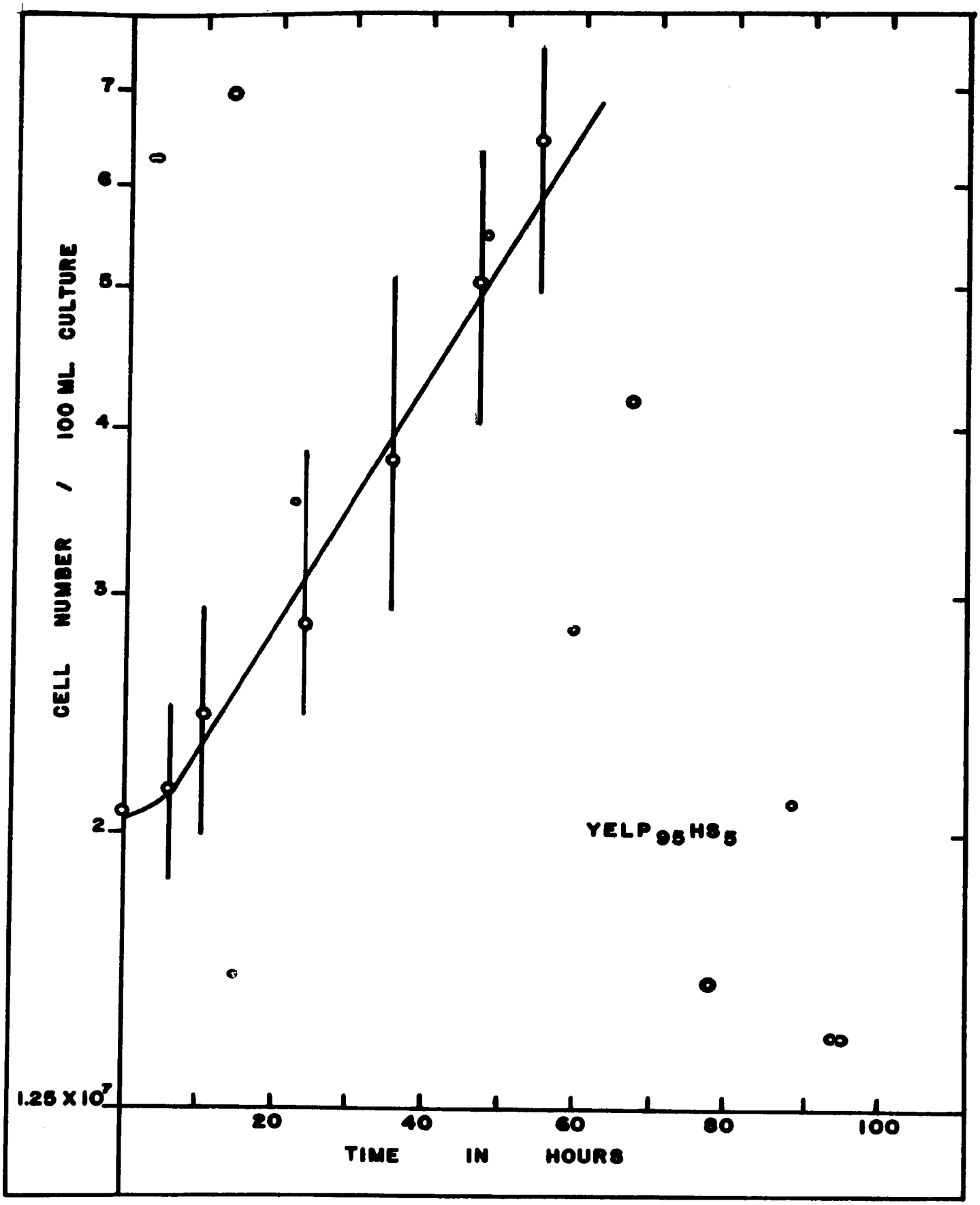



FIGURE 22. THE APPLICATION OF THE HEMOCYTOMETER TO ANALYZE THE GROWTH OF A MOUSE FIBROBLAST POPULATION.

## DISCUSSION

Numerous advantages became apparent in using the fluid suspension culture for the study of mammalian cell population growth *in vitro*. In the first place, when such a culture was established the cells grew as a uniform single-cell suspension. Periodic sampling for the purpose of making cell counts could be carried out on a single culture throughout the growth cycle; thus many separate individual populations were studied. A second advantage following from the first, was that a small sample could be extracted from the culture which was representative of the population. Thirdly, the entire cell surface was exposed in a uniform manner, consequently the validity of quantitative study was improved.

The capacity of animal cells to function as individual units was recognized by Sanford *et al.* (75) when strain L was first isolated as a clone. With the development of the plating technique, indications were that every isolated mammalian cell could develop into a population of free-living cells. The efficiency of development was directly related to the nutritional adequacy of the medium (71). The fact that strain L fibroblasts grew as free-living cells in agitated suspension cultures could be surmised from the fact that the rate of growth in the logarithmic phase was not influenced by variations of inoculum size ranging from $5 \times 10^4$ to $5 \times 10^5$ cells/ml.

This indicated that synthetic events in different individual cells during the logarithmic phase occurred at similar rates and thus animal cells were not interdependent under these conditions. This uniformity of generation time in HeLa cell populations during logarithmic growth has been observed when the initial cell inoculum varied from one cell upward (28, 71).

The development of a mouse-fibroblast population was therefore analogous to the growth of microbial populations, since these cells functioned as individual units and the increase in fibroblast numbers was described with a sigmoid curve. Physically, a single fibroblast introduced into an adequate medium would constitute an open or steady-state biological system, since substrate would flow continuously into the cell, undergo changes, and flow out in a modified form. The conception of the open system and general kinetic principles as applied to biological systems was advanced by von Bertalanffy in 1932 (3, 4) and has been elaborated by him since then. In the present study, it was not feasible to treat the system as open, because of the undefined nature of the media which support fibroblast growth. The term medium as used here is not limited to nutritional factors. Rather, a simpler but less quantitative approach was used, which would provide elementary information to be used later in open-system studies. A population of fibroblasts plus the environment within a stoppered erlenmeyer flask was treated as a closed biological system. These cells would function at the expense of their environment which would become degraded and the organisms

would no longer function (53). Such an idealized closed-system approach has provided a wealth of information in microbial cultures (48); it was thought that a growing population of fibroblasts might first be studied in such a manner, to obtain data on growth rates under various conditions and perhaps determine the optimal growth system.

Population development of fibroblasts in this system could be separated into two major cyclical trends (1) increases in cell numbers and (2) increases in cell protoplasmic mass (53). When plateau phase cells were used as the inoculum, the population failed to increase in number in the first fifteen hours; this period has been called true growth lag. However, during this interval, there was a heightened activity in cellular synthetic processes. This striking phenomenon was manifested by increases in packed cell mass, in dried cell weight, in protein synthesis, and in RNA synthesis. These observations indicated that the lag phase was not a period of inactivity, rather a period when biosynthetic events were prominent. Such an observation presupposed a study of cell sizes related to protoplasmic increases, so that the cyclical trends of cell number increases and cell protoplasmic increases might be incorporated into a unified developmental relationship. Such an increase in cell size in the late lag phase of most bacterial species was noted early by Clark and Ruehl (16).

Measurements of cell diameter were made at periodic intervals throughout the growth cycle. Observations at zero time on cells taken from plateau phase cultures showed that

the majority of cells measured approximately 14μ in diameter; this indicated a cell volume of 1500$\mu^3$. As the population developed, it was observed that toward the end of the lag phase in cell numbers, the majority of cells were in the range of 18μ in diameter, indicating a cell volume of 3000$\mu^3$.

From this simple observation a working hypothesis was formulated. It was assumed that a cell 14μ in diameter might be a resting cell; when the cell was placed in growth medium, it synthesized protoplasm and thus increased in size until the diameter was 18μ and the volume 3000$\mu^3$, or double the volume of the initial cell. At this point division occurred and two cells were formed, each 14μ in diameter (1500$\mu^3$). This evidence led to the hypothesis that cells in the plateau phase somehow attained a uniform size, possibly their resting size, which may be 1500$\mu^3$ or the size just after division. The supposition that uniformity of size was a product of the plateau phase was supported by the following evidence: Cell number increases toward the end of the logarithmic phase did not result in increased packed-cell volume; in some instances the volume remained constant while the cell number almost doubled. From this evidence, it was hypothesized that during a division cycle a cell reaches an energy level which permits the ensuing division to proceed independently of the total energy of the system. Support for such a theory has been provided by Swann (80), in studying the mechanism of division in sea urchin eggs. He suggested the possibility that a cell contained some sort of reservoir which was filled by normal

aerobic respiration. When the reservoir was full (at about 2/3 the interphase time), a critical point was reached which may be related to energy storage or to the synthesis of a vital cell component. After this critical point, the cell can be likened to a siphon; the cell utilizes the stored material to carry out the process of mitosis and cleavage. Other studies have substantiated the reservoir theory by showing that inhibition of aerobic metabolism after some critical point did not interfere with the completion of cleavage (5, 73). Hence, a cell that has not reached the activated level functions as a dynamic chemical system following the principles of chemical equilibrium stated by LeChatelier. Thus, when nutrients become limiting, cells that have reached the activated state progress to division; cells that have not reached this state move in the direction of equilibrium, which is analogous to the resting cell size, and soon many of the cells in the population approach the same size range.

The phenomenon of the rapid rate of RNA synthesis in the early lag phase, as observed in our studies, was analogous to findings in regenerating liver in vivo (70) and of various microbial cultures (59). The sudden increase in RNA and packed cell volume was believed to be a reflection of the uniformity of cell size obtained in the plateau phase and may be related in time to the saturation of the reservoir described above, or specifically, RNA might be the critical cell component.

Returning to further observations on the growing population, it had been noted that at the end of the lag period,

as the cell population began to increase in numbers, occasionally a sudden spurt in cell division occurred. After such a mitotic spurt, the majority of the cells were 14μ in diameter (1500$\mu^3$). A continual changing in cell size could be noted during the subsequent 30 hours. The shift in cell sizes was not as striking during the second period, because the population became more randomly distributed as to size. This randomness increased during the third period, or after two and one-half days from the time the cells were placed in fresh medium, indicating an exponential slope from then on. The results shown in figure 19 support the idea of uniformity of cell size in the plateau phase.

The role of pH in the experiments reported appeared to be complex. It had been noted in earlier studies that pH 7.5 or above was required to assure sticking of strain L cells to a glass surface. When the pH fell significantly below this value, many cells failed to attach and remained floating in the medium. Low pH levels, however, while they inhibited cell attachment did not interfere with the growth of previously attached cells. This fact was not at first appreciated and it was assumed that pH 7.5 was optimal for growth of these cells. When attempts were made to establish suspension cultures at pH 7.5, it was learned that this was not the case. Rather, the medium assumed a pH which varied with the serum concentration (ranging from 5 to 40% horse serum) and it was found that logarithmic growth proceeded at similar rates between pH 6.8 and 7.4. The maximum population

density was lower at 5% levels, but at a 15% serum concentration and upward the population densities were similar. It was concluded that for this system 20% serum represented the most efficient level in terms of utilization of available nutrient. The pH then remained constant during logarithmic growth and dropped when cells entered the plateau phase. The apparent independence of pH optima for cell adhesion and for cell growth was of interest both practically and theoretically. It was suggested from other work in this laboratory that the role of pH in determining attachment of cells to glass depended on the existence of a divalent cationic bridge between the glass and a fibrous protein on the surface of the cell. Further, it was believed that the attaching group on the protein was a carboxyl group, because the pH range for maximum cell attachment is comparable to the pH range for maximum absorption of cations by carboxylic groups on exchange resins, as shown by Zago (86).

Various tissue culture media were analyzed and supplements were added in order to obtain the most economical medium that would provide optimal conditions for the growth of mass cultures. It was learned that medium 199, supplemented with 0.5% peptone, required only 5% serum to sustain fibroblast growth. The growth rate was increased and the generation time was reduced to about 28 hours, which appeared to be optimal, since it has been reported by others (20, 31, 78) in studies using the best available media. The maximum cell level was slightly increased and the lag phase was reduced.

Cells had to be harvested sooner in this medium, because of the increased rate of metabolism and because the 5% horse serum represented a reduced buffering capacity. Another medium which served the same purpose was a lactalbumin - yeast extract - Eagles - peptone medium (YELP) supplemented with 5% horse serum. In fact it was a better medium for mass culture, since the ingredients were quite inexpensive.

Further studies with media supplemented with peptone have shown that fibroblasts would grow in such media without serum (68,82). The role of serum as a buffer has been established, and peptone probably has this capacity also. However, it was found in these studies that serum played another role, because when it was omitted from suspension cultures, the surface tension increased and fibroblasts collected on the liquid surface and began growing as a loose network of bound cells. This striking property resembled that of embryonic mesenchyme (61). When serum was added to the culture medium and an inoculum of dispersed cells was added, surface tension was reduced and the cells were sustained as individual rounded units. It was further indicated that serum was bound by individual cells, so that binding between cells occurred only minimally. The binding of serum by animal cells has also been evidenced by the fact that antiserum prepared against these cells (even after repeated washings) reacts in an immunological reaction with horse serum (51).

When cells were taken from a population growing in the exponential phase, where the division events were random,

the hypothetical model would indicate that logarithmic growth must ensue (i.e. the slope of the line must be straight). However, this was not always true; for log growth to continue, the medium had to be adequate chemically and physically. When cells were inoculated into non-equilibrated (pH and temperature) medium, a slowdown in cell number increases occurred; this, however, was not a true lag in terms of Hinshelwood (48) for there were cell divisions occurring. Rather there was a slowdown in the rate of some one or many enzymatic reactions, and the time interval before the maximum rate was regained was about 15 hours, at which time the maximum rate of development was approached, as depicted by the exponential slope in the logarithmic phase. During this slowdown process, there was no indication that cell size distribution was altered from randomness. The previous finding was further substantiated by the fact that when logarithmic phase cells were inoculated into medium pre-adjusted for pH and temperature, the population did not change in distribution according to size, but remained random and the slope was exponential from zero time.

The growth of cells must eventually be slowed and stopped in such a modified "closed system." At the point of cessation of positive growth the system is in a low free energy state. The cells are still viable and continue to respire with their endogenous nutrient supply. When the internal cell energy is degraded, cell death occurs, but cell death may also occur because of a toxic metabolic by-product (53).

Populations have been studied in the later plateau phase. With the inoculation of such cells, it was noted that cell number decreases occurred during early lag phase. Although this point was not critically investigated, it was believed that the decrease was a function of cells in the logarithmic death phase. This was difficult to assess, because there was no simple method to measure viability in the late plateau phase. Dying cells in this growth phase were microscopically visible and were included in cell counts. When these cells were placed in fresh medium, the dying cells degenerated. Populations were noted which decreased by one-half or more in number before log growth began.

Graham and Siminovitch (40) have provided a method to analyze this problem, but in their studies only logarithmic phase cells were used. Fibroblasts were grown in medium containing $P^{32}$, which became incorporated into DNA at division. These DNA-labeled cells were transplanted during the log phase to fresh non-labeled medium. As growth proceeded, $P^{32}$ was never found in the supernate. It was suggested, therefore, that cell breakage did not occur when log-phase cells were transferred to fresh medium. This method could be used to extend studies of plateau-phase cells; however, it has a pitfall in that nucleoproteins have been observed to be preferentially and rapidly incorporated into cells (41).

From experience in this laboratory, it appeared that cell breakage could easily be noted microscopically, because cell fragments were visible where population decreases had

occurred. This was the crude criterion used to measure viability in newly established cultures.

The characteristics of growth of cells and populations of fibroblasts under the conditions described exhibited many similarities to development of microbial populations and to regenerating animal cells *in vivo*. Growth phases based on the rate of cell division were noted in these free-living cell populations. The attractive feature of this system was that it demonstrated the correlation between cell morphological changes and synthetic events. The electronic counter increased the validity of cell counts and thus improved the precision of the system. This closed system itself can be used profitably to study the synthesis of animal cell virus and the kinetics of growth inhibitors, and such studies should eventually lead to the development of an optimal growth medium. Data obtained from an approach such as was used in this study will provide the information needed to study the growing animal cell in its natural environment which is the open system, and this work will pave the way for exact and specific studies of animal cells.

## SUMMARY

With the propagation of strain L in suspension culture, it was noted that populations of these fibroblasts could be characterized by a growth culture cycle similar to population development described for unicellular microbial cultures. The lag phase was eliminated in these cultures when logarithmic phase cells were used as the inoculum in media pre-adjusted for pH and temperature. This made possible the establishment of transfer methods which were highly efficient and which yielded populations with a considerable degree of uniformity.

Further application of the growth cycle attempted to define the optimal conditions for fibroblast growth. Various nutrient mixtures were studied for the ability to support and enhance growth. The minimal generation time achieved was 28 hours; the maximum number of cells was about $2 \times 10^6$ cells/ml. Growth rate was independent of the serum concentration in the medium over a wide range. In Medium 199, maximum population density increased with the serum content to about 15%; beyond this level the extent of growth was rather constant. The rate and extent of fibroblast growth also appeared to be independent of the inoculum size.

The development of the cell population in suspension culture was also followed during the growth cycle relating the number of cells to packed cell volume. Measurements of

packed cell volume showed that protoplasm increased exponentially from zero time, though a lag in the increases in number of cells was noted. The range in cell volumes of growing fibroblasts, as determined from measurements of cell diameter, [illegible] 3000$\mu^3$. An increase in cell size during the lag phase of growth accounted for the increase in cell mass in the absence of an increase in the number of cells. Measurements were made of DNA, RNA, total nitrogen, and dry weight during the growth cycle, and were correlated with the increases in number of cells and in packed cell volume. DNA was found to correlate directly with cell number, while RNA, total nitrogen, and dry weight increases during the lag phase were related to the increases in cell volume.

To increase the speed and precision obtained in counting fibroblasts, an electronic blood cell counter was employed. It has been shown that the instrument reduces the degree of variation in cell counts.

# BIBLIOGRAPHY


1. Baker, L. E. and Carrel, A. 1928 Nitrogen metabolism of normal and sarcomatous fibroblasts in pure cultures. J. Exper. Med., 48, 533.

2. Berenblum, I., Chain, E. and Heatley, N. G. 1939 The study of metabolic activities of small amounts of surviving tissues. Biochem. J., 33,68.

3. Bertalanffy, L. von. 1938 A quantitative theory of organic growth (II. Inquiries of growth laws). Human Biol., 10, 181.

4. Bertalanffy, L. von. 1950 The theory of open systems in physics and biology. Science, 111,23.

5. Blumenthal, R. 1939 Mitotic development of arbacia eggs in cyanides. Physiol. Zool., 3,539.

6. Brecher, G., Schneiderman, M. and Williams, G. Z. 1956 Evaluation of electronic red blood cell counter. Am. J. Clinc. Path., 26, 1439.

7. Buchanan, R. L. 1918 Life phases in bacterial cultures. J. Inf. Dis., 23,109.

8. Burrows, M. T. 1910 The cultivation of tissues of the chick embryo outside the body. J. Am. Med. Assn., 55,2057.

9. Carrel, A. 1914 Present condition of a strain of connective tissue twenty-eight months old. J. Exper. Med., 20,1.

10. Carrel, A. and Burrows, M. T. 1911 Cultivation of tissues in vitro and its techniques. J. Exper. Med., 13,387.

11. Caspersson, T. 1940 Methods for the determination of the absorption spectra of cell structures. J. Royal Microsp. Soc., 60,8.

12. Caspersson, T. 1941 Studien über den Eiweissumsatz der Zelle. Naturwissenschaften, 29,33.

13. Champy, C. 1922 L'action de l'extrait thyroidlen sur la multiplication cellulaire. Caractere electif de cette action. Arch. morphol. gen. et exper., fasc. 4.

14. Chargaff, E. and Davidson, J. N. 1955 The Nucleic Acids, vol. I. Acad. Press., New York.

15. Cherry, W. R. and Hull, R. N. 1956 Studies on the growth of mammalian cells in agitated fluid media. Anat. Rec., 124,7.

16. Clark, P. F. and Ruehl, W. H. 1919 Morphologic changes during the growth of bacteria. J. Bact., 4, 615.

17. Colwick, S. P. and Kaplan, N. O. 1957 Methods in Enzymology. Acad. Press, New York.

18. Cooper, P. D. 1955 A method for producing plaques in agar suspensions of cells. Virology, 1,397.

19. Cunningham, B. and Kirk, P. L. 1942 Measure of "growth" in tissue culture. J. Cell and Comp. Physiol., 20,343.

20. Danes, B. S. 1957 Suspension Cultures of strain L mouse fibroblasts. Exper. Cell Research, 12,169.

21. Davidson, J. N. and Waymouth, C. 1942 The effect of certain substances on growth of tissues in vitro. Biochem. J. 36,1(nos. 3 and 4).

22. Davidson, J. N. and Waymouth, C. 1943 Factors influencing the nucleoprotein content of fibroblasts growing in vitro. Biochem. J., 37,271.

23. Davidson, J. N. and Waymouth, C. 1944 Nucleic acids and tissue growth. Nut. Abs. and Rev., 14,1.

24. Davidson, J. N. and Waymouth, C. 1949 The nucleoprotein content of fibroblasts growing in vitro. 4. Changes in the ribonucleic acid phosphorus (RNAP) and desoxyribonucleic acid phosphorus (DNAP) content. Biochem. J., 44,5.

25. Demuth, F. and Meier, R. 1929 Milchsaurebildung in Gewebekulturen. Biochem. Ztschr., 212,399.

26. Dounce, A. L. 1955 The isolation and composition of cell nuclei and Nucleoli. The Nucleic Acids, chap. 18, vol. II. Acad. Press, New York.

27. Dounce, A. L. 1944 The preparation of isolated cell nuclei of rat liver. Science, 99,512.

28. Eagle, H. 1955 The specific amino acid requirements of mammalian (strain L) in tissue culture. J. Biol. Chem., 214,839.

29. Eagle, H. 1955 Nutrition needs of mammalian cells in vitro. Science, 122,501.

30. Earle, W. R. 1943 Changes induced in a strain of fibroblasts from a strain $C_3H$ mouse by the action of 20-methylcholanthrene. J. Nat. Canc. Inst., 3,555.

31. Earle, W. R., Bryant, J. C. and Shilling, E. L. 1954 Certain factors limiting the size of the tissue culture and the development of massive cultures. Ann. N. Y. Acad. Sci., 58,1000.

32. Earle, W. R., Schilling, E. L., Bryant, J. C. and Evans, V. J. 1954 The growth of pure L strain mouse cells in fluid suspension cultures. J. Nat. Canc. Inst., 14, 1159.

33. Ebeling, A. H. 1921 Measurement of the growth of tissues in vitro. J. Exper. Med., 34,231.

34. Ebeling, A. H. 1936 Effect of high concentrations of glucose on fibroblasts, leucocytes and epithelial cells. Proc. Soc. Exper. Biol. Med., 34,886.

35. Evans, V. J. and Earle, W. R. 1947 The use of perforated cellophane for the growth of cells in tissue culture. J. Nat. Canc. Inst., 8,103.

36. Evans, V. J., Earle, W. R., Sanford, K. K., Shannon, J. E. Jr. and Waltz, H. K. 1951 The preparation and handling of replicate tissue cultures for quantitative studies. J. Nat. Canc. Inst., 11,907.

37. Fisher, A., Astrup, T., Ehrensvard, G. and Oehlenschlager, V. 1948 Growth of animal tissue cells in artificial media. Proc. Soc. Exper. Biol. Med., 67,40.

38. Feulgen, R. and Rossenbeck, H. 1924 Mikroskopisch Nachweis einer Nucleinsaure vom Typus der Thymonucleinsaure und die daruf beruhende elektive Fabung von Zellkernen in Mikroskopischen Praparaten. Z. physiol. Chem., 135, 203.

39. Graham, A. F. and Siminovitch, L. 1955 Proliferation of monkey kidney cells in rotating cultures. Proc. Soc. Exper. Biol. Med., 89,326.

40. Graham, A. F. 1957 Multiplication of animal cells in suspension. Tissue Culture Association Meetings, Baltimore.

41. Harris, M. and Kutsky, R. F. 1954 Synergism of nucleoprotein and dialysate growth factors in chick embryo extract. Exper. Cell Res., 6,327.

42. Harrison, R. G. 1908 Embryonic transplantation and the development of the nervous system. Anat. Rec. 2,385.

43. Henrici, A. T. 1928 Morphological Variation and the Rate of Growth of Bacteria. Charles C. Thomas, Springfield.

44. Hershey, A. D. and Bronfenbrenner, J. 1937 I. On factors limiting bacterial growth. Proc. Soc. Exper. Biol. Med., 36,556.

45. Hershey, A. D. and Bronfenbrenner, J. 1937 Factors limiting bacterial growth. II. Growth without lag in B. coli cultures. Proc. Soc. Exper. Biol. Med., 38,127.

46. Hershey, A. D. and Bronfenbrenner, J. 1938 Factors limiting bacterial growth. III. Cell size and "physiologic youth" in B. coli cultures. J. Gen. Physiol., 21,721.

47. Hershey, A. D. and Bronfenbrenner, J. 1939 Factors limiting bacterial growth. IV. Equations describing the early periods of increase. J. Gen. Physiol., 23,11.

48. Hinshelwood, C. N. 1946 The Chemical Kinetics of the Bacterial Cell. The Clarendon Press, Oxford.

49. Hull, R. N. 1953 Establishing long-term cultures of mammalian normal, solid tumor and ascites tumor cells on glass. Science, 117,223.

50. Hull, W. and Kirk, P. L. 1950 Tissue culture studies. II. The relationship of incorporation of radioactive phosphorus in nucleic acid to the growth of cells in vitro. J. Gen. Physiol., 33,335.

51. Kite, J. H., Kuchler, R. J. and Merchant, D. J. 1957 Conditions for macroscopic agglutination of mammalian tissue cells grown in vitro. Am. J. Clin. Path., 28, 174.

52. Krontowski, A. A., 1931 Grundvorgange des energieliefernden Umsatzes in Gewebskulturen und ihre biologische Bedeutung. Arch. f. exper. Zellforsch., 11,93.

53. Lamanna, C. and Mallette, M. F. 1953 Basic Bacteriology, The Williams and Wilkens Co., Baltimore.

54. Lane-Claypon, J. E. 1909 Multiplication of bacteria and the influence of temperature and some other conditions thereon. J. Hyg., 9,239.

55. Laser, H. 1932 Eine Methode zur manometrischen Messung des Stoffwechsels von Gewebekulturen wahrend des Wachstums. Biochem. Ztschr., 251,2.

56. Lebensohn, E. G. 1934 Zur Frage der Abhangigkeit des Zellstoffwechsels und der Wachstumsintensitat in vitro vom Alter. Arch. f. Exper. Zellforsch., 16, 264.

57. Levene, P. A. and Bass, L. W. 1931 Nucleic Acids, Chemical Catalog Co., New York.

58. Levene, P. A., Mikeska, A. and Mori, T. 1930 On the carbonhydrate of thymonucleic acid. J. Biol. Chem., 85,785.

59. Malmgren, B. and Heden, C. G. 1947 Studies of the nucleotide metabolism of bacteria. Acta. Path. Microbiol. Scandinav., 24,417.

60. Mejbaum, W. 1939 Uber die Bestimmung kleiner Pentosemengen inbesondere in Derivaten der Adenylsaure. Zeitsch. f. physiol. Chemie., 258,117.

61. Merchant, D. J. and Kahn, R. 1957 Fiber formation in suspension cultures of L strain fibroblasts. Submitted to Proc. Soc. Exper. Biol. Med.

62. Meier, R. 1931 Zur Methodik der Stoffwechseluntersuchungen an Gewebekulturen. I. Atmungsmessung an Gewebekulturen. Biochem. Ztschr., 231,247.

63. Monod, J. 1949 The growth of basterial cultures. Ann. Rev. Microbiol., 3,371.

64. Morgan, J. F., Morton, H. J. and Parker, R. C. 1950 Nutrition of animal cells in tissue culture. I. Initial studies on a synthetic media. Proc. Soc. Exper. Biol. Med., 73,1.

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

66. Owens, O. von H., Gey, M. K. and Gey, G. O. 1954 Growth of cells in agitated fluid medium. Ann. N. Y. Acad. Sci., 58,1039.

67. Parker, R. C. 1950 Methods of Tissue Culture, Paul B. Hoeber Inc., New York.

68. Parker, R. C. Unpublished personal communication.

69. Penfold, W. J. 1914 On the nature of the bacterial lag. J. Hyg., 14,215.

70. Potter, V. In press.

71. Puck, T. T., Marcus, P. I. and Cieciura, S. J. 1956 Clonal growth of mammalian cells in vitro. J. Exper. Med., 103,273.

72. Rinaldini, L. M. 1954 A quantitative method for growing animal cells. Nature, 173,1134.

73. Runström, J. 1953 Stoffwechselvorgänge während der ersten Mitose des Seeigeleies. Protoplasma, 20,1.

74. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K. and Shannon, J. E. Jr. 1951 The measurement of proliferation of tissue cultures by the enumeration of cell nuclei. J. Nat. Cancer Inst., 11,773.

75. Sanford, K. K., Earle, W. R. and Likely, G. D. 1948 The growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst., 9,229.

76. Schneider, W. C. 1945 Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxyribonucleic acid and ribonucleic acid. J. Biol. Chem. 161, 293.

77. Shannon, J. E. Jr., and Earle, W. R. 1951 Qualitative comparison of the growth of chick-heart and strain L fibroblasts planted as suspensions on pyrex glass and perforated cellophane substrates. J. Nat. Cancer Inst., 12.155.

78. Siminovitch, L., Graham, A. F., Lesley, S. M. and Nevill, A. 1957 Propagation of L strain mouse cells in suspension. Exper. Cell Res., 12,299.

79. Schmidt, G. and Thannhauser, S. J., 1945 A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J. Biol. Chem., 161,83.

80. Swann, M. M. 1954 The mechanism of cell division: Experiments with ether on the sea urchin egg. Exper. Cell Res., 7,505.

81. Swift, H. 1955 Cytochemical techniques for nucleic acids. The Nucleic Acids, chap. 17, vol. II, Acad. Press, New York.

82. Waymouth, C. Unpublished personal communication.

83. Willmer, E. N. 1942 Carbohydrate metabolism of chick fibroblasts in vitro. J. Exper. Biol., 18,237.

84. Willmer, E. N. and Jacoby, F. 1936 Studies on growth of tissues in vitro. IV. On the manner in which growth is stimulated by extracts of embryo tissues. J. Exper. Biol., 13,237.

85. Wilson H., Jackson, E. B. and Brues, A. M. 1942 The metabolism of tissue cultures. I. Preliminary studies on chick embryo. J. Gen. Physiol., 25,689.

86. Zago, F. 1956 Studies of the Mechanism of Adsorption on Neurotropic Viruses, Thesis, University of Michigan.