Trypsinization of Animal Tissues for Cell Culture: Theoretical Considerations and Automatic Apparatus

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ABSTRACT

GORI, GIO B. (Microbiological Associates, Inc., Bethesda, Md.). Trypsinization of animal tissues for cell culture: theoretical considerations and automatic apparatus. Appl. Microbiol. 12: 115–121. 1964.—The theoretical background and the experimental feasibility of an automatic control for the continuous trypsinization process are discussed. An automatic apparatus is described with experimental evidence that the optimal mean residence time of monkey kidney cells in the trypsinization flask is between 5 and 8 min, in a volume of fluid approximately ten times that of the tissue processed. A temperature of 36°C and a pH of 7.8 provide optimal conditions for cell viability.

Monodisperse living cells can be obtained from animal tissues by trypsinization because the enzyme differentially degrades the protein matrix which binds the cells in the tissue, and releases these cells in suspension before they are seriously damaged in the process. The cells so dispersed can be separated by centrifugation from the trypsin solution and resuspended in a suitable growth medium.

Although this trypsinization technique has been widely used, little is known with respect to the intimate nature of the process, and the extent of its application has been limited in part by the empirical methods in use. The present study suggests an automatic method to insure the optimal yield of viable cells. This has obvious relevance to the mass production of cell cultures now necessary for the preparation of human and veterinary vaccines.

The first part of the paper will deal with a simple theoretical analysis, and the last will present experimental results obtained through use of an automatic apparatus.

THEORETICAL ANALYSIS

The Continuous Process

The first trypsinization procedures were discontinuous batch methods requiring frequent handling and giving low yields (Dulbecco and Vogt, 1954; Youngner, 1954; Melnick et al., 1955; Bodian, 1956). As demand increased, new devices were designed involving continuously working systems (Barski, 1956; Rappaport, 1956; Gori, 1958; Bishop, Smith, and Beale, 1960), and the variables of the process were analyzed (Rappaport, 1956; Gori, 1958). Such continuously operating systems consist basically of a reservoir from which the enzyme solution flows at a controlled rate to the trypsinization vessel (reactor) in which the process takes place. The resulting cell suspension and side products of the digestion overflow from the reactor to a collecting flask where the action of trypsin is arrested usually by low temperature and the addition of excess serum proteins (Barski, 1956; Rappaport, 1956; Gori, 1958). The hydrodynamics of this type of continuous process has been extensively studied, and the importance of such environmental variables as temperature and pH has been recognized (Monod, 1950; Novick and Szilard, 1950).

The independent variable of the trypsinization process is the amount of substrate, i.e., the amount of tissue present in the reactor. This determines the concentration of active enzyme required in the reactor, the rate of its inactivation, and, thus, the rate of flow necessary both to carry off the products and to replenish the enzyme.

Flow Rate and Mean Residence Time

The cells are first released from the tissue in clumps, and then these clumps are further digested until the cells are monodispersed. If such monodispersed cells are left in the reactor for a long time, they will be in turn digested and injured by the enzyme.

Variation in the mean residence time will affect both the degree of dispersion and the integrity of the cells, thus determining the suitability of the cells for tissue culture purposes.

The usual reactor has a constant level outlet so that the volume of fluid in the reactor remains constant. If \( W \) is the flow rate in ml/min and \( V \) is volume of fluid, the washout rate \( W/V \) indicates the number of changes of the fluid in the reactor per time unit. The mean residence time \( T \) of the cells in the reactor is \( V/W \) and can obviously be decreased by increasing the flow rate \( W \) or decreasing the volume of the reactor \( V \).

Enzyme Concentration and Kinetics of the Reaction

One may define a tissue digestion speed coefficient as:

\[
\alpha = \frac{\text{cells}}{\text{minute}}
\]

When temperature and pH are kept at optimal values, the speed of the digestion increases with the enzyme concentration, up to a limiting saturation value. Since the enzyme is inactivated during the process, the concentration \( C \) of active trypsin entering the reactor must be higher
than the concentration $c$ required in the reactor itself. The concentration $c$ will be determined by the value of $C$, by the inactivation rate and by the flow rate.

**Substrate**

During trypsinization, the surface layer of cells is successively exposed to enzymatic action. The number $m$ of cells present on the surface and, therefore, capable of immediate release will be a maximum at the beginning, decreasing to zero at the end of the process. Since the tissue is usually minced before trypsinization, and assuming that the tissue fragments have approximately the same size, spherical shape, and uniform cell density, the following relation can be written:

$$m = P \times \frac{4}{3} \pi (R - ad)^3$$

(2)

which is a decreasing function of time, and where $P$ is the number of tissue fragments, $s$ is the monolayer density of cells, $R$ is the initial radius of the fragments, $a$ is the digestion speed coefficient, $d$ is the cell diameter, and $t$ is the time in seconds. (Since the volume of tissue decreases with time unless new tissue is added, the volume $V$ of the reactor will increase with time, thus affecting the residence time $T = V/W$, where $W$ is the flow rate.)

**Steady-State Continuous Process**

The ideal self-controlling continuous apparatus should maintain all variables in a steady-state and at optimal values. One or more variables are monitored by a feed-back controlling device which in turn modulates these and other variables.

The concentration $N$ of free cells in the reactor can be monitored turbidimetrically and has the following relation:

$$\frac{dN}{dt} = \frac{am}{V} - \frac{W}{V} N$$

(3)

where $t$ is time, $a$ is the digestion speed coefficient, $m$ is the number of cells on the surface of the tissue fragments, $V$ is the volume of fluid in the reactor, and $W$ is the flow.

At the steady state of the process, both $m$ and $N$ should be constant giving $dN/dt = 0$ and

$$N = \frac{am}{W}$$

(4)

The flow $W$ will be preset to the constant value that assures the optimal mean residence time ($T = V/W$) required for the best quality of the cells.

Since the digestion speed coefficient $a$ also will be constant, it is necessary only to have a controlling device which will monitor $N$ turbidimetrically and will keep both $N$ and $m$ constant through a regulated supply of tissue to the reactor.

Once this regulation is accomplished, the constant value of $m$ will require a particular concentration $c$ of active enzyme in the reactor. The change of $c$ with time is expressed as follows:

$$\frac{dc}{dt} = (C - c) \frac{W}{V} - \frac{nm}{W}$$

(5)

where $c$ is the concentration of active enzyme in the solution entering the reactor, $W$ is the flow, $V$ is the volume of fluid in the reactor, $u$ is a utilization constant expressing the number of moles of trypsin inactivated after the release of a single cell, $a$ is the digestion speed coefficient, and $m$ is the number of cells exposed to the enzyme. Since the value of $c$ has to be maintained constant at the optimal value that insures the maximal digestion speed of $a$, it is $dc/dt = 0$, and the concentration $C$ of enzyme in the solution entering the reactor will be defined as:

$$C = c + \frac{nm}{W}$$

where $A$ is the maximal value of $a$ and where the flow is preset to the constant value that assures the best mean residence time $T = V/W$.

**Automatic Control**

**Substrate as the controlling variable.** The free cell concentration $N$ ($m$) in the reactor is monitored through the turbidity $G$, the measurement of which is then related to the amount of tissue in the reactor, or rather to the number $m$ of cells exposed to the enzyme. The turbidity can be accurately measured and, as the amount of tissue in the reactor and the number $m$ of cells available to enzyme digestion decreases, the turbidity and the concentration $N$ of free cells will decrease correspondingly.

The electronic control unit measures this decrease and immediately introduces tissue in the reactor, restoring the concentration $N$ and the turbidity $G$ to their preset constant values.

In actual operation, the constant value $G_0$ will never be stationary, due to the inertia of the system and to the difficulty in obtaining infinitesimal variations of $m$. Therefore, the control maintains this variable within a range around the vicinity of the ideal value $G_0$.

A possible apparatus is shown diagrammatically in Fig. 1. The trypsin solution flows from the reservoir (A) through the measuring pump (B) into the reactor (D). The reactor is surrounded by a water jacket, and a magnetic stirrer (E) provides for the mixing of the fluid in the reactor itself. The overflow leaves the reactor through a constant-level type outlet to a collecting flask (F).

A lamp (G) is at one side of the reactor, and a photocell (H) is at the opposite side. A second photocell (H1) is directly lighted by the lamp through the diaphragm (I), which can be adjusted to the required turbidity of the mixture in the reactor. The control unit (L) operates a continuous-screw feeding device (C) which regulates the transfer of the minced tissue from the reservoir (K) to the reactor (D).

**Flow rate as the controlling variable.** The apparatus just
described may prove useful where a large amount of primary cell suspension is required for large-scale production of viruses, but it is not suitable for limited operations where the isolation of tissues from individual animals is required, as in the processing of monkey kidney tissues for the production of vaccines for human use.

For this purpose, the flow rate $W$ can be adjusted to the amount $m$ of cells exposed to the enzyme. In this case, referring to equation 4:

$$W = \frac{am}{N}$$ (7)

where $W$ is the flow, $a$ is the digestion speed coefficient, $m$ is the number of cells capable of being released from the tissue, and $N$ is the concentration of free cells in the reactor.

If the controlling device is to maintain a stationary value of the cell concentration $N$, then, since the coefficient $a$ is constant, the flow $W$ is a direct function of $m$, and both will decrease with time as tissue is digested.

Furthermore, the volume $V$ of fluid in the reactor also increases with time. It follows that the mean residence time $T = V/W$ is not constant, but also increases with time, and the average of the mean residence times during the entire process is defined as grand mean residence time ($GMRT$).

In this modified apparatus, the controlling device reads any deviation from the preset and constant turbidity $G_0$ corresponding to the stationary cell concentration $N_0$, and appropriately adjusts the flow $W$.

The diagram of the modified apparatus is shown in Fig. 2, and is essentially similar to the one previously described in Fig. 1.

The feeding solution flows through the electromagnetic valve (B) from the reservoir (A). The faucet (C) allows hand regulation of the maximal flow required through the pipes. The reactor (D; Fig. 3) is surrounded by a water
jacket arranged in the lower part of the vessel. The magnetic stirrer (E) provides for the mixing of the fluid in the reactor. The overflow leaves the reactor through a constant level outlet to a collecting flask (F).

At one side of the reactor (Fig. 2), there is a lamp (G), and a photocell (H) is at the opposite side. A second photocell (H1) is directly lighted by the lamp through the diaphragm (I) which can be adjusted to the required turbidity. The control unit (I) operates the valve opening according to the impulses received from the photocells.

The electric diagram of the control unit is shown in Fig. 3. The photocells are connected with a difference amplifier. At predetermined intervals, and for a given length of time, the timer (T) turns on the switch (S), inserting an alternating current into the anode circuit of the thyatron (V3). If photocell H is receiving less light than H1, the plate of pentode V2 has a higher voltage than pentode V1, the thyatron fires, and the electromagnetic valve (B) is energized.

**Materials and Methods**

**Tissue.** The experiments described here were carried out with kidneys of rhesus monkeys (*Macaca mulatta*). Healthy animals of uniform weight (3 kg) were used in three different batches procured from the same source. The animals were anesthetized by intravenous injections of 25 mg of Nembutal (Abbott Laboratories, North Chicago, I1.), per kg of body weight, and exsanguinated by cardiac puncture. Whole decapsulated kidneys were minced with scalpel blades, washed in Hanks’ balanced salt solution until clear of erythrocytes, and subsequently rinsed twice in trypsin solution. For each set of experiments, the minced kidney tissue from several monkeys was pooled and divided into 10 to 12 g portions for use in individual experiments.

**Trypsin solution.** Trypsin (Difco; 1:250) was prepared in phosphate-buffered saline (Dulbecco and Vogt, 1954), and the solution was sterilized by Seitz filtration. The pH was modified by appropriate changes in buffer concentrations.

In one experiment, where the effect of pH values higher than 7.4 were studied, the trypsin solution was prepared in Earle’s balanced salt solution, and the pH was adjusted by gassing with sterile air or carbon dioxide.

**Trypsinization.** Temperature and pH values were indicated. The magnetic stirrer was set at 300 rev/min, and the monitoring device was activated every 90 sec for 3 sec in all experiments.

A Pyrex reactor (100 ml; outside diameter, 4 cm; Fig. 1) was used in all instances. The minced tissue was not pre-digested, but was placed in the reactor immediately after washing in trypsin solution. The cells were collected in a chilled flask (2,000 ml) to which 100 ml of calf serum was added.

**Cell counting.** Nuclei were counted by staining one part of the digested cell suspension with two parts of a 0.1%
solution of crystal violet in 0.1 m citric acid. After trypsinization, the cells were washed twice in Hanks' balanced salt solution supplemented with 10% calf serum, and collected each time by centrifugation in 250-ml bottles in a PR2 International centrifuge at 700 rev/min (165 X g) for 10 min. The cells were then packed in 20-ml graduated centrifuge tubes at 500 rev/min (85 X g) for 10 min.

Cell-viability evaluation. The "minimal inoculum" method was used to test the viability of the cells after trypsinization. As a rule, the packed cells were diluted at 1:250, 1:500, and 1:1,000, and 1 ml of each dilution was seeded in each of 20 tubes at each dilution.

The growth medium throughout was Hanks' balanced salt solution with 5% inactivated calf serum and 0.5% lactalbumin hydrolysate, with penicillin and streptomycin added, each at 100 units per ml. The growth of each culture was scored at 0, 25, 50, 75, or 100% confluence. The readings were made at 2, 8, and 16 days after cell planting. The average of all readings was taken as an index of cell viability for the particular experiment.

At each reading, except the last, the cultures were fed with Earle's balanced salt solution with 0.5% lactalbumin hydrolysate, 1% inactivated calf serum, and antibiotics as above.

Recording. The electrical circuit of the electromagnetic valve regulating the flow was connected to a writing recorder. This allowed the registration of the flow integral.

Statistics. The GMRT was calculated by the formula:

\[
GMRT = \frac{\text{total trypsinization time (min) \times 100}}{\text{total trypsinization solution used (ml)}}
\]

In comparing different experiments, the cell viability was scored as a percentage of the highest value observed in the particular set of experiments. All other values are as experimentally recorded.

RESULTS

Flow Rate and GMRT. In Fig. 4, the integral of the flow is recorded for a trypsinization at 36 C and pH 7.4 with 45% light absorption and 14.5 g of original tissue. The slope decreases with time, approximately as predicted by equations 2 and 7, reflecting the progressive decrease of the amount of tissue in the reactor. The variations of the mean residence time \( T \) and of the GMRT are also interpolated in the same figure, where their values increase with time as expected.

It is evident that, if the amount of tissue in the reactor was maintained constant by a continuing provision of tissue, the GMRT would essentially coincide with \( T \). This is better indicated in Fig. 5, where the mean residence time is recorded as a function of the ratio of the volume of tissue to the total volume of the reactor.

Variation in the GMRT affects the degree of digestion of cell aggregates and, therefore, their size. The size of these aggregates as a function of the GMRT is indicated in Fig. 6 and, as shown, the larger the GMRT, the fewer the cells per clump and the larger the proportion of monodisperse cells.

Cell viability. Excessively large cell aggregates result in
lowered cell viability (Fig. 7). As the GMRT becomes longer than 5 min, and as the cell dispersion improves (see Fig. 6), the cell viability increases up to a GMRT of about 9 min, after which it sharply drops, probably due to direct digestion of the cells. These results in conjunction with those in Fig. 5 show that for an optimal mean residence time, and, therefore, for optimal cell viability, the volume of tissue processed should be approximately one-tenth of the reactor's volume.

In Fig. 7, the lower amount of cells harvested at a short GMRT reflects the inaccurate cell counting due to the presence of aggregates, but for a GMRT above 11 min the slight decrease in the cell number is probably due to a true loss of cells through direct digestion. It is clear in Fig. 7 also that the volume of the harvested cells decreases as the GMRT increases and the digestion of tissue structures is more effective.

**Temperature.** The efficiency of trypsinization improves by increasing the temperature between 29 and 39°C at pH 7.4, and is reflected in the decreased trypsinization time, decreased consumption of trypsin solution (see Fig. 8), and shorter GMRT.

Long GMRT causes aggregates at trypsinization temperatures between 29 and 33°C and, therefore, low cell viability, which gradually improves up to approximately 36°C, after which it sharply decreases, probably because of direct cell digestion at higher temperatures.

**Hydrogen ion concentration.** The digestion efficiency increases with pH in the range of 7.13 to 7.85, the highest value tested (see Fig. 9). The GMRT is also shortened, making for an increased cell viability which attains a maximum at pH 7.6 to 7.85 (see Fig. 9).

The data presented in this paper are obtained through the use of carefully selected animals. Tissue from different animal species, and even kidney tissue from rhesus monkeys of different ages and states of health, may require somewhat different conditions than those here presented. These conditions will have to be identified experimentally in each instance. However, it is possible to generalize with reasonable confidence that a turbidimetric automatic con-
control of the trypsination process is feasible and can consistently provide an optimal homogeneous product.

The optimal conditions for processing rhesus kidney tissue are at 36°C and pH 7.8, with a mean residence time of 5 to 8 min, and with a reactor with a volume of fluid approximately ten times that of the tissue processed.

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LITERATURE CITED


