Rhythmic and Synthetic Processes in Growth

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RHYTHMIC AND SYNTHETIC PROCESSES IN GROWTH

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Executive Committee, 1956

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FOREWORD

DIDEROT: *Le soleil eteint, qu'en arrivera-t-il? Les plantes periront, les animaux periront, et voila la terre solitaire et muette. Rallumez cet astre, et a l'instant vous retablissez la cause necessaire d'une infinité de generations nouvelles entre lesquelles je n'oserais assurer qu'a la suite des siecles nos plantes, nos animaux d'aujourd'huy se reproduiront ou ne se reproduiront pas.*

D'ALEMBERT: *Et pourquoi les memes elemens epars venant a se reunir, ne rendroient-ils pas les memes resultats?*

DIDEROT: *C'est que tout tient dans la nature, et que celui qui suppose un nouveau phenomene ou ramene un instant passé, recrée un nouveau monde.*

LE RÊVE DE D'ALEMBERT

THE Fifteenth Symposium of the Society for the Study of Growth and Development was held at Brown University, Providence, Rhode Island, on July 18–20, 1956. The Society is deeply indebted to the National Science Foundation which subsidized the Symposium and to Brown University and the local committee who made the occasion so agreeable and so fruitful. Most sincere thanks are due all those, within and without the Society, who contributed to this success, as well as to all those, both within and without the Princeton Press, who have brought the present volume to completion.

The program was arranged in the form of three subsymposia, each coherent within itself. The subject of the first day was the recent great advances in tissue culture methods which have opened such vast new experimental possibilities in cell biology. Theodore T. Puck discussed his innovations in the field of clonal cultures of animal cells; R. Dulbecco his progress into the stimulating problems of virus reproduction; and Richard M. Klein the equally active field of plant tissue cultures.

The second day was occupied with problems of cyclic activity: the growth-division cycle in Amoeba (David M. Prescott); time-measurement by organisms (Colin S. Pittendrigh); and diurnal rhythms in vascular plants (E. Bünning). The last group of speakers—H. Gaffron, Harold F. Blum, B. L. Strehler, and Harlow Shapley—devoted themselves to questions of biochemical evolution, and indeed, as the reader will observe, did not hesitate to take off in the grand manner to primordial time and galactic space. The morning and the evening were the third day.

The Executive Committee feels that the Fifteenth Symposium marks an appropriate point for a backward look at the Society's efforts and progress thus far. This volume includes, therefore, an index, by authors, of articles published in the first fifteen Growth Symposia, beginning with the first one in the summer of 1939.

DOROTHEA RUDNICK

Yale University

CONTENTS

RHYTHMIC AND SYNTHETIC PROCESSES IN GROWTH

I. THE MAMMALIAN CELL AS MICROORGANISM

BY THEODORE T. PUCK[1]

I. INTRODUCTION

MAMMALIAN biology for the most part has been the biology of the *macro*organism—study of the body as an architectural structure with specialized functions lodged in various tissues and organs. The cell has been recognized as the ultimate unit of this structure, and the study of individual cell morphologies has contributed enormously to the understanding of how different tissues are constituted so as to perform their specific functions. Yet in no case is it clear to what extent a given cell is autonomous and to what extent it is governed by its status as a sub-unit of a particular tissue, which is itself tightly integrated into the over-all economy of the body.

Detailed morphological studies of individual cell types have yielded exceedingly important data, much of which has become indispensable in many procedures of medicine. A wealth of biochemical information has also accumulated describing the function and distribution of enzymes, nucleic acids, specific proteins and other molecules in specific regions of special cells. But there remain largely unanswered questions like the following, relating the metabolism of the individual cell to the total body economy:

1. What proportion of the cells of various normal tissues are capable of independent growth and multiplication in isolation if furnished with the proper nutrient environment? I.e., which cells are capable of existence as true microorganisms, and which are dependent for continued metabolism and reproduction on close association in a community with other cells of similar or different constitution?

2. Which of the different cell types arising during normal embryonic differentiation represent changes in genetic constitution which are relatively fixed, and to what extent are these merely adaptive responses which all cells may exhibit when properly stimulated but which affect different cells differently, simply because of factors depending on physicochemical, temporal and spatial relationships?

[1] Department of Biophysics, Florence R. Sabin Laboratories, University of Colorado Medical Center, Denver. Contribution No. 47.

3. May mammalian cells undergo genetic modification by the processes of transformation, transduction, or direct sexual exchange of nuclear or cytoplasmic genetic determinants?

4. What is the distribution of nuclear genes in the mammalian chromosomes; what is the frequency of processes like crossing-over and spontaneous gene mutation; and how do mutagenic agents like high energy radiation affect the growth potential and genetic constitution of individual mammalian somatic cells?

5. What is the chromosomal and genic basis of the various defects responsible for mammalian genetic diseases?

6. To what extent do the processes like cancer formation and ageing represent changes in the genetic or the physiologic components of the cell?

Answers to questions like these require means for study of the growth and genetic processes of mammalian cells in a manner similar to that which has been so rewarding in microorganisms like *Neurospora* and *E. coli*. An experimental program directed toward this end is in progress in our laboratory.[2] Study was initiated to obtain quantitative growth of single cells, i.e. to find conditions such that practically every cell of a mammalian population will reproduce in isolation to form colonies of any desired size. It must be possible to carry out such quantitative operations rapidly and simply, and on a very large scale, to permit discovery and isolation of the genetic mutants which can serve as markers to illuminate hereditary processes. Since gene mutations generally occur with frequencies in the neighborhood of one in a million, methods requiring separate manipulation of each individual cell cannot be employed. Hence, attention was devoted to devising methods for growing single mammalian cells into colonies in a fashion completely analogous to the growth of single bacteria plated on solid nutrient media.

Growth of mammalian cells in vitro by conventional tissue culture methods is by this time a commonplace operation. These procedures, requiring as they do an inoculum of approximately 10^5 cells in order for self-sustaining growth to be initiated (Earle et al., 1951), make it impossible to determine whether, under the conditions employed, a cell *community* is needed to initiate continuing growth, or whether only a rare cell is capable of reproduction. In the latter case, the magnitude of the required inoculum would be a measure of the number of cells needed

[2] The experiments described are the results of a joint program by the author and the following co-workers: Steven Cieciura, Harold Fisher, and Philip Marcus, who are candidates for the Ph.D. degree.

to insure the presence of at least one reproducing individual. Earle and his co-workers who emphasized the need for cells to "condition" the conventional tissue culture media in order to make them able to support growth, succeeded in inducing self-sustaining multiplication from several different mouse cells, by introducing single cells into fine capillaries which were then sealed and incubated (Sanford, Earle, and Likely, 1948). These critical experiments at once established that at least some single mammalian cells can multiply in isolation, and permitted the establishment for the first time of true clonal stocks. (A clone is a population all of whose members have descended from the same single member by asexual reproduction.) However, only a small proportion of the cells so isolated did multiply. Further, the necessity for separate manipulation of each individual cell precludes the routine screening of large numbers of individuals which is prerequisite for systematic genetic studies. Growth of single *ascites* tumor cells into large populations has been demonstrated in vivo (Yoshida, 1952) but this technique also is not readily applicable to the large populations required by genetic studies. Moreover, since this growth takes place in vivo, the possibility of genetic interaction between the inoculated cell and the host always adds uncertainties to the interpretation of such experiments.

II. QUANTITATIVE GROWTH OF SINGLE CELLS

The successful plating of single mammalian cells on petri dishes under conditions leading to colony formation with an efficiency close to 100% was achieved first with cells of the HeLa Strain (Scherer, Syverton, and Gey, 1953), originally isolated from a human cervical carcinoma. The technique for this single cell plating has been published in detail (Puck, Marcus, and Cieciura, 1956). The only significant difference between this method for plating animal cells and that which is the basis of quantitative bacteriology, lies in the additional necessity for dispersing into single cells the aggregates in which mammalian cells characteristically tend to grow. If the reproductive potential of each single cell is to be preserved, this procedure must be carried out without trauma. Either trypsin or a chelating agent may be used for this purpose, but it is imperative to follow faithfully a procedure which insures complete dispersal without significant damage to the cells (Puck, Marcus, and Cieciura, 1956). If the medium available is nutritionally adequate, it is sufficient simply to pipette an inoculum containing the desired number of dispersed cells onto a petri dish containing several cc of the growth medium. The cells attach to the glass, and since these are initially well

mixed in the medium, and usually present in numbers less than 200, each cell is sufficiently far from its neighbors to produce a discrete colony of about 2 mm in diameter after 9 or 10 days incubation. On Plate I, 1-3, are presented some typical colonies arising from the plating of single cells originating from a variety of human tissues.

While this method is satisfactory for some cells in the Complete Growth Medium which we have adopted as a standard in our laboratory (Marcus, Cieciura, and Puck, 1956), other cells fail to multiply when plated therein after dispersal. Yet these same cells can multiply indefinitely in the same medium if seeded in a large inoculum instead of in the single state. For example, a massive inoculum of HeLa S_3 cells will multiply rapidly and continuously in a basal medium to which no cholesterol has been added, but single cells require this metabolite added to the medium, in order to reproduce. This constitutes a model situation in which cell reproduction requires cooperation between several individuals either through the need to modify the medium as Earle has suggested, or possibly also through some other more direct type of cell-cell interaction.

The alternative plating technique devised to permit colony formation from single cells which fail to multiply in the usual nutrient media utilizes a "feeder" system of cells whose own reproduction has been terminated by a previous exposure to x-irradiation (Puck, Marcus, and Cieciura, 1956). The use of high energy radiation is particularly well suited to this purpose because of its enormously greater potency in blocking reproductive as opposed to other metabolic activities. We have demonstrated that the mean lethal dose of x-rays for a mammalian cell involves an energy absorption equivalent to a temperature rise of only 0.0001°C (Puck and Marcus, 1956). It was demonstrated that the addition of such x-rayed cells to a plate seeded with a measured inoculum of single cells which by themselves show no growth, results in 100% efficiency of colony formation. Hence, the use of such a "feeder" system makes possible extension of this plating technique even to cells otherwise deficient in the ability to grow as independent microorganisms in the media available (Plate I, 4).

Experiments have demonstrated that cells differ greatly in their capacity to act as feeders. Thus, while the irradiated HeLa cell can act as an effective feeder for normal HeLa cells in a nutritionally deficient medium, it can not feed human fibroblasts. These latter, however, can feed either themselves or the HeLa cell. Thus, the use of a feeder system furnishes a new investigative tool for various phases of cell–cell inter-

action and, indeed, suggests applications that may fruitfully be applied to studies with molds and bacteria.[3]

The advantages of a quantitative method for producing growth of single cells by a rapid and convenient plating procedure may be summarized: (1) It provides means for accurate titration of the reproductive powers of the individual cells of a population. Thus it furnishes a much more potent tool for study of the dynamics of cell growth and the effects thereon of physical and chemical agents, including nutritional and toxic factors. (2) It makes possible ready recognition and isolation of genetic mutations, and thus furnishes the means for quantitative study of an enormous variety of genetic processes in animal cell populations. (3) With the aid of the feeder system it permits new kinds of experimentation on cooperation and antagonisms in the interaction of cells with other cells of the same and different kind.

The remainder of this discussion will deal with representative experiments which have been carried out in our laboratory, illustrating applications of this plating technique to specific problems.

A. Establishment of clones from different tissues. A series of experiments is in progress testing how wide a variety of human cell types can be plated by these means. Cells of both epithelial and fibroblastic morphology have already been plated in this way with efficiencies in the neighborhood of 50–100%. The tissues represented include human skin, liver, conjunctiva, appendix, kidney, spleen and bone marrow. Good results have been obtained with cells recently isolated from human subjects as well as those previously cultivated for long periods in tissue culture. So far, we have not encountered a human cell type which has absolutely failed to grow under conditions of plating as single cells—i.e. which has not retained the capacity to be an independent microorganism. It becomes of primary importance to determine which, if any, of the body cells have indeed irreversibly lost this potentiality. Current studies include measurement by this same means of the growth potentials of cells from a variety of human tissues taken from subjects of different ages, and exhibiting a variety of different disease conditions.

B. Study of agents which affect growth—the action of x-rays on mammalian cells. This technique has been applied to analysis of the action of various agents on the cellular growth process. A typical

[3] It is of interest that the basic principle of feeder cells had been used for the cultivation of single plant cells (Muir, Hildebrandt, and Riker, 1954). These investigators did not use irradiated cells for their feeder system but rather laid a filter paper inoculated with single plant cells on top of a macroscopic segment of living plant tissue, the molecular exchange then progressing by diffusion through the filter paper.

growth curve obtained from a single plate by counting the cells per colony in 20 or more colonies after various incubation periods is pre-

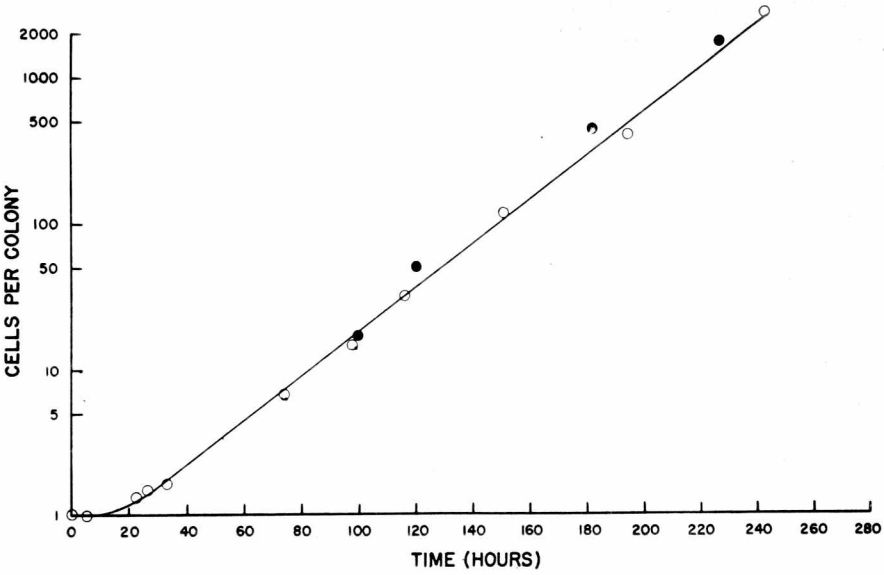

Fig. 1. Typical growth curve of single HeLa S3 cells when plated by the technique here discussed. In optimal medium, an almost identical curve is obtained from cells of conjunctiva, liver and kidney.

sented in Fig. 1. Such curves have been shown to be highly reproducible for a given cell in a given medium, even after the lapse of more than a year (Puck, Marcus, and Cieciura, 1956). It is evident, then, that a curve derived from such a single plating procedure can yield three types of quantitative information about the nature of the growth process: (1) the percent of the individuals of the population capable of forming colonies under the particular conditions employed; (2) the average lag period before growth begins, which presumably represents the time needed for adaptation to the given environment; and (3) the generation time—i.e. the time taken for the population to double when the colony is in its logarithmic growth phase. Thus, by providing different quantitative measures of various aspects of cell growth, this technique affords more comprehensive analysis of the action of agents which can affect the growth process. For example, it has been demonstrated that decreasing the concentration of human or porcine serum from 15% to 3% in the Growth Medium, reduces the growth rate of S3 cells but does not change the plating efficiency—i.e. the percentage

of the single cells which can form colonies. If the serum concentration falls below 2%, then the number of individuals which can reproduce also drops sharply. Similar studies are in progress on the effects of temperature, and a variety of drugs, and other agents of chemical and biological origin.

It is of interest that every cell type with which we have used this technique, originating from a variety of human tissues, and including both normal and carcinomatous specimens, has exhibited the same maximum growth rate, equivalent to a generation time of 18-20 hours (Marcus, Cieciura, Puck, 1956). All these cells also exhibit a similar mitotic time of about 40–50 minutes under these conditions. These observations suggest a limiting maximal reproductive rate for human cells, governed primarily by some rate-limiting process during the interkinesis period.

One study which merits special consideration involves the action of high energy radiation on the growth of mammalian cells (Puck and Marcus, 1956). Other techniques used in attempt to quantitate the destructive action of ionizing radiation on mammalian cells have yielded values ranging from several hundred to several hundred thousand roentgens as the mean lethal dose, a divergence so great as to render the data almost meaningless for many purposes where accurate information is needed. The present plating technique makes possible exactly the same kind of precise, survival-curve determination for the reproductive potential of mammalian cells as has become standard for bacteria. Plates inoculated with known numbers of single cells were irradiated with a series of different doses of x-irradiation, then incubated in the standard manner. The survivors which had retained the ability to form macroscopic colonies were then counted. A typical survival curve for the clonal HeLa cell is presented in Fig. 2. The shape of this curve indicates a 2-hit process as the one responsible for destruction of the reproductive capacity, and combined with other data, leads to the conclusion that the critical events have taken place in the cellular genetic apparatus (Puck and Marcus, 1956). The value for the mean lethal dose obtained from this curve is 96r, an astonishingly low one. The original study was carried out on a cell from the HeLa carcinoma of the cervix, and it was at first thought that this cell might have a radiation sensitivity far greater than that of cells from normal tissues. However, repeated determination of the survival curve on cells originating from normal tissues, has yielded values for the lethal dose which in no case so far exceed that of the HeLa cell by more than 20-30%.