

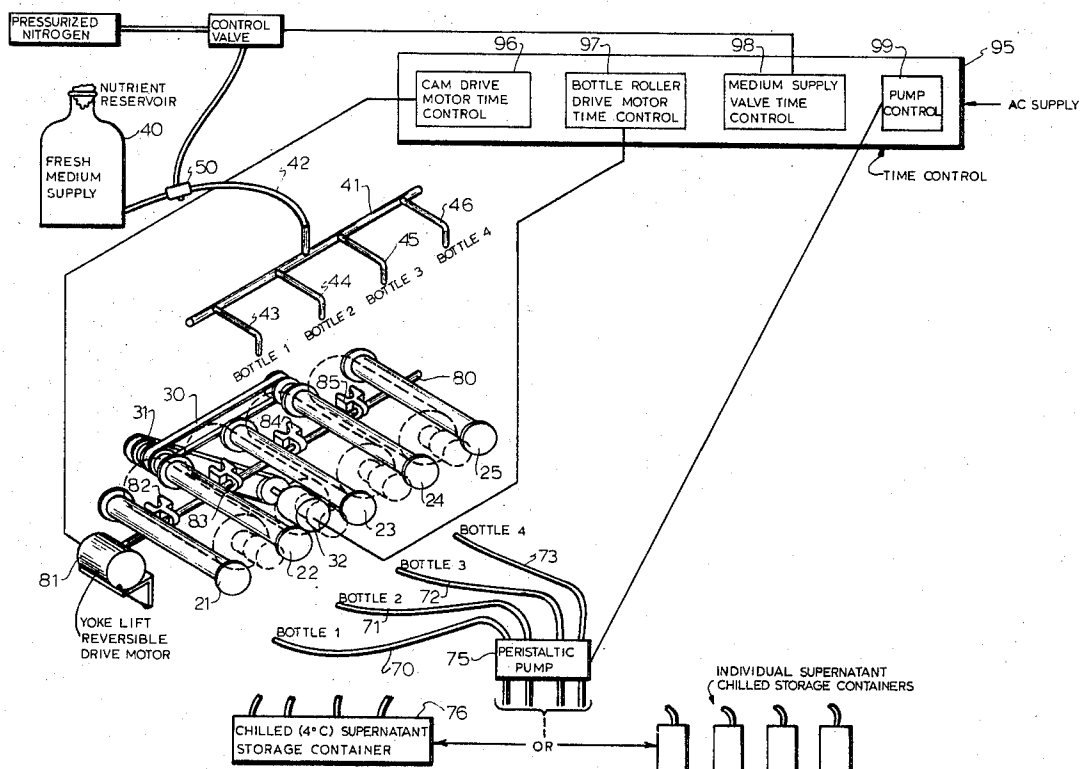
[54] **APPARATUS AND METHOD FOR RAPID HARVESTING OF ROLLER CULTURE SUPERNATANT FLUID**[75] Inventors: **Ralph E. Smith; Frank E. Kozoman,**
both of Durham, N.C.[73] Assignee: **Duke University, Inc.,** Durham, N.C.[22] Filed: **July 9, 1973**[21] Appl. No.: **377,806**[52] U.S. Cl. **195/127, 195/1.7, 195/143**[51] Int. Cl. **C12b 1/00**[58] Field of Search **195/127, 143**[56] **References Cited**
UNITED STATES PATENTS

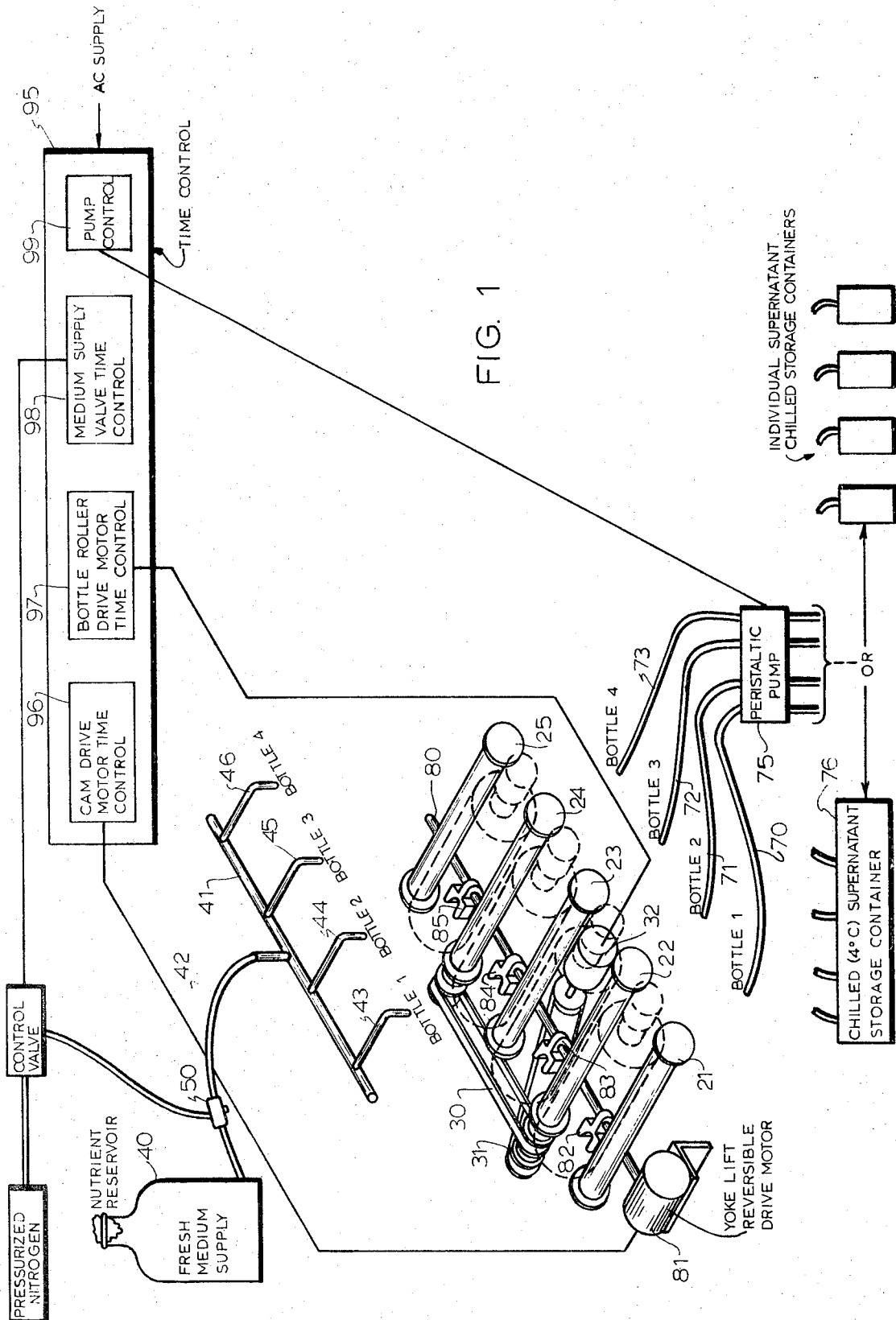
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[57] **ABSTRACT**

A conventional perfusion tissue culture apparatus using roller bottles is modified such that the supernatant fluids may be removed automatically and at frequent intervals from the roller culture bottles. The modified apparatus is programmed such that the bottles are periodically stopped in their rotative movement, are tilted, are drained of their respective supernatant fluids, are replenished with a predetermined amount of fresh medium, are then lowered and rotation is resumed until the fluid removing cycle is repeated.

4 Claims, 5 Drawing Figures



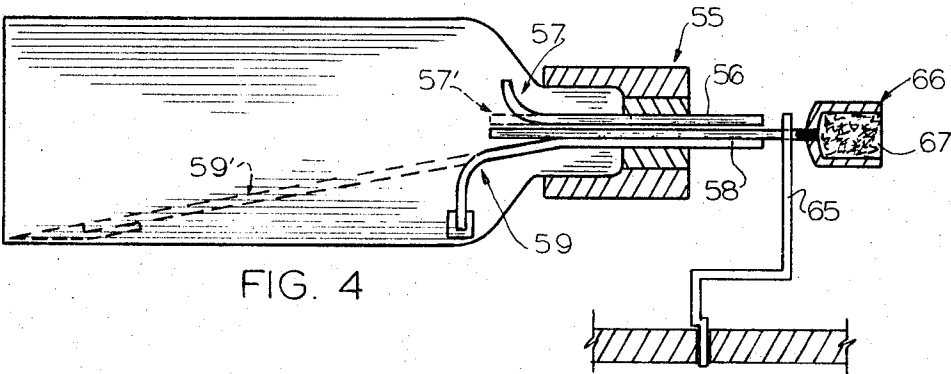


FIG. 4

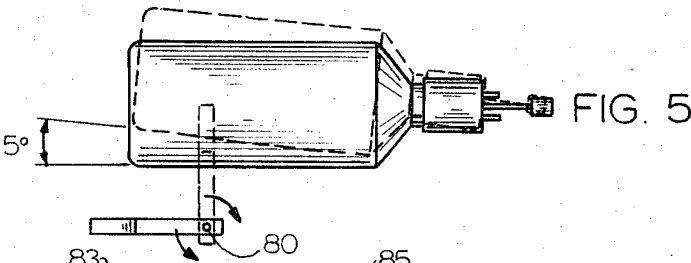


FIG. 5

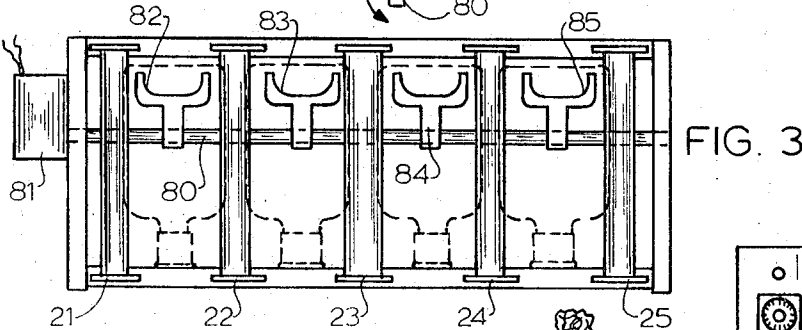


FIG. 3

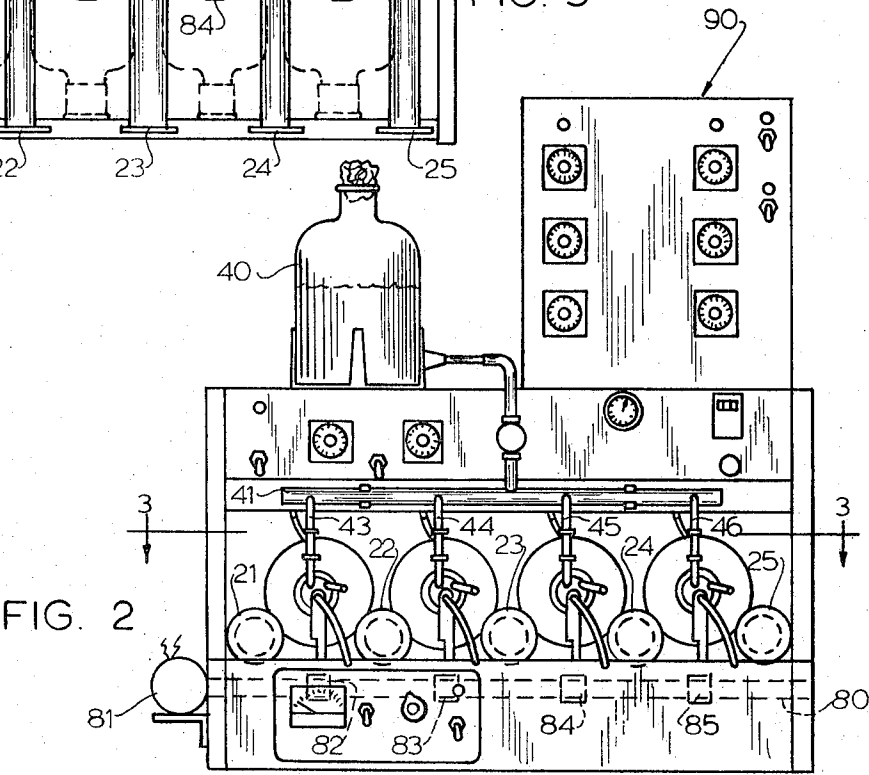


FIG. 2

APPARATUS AND METHOD FOR RAPID HARVESTING OF ROLLER CULTURE SUPERNATANT FLUID

The invention described herein was made in the course of work under a grant or award from the Department of Health, Education and Welfare.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The invention relates broadly to tissue culture and more specifically to apparatus and methods used in perfusion tissue culture wherein the cells are grown in bottles which rotate during the growth of the cells and are therefore referred to as roller bottles. Perfusion culture refers to the periodic replacement of nutrient solution to prevent cell starvation and waste product accumulation.

2. Description of the Prior Art

The technique of growing cells in a medium which is placed in a bottle which is rotated during the growth of the cells has been recognized. Also, commercial tissue culture apparatus using this technique has been available in the marketplace. The perfusion tissue culture technique has also been widely practiced. Typical literature references which in turn cite a multitude of other references include the following:

"Production and Purification of Large Amounts of Rous Sarcoma Virus," Ralph E. Smith and Eugene H. Bernstein, *Applied Microbiology*, Mar. 1973, pp. 346-353.

"Comparison of Immature (Rapid Harvest) and Mature Rous Sarcoma Virus Particles," Kwok-Sing Cheung, Ralph E. Smith, Marie P. Stone, and Wolfgang K. Joklik, *Virology*, Dec. 1972, pp. 851-864.

"Multi-Layer Perfusion Tissue Culture," R. Elsworth, *Process Biochemistry*, Mar. 1970.

The commercial tissue culture roller bottle apparatus which has thus far been available has not provided a satisfactory means or method for removing the supernatant fluids automatically and at frequent intervals to enable rapid harvesting of the cells. As an example, the New Brunswick perfusion control apparatus which is referred to in the above references has been provided with a so-called perfusion swivel cap which allows fluids to be admitted to and removed from the bottles while the bottles are rotated but the conventional swivel cap arrangement does not allow for the complete removal of the supernatant fluids. Further, this type of conventional roller bottle tissue culture apparatus does not provide an overall system and method for operation on a programmed basis whereby the supernatant fluids from a plurality of roller bottles may be automatically and periodically collected in chilled flasks to arrest viral maturation, the medium automatically replaced and the virus cells maintained at physiological temperatures.

SUMMARY OF THE INVENTION

According to the method and apparatus of the invention the plurality of roller bottles conventionally employed in perfusion culture apparatus are mounted on and rotated by conventional bottle roller apparatus. Through use of a timing control the bottle rotating apparatus is periodically stopped and the bottles are tilted slightly by means of a plurality of fork or yoke like arms which are mounted on a common shaft turned by a re-

versible motor. In one direction of movement of the fork or yoke arms, the rear of each bottle is engaged by a fork arm which slides on the bottle surface and gradually tilts the bottle in some predetermined amount at which point the forks are brought to a rest with all of the bottles in a tilted position. Each bottle connects through an outlet tube to a suitable storage container. A pump is activated after the forks bring the bottles to a full tilt and the supernatant fluid in the bottles is then drained through the respective outlet tubes and collected either in a common or individual chilled storage flasks. To insure essentially complete emptying of each bottle the conventional straight entrance end of the outlet tube is given a relatively sharp bend within the swivel cap so that when the bottle is tilted essentially all of the fluid can be drained. The timing control now causes a predetermined amount of fresh medium to be added through a commercially available manifold-pinch valve arrangement and through inlet tubes passing through the swivel cap of each respective bottle. The reversible motor is then energized to cause the shaft on which the fork arms are mounted to rotate in a direction to allow the bottles to return to their normal horizontal position. The described cycle is repeated whenever it is desired to drain the supernatant fluid and add fresh medium.

DESCRIPTION OF THE DRAWINGS

FIG. 1 is a somewhat generalized schematic view of a roller tissue culture system according to the invention.

FIG. 2 is a front view of a modified commercially available roller tissue culture apparatus incorporating the invention.

FIG. 3 is a plan section view taken in the direction of line 3-3 of FIG. 2 and showing the yoke lifting arrangement.

FIG. 4 is a partial sectional, partial schematic view illustrating the modified swivel cap arrangement used in the invention apparatus.

FIG. 5 is a partial side elevation view of one bottle and one yoke lifter showing how the bottle is tilted for draining purposes.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The invention apparatus has been arrived at by modifying a conventional perfusion control apparatus as sold by New Brunswick Scientific Co., Inc. of New Brunswick, New Jersey. Such an apparatus is fully described in the company catalog entitled "Perfusion Control Apparatus, Models PF-4 and PF-8" dated Oct. 22, 1971 and labeled "M1006-5050." The operation and construction of this apparatus is fully described in that catalog. Therefore, the present description will go only into enough detail for those skilled in the art to understand how the method and apparatus of the invention are related to the conventional method and apparatus and the mentioned New Brunswick apparatus will be used as a basis of comparison.

Referring to FIG. 1, there is shown a schematic diagram of the invention apparatus as well as the conventional apparatus with which it most closely relates. The conventional perfusion control apparatus includes five rollers, 21, 22, 23, 24 and 25. Rollers 22 and 24 are driven through belts 30 and 31 by a roller driven motor 32. Rollers 21, 23 and 25 are thus idler rollers and four

bottles are received as indicated in dashed lines in FIG. 1 between each pair of rollers. When this roller driven motor 32 is energized, each of the four bottles tends to rotate in a conventional manner.

The nutrient is retained in a reservoir 40 which is connected to a conventional manifold 41 through a supply tube 42 and each of the four bottles are fed through individual supply tubes 43, 44, 45 and 46.

A pinch cock valve 50 controls the gravity flow between reservoir 40 and manifold 41. As best seen in FIG. 4, each bottle is provided with a modified swivel cap 55. The modified swivel cap includes an individual bottle inlet tube 56 which has a sharp upward bend 57 at the fluid discharge end as compared to the normally straight configuration, 57', shown in dashed lines. The manifold swivel cap also includes a fluid drain line 58 whose entrance end 59 is bent sharply downwardly as compared to the normally longer and straight configuration 59'. The so called inoculation or gas exhaust line is stuffed with a suitable cotton wadding or other air filtering material 67. A thin metal bracket 65 is conventionally used on each bottle and supports the front of the bottle during rotation as shown.

The fluid drain line 58 from each respective bottle connects to respective storage drain lines 70, 71, 72 and 73. Lines 70, 71, 72 and 73 in turn pass through a peristaltic pump 75 and then to a suitable chilled supernatant storage container 76.

A horizontal shaft 80 extends below and perpendicular to and rearwardly of the rollers 21, 22, 23, 24 and 25. Shaft 80 is in turn connected through a suitable reversible motor gear drive 81 such that shaft 80 can be rotated back and forth upon suitable energization of motor drive 81. Between each pair of rollers there are mounted on shaft 80 lifting yokes 82, 83, 84 and 85. Referring particularly to FIGS. 1, 3 and 5 it can be seen that when shaft 80 rotates in a clockwise direction, as seen in FIG. 5, each respective yoke lifts the rear portion of each respective bottle and causes the bottle itself to be tilted in some predetermined amount, e.g. 5°. Then when shaft 80 is rotated in a counterclockwise direction as seen in FIG. 5 the bottle is lowered back to a horizontal position. Motor drive 81 is purposely chosen so that this movement is slow and gradual and the timing of motor drive 81 is such that shaft 80 stops turning when the respective bottles have reached a maximum tilt. Further, each yoke is formed with its bottle engaging surface relatively smooth so that the yoke tends to slide along the length of the bottle during the tilting process. Since the rollers 21-25 are normally surfaced with a friction material the bottles do not tend to slide axially of the rollers 21-25. Also the previously mentioned brackets 65 tend to maintain the bottles in their proper position. Thus, the bottles can be tilted, drained, replenished with medium, and lowered back many times without changing the relative position of the bottles on the rollers.

Since those skilled in the art will readily see the general type of timing controls called for by the invention, the same are only generally represented in FIG. 1. In FIG. 2 the auxiliary timing controls are also shown at 90 as they might appear in an actual apparatus embodying the invention. Also in FIG. 2, since many of the parts shown are conventional, only those parts of particular interest to the invention have been identified by numbers since those skilled in the art will readily see

how the invention is incorporated into a conventional apparatus.

Referring back to FIG. 1, the overall timing control generally designated 95 includes a time control for the yoke drive motor designated 96, a time control for the bottle roller drive motor designated 97, a time control for the nutrient supply control valve 98 and a time control designated 99 for the peristaltic pump designated 75. The necessary timing circuitry to coordinate, interlock, etc. the individual timers is not shown, since those skilled in the art will readily see a great variety of timing and programming circuits suitable to the invention. In one embodiment, eight timers (Industrial Timers, Inc., Parsippany, N.J.) were required to allow the necessary operations. The main timer regulated the length of time between medium/ladd cycles. Two timers were provided: a 3-hour timer was used for intervals of from 5 minutes to 3 hours, and a 5-minute timer was used for intervals of 5 minutes or less. The minimum collection time was 30 seconds, in which bottles were rotated for 12 seconds, and the balance of time was required to complete the collection cycle. The bottle up timer regulated the length of time that the motor driving the cam shaft was in operation. This timer was regulated to stop the cams at their maximum vertical elevation. The delay timer provided a 5 second delay to allow medium drainage to the front of the bottles after they were tilted forward, and reversed the circuit to the motor driving the cam device. The pump-on timer activated a Kraft automatic pipettor (Model P300, Kraft Apparatus, Inc., Queens, N.Y.).

In operation, medium was pumped into prechilled collection vessels maintained either in an ice bath in the same room as the perfusion apparatus, or in an adjacent cold room maintained at 4° Centigrade (a hold was cut in the wall separating the rooms). The medium/add timer served the same function as in the unmodified perfusion system, namely, to activate a solenoid, which released pressure (provided by bottled nitrogen) on the automatic pinchcocks and allowed the addition of fresh medium by gravity flow. The amount of medium added to each bottle varied according to the frequency of collection. For example, 10 ml of medium was added per bottle during collection at 5 minute intervals, and 25 ml of medium was added during collection at 2 hour intervals. During extended perfusion cultures, the pH of the medium in the reservoir was readjusted daily by bubbling with carbon dioxide passed through a sterile cotton-plugged pipette. The bottle down timer lowered the bottles, and the reset timer prepared the electronic circuitry for the next cycle and started rotation of the apparatus of the invention.

A prototype apparatus has performed satisfactorily during numerous experiments for long-term collections of virus at frequent intervals. For example, roller culture bottles containing chick embryo fibroblasts transformed with Rous sarcoma virus (RSV) were maintained on the apparatus for over 3 weeks, with collection of supernatant fluid every 2 hours. The bottles were of contamination during the entire period of collection, and no malfunction was encountered. Cell growth was vigorous under the perfusion conditions outlined. For example, a roller culture bottle maintained by perfusion culture yielded more than twice the number of cells obtained from a roller culture bottle maintained by daily medium changes.

The device was designed to study virus particles recently budded from the cell surface. During the course of an investigation reported elsewhere it was found that ribonucleic acid (RNA) purified from RSV harvested at intervals of 2 hours or shorter was more uniform in size than the virus harvested at longer intervals (12 to 24 hours). The manual harvesting of supernatant fluids every 2 hours is tedious, and can be done for only a portion of the day. Since the modified perfusion allows automatic harvesting during the entire day, including weekends, the apparatus is now proving useful for obtaining large quantities of high quality RNA with relative ease. The perfusion culture technique outlined could also be used whenever sequential harvests of a virus, cell metabolite, or other extracellular material is desired.

A number of advantages can be seen in the method and apparatus just described. A principal advantage is that rapid harvesting can be accomplished automatically and with essentially complete removal of the supernatant fluids. The use of the peristaltic pump provides positive removal of the supernatant fluids and allows the various drain lines to be purged as required. Since the bottles are not shifted axially and lifted, and since the tilting is accomplished slowly and gradually, the cell growth is not substantially disturbed during the tilting and draining cycles. A typical work schedule according to the method of the invention would include the following steps:

1. Fill the fresh nutrient reservoir;
2. Admit to the bottles a predetermined amount of fresh nutrient and, if not already accomplished, simultaneously pre-chill the storage container or containers;
3. Warm the bottle surroundings if not already done;
4. Start the bottle rollers;
5. Rotate the rollers for a predetermined time;
6. Stop the bottle rollers;
7. Tilt the bottles and hold tilted for a predetermined time;
8. Drain the supernatant fluids by positive pump action from the bottles while they are tilted;
9. Replenish the bottles with a predetermined amount of fresh nutrient while the bottles are tilted;
10. Lower bottles;
11. Resume rotation.

Those skilled in the art will readily appreciate that variations on the above method of using the invention can be adopted. For example, the fresh nutrient can be restored either with the bottles in a tilted position or in a lowered position. It is preferred to do this when tilted to avoid a sudden rush of nutrient and a damaging action were the nutrient to suddenly flow as a wave from one end of the bottle to the other. Also the bottle warming and storage chilling steps can be accomplished at any suitable time. Considering that the prior art method required manual operations for all of the filling and removing steps indicated, it can be seen that the researcher is given a new tool and an extremely valuable apparatus and method for rapid harvesting.

What is claimed is:

1. In a roller tissue culture apparatus of the type having a plurality of parallel rollers, means to drive at least some of the rollers while others of the rollers are idled, a plurality of culture bottles adapted to be placed on

the rollers and to be turned by the driven rollers and to in turn rotate the idled rollers, said bottles each having a swivel cap adapted to mount influent, effluent, and gas inlet-exhaust pipes, having a nutrient supply, piping connecting said nutrient supply to respective bottle influent pipes and with valve means to control the flow of said nutrient to said influent pipes whereby to control the flow of nutrient to said bottles, the improvement comprising:

- a. tilting means provided below each of said bottles and operable to engage, gradually raise and tilt each of said bottles simultaneously in some predetermined angular amount while at least a portion of each of said bottles remains on its respective said rollers, being adapted to hold each of said bottles in said tilted position for some predetermined time and then to lower said bottles back to their normal position on said rollers;
- b. electrical operator means connected to said tilting means for operating said tilting means and energizable in one mode to cause said tilting means to gradually raise and tilt said bottles, in another mode to cause said tilting means to hold said bottles tilted and in a third mode to cause said tilting means to gradually lower said bottles and restore said bottles to their normal position on said rollers;
- c. a swivel cap like piping arrangement in each said bottle including an influent pipe connected to receive nutrient from said supply and to discharge the same through a section of said influent pipe within the respective said bottle, a gas inlet-exhaust pipe having one end communicating with the interior of said bottle and an opposite end communicating with the environment external of the respective said bottle and having an effluent pipe with one end terminating proximate the forward end of the respective bottle and adjacent the interior surface thereof;
- d. a plurality of effluent piping means connected to said respective said swivel cap effluent pipes at one end and to suitable chilled supernatant storage container means at the opposite end;
- e. pumping means connected to and operative on said effluent drain pipes to pump fluids from said bottles to said storage container means; and
- f. electrical timing control means connected to said operator means, said pumping means, said nutrient supply valve means and said roller drive means whereby said bottles once filled with a predetermined amount of said nutrient may be caused to be rotated for a predetermined time, be stopped, be tilted, be held in a tilted position, be drained of the supernatant fluids in said bottles, be replenished with said nutrient, be gradually lowered to the normal position on said rollers and then again be rotated on said rollers, to repeat the cycle on some predetermined sequence whereby the supernatant fluid and its contents in said bottles may be automatically harvested in said storage container means according to said sequence.

2. In a roller tissue culture apparatus as claimed in claim 1 wherein said tilting means includes a shaft extending below and perpendicular to the axis of said bottles and on said shaft a plurality of yokes adapted to be rotated by said shaft to engage, tilt and lower said bottles.

7

3. In a roller tissue culture apparatus as claimed in claim 2 wherein said operator means includes a reversible drive motor connected to said shaft.
4. In a roller tissue culture apparatus as claimed in claim 3 wherein said swivel cap influent pipe includes an upturned section within and near the mouth of each

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said respective bottle and said swivel cap effluent pipe includes a section turned sharply down within and near the mouth of the respective said bottle and terminating proximate the interior surface thereof.
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