after isolation from bacterial cells which have been treated with mitomycin C. The authors had no evidence of a direct effect by the agent on purified DNA. Although their results support the argument that mitomycin C requires a metabolic trigger, evidence is still lacking that mitomycin C itself forms the bridge between strands, or even reacts to form a covalent bond with any cellular component.

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10 September 1963

Continuous Recording of Cell Number in Logarithmic and Synchronized Cultures

Abstract. *An instrument for the continuous recording of cell number has been developed and is being used to record changes in populations of logarithmic and synchronized cultures of protozoan flagellates. A Coulter cell counter is used in conjunction with a counting chamber that is fitted with a flexible polyethylene aperture (75 μ in diameter). This aperture rarely becomes blocked and appears to be self-clearing. The unit consists of a proportioning pump, a cell counter, a counting chamber, a rate meter, and a recorder.*

There are two common methods of detecting unequivocally cell division in a population of cells. One is by direct microscopic observation of cell division and the other is by counting the cells as a function of time. All other methods, such as those that use optical density, turbidity, mass, or DNA content, are less satisfactory since they rely on variables that may change in the absence of cell multiplication. Consequently, to detect and measure synchronous cell division, one must rely on counting the cells. Fortunately, electronic methods (*1, 2*) have been developed which are reliable, but to measure growth rates or division synchrony, one must use a batch method of counting. This is because the aperture or window in the Coulter counter through which the cells must pass is small, between 30 and 200 μ in diameter, depending on the cell being counted, and it is subject to plugging. The window surface can be cleaned between batch counts when plugging occurs. The plugging usually results from debris that is present in even the most carefully prepared culture medium. Thus, counting nonsterile aliquots is readily accomplished, but counting a sterile system directly becomes a difficult operation.

We have now developed an instrument for counting continuously the cells in a sterile, growing culture of *Astasia longa*. By means of a proportioning pump (*3*), a small part of the stirred parent culture is continuously removed, diluted, mixed, and pumped into a counting chamber (Fig. 1) whose leads are attached to a Coulter counter, model B. The electrical output of this instrument is fed into a rate meter and then to a recorder. Since the rate meter (*4*) requires a negative signal pulse, an auxiliary circuit is used to reverse the polarity of the signal. The recorder (*5*) is set at 0.66 in./hr (1.67 cm/hr). With this recorder, other signals, such as culture temperature, can be monitored along with cell number.

Figure 1 shows a schematic diagram of the apparatus and includes details of the counting chamber. The window or aperture of this unit is a perforation in a flexible polyethylene or Teflon membrane. It is mounted in an "O" ring ball joint (*6*) and held in position with a Teflon or Neoprene "O" ring. The aperture is made in the membrane with a tapered glass needle drawn to cover the range of diameters needed to count specific cells. The membrane is perforated to the appropriate diameter under a dissecting microscope. When continuous counting was attempted with a solid aperture, such as the one supplied with the counter, plugging occurred frequently and the system could not be used. The best apertures could be made in polyethylene membranes, 25 to 35 μ thick. We have used several of these for 2 weeks of continuous counting without their showing signs of deterioration. Teflon membranes of the same thickness appear to change their electric characteristics with time.

Our design is based on several factors that we consider important for the continued use of such a unit. With an aperture of about 75 μ, the flow rate must not exceed 3 ml/min or high pressure will break the membrane. To reduce coincidence error to a minimum, the output from the parent culture is diluted with 0.3 percent sodium chloride as the electrolyte. To obtain a dilution ratio of one part of the culture to 8.3 parts of the diluent, and to maintain the total flow rate at 2.8 ml/min, tubes of 0.030 inch (0.08 cm) and 0.081 inch (0.21 cm) diameter are used with the Autotechnicon proportioning pump. Since the linear flow rate must be such that settling of the cells in the tubing is reduced to a minimum, the culture input line is injected into the vertically directed diluent stream in the counting chamber. The diluent flow rate is eight times greater than the culture input flow rate, consequently the cells are washed upward, settling does not occur, and mixing is optimal in this region of the

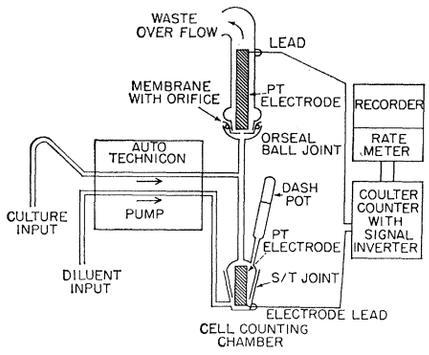

Fig. 1. Schematic diagram of the apparatus for the continuous recording of cell number, showing details of the counting chamber.

counting chamber. The cells are living and motile until they are diluted in the chamber, so that the tendency to stick or settle in various regions of the input line is minimal. Furthermore, Teflon tubing with a 0.75-mm inner diameter (*7*) is used to withdraw cells from the culture and is superior to glass for this purpose. A small amount of settling and sticking occurs wherever glass is introduced into the system. At our rate of pumping, however, tubing of this diameter gives a linear flow rate of 2.5 ft/min (0.76 m/min), and no detectable settling or sticking of cells has occurred over the period of a month.

Each electrode is made of platinum foil, 6.5 cm square, welded to platinum wire leads. The electrode chambers are large enough to accommodate the foil and are arranged to reduce the likelihood of collecting cells or contamination. To avoid contamination with other microorganisms, the diluent is also supplemented with formaldehyde (5 percent). The dash pot (5 ml) on the lower electrode vessel is placed so that the inherent pulsing of the pump will be smoothed out, thus preventing periodicity in the count rate. The size of the air space in the dash pot can be used to estimate the changes in the diameter of the aperture and to determine when partial plugging occurs.

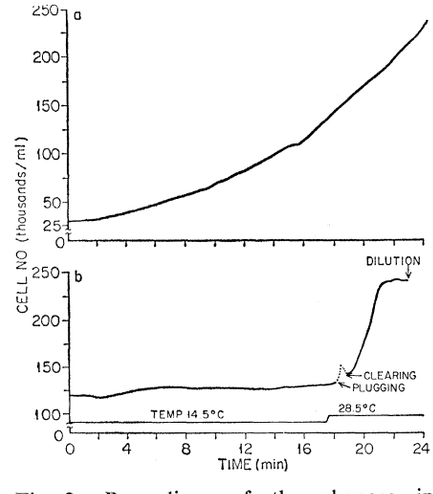

Fig. 2. Recordings of the changes in cell number over (*a*) a logarithmic growth period, and (*b*) a synchronous growth cycle of *Astasia longa*.

In its present form, the cell number monitor can be used to obtain growth curves starting from about 25,000 cells per milliliter to about 250,000 cells per milliliter in the parent culture. The upper portion of Fig. 2 shows a curve obtained during the growth of *A. longa* over 24 hours at 28.5°C. It shows the logarithmic character of population growth, and when plotted on semilogarithmic paper, a straight line of good fit is obtained. Alternatively, a log-linear recorder can be used to obtain a straight line plot if the populations are such that the coincidence error is low (*1*). The lower portion of Fig. 2 is a recording of cell number in a synchronized culture of this flagellate. The temperature cycle used to induce this synchrony is indicated by the lower curve, while the upper curve is the plot of cell number. The shape of the burst of cell division is clearly plotted showing the time of maximum rate of division, and the dispersion of the division burst relative to the total cycle length, 24 hours. The doubling in number can also be appraised from the graph. This synchronizing procedure is a repetitive method of synchronizing the cell division of this organism (*8*) and the recording represents only one cycle in a continuous series in which the media are replenished daily.

There are at least three sources of error that must be taken into account in designing a cell number monitor. They include the coincident error, the overall time constant of the system, and the counting error. The errors introduced by coincidence of particles in the window has been carefully examined for the Coulter counter and correction factors can easily be determined for any given window (*1*). The time required for a 63-percent response [1-(1/*e*)] to an abrupt dilution of the culture is about 5 minutes. This time constant was evaluated empirically and can be decreased or increased by attention to the flow rate, volume of input lines, and volume of the mixing region. These factors are the largest source of delay between a change in the culture and the appearance of the change on the recorder. The rate meter time constant is generally set at 100 seconds, but this factor is not the only one determining the overall time constant.

The counting error—that is, the standard deviation of the continuous record of number, can be estimated by considering the width of the recording line over a region of constant number. In a recording rate meter the fluctuations of the line about the average will approximate a range of slightly more than plus and minus two standard deviations (*9*). The records, of which Fig. 2 is a tracing, indicate a standard deviation at an average cell number of 100,000 per milliliter, ± 1200 cells, or a coefficient of variation of ± 1.2 percent.

Although the biggest drawback to the present system is that part of the culture is used up in the operation, our experience has shown that whenever we monitor cell number over a synchronous burst of cell division, the loss from the culture by batch-counting methods is considerably greater than by continuous counting because of the sampling waste. Moreover, the statistics with batch counting are less favorable.

We feel that the reason for the success of this continuous counting device is the flexible polyethylene aperture or window. It rarely plugs up and even then it appears to be self-clearing (see Fig. 2). The system generally can be used for following not only logarithmic and synchronized cultures, but also the cell number in chemostats or for control of cell numbers in other types of continuous cultures. We are now using it for recording data from an experimental cell-division synchronizer. In this application it provides an accurate record of population events following a manipulation of, or the addition of agents to, the culture.

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Immune Response and Mitosis of Human Peripheral Blood Lymphocytes in vitro

Abstract. *Phytohemagglutinin causes cultured lymphocytes to agglutinate, divide, and produce γ-globulin. Most cells are transformed into large lymphocytes, some resembling plasmocytes. Actinomycin D stops γ-globulin production after 2 hours. When specific antigens are added to lymphocytes from sensitized individuals, only some cells undergo morphological transformation, produce γ-globulin, and divide. When cells or cell extracts from an unrelated individual are added to a culture, a similar reaction occurs.*

Phytohemagglutinin, an extract of the kidney bean, *Phaseolus vulgaris,* has been known to be a hemagglutinating agent since 1949 (*1*). In 1959 it was discovered that this extract induces mitosis of human peripheral blood lymphocytes in vitro (*2*). In using a modification of the technique for chromosome study from lymphocytes (*3*), we noticed that mitoses seemed to appear within clumps of lymphocytes. Such clumps did not appear in the absence of the bean extract. The hemagglutinating and mitogenic properties of the extract could be separated, but the latter could not be separated from the ability to agglutinate white cells (*4*). We and others (*5*) observed that during the 72 hours required for mitoses to appear, the vast majority of small lymphocytes were morphologically transformed to large cells, some of which resembled plasma cells. Since, in our method, 80 to 90 percent of the cells at the beginning of culture are small lymphocytes, and since there is no loss of cells during the culture period, it must be these cells which undergo transformation and eventually divide.

The mechanism of action of phytohemagglutinin is not understood. In view of the aforementioned findings, we suspected that some form of immunological reaction occurs in response to the addition of the bean extract to these cells. Pearmain and Lycette, by adding purified protein derivative of tuberculin to the lymphocytes from individuals sensitive to tuberculin, demonstrated that mitoses of peripheral lymphocytes can result from an in vitro immune stimulus (*6*). Bain has reported that DNA is synthesized when a mixture of peripheral blood leukocytes from two unrelated individuals is cultured in a tube (*7*).

We have now studied the possible immune response to phytohemagglutinin and have compared this with the response to specific antigens. All lymphocyte cultures were set up according to the method of Hastings *et al.* (*3*), with the following exceptions: the mitotic arresting agent was vincaleucoblastine (Velban, Eli Lilly) at a final concentration of 7.5×10^{-3} μg/ml (stock, 0.5 μg/ml) for the final 2 hours of culture, and cultures with specific antigens were permitted to grow for 5 days; C^{14}-labeled phenylalanine was used in the incorporation experiments, which were evaluated by standard methods of precipitation with trichloroacetic acid for protein assay and paper electrophoresis.

When phytohemagglutinin was treated by multiple passages through red blood cells until no further hemagglutination occurred, its leukoagglutinating and mitogenic potency was not diminished. This result was independent of the ABO blood type of the donor. When phytohemagglutinin was similarly treated with lymphocytes until no further leukoagglutination occurred, its mitogenic property was lost.

The addition of C^{14}-labeled phenylalanine to the medium at the beginning of the culture period resulted in incorporation of radioactivity into protein found in the supernatant medium when phytohemagglutinin was present. In the absence of phytohemagglutinin only a minimal amount (approximately 20 percent) of such incorporation was found. When actinomycin D(3.2 μg/ml) was added along with the bean extract, the amount of protein produced was the same as that obtained without the addition of extract (see Fig. 1). When the supernatant medium was dialyzed to remove free labeled amino

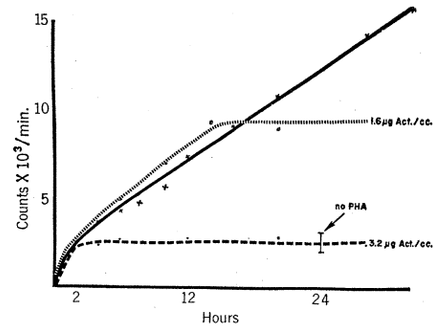

Fig. 1. Incorporation of C^{14}-labeled phenylalanine into protein. (Solid line) Protein production in a culture to which phytohemagglutinin (PHA) was added at time zero. The other two lines represent similar cultures to which phytohemagglutinin and actinomycin D were added at time zero. The vertical bar at 24 hours represents the range of total protein produced by a culture (several experiments) to which no phytohemagglutinin or actinomycin D was added.

acids and the dialysate was subjected to paper electrophoresis, all the radio-activity as read on a chromatogram scanner remained in the γ-globulin region. Since phenylalanine represents approximately 4 percent of human γ-globulin, a calculation based on the radioactivity of the protein obtained by precipitation of the medium with trichloroacetic acid after 24 hours of culture indicates that in the presence of phytohemagglutinin approximately 1 million lymphocytes produced approximately 10 μg of protein as γ-globulin.

Incubation of the cells after the first 24 hours of culture with fluorescein-conjugated rabbit anti-human γ-globulin resulted in specific fluorescence of almost all the lymphocytes in culture. No such fluorescence was observed in the absence of phytohemagglutinin.

In two experiments with lymphocytes from an individual with sex-linked congenital agammaglobulinemia, phytohemagglutinin produced the usual mitotic rate in 72 hours. The usual morphological transformation of cells was observed, but γ-globulin production, as judged by immunofluorescence, could not be detected.

Utilizing lymphocytes from sensitized and nonsensitized individuals, we substituted the following antigens for phytohemagglutinin and examined the cultures for mitotic rate on the 5th day. The antigens and amounts used (per 2×10^6 cells) were purified protein derivative (Parke Davis, 2×10^{-5} to 1.2×10^{-1} mg), diphtheria toxoid (2×10^{-4} mg), pertussis vaccine (Eli Lilly, 5×10^{-3} ml), and penicillin (200