Reports and Letters

Study of Tumor Cell Populations by Monte Carlo Methods

A method for calculating the growth of cell populations has been developed with the aid of the Los Alamos digital computer known as the MANIAC (*1*). One of the major obstacles to the estimation of the growth of a cell population, such as occurs in transplantable mouse tumors, has been the randomness in the intermitotic time. The chain reaction arising from binary fission of cells is very sensitive to the intermitotic time.

The digital computer is given a probability distribution for the intermitotic times and, by the Monte Carlo method, grows the hypothetical population, or "tumor," beginning with one cell. As this cell grows, the machine plays the game of chance determining when division occurs, the rules of the game being given by the probability distribution. At the instant that division starts a probability distribution of mitotic durations takes over, and another game of chance is played to specify when the division is complete. The cycle is repeated for all daughter cells until the allotted time interval has elapsed. This is a free birth process. Cells live independently; none die; and the population increases exponentially with time.

Population growth is followed by having the computer print out at prescribed time intervals the number of cells in mitosis and those intermitotic, the number of cells having different intermitotic ages, and the number of cells in the different stages of mitosis.

Three major distributions of the intermitotic times have been explored, each representing widely different models of cell growth. The first model was the analogy with radioactive decay that has been applied by Iversen and Arley (*2*) to chemically induced tumor growth in mice. The cell has a finite probability of dividing as soon as it is formed, and thereafter the probability that it has not divided decreases exponentially. The second model was designed to avoid the possibility of having a cell divide immediately upon birth. The first model based on radioactive decay was modified by introducing a rest period after birth. Following the "rest" period there is a finite probability of dividing, which also decays exponentially. The third model was based on the spread of intermitotic times measured by direct observation of mouse spleen cells in tissue cultures by Fell and Hughes (*3*). Their data show a spread of a factor of 2. Although they are too scant to describe a complete probability distribution, they provide an estimate on which to construct a tentative model.

A notable result of the computations is the long time required for the cell populations to reach a steady growth. In all three models tumors of 50,000 cells each were grown to establish the average cell generation times and the mitotic index. Wide fluctuations reflecting the random incidence of division about the average occurred during the first nine average generation times. A cell and its two daughters may take long times before dividing. Although two generations have divided, they may have taken as much as five average generation times to do so. This means that there is a fundamental latent period of growth when one cell starts out in a random growth cycle.

The effect of the randomness is that the growth curve of the population stemming from one cell does not necessarily extrapolate back to one cell at zero time. Statistically, single cells generate widely differing numbers of cells in a fixed time interval. In the first model of growth six single cells grew populations ranging from 41 to 2300 cells in an interval of nine average generation times. In the third model, based on mouse spleen cell data, fluctuations of 30 percent appeared in the time required to generate 1000 cells along with variations in the mitotic index up to 4 times the average.

The computed incidence of mitosis is found to follow a Poisson distribution, which has been reported in experimental data on mouse ear skin and on mouse tumors. An unexpected finding in the computations was that the average mitotic index in freely growing cell populations is not always simply related to the time parameters of the life-cycle. When a rest period, during which no division can occur, is introduced into the early life of the cell, the mitotic index is less than that based on the ratio of the average duration time to the average generation time. This means that even in the exponential growth of independent cells as carried out on the computer, the mitotic index is a complex function of the cell's time parameters.

The sizes of the cell populations generated in the random birth process are sensitive to the distribution of intermitotic times. If the latter has a narrow range of possible values, then the "tumors" grown by the computer fall within narrow limits. In the obvious case where cells have but one possible intermitotic time they multiply with a clocklike regularity and always produce the same sized tumor in a given time interval.

At the other extreme wherein the intermitotic time scatters by a factor of 5, the tumors grown in a fixed interval vary by a factor of 56 in size. The sensitivity between tumor sizes and the intermitotic times should provide a method for determining the latter from experimental data on the former, especially with single-cell inoculations, since the computer can indicate the expected tumor sizes in idealized models of growth.

The Monte Carlo method affords a means of bridging the gap between cell sizes and time parameters in a cell's life. This hinges on the fact that the age structure of the population determines the distribution of sizes, both of cells and of nuclei. Thus in the mouse tumors nuclei and the entire cell may grow exponentially (*4*) with intermitotic age. Therefore the volume distribution of nuclei permits inferences about the distribution of intermitotic times by the computer method.

Of fundamental interest are the events that cause randomness in a cell's growth period. The method makes possible estimates concerning the sequence of events in that period. The model based on radioactive decay is basically one in which a single random event sets off the division process. Our second model merely introduced a rest period before this single event was allowed. The third model had inherent a mechanism of about 20 random events that had to occur in a time sequence before division could be started.

Such events may be speculatively associated with chromosomes or large groups of molecules in growth. During the intermitotic growth a cell must undergo a characteristic series of operations. Kendall (*5*) analyzed the data on *B. aerogenes* of Kelly and Rahn and estimated that 20 discrete events occur before division. Rahn's (*6*) original hypothesis about this bacterium was that simultaneous submicroscopic processes, whose number was identified with the gene number, took place. In the Dba mouse tumor grown subcutaneously there appear to be 18 random events in sequence

that determine cell division. The meaning of these numbers has yet to be clarified. But the Monte Carlo method affords a means for estimating them from data on various aspects of individual cell life.

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7 January 1955

Nonbicarbonate Buffers in Cell Culture Media

The use of cell cultures in studying fundamental problems in many fields of experimental biology has increased considerably in the last few years. It is frequently desirable to employ cells that have been cultivated under conditions that are as standardized as possible. From the beginning of "tissue culture," cells of mammalian and avian origin have been cultivated *in vitro* in media composed of a balanced salt solution containing sodium bicarbonate as the principal buffer, supplemented with embryo extract and serum. Adequate control of the *p*H of such cultures introduces a number of technical problems, for it is necessary to maintain a specified carbon dioxide tension in the atmosphere by employing gassing procedures and closed culture flasks. In an attempt to circumvent some of these difficulties, studies were initiated to test the possibility of growing cells in media containing a fixed buffer instead of the conventional bicarbonate system. In addition to facilitating the control of *p*H and reducing the labor required for maintaining cultures, fixed-buffer media have a number of obvious applications in mass culture techniques and in metabolic and viral studies with cultured cells.

The following cell cultures were employed: fibroblasts derived from normal human foreskin, testicle, human embryonic kidney, and lung, as previously described (*1*); epithelial cells, strain HeLa (*2*); mouse fibroblasts, strain L (*3*). All cultures were grown directly on glass. The medium for foreskin, embryonic kidney, lung, and testicle cells consisted of mixture No. 703 (*4*) supplemented with 5-percent beef embryo extract and 10- to 20-percent horse serum. Human serum (20 percent) was employed instead of horse serum for HeLa cultures, and the L cells were grown on mixture No. 703 supplemented with 10-percent beef embryo extract.

The sodium bicarbonate of mixture No. 703 was replaced with tris(hydroxymethyl)aminomethane (5, tris) at concentrations of 2.0 to 3.6 g/lit, and the *p*H was adjusted to the desired value by the addition of hydrochloric acid. This mixture was supplemented with embryo extract and serum in the same concentrations as were employed for the stock cultures. Foreskin, embryonic kidney, lung, and testicle cultures grew as well as the controls (bicarbonate medium) with all levels of tris when the initial *p*H of the nutrient fluid is 7.8 to 8.1. All cultures were propagated serially with weekly subcultures. Foreskin and testicle cells have been maintained for the longest period and continue to do well after 3 mo. These cultures have been maintained in both stoppered flasks and in flasks closed only by means of cotton plugs or loose-fitting metal caps. A number of differences between cells grown in tris and those grown in bicarbonate have been observed. For example, it appears that the number of cells required per flask is a more critical factor in tris fluids than it is in bicarbonate fluids. The only change in the appearance of cells cultured in tris fluids noted to date is a small increase in the number of intracellular granules. The results of a limited number of experiments indicate that prolonged cultivation of cells in fluids containing tris does not alter their susceptibility to infection with polio virus or the yield of virus. In contrast with the growth rate of normal human fibroblasts, the growth rate of HeLa and L cells was somewhat inhibited with higher levels of tris. The HeLa cells were smaller and more granular than those in the control cultures and there was a tendency for the cells to grow in clumps rather than as a continuous monolayer. The L cells also exhibited changes in morphology when they were cultured in media containing tris. Despite alterations in morphology and decreased rate of proliferation, both strains have been maintained through 14 subcultures and continue to proliferate at a constant rate.

The results of short-term experiments indicate that glycylglycine is less toxic than tris for HeLa cultures. Under these conditions, the cells grew rapidly without any appreciable alteration in morphology. Cultures of foreskin cells, however, did not proliferate as well in glycylglycine as they did when tris was employed as the principal buffer.

Although the results obtained to date with tris are particularly promising with cultures of normal human fibroblasts, factors such as concentrations of tris, optimal *p*H, and the effect of other constituents of the medium require more detailed study before a final evaluation can be made. Several organic and inorganic compounds with a pK_a in the range of 6.5 to 8.5 that are compatible with other components of cell culture media are now under investigation in an attempt to replace tris in cultures where it is toxic or where the optimal *p*H for growth is below the effective limit of this buffer system.

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* Aided by a grant from The National Foundation for Infantile Paralysis.

9 May 1955

Substrate Specificity of Peroxidase

A current view of enzyme specificity is that compounds of certain specific configurations can be bound by the enzyme and can lead to formation of the activated complex. The substrate specificity of catalases and peroxidases for hydrogen peroxide has been recognized for some time, and Theorell has recently suggested the term *hydroperoxidases* for this class of enzymes (*1*).

Such a definition of specificity includes reactions with homologs of the substrate, methyl or ethyl hydrogen peroxide, which have been shown to react in a second-order fashion with peroxidase and to form the same series of intermediates as does hydrogen peroxide (*2*). But the possibility of forming these reactive intermediates by a wide variety of inorganic oxidizing agents, recently pointed out by P. George (*3*), would seem to vitiate current ideas of formation of the enzyme-substrate compound and to favor the idea that the spectroscopically detectable peroxidase intermediate (complex I) is only the product of some rapid, unobserved oxidation reaction caused by a great variety of nonspecific oxidants.

This possibility lead George to the view that peroxide would react by a similar mechanism and would not therefore be a component of the structure of complex I, nor would it, per se, be required for