The distribution of tracer is not that which is expected if two equal triose phosphates combined to yield a hexose phosphate.

The authors are indebted to Dr. R. C. Fuller for discussion, helpful suggestions, and assistance in carrying out the short-time experiments. They are also grateful to Dr. Jerome Schiff and Dr. G. Kandler for laboratory assistance.

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MULTIPLICATION OF ANIMAL CELLS IN SUSPENSION MEASURED BY COLONY COUNTS*

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Communicated by A. Lwoff, April 11, 1957

During the past few years the development of new techniques for the cultivation of animal cells in vitro has facilitated the quantitative study of many aspects of cell biology. At present the most commonly used method of propagating cell strains is based on the ability of cells to multiply while attached to a glass surface. The cells may be subcultured by removing them from the surface into suspension and then distributing them into other vessels, where they will again adhere to the glass and populate the surface. This procedure has been developed by Earle and his associates1, 2 into the so-called quantitative replicate culture technique and applied to a variety of studies with animal cells. Despite the technical advance represented by this method, there are, nevertheless, serious experimental limitations
inherent in the use of glass surfaces for cultivating large cell populations. Perhaps the most obvious of these is the problem of removing representative samples at will during the growth of a cell population. In addition, subculture requires the removal of cells from the surface, with consequent risk of cell injury.

In order to obviate these restrictions, efforts have been made by several investigators to propagate animal cells in suspension. Previous communications from this laboratory have described a technique that accomplishes this purpose. It was shown that, for several strains, the growth of animal cell populations in suspension, estimated by enumeration of cells in a counting chamber, is similar to that of bacteria, in that lag, logarithmic, and stationary phases were observed. Cells could be kept continuously in the logarithmic phase of multiplication by subculturing at frequent intervals.

While such a system would seem to be ideally suited to biochemical studies, its full usefulness cannot be realized without some knowledge of the number of viable cells in the cultures. To obtain this information, we have used the clone technique recently developed by Puck and his associates. In the present paper it will be shown that cells grown in suspension readily form colonies on a glass surface and that, for two strains at least, they will plate with a high and constant efficiency during the logarithmic phase of growth.

Two strains of cells were used in this work: (1) Earle's L strain, a clone derived originally from a culture of adult mouse connective-tissue cells, and (2) a clone, selected in this laboratory, from an altered monkey kidney cell population provided by Dr. R. C. Parker. The method for cultivating cells in suspension, and the medium, have been described in detail elsewhere. In brief, large test tubes containing the cell suspensions were rotated at 60 rpm around their long axes at 37°C.

The medium contained 20 per cent horse serum in solution CMRL-1066, which is a defined mixture of salts, amino acids, vitamins, coenzymes, and other metabolites. Cells of both strains multiplied readily under these conditions, with generation times of 20–25 hours in the logarithmic phase of growth. Stock cultures were kept continuously in the logarithmic phase by frequent subculture.

When cells from a logarithmic-phase culture were placed in a stationary container, macroscopically visible colonies appeared on the glass surface after 6–10 days' incubation. A linear relationship was obtained between the number of cells plated (10–500) and the number of colonies formed. The procedure for plating the cells was similar to that used by Puck, Marcus, and Cieciura for suspensions of cells from trypsin-treated monolayers. For the L strain, daily enumeration of the cells in ten randomly selected colonies showed that multiplication of the individual cells began 12 hours after plating and continued logarithmically, with a doubling time of 18 hours, until the colonial populations reached 500 cells or more. Since we found in these experiments that both the plating efficiency, i.e., the percentage of plated cells that developed into colonies, and the rate of cell increase within colonies varied greatly with the particular lot of horse serum employed, a selected serum was used in the remainder of the work.

In order to determine the increase in a cell population by the colony-count method a rotating culture containing about 5 × 10⁴ cells per milliliter was prepared from a logarithmic-phase stock suspension. At intervals samples were removed and the cell densities estimated with a hemocytometer. Serial dilutions of the sample
were made in solution 1066 at room temperature. One hundred cells were plated in each of six Petri dishes (6-cm. diameter) containing 5 ml. of complete medium (solution 1066 supplemented with horse serum) previously equilibrated at 37° C. in an atmosphere of 5 per cent CO₂. After incubation of the plates at 37° C. for 8–10 days in the 5 per cent CO₂ atmosphere, the colonies were washed with buffered saline, fixed with Bouin's solution, treated with Giemsa's stain, and scored. The colonies of L cells on a representative plate are shown in Figure 1.

Figure 2 shows the growth curves obtained for L cells by hemocytometer count (upper curve) and by plate count (lower curve). Each point on the lower curve represents the average of six plates, or 400–600 colonies. The method of least squares was used to fit the exponential portion of this curve and indicated that the standard deviation of the points from the line was about 12 per cent. A similar set of curves is shown in Figure 2 for a suspension of the monkey kidney cells. The average plate counts deviate from the linear part of the curve by 8 per cent. The errors, which include day-to-day variation and sampling errors, are comparable to those obtained in colony counts with bacteria.

It will be seen that the plating efficiencies in both experiments remained constant at about 75 per cent throughout the logarithmic phase of growth. During the stationary phase, however, the plating efficiencies may decrease significantly (Fig. 3). This decrease was very marked in some other experiments, indicating that the cells may undergo an extensive physiological change during this period.
It is of interest now to inquire whether the 75 per cent plating efficiency observed during logarithmic growth represents all the viable cells in the population or whether some inadequacy in the plating procedure or in the medium permits only 75 per cent of the cells to form colonies. In an effort to answer this question, we have utilized the feeder-layer technique developed by Puck et al. Table 1 shows the results of several experiments in which monkey kidney cells from loga-
FIG. 3.—Multiplication of altered monkey kidney cells in suspension. Upper curve: hemocytometer count; lower curve: colony count.

logarithmic-phase suspensions were plated over feeder layers of giant cells obtained from X-irradiated cultures of the same cell strain. In each case the plating efficiency was higher in the presence of feeder cells than in their absence and was frequently in the neighborhood of 100 per cent. These observations indicate that, during the logarithmic phase of multiplication, nearly all the cells possess the ability to form colonies under the improved plating conditions provided by the presence of feeder cells. It should be mentioned, however, that for many practical purposes, e.g., studies on virus and radiation sensitivity of cells during the period of logarithmic increase, a plating efficiency of 75 per cent achieved without the use of feeder cells is adequate.
TABLE 1.

Plating Efficiency of Altered Monkey Kidney Cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Without Feeder Cells (Per Cent)</th>
<th>With Feeder Cells* (Per Cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>98</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>92</td>
</tr>
<tr>
<td>3</td>
<td>62</td>
<td>102</td>
</tr>
<tr>
<td>4</td>
<td>66</td>
<td>90</td>
</tr>
</tbody>
</table>

* 5 x 10^4 irradiated cells were added to each Petri dish to form the feeder layers.

It is evident that the culture systems described here have the essential properties that will allow a highly versatile and precise experimental approach to many problems of animal cell physiology and biochemistry. They provide populations of individual cells that multiply in suspension, from which representative samples may be taken at will. During the logarithmic phase the number of cells that will form colonies on a glass surface without feeder cells is a constant and high proportion of the total population, and the amounts of RNA, DNA, and protein per cell are constant. In these respects there is a marked similarity between these populations of animal cells and many bacterial populations.

Summary.—When animal cells of two different strains were withdrawn from populations growing in suspension, they multiplied, without an appreciable lag, to form colonies. While the percentage of cells that formed colonies varied widely when they were plated from the stationary phase of growth, 75 per cent of the cells gave to colonies when plated at any time during the logarithmic period. Almost 100 per cent of the cells in the logarithmic phase grew into colonies if they were plated over feeder layers of X-irradiated cells, showing that all, or nearly all, of the cells were capable of multiplying if provided with the proper nutritional environment. The cell-culture systems described should facilitate a variety of studies on the physiology and biochemistry of animal cells.

We wish to acknowledge our gratitude to Dr. R. C. Parker for a great deal of helpful discussion and advice during the course of this work.

* Aided in part by grants from the National Foundation for Infantile Paralysis, the Public Health Service of the National Institutes of Health of the United States, the National Cancer Institute of Canada, and the W. B. Boyd Memorial Fund.
8 T. T. Puck and P. I. Marcus, these PROCEEDINGS, 41, 432, 1955.
11 The phenomenon of alteration in normal and-malignant cell cultures has been described by R. C. Parker, L. N. Castor, and E. A. McCulloch ("New York Academy of Sciences Special Publications," No. 5 [in press]).
In our previous work on the growth of animal cells in suspension (see nn. 6 and 7), cell densities were determined in a Petroff-Hauser bacterial counter rather than with a hemocytometer. Subsequent comparison of the two types of counter showed that estimates made with the hemocytometer are correct, while those determined with the bacterial counter were high. Cell densities reported in previous publications may be corrected by multiplying them by the factor 0.65.

ON THE DISTRIBUTION OF PURINE AND PYRIMIDINE BASES IN THE NUCLEIC ACID OF TOBACCO MOSAIC VIRUS

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Communicated by W. M. Stanley, March 22, 1967

Reddi and Knight\(^1\) have recently investigated the products of ribonuclease digestion of the nucleic acids of several strains of tobacco mosaic virus. After the enzyme had been allowed to act on the isolated ribonucleic acids, the trichloroacetic acid–precipitable fraction (hereafter called "core") of each digest was analyzed to determine the molar ratios of purine and pyrimidine bases. As might be expected from the specificity of pancreatic ribonuclease,\(^2\) the core material was found to contain several times as many purines as pyrimidines, whereas the ratio of purines to pyrimidines is close to unity in the whole nucleic acids. And just as no significant differences have been found in the over-all composition of the nucleic acids of the strains of tobacco mosaic virus,\(^3\) no significant differences were observed in the base compositions of the cores.

In the present study an attempt is made to determine from theoretical considerations whether the similar compositions of the cores are indicative of some common arrangement of purines and pyrimidines in the nucleic acids. In order to do this, a hypothetical nucleic acid is considered to undergo the treatments described by Reddi and Knight and to serve as a control in their experiments. The control material is assumed to be a single long-chain nucleotide polymer with the characteristic ribose-to-phosphate linkages of a ribonucleic acid and to have the same relative numbers of adenylic, cytidylic, guanylic, and uridylic acid residues as do the nucleic acids of the viral strains. However, the sequence of these residues along the hypothetical polymer is assumed to be completely random, and for this reason it serves as a control; if the core compositions for the viral-strain nucleic acids do not differ from the core composition which would be obtained for the random polymer, then they are not necessarily indicative of any specific nucleotide sequence common to the strains. The validity of this test can be appreciated if it is remembered that the random arrangement is not any particular sequence of nucleotide residues but is a mathematical composite resembling a majority of the vast number of possible sequences. If the nucleic acids of the viral strains have core compositions consistent with such a model, it can be concluded that they are typical by this criterion, but not that the strains are identical or even similar with respect to their particular nucleotide sequences.

The question of how closely a viral nucleic acid resembles a random polymer has another, more general significance. Recent experiments\(^4\) have indicated that a