

[54] **CELL CULTURE SYSTEMS**
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[51] **Int. Cl.**..... **C12b 1/00**

[58] **Field of Search**..... **195/127, 139, 140, 142,**
 195/143, 109, 1.7, 1.8

[56] **References Cited**

UNITED STATES PATENTS

2,522,947 9/1950 Hatch et al. 195/143 X

3,102,082 8/1963 Brewer..... 195/139
 3,281,307 10/1966 Moeller et al. 195/142 X
 3,740,321 6/1973 Pagano et al. 195/127

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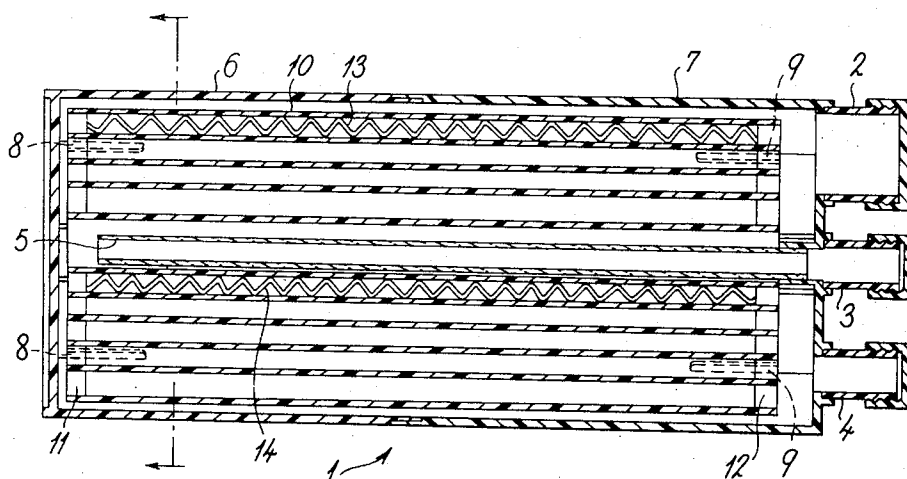
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[57]

ABSTRACT

The present invention comprises a method for cultur-
 ing animal cells in which cells adhering to the surface
 of a flexible strip wound or otherwise formed by suc-
 cessive changes of direction into a compact cell sup-
 port are contacted with culture medium. The present
 invention also comprises apparatus for carrying the
 method into effect, in which apparatus neighbouring
 surfaces of the support may be spaced apart to pro-
 vide one or more passageways whereby in operation
 culture medium has access to the support to nourish
 cells at the surface thereof.

14 Claims, 11 Drawing Figures



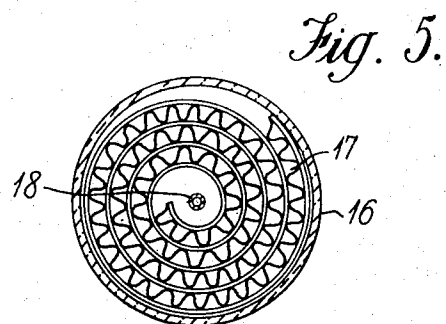
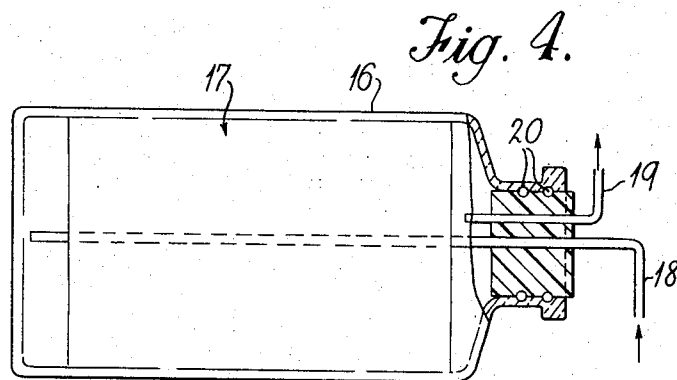
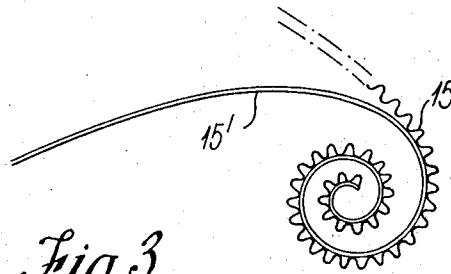


Fig. 6.

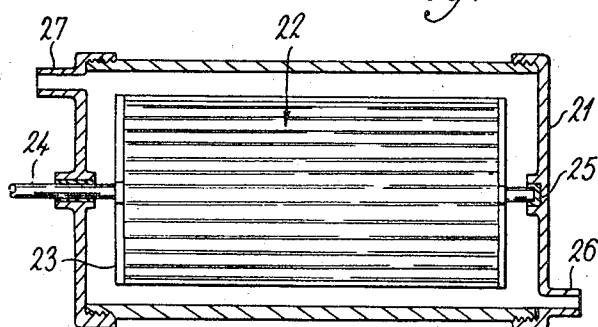


Fig. 7.

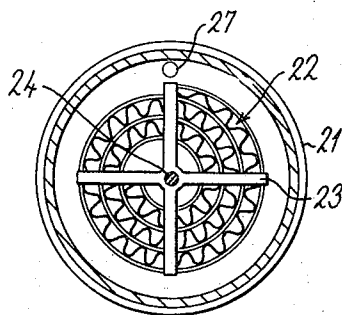


Fig. 10.

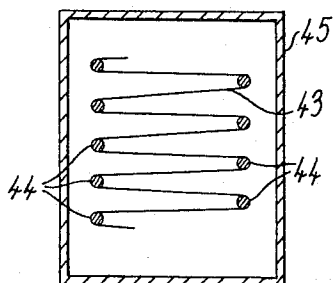


Fig. 11.

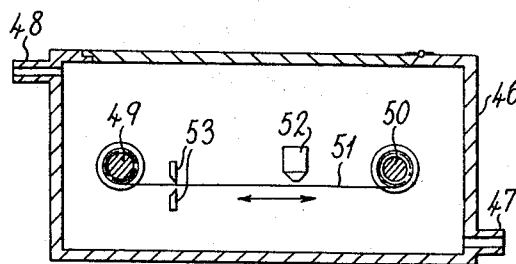


Fig. 8.

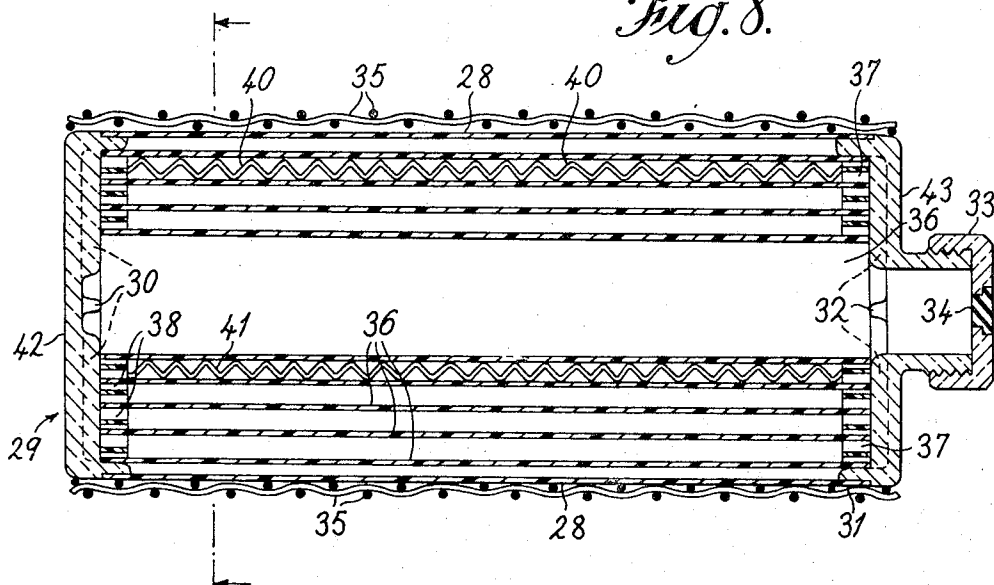
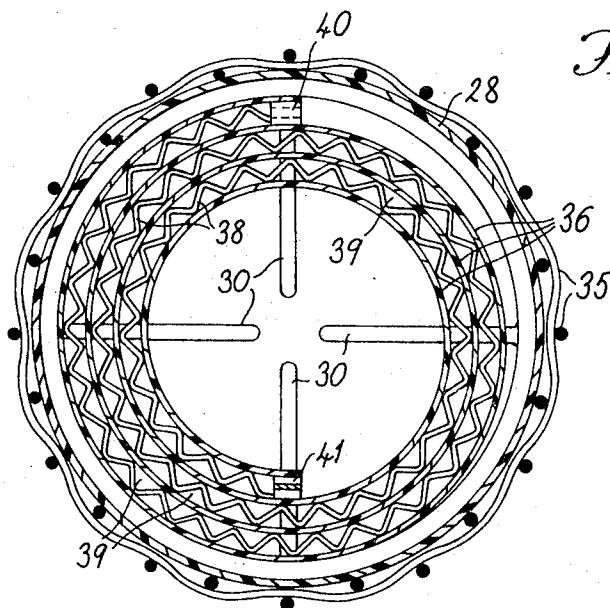


Fig. 9.



CELL CULTURE SYSTEMS

This invention relates to the culture of animal and in particular mammalian cells and apparatus therefor.

Certain of the cell lines used in tissue culture, such as BHK21, grow by adhering to and spreading over a suitable surface. Surfaces which have hitherto been employed for large scale growth of such cell lines include the inner surfaces of glass bottles (roller bottles) and the outer surfaces of beads, e.g., of porous silica or dextran, glass helices, glass or stainless steel plates and ion exchange beads which are packed or stacked in a suitable vessel.

It has now been found that animal cells can be cultured with advantage on other supports.

According to the present invention, a method for culturing animal cells comprises contacting with culture medium cells adhering to the surface of a flexible strip wound or otherwise formed by successive changes of direction into a compact cell support.

It has been found that in this method excellent results are obtained when the strip material is deformed into a variety of configurations in order to provide a support of large surface area per volume of the support as compared with the supports hitherto employed. Growth and multiplication of the cells proceeds with transfer to the cells of nutrient medium in the bath, the medium also providing a suitable pH and gaseous environment for growth.

The strip material should possess a non-toxic surface to which the cells are adhere and preferably has a thickness within the range 10–1000 microns. Unplasticised polymers which possess a relatively hydrophilic surface, e.g., polyesters such as "Melinex" (a Registered Trade Mark), are suitable as materials for the support as also are more hydrophobic materials (e.g., polypropylene and polystyrene), which can be treated so as to possess hydrophilic surfaces, for example "Bexphane" and "Polyflex" (Registered Trade Marks). "Polyflex," which is particularly suitable, is an oriented polystyrene material which can be treated by corona discharge to give a hydrophilic surface. These materials preferably satisfy the criterion for choice of good cell adhesion, low toxicity, high strength, low weight, low cost and ready availability. The surface tension is preferably at least 40 e.g., at least 50 dynes/cm. Strip material such as aluminised polymers, stainless steel or aluminium may be alternatively employed if so desired.

In a preferred embodiment of the method particularly suitable for batch wise culture, neighbouring surfaces of the support are spaced apart to provide one or more passageways whereby culture medium has access to the support to nourish cells at the surface thereof.

The support in the form of a roll the turns of which are preferably spaced apart by 1 to 10 mm. e.g., 3 to 5mm. to allow access of nutrient medium to the cells, may be formed by winding a length of corrugated strip material and a length of smooth, planar strip material together into a self-spacing spiral, the corrugated strip being preferably much narrower than the non-corrugated strip. If so desired the corrugated strip material may be replaced by a non-corrugated strip possessing a recessed, e.g., a dimpled, surface. Layers of strip material may alternatively be spaced apart by other means e.g., by employing alternating layers of chain, rod, string, beads or the like. When smooth, planar strip material is employed moreover, support may

be received from a frame, which can serve to space apart the surfaces of the strip.

The containers employed may of course vary considerably in shape and size. The glass roller-bottles commonly used in tissue culture or other vessels of similar dimensions may be employed, and inclusion of self-spacing spirals may then increase the effective surface area by 5 to 20 fold e.g., 10 fold. When the increase in surface is larger than 5-fold however it is generally necessary to replace the oxygen and in some cases the nutrient medium either batchwise or continuously via an inlet and outlet which, when rotation of the bottle together with the support is desired, may pass through a closure provided with a rotary seal.

In preferred embodiments of the present invention however the container is fabricated from a suitable plastics material e.g., polystyrene, is preferably generally cylindrical in shape and 5 to 50 cm. in diameter by 20 to 50 cm. long e.g., 10.2×30.5 cm., with capacity 500 to 20,000 ml. e.g., 500 to 2,000 ml. The vessel is provided with an inlet for nutrient medium and cells, and a sparge tube and encloses a cell support of flexible strip material preferably 50 to 100 microns thick. It is envisaged that the cell culture apparatus will be supplied to the user with a support in place as a disposable irradiation-sterilised unit. Such units can obviate the handling costs involved in washing and sterilising the glass roller-bottles at present used. Incubation space may be substantially reduced as the units allow the realisation of improved cell growth at a relatively reproducible and uniform surface. Surface/volume ratios of 0.5 to 10 cm^{-1} can be achieved, typically 3 to 5 cm^{-1} , e.g., 4 cm^{-1} .

Whilst rotation of the support is advantageous in promoting inoculation it is not essential for growth. It is possible to obtain good growth by first allowing cells to settle on to a support which is horizontally disposed, following which the vessel containing the support is moved to a position wherein the support surface is vertical. In some cases it may be necessary to provide an inlet and outlet for continuous replenishment of medium. As an alternative to aerating the medium by sparging, recirculated medium may be externally aerated.

In further embodiments of the present invention, the container has a wall portion through which the medium can be aerated. The gas permeable container may be conveniently fabricated from a plastic material e.g., polyethylene, polypropylene, a silicone elastomer, fluorocarbons e.g., polytetrafluoroethylene, of such thickness as to allow adequate supply of oxygen and carbon dioxide across the container walls to sustain respiration of the cells within. In practice the container is generally fabricated at least in part from a film of the plastics material, the thickness of which preferably lies within the range 0.001 to 0.01 inches, the film preferably receiving support from an external or internal mesh. The container may be fabricated in a large number of sizes, the upper size limit being set by the rate of gas transport across the membranous container walls required to sustain growth of the cells within the unit. Typically however the container is of a size such that the growth of 0.5×10^9 to 5×10^9 cells e.g., 1.0×10^9 cells can be supported, and such that it can be rotated on a commercial rolling machine, units of size 4 inches $\times$ 12 inches having given satisfactory results. Rotation about the longitudinal axis causes the nutrient to flow from

the outside to the inside of the cell support following a spiral pathway. When the support is in the form of a roll it is in some cases desirable for the innermost and/or outermost end of the strip to be spaced from the nearest portion of support surface by means of corrugated strips disposed along the length of the roll to facilitate through flow of medium.

In use units comprising gas permeable wall portions and containing cells and culture medium to which has been added a suitable buffer, e.g., a bicarbonate buffer, may be slowly rotated in an incubator containing a carbon dioxide/air mixture at a temperature such as 37° centigrade. The rate of rotation should be controlled at 1 - 10 revolutions per hour e.g., 4 revolutions per hour to prevent shearing of the cells from the spiral. Rotation about the longitudinal axis of the unit causes nutrient medium to circulate from the exterior to the interior and thence to the ends of the spiral. After cell growth is completed the cells may be harvested by rotation of the unit which is preferably effected manually and at greater speed than that employed during incubation; the shearing action thereby induced in the washing fluid induces transfer of the cells from the spiral thereto. If so desired, harvesting may be effected enzymically. The cells are then detached from their support by washing with a proteolytic enzyme such as Trypsin in conjunction with a chelating agent e.g., EDTA. Inoculation with a virus after cell growth has taken place can be effected by injection through an elastomeric septum which may be incorporated in a portion of the container wall.

In yet further embodiments of the present invention an incubation vessel contains a support e.g., a self-spacing spiral support which is secured to a frame rotatable with respect to the vessel. Medium can be continuously supplied to and discharged from the vessel via an inlet and outlet.

Also included within the scope of the present invention are cell culture processes which employ looped or folded strip, particularly planar strip, to form part of a conveyor system, the support members of the frame employed to support the strip being preferably rotatable. The conveyor system can carry cells from one set of incubating conditions to another and may be continuously inoculated with fresh cells at one end with harvesting of cells of a precisely determined age and growth regime at the other. The conveyor can either be supplied with continuously fresh strip when it is desirable to harvest both the cells and their support or alternatively can form a belt i.e., a continuous re-entrant loop, with inoculation at the head-end and harvesting at the later stage. In the latter case a part of the cells may be left on the conveyor belt after harvesting for return to the head-end as inoculum.

In particular mitotic cells may be preferentially removed from the strip material by washing and used as an inoculum to provide a synchronous population.

Cells can be efficiently and easily harvested from extended surfaces simply by scraping, with the use of water jets or both operations simultaneously. In the latter case the surface is wiped in a manner analogous to the operation of a windscreen wiper.

The foregoing general requirement for spacing apart the film surfaces to allow access of nutrient medium to the adherent cells during growth may be avoided if desired. In accordance with a second aspect of the present invention, a surface portion of the support is inter-

mittently exposed to a bath of nutrient medium for a period such that sufficient nutrient is taken up to nourish cells adhering to said portion during the interval between exposures, the portion being incorporated for said interval into a configuration in which access of nutrient to said portion is relatively reduced. The latter process may be operated by employing two spools which may be enclosed within the vessel containing nutrient and between which the strip material is transferred through the bath of nutrient medium by periodic reversal of the direction of rotation. When this system is employed, inoculation and harvesting can be effected with great convenience at e.g., the end of the growth cycle by the action of a wiper blade or water jet or both together. The support can be conveniently inoculated by means of a jet.

Embodiments of the invention will now be described by way of example only with reference to the accompanying drawings of which:

FIG. 1 shows a longitudinal cross-section of a cell culture unit suitable for aeration by sparging;

FIG. 2 shows view in cross section of the unit shown in FIG. 1;

FIG. 3 represents diagrammatically a method for forming a support;

FIG. 4 shows a side view of a second embodiment with the support shown in FIG. 3 in place;

FIG. 5 shows an end view of the embodiment shown in FIG. 4;

FIG. 6 shows a side view of a third embodiment of the invention;

FIG. 7 shows a sectional view of the third embodiment shown in FIG. 6;

FIG. 8 shows a longitudinal cross-section of a cell culture unit possessing a gas permeable wall portion;

FIG. 9 shows a view along the line II-II of FIG. 8;

FIG. 10 shows a sectional view of a fifth embodiment of the invention; and

FIG. 11 represents diagrammatically a sixth embodiment of the invention.

Referring now to FIGS. 1 and 2, a generally cylindrical vessel 1 of polystyrene is provided at one end with an inlet port for medium 2 and for gas 3, e.g., an air/CO₂ mixture and an exhaust port 4 for waste gas. The gas inlet port communicates with an axially disposed sparge tube 5 of plastics material which extends from one end of the vessel to the region of the other end. The vessel is formed from two generally cylindrical portions 6, 7 of approximately equal length which are joined at their rims by waterproof tape (not shown). The vessel bears on each end face a cruciform arrangement of four L-shaped ribs 8, 9, the limbs of which serve to space a cell support 10 enclosed within the vessel from the end faces and curved surface of the vessel. The support 10 takes the form of a roll of polystyrene film the turns of which are spaced 4 mm. apart by two narrow corrugated strips 11, 12 of "Melinex," one at each end of the roll. Spacing of the ends of the polystyrene sheet from the surface of the neighbouring layer is additionally assisted by inclusion of two corrugated strips 13, 14 arranged along the length of the roll, the interior of which is penetrated by the centrally arranged sparge tube 5.

The unit as supplied is sterile and disposable and the inlet ports 2, 3, 4 are screw threaded to take caps which are removed before use. Removal of the tape after cell culture permits the cell support to be removed and the

cells to be harvested by unwinding the roll followed by squeegee action.

If so desired, however, the cells may be washed from the support without dismantling the apparatus. In this case, or when for example the cells on the support are to be infected by contact with a small volume of a virus suspension, the circumferentially disposed limbs of the L-shaped ribs 8, 9 preferably vary in thickness e.g., logarithmically so that the outer edge of the support 10 contacts the wall of the vessel 1 so that it can act as a scoop. On rotation, a small volume of liquid introduced into the vessel will then flow preferentially to the inside of the support.

FIG. 3 represents a diagrammatic method of forming the cell support by winding together a length of corrugated strip material 15 and a length of smooth planar strip material 15. The corrugated strip material may be made by passing Melinex, 1 cm wide by 125 microns thick, firstly through the air from a hot-air blower of exit temperature 300°C, then through a pair of spur gears of pitch 4 mm (British 12 P.C.D., ½ inch wide by 1½ inches in diameter) on a hand-operated rotary crimper, and finally through a blast of cold compressed air. To produce corrugations which withstand subsequent autoclaving, it is necessary to have the tape almost melting as it enters the gears, and to cool it immediately upon exit.

An example of the use of the apparatus now follows:

Procedure

To inoculate with cells, medium is added, followed by a suspension of cells to a final volume of 2 litres, the unit inverted several times to distribute the cells evenly throughout the windings of the spiral support. The cells are then allowed to settle and spread evenly over both sides of the film, by rolling on a very slow machine (about 2 revolutions per hour). Normal roller speeds (10 to 60 r.p.h.) tend to wash cells off the surface and produce aggregates (presumably because of the increased hydrodynamic shear due to the narrower spacing in the spiral, as compared with a normal roller bottle). Cells are deposited uniformly, on both sides of the film, over its whole length, by selecting the optimum roller speed, non-uniform spreading being found to be the most frequent cause of low cell yield. For routine inspection, the outer layer of the spiral can be examined by placing the bottles on an inverted microscope in the usual way.

When the attachment and spreading of the cells is complete (from 3 to 18 hours, depending on the type of cell) the bottles are removed from the rollers and placed upright for aeration.

Both the inlet and the outlet tube for air are fitted with a glass tube containing a cotton-wool plug. In the absence of anti-foam, the rate of aeration is of necessity very low, about ten bubbles per minute. However, to ensure uniform stirring throughout the spiral, a more vigorous gassing, with added anti-foam (Midland Silicone, Anti-foam Emulsion RD) is preferred. Spacing of the roll from the end of the vessel distal from the inlet port 2 by the L-shaped ribs 8 allows free recirculation of medium through the turns of the roll from one end to the other when the container and support are sprayed in a vertical position.

The medium used is Dulbecco's modification of Eagle's medium, with 10 percent calf serum and aeration is usually done with 10 percent CO₂/air mixture, at

100 to 300 cc per minute per bottle. In some experiments a pH-stat can be used to operate a solenoid valve on the CO₂ line; with this device it is possible to maintain the required pH within ±0.1 unit if required; however, for the comparative growth studies described below, the gas composition is kept constant at 10 percent CO₂.

Harvesting is done with trypsin in the same way as from a normal roller bottle, but with the volumes of trypsin and other reagents being scaled up proportionately to the number of cells. Mechanical harvesting is also possible by scraping or washing the cells off the plastic film, but may produce sheets and clumps of cells.

Results

8 × 10⁷ BHK C13 are cultured in 2 litres of medium. After 5 days 1.0 × 10⁹ cells are harvested. In comparative tests, with primary cells (whole mouse embryo), 10⁹ cells are routinely obtained with a 2 litre vessel containing a spiral support, compared to 10⁸ cells from an ordinary roller bottle containing 200 ml of medium.

If so desired, when cells have become confluent, the medium can be removed from the culture vessel and replaced by a much smaller amount of a liquid e.g., a virus suspension which can be efficiently distributed over the surface of the support when the vessel is rotated on a roller machine.

Referring now to FIGS. 4 and 5, a glass roller-bottle 16 contains a self-spacing spiral support 17 and is provided with an inlet for medium 18 and outlet 19. Rotation of the bottle 16 together with the support 17 is made possible by a rotary seal 20.

Referring to FIGS. 6 and 7, a cylindrical incubation vessel 21 contains a self-spacing spiral support 22 mounted on a frame 23 which is rotatable with respect to the vessel 21 on a shaft 24 provided with a bearing 25. Medium can be continuously supplied and discharged through an inlet 26 and outlet 27.

Referring now to FIGS. 8 and 9, a generally cylindrical container for culture medium comprises a cylindrical polythene membrane 0.001 inches thick 28 sealed at one end to a thick walled polyethylene cap 29 provided with a generally cruciform arrangement of four ribs 30 and sealed at the other end to a thick walled end portion 31 also bearing a cruciform rib arrangement 32 and bearing a screw closure 33 provided with an elastomeric insert 34 for injection of liquids. The container is enclosed and thereby protected by a ¾ inch plastics mesh work 35 and contains a 0.006 inch thick sheet of polystyrene or Melinex (a Registered Trade Mark) formed into a roll 36 the layers of which are spaced 4 mm apart by incorporation of two narrower corrugated strips 37, 38 one at each end of the roll, to provide spiral passageways 39 for culture medium. Spacing of the ends of the polystyrene sheet from the surface of the neighbouring layer is additionally assisted by inclusion of two corrugated strips 40, 41 which arranged along the length of the roll, the outer surface of which is supported and spaced 4 mm from the membrane 28 by the end portion 31 and cap 29, and the ends of which are spaced from the end walls 42, 43 of the end portion 31 and cap 29 by means of the two cruciform sets of ribs 30, 32.

An example of the use of the latter embodiment for cell culture will now be described.

1,600 ml Dulbecco's modification of Eagle's medium with 10 percent calf serum and 8 × 10⁷ cells (BHK

C13) are introduced into the sterilised cell culture unit. The stopper is flamed and replaced tightly, the bottle is placed on a Luckham roller machine, inside a cabinet heated to 37° and providing an atmosphere of 10 percent CO₂/90 percent air and rolled at four revolutions per hour. After five days the cells are confluent and the bottle is then removed, the medium discarded and trypsin/EDTA (200 ml) added to the unit and the cell removed by manual rotation of the bottle. 7×10^8 cells are produced representing an approximately 9 fold increase. As hereinbefore described, in some cases it may be desirable for the outer longitudinal edge of the roll to contact the wall of the vessel so as to act as a scoop when small volumes of liquid are introduced, and facilitate distribution to the inside of the roll.

Referring to FIG. 10, a length of non-corrugated strip 43 is wound upon support members or circular cross-section 44 in a vessel 45. When the support members 44 are rotatable, the support can form part of a conveyor system possessing a large area per unit volume of container.

Referring to FIG. 11 a vessel 46 is provided with an inlet 47 and outlet 48 for nutrient medium, and encloses capstans 49 and 50 on to which strip material 51 (which may be flat surfaced, dimpled or crimped) is wound. An inoculator 52 and harvester 53 are provided. In operation, the film is wound between the two capstans which periodically reverse their direction of rotation. The cells adhering to the film are thus periodically exposed to fresh medium from the vessel. The speed and tension of the capstans and length of film exposed to the medium are adjusted so that the amount of fresh medium picked up by any portion of the film in its travel between the capstans is sufficient to nourish the cells adhering to that portion for the period during which the portion spends on the capstan i.e., during which it is not fully exposed to the nutrient medium in the vessel and the speed at which the exposed film moves should be sufficiently slow that cells are not washed off into the medium.

We claim:

1. Cell culture apparatus comprising a container for culture medium which encloses a compact cell support of a flexible strip material formed into turns which are spaced one from another to provide a continuous passageway through which cells and medium can travel

progressively and continuously on rotary movement of the support during inoculation.

2. Apparatus according to claim 1 which incorporates a sparge tube for aeration of the medium during cell growth said sparge tube being so disposed within the container that aeration promotes circulation of culture medium along the length of the support between the turns.

3. Apparatus according to claim 1 in which the support is a roll of flexible strip material the turns of which in cross-section approximate to a spiral.

4. Apparatus according to claim 1 in which the turns of the support are spaced apart by corrugated strips located in the region of the end of the support.

5. Apparatus according to claim 2 in which the sparge tube is axially located within a support in the form of a roll of flexible strip material having an approximately spiral cross-section.

6. Apparatus according to claim 1 in which the strip material is formed of a material selected from the group consisting of polyester and polypropylene.

7. Apparatus according to claim 1 in which the strip material is formed of polystyrene which has been treated by corona discharge so as to produce a hydrophilic surface.

8. Apparatus according to claim 1 in which the surface tension of the strip material is not less than 40 dynes/cm.

9. Apparatus according to claim 3 in which the turns of the roll are spaced 1 to 10 mm. apart.

10. Apparatus according to claim 1 in which the ratio of the surface of the support and container to the volume of the container is between 0.5 and 10 cm⁻¹.

11. Apparatus according to claim 1 in which the container has a gas permeable wall portion through which the medium can be aerated.

12. Apparatus according to claim 11 in which the wall portion is of film 0.001 to 0.01 inches in thickness.

13. Apparatus according to claim 3 in which the outermost longitudinal edge of the roll contacts the wall of the container.

14. Apparatus according to claim 1 in which the container and cell support form a sterile unit.

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