A METHOD FOR CONTINUOUS CULTIVATION OF SUSPENDED ANIMAL CELLS IN A FLOWING SYSTEM

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Tissue culture in vitro, invaluable in the study of numerous cytological, biochemical, embryological and virological problems, also facilitates investigations into the processes of growth and tissue differentiation. However, the many difficulties encountered just in maintaining cell cultures in vitro have relegated to a secondary position the even more important problem of simulating the conditions under which the cells are found in vivo. The most important aspects of this latter problem are how to maintain certain basic parameters of the culture medium at constant levels and how to effect continuous changes in its composition. These problems have to be solved, since only when they are will we obtain cell populations which at any given moment are equivalent to in vivo populations and therefore can serve as experimental models for phenomena that occur in living organisms. The cultivation of cells in a suspended state [1-12] is a case in point.

We attempted to develop instrumentation for continuous cultivation of live cells in suspension, in order to establish and regulate basic physicochemical parameters for growth and metabolic activities of a culture. The apparatus used must satisfy the following basic conditions: the possibility of controlling and recording of the basic parameters for cultivating, the possibility for changing parameters in a wide range, and the ability to maintain the parameters at a required level.

The following conditions must be stabilized and regulated: cell concentration, the composition of the used gas mixture, the presence of serum in the nutrient medium, the availability of nutrient medium in relation to the speed of cell growth, pH of the medium, temperature and the flow rate of cells in the system.

Apparatus and the Operation Procedure

A general diagram of the apparatus is presented in Fig. 1. A 2-liter ellipsoidal container Ru, made from neutral glass, is used for cultivation; the assembly for delivery of medium has an automatic dosimeter, one cycle of which pours a given quantity of a medium (7-15 ml). There is also a facility for uninterrupted delivery of the medium through a dropping device. The assembly for delivery of a gaseous mixture contains special values regulating the rate of flow of a given gas. Electronic impulses from sources of gas bubbles, connected to counters, serve as controls for uniform volume of supplied gas in unit time. The three gases are mixed prior to entering the growth chamber, the mixture is humidified by passing it through an area of doubly distilled water at 37°C. The growth chamber was placed in an aluminum container and immersed in a water bath kept at the desired temperature. Warburg's apparatus was used for mixing and incubating the cell suspension. The speed of the shaker for mixing was set at 6600 times per hour. Overheating of the shaker's motor was prevented by automatic shut-off of the motor for 15 min every 45 min, by means of a watch mechanism.

The growth cells were removed, as were the waste products, at desired rate by micropump or by establishing negative pressure in the receiving flasks by means of a vacuum pump. The last flask, containing the poured out suspension, was removed from the system and replaced with a new one, using aseptic technique.

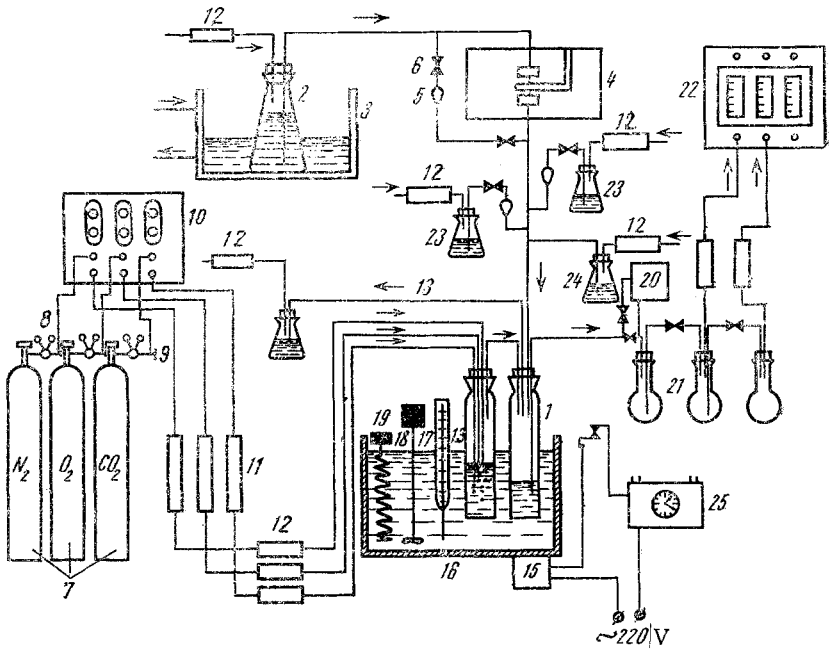

Fig. 1. Diagram of an apparatus for continuous flow cultivation of animal cells in suspension. 1) Cultivator; 2) a container for medium; 3) reservoir for cooling; 4) automatic medium dispensing unit; 5) dropping device; 6) clamp; 7) gas cylinder; 8) reduction valve; 9) valve; 10) electronic impulse counter of gas bubbles; 11) carbon filter; 12) cotton filter; 13) gas mixer; 14) exit of gas from the cultivator; 15) a mixing shaker; 16) water bath incubator; 17) contact thermometer; 18) mixing paddle; 19) heating element; 20) micropump; 21) a battery of containers for decanting of grown cells; 22) vacuum pump; 23) assembly for regulating pH; 24) inoculated container; 25) time clock.

The regulation and maintenance of pH was carried out by two procedures: change in content of CO_2 in the gas mixture or addition of drops of 3% solution of acetic acid and 7.5% of sodium bicarbonate from special containers, connected by siphon to the line supplying the medium. All glass and rubber parts of the reactor in contact with the culture or culture medium were carefully treated by Paul's method [9] to insure absolute cleanliness of the surface, after which the apparatus was assembled. The culture chamber, the assembly for addition of the medium, the assembly for removal of the contents, the assembly for delivery and removal of gas and the assembly for regulating pH were sterilized in an autoclave for 20 min at 1.5 atmospheres and subsequently were attached to the assembled unit of aluminum elbows and organic glass. The assembly of sterile parts of the reactor, as well as the insertion of the inoculated container for introduction of cell mixture into the cultivator, was carried out under aseptic conditions. The transfer of cell suspension from the inoculated container into the cultivator was brought about by means of an air compressor.

A series of experiments have been carried out in the apparatus. One of these, used to determine the functional properties of a system and to demonstrate stable development of the culture, is described below.

Heart muscle of 15-day-old chick embryos served as starting material for cultivation. A cell suspension was prepared by trypsinization. The reactor was turned on with the initial cell concentration of $7 \cdot 10^6$/ml and 350 ml volume. The cultivation was carried out in medium No. 199, to which was added 10% calf serum. Antibiotics were added to the medium (100 units of penicillin and 100 mg streptomycin per ml of medium). The gas assembly was regulated to deliver 3600 ml/hour (CO_2, 5%, O_2 15%, N_2 80%). The temperature of the culture was 37 $\pm$0.1°. The shaker constantly mixed the developed cells. Removal and addition of 40-50 ml was carried out 4 times a day (except during the first day). A sample of suspended cells was taken for analysis, the excess of cells was decanted. Total cell concentration in suspension was determined by counting in a hemocytometer, using crystal violet, and the

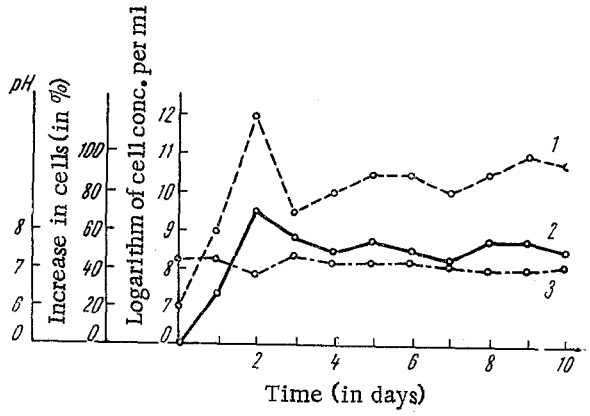

Fig. 2. Growth curves of cells. 1) Increase in number of cells; 2) number of cells; 3) pH.

viable count was determined using trypan blue; the pH of the medium was determined electrometrically using an LP-58 potentiometer with a small glass electrode. The morphological study of growing cells stained according to May-Grunwald–Giemsa and Romanovsky–Giemsa was carried out after a prior fixation with methyl alcohol and formalin vapor.

RESULTS

The initial cell concentration of $7 \cdot 10^6$/ml after 24 h and to $12 \cdot 10^6$ cells/ml after 48 h. On the third day of the experiment the cell concentration decreased to $9.5 \cdot 10^6$/ml, because of decrease in the level of cell division. The reason for this turned out to be a decrease in culture temperature to 25°-27° for 4 h because of an interruption in electric power. In the future, the cell concentration increased to $10.5 \cdot 10^6$/ml and was maintained to the end of the experiment at a concentration of from 10 to $11 \cdot 10^6$/ml.

The relative percentage of live cells was high, reaching 95-97 (their level on the 4th experimental day is an exception, when the number of dead cells increased to 8%, evidently as a result of an upset in the temperature).

The reaction of the medium was stabilized (pH 7.2-7.3). A certain decrease in pH took place on the second experimental day, as a result of a sharp increase in cell number and in connection with it accumulation of acid metabolic products. The other parameters (the composition of gas mixture, the content of serum in the nutrient medium, the use of nutrient medium corresponding to the growth of cells, the speed of cell circulation in the system) were maintained constant for the duration of the experiment. Let us note that the dynamics of the daily addition of cells was dependent on maintenance of the enumerated parameters (Fig. 2). Temporary reduction of temperature from 37 to 25° lowered the daily increase in cells from 70 to 50%. It is probable that elimination of oxygen from the gaseous mixture for one hour during the seventh experimental day had a similar effect on the dynamic aspect of daily increase in cells. The experiment was terminated on the 10th day for technical reasons, but the condition of the cells allowed us to continue subsequent cultivation and for a longer period of time.

The results of the study indicate an absence of biological obstacles of any kind to prolonged cultivation of a constant–equal weight population of chick embryo heart fibroblasts in a continuous liquid culture grown under stabilized environmental conditions.

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