A CULTURE FLASK FOR THE CIRCULATION OF A LARGE QUANTITY OF FLUID MEDIUM*

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The apparatus described in this paper was developed for the purpose of cultivating a large number of tissue fragments in a single flask, and in a thin layer of well oxygenated and constantly circulating medium.[1]

The flask is made of a single piece of pyrex glass and contains two chambers, one above the other (Fig. 1). The culture medium is inserted in the upper chamber (reservoir chamber). The tissue fragments are inserted in the lower chamber (culture chamber). The circulation and oxygenation of the culture medium are maintained by the control gas which is passed through the flask.

After the flask has been connected to the control gas line and the rate of flow adjusted, only routine inspection is required. It is the practice in this laboratory to readjust the flow of control gas once every 24 or 48 hours. Sterility is maintained by passing the control gas through cotton filters.

Description of the Flask

A glass tube, with an inside diameter of 2.5 mm., leads from a point about 2.5 mm. above the floor of the culture chamber to a point 10 mm. from the dome of the reservoir chamber. This tube carries the culture medium from the culture chamber to the reservoir chamber. A second glass tube, containing two holes near the lower end, surrounds the first tube. The inside diameter of the second tube should be at least 3 mm. larger than the outside diameter of the first tube. The two tubes are sealed together at a point approximately 1 cm. above the bottom of the first, or inner tube. The top of the second tube is attached to the bottom of the reservoir chamber, so that all fluid leaving the reservoir chamber must pass through the second tube. A third glass tube surrounds the second and is sealed to it at a point just below the two holes at the bottom of the second, or middle tube. The inside diameter of the third, or outer glass tube should be at least 2 mm. larger than the outside diameter of the middle glass tube. The top of the outer glass tube is open, and the edge is turned over to

* This apparatus has been used successfully in connection with unpublished experiments carried out in these laboratories by Dr. Raymond C. Parker.

[1] For the description of earlier devices the reader is referred to Parker, R. C., Methods of tissue culture, New York, Paul B. Hoeber, Inc., 1938, pages 117–124.

form a lip. It is desirable to use glass tubing with thin walls for the construction of the flask. If thick walled tubing is used, or if the clearances are larger than those stated above, the neck of the flask becomes unnecessarily bulky.

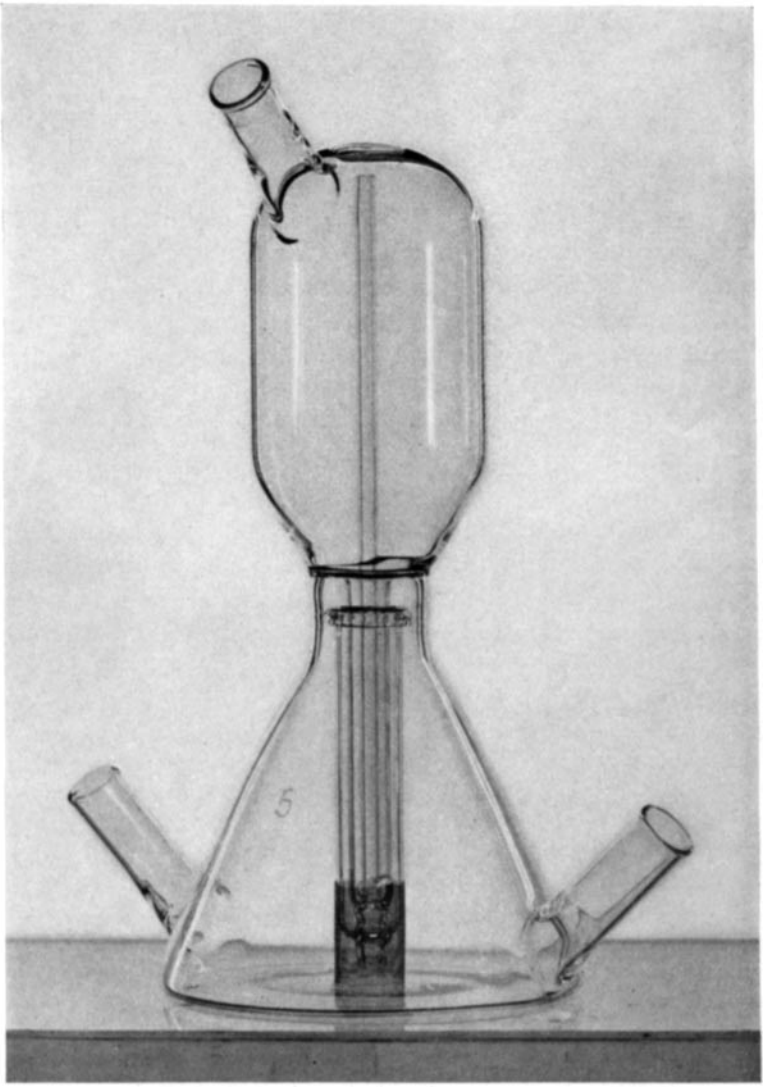

FIG. 1. Culture flask.

The middle glass tube is filled with fine silica sand, so that fluid from the reservoir chamber must pass down through the sand, out through the holes at the bottom of the tube, and up through the outer tube, before it reaches the culture chamber. The lip

at the top of the outer tube must extend to a point approximately 1 mm. from the neck of the culture chamber, and must be curved down until it is hook-shaped in cross section.[2] This causes the fluid to run down the wall of the culture chamber and assures thorough oxygenation and circulation of the fluid medium.

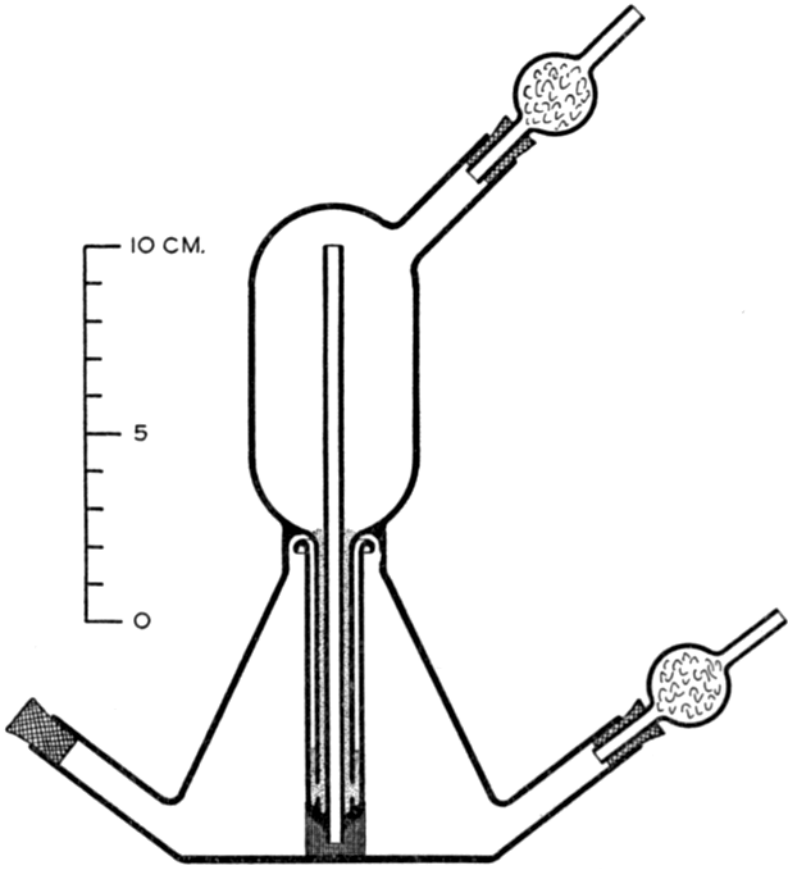

FIG. 2. Cross section of culture flask.

A cylindrical platinum screen (80 mesh, 0.003 gauge wire) extends from the floor of the culture chamber to the bottom of the outer glass tube. This screen has an

[2] If these directions are not followed carefully, the fluid passing over the lip of the outer glass tube will not run down the wall of the culture chamber, but instead, will run down the outside of the tube itself, thereby short circuiting the circulation in the culture chamber. The lip at the top of the outer glass tube must be bent down until it forms an acute angle with the wall of the tube. The lip must be so close to the neck of the flask that fluid passing over the lip touches the neck, regardless of the point on the circumference of the lip that the fluid passes over first.

inside diameter very slightly exceeding the outside diameter of the outer glass tube, so that the top of the screen can be slipped over the end of the glass tube. The bottom of the platinum screen is sealed into the glass floor of the culture chamber. This screen prevents fragments of tissue from passing into the inner glass tube.

There are two openings to the culture chamber, 180° apart. One opening is essential for the insertion and removal of culture medium and tissue fragments; the other is for the introduction of the control gas which oxygenates the fluid, regulates the pH, and actuates the circulation. The reservoir chamber has one opening. This is for the insertion or removal of the silica sand, and the main bulk of the culture medium. It also serves as an outlet for the control gas. All three openings are set at an angle which facilitates the insertion of the culture medium and, at the same time, lessens the horizontal area exposed to infection by falling particles from the air. One of the two openings in the culture chamber is closed with a rubber stopper. The other opening to the culture chamber and the opening to the reservoir chamber, are closed with rubber stoppers to which cotton filter bulbs have been attached.

The type of flask now in use has a maximum working capacity of about 80 cc. of fluid. It will not operate satisfactorily with less than 25 cc. of fluid. The culture chamber floor is 8.5 cm. in diameter. The reservoir chamber is 9 cm. in height, and has a diameter of 4.5 cm. The overall height of the flask, including the reservoir chamber filter bulb, is 23 cm. The inner glass tube has an inside diameter of 2.5 mm., and an outside diameter of 4 mm. The middle glass tube has an I.D. of 7 mm., and an O.D. of 9 mm. The outer glass tube has an I.D. of 11 mm. and an O.D. of 13 mm. The glass tubes which form the openings to the culture and reservoir chambers have an I.D. of 10 mm., and are 45 mm. in length. The filter bulbs which hold the non-absorbent cotton, have an I.D. of 20 mm. The filter bulb tubes have an I.D. of 3 mm., and an O.D. of 5 mm. They are 20 mm. long.

The circulating flasks can be made in different sizes, with varying capacities in the culture and reservoir chambers. However, if the dimensions given in this paper are changed, it is important not to increase the inside diameter of the inner glass tube.[3] The height of fluid in the inner glass tube, when it is full, must not be very much greater than the distance from the holes at the bottom of the middle glass tube to the fluid level in the reservoir chamber at the time the fluid in the culture chamber touches the bottom of the inner glass tube. This is to avoid the possibility of control gas passing from the culture chamber through the sand in the middle glass tube, and bubbling up through the reservoir chamber.

If higher flasks are used, with longer inner glass tubes, it is necessary to increase the total depth of water in the pressure-regulating bottles to be described later. The total depth of water which the control gas bubbles through in the pressure-regulating bottles must always be substantially greater than the height of the inner glass tube in the highest circulating flask which is being operated.

Principle of Operation

The circulation of the fluid medium is actuated by a very slow flow of control gas through the cotton filter bulb leading to the culture chamber.

[3] If the inside diameter of the inner glass tube is too large, the control gas will bubble through the culture medium, instead of carrying the column of medium through the tube and into the reservoir chamber.

This gas passes through the inner glass tube, leading from the bottom of the culture chamber to the top of the reservoir chamber, and out through the cotton filter bulb which protects the opening to the reservoir chamber. This flow of control gas is unobstructed as long as the fluid level in the culture chamber is below the opening of the inner glass tube (about 2.5 mm. from the bottom of the culture chamber). As long as the flow of control gas is unobstructed, the fluid medium from the reservoir chamber passes slowly down through the fine silica sand in the middle glass tube, out through the holes at the bottom of the tube, up through the outer glass tube to the lip at the top, and down the inner wall of the culture chamber, until it reaches the fluid layer at the bottom of the culture chamber. When the fluid level in the culture chamber rises sufficiently to close the opening at the bottom of the inner glass tube, the control gas entering the culture chamber slowly raises the pressure in the chamber. This pressure forces the fluid medium up in the inner glass tube, and down in the outer glass tube. The fluid level continues to drop in the outer glass tube until it reaches the sand at the bottom of the tube. The fluid level continues to rise in the inner glass tube until it reaches the top of the tube and overflows into the reservoir chamber. After the fluid level in the culture chamber touches the bottom of the inner glass tube, the fluid maintains contact with this tube until the fluid level in the culture chamber has fallen appreciably below the bottom of the tube. Consequently, the depth of fluid covering the tissue fragments is constantly changing during the cycle of operation of the flask. The minimum and maximum depths of fluid are dependent on the distance of the bottom of the inner glass tube from the floor of the culture chamber.

When the fluid level in the culture chamber drops far enough to break contact with the bottom of the inner glass tube, the pressure of control gas in the chamber quickly lifts the column of fluid left in the inner glass tube, and carries it into the reservoir chamber. As soon as the inner glass tube is open again, and the culture chamber is back to atmospheric pressure, the fluid in the reservoir chamber begins to flow through the silica sand in the middle glass tube, up over the lip of the outer glass tube, and down the inner wall of the culture chamber, thereby completing the cycle of circulation.

The rate of circulation of the fluid medium is governed by the size of the grains of sand in the middle glass tube, by the height of sand in the tube, and by the amount of fluid in the reservoir chamber. The rate of flow of control gas through the flask also affects the rapidity of circulation.

Sterility is maintained by protecting all openings with cotton filter bulbs. No portion of the apparatus beyond the filter bulbs is kept sterile.

Assembly of the Apparatus

A gas cylinder, containing the desired mixture of gases, is placed in a convenient location near the incubator in which the flasks are to be operated. A pressure gauge and needle valve should be attached to the cylinder outlet. A thick wall, pressure type, rubber tube is attached to the needle valve, and carried into the incubator to a saturation bottle. The outlet of the saturation bottle is connected to a glass T. One of the openings from this T is connected with two saturation bottles, connected in series, which form a pressure-regulating valve for the control gas. The other

FIG. 3. Assembled apparatus.

opening from the T is connected first to a condensation bottle, then to a rubber tube containing a series of glass T's (one for each circulating flask). Each of these T's is connected to a very fine bore capillary glass tube, approximately 20 cm. in length.[4] These capillary glass tubes are connected to the circulating flasks by a very flexible gum rubber tube, which is attached to the cotton filter bulb leading to the culture chamber.

All rubber tubing used between the capillary glass tubes and the control gas cylinder is of a thick wall pressure type, with an inside diameter of 4 mm. and an outside diameter of 13 mm. The tubing used to connect the capillary glass tubes to the circulating flasks is of a gum rubber type, with an inside diameter of 4 mm. and an outside diameter of 8 mm.

[4] The rate of flow of control gas through the flask is regulated by the diameter of the capillary and the length of the tube. It is the practice in this laboratory to pass about 2 cc. of control gas per minute through each flask.

The first saturation bottle should be high enough to permit the control gas to be thoroughly saturated with water vapor as it bubbles through. The two saturation bottles which are used as a control gas pressure-regulating valve should be high enough to cause the control gas to bubble through a total of approximately 25 cm. of water. (If it is more convenient, one saturation bottle of double height may be used in place of the two described here.) The condensation bottle is simply an empty bottle, placed between the saturation bottle and the capillary glass tubes, to prevent the possibility of any water getting into the latter.

Operating Directions

The apparatus should be started before the circulating flasks are attached, in order to replace the air which is in the saturation bottle and in the rubber tubes after it is first assembled.

The needle valve on the control gas cylinder should be turned off. Pinch-cocks should be placed on the rubber tube outlets from all of the capillary glass tubes which are not to be used. Only the capillary glass tubes which will be attached to circulation flasks should be left open. The valve on top of the control gas cylinder should then be opened, and the needle valve opened until control gas bubbles very slowly through the two pressure-regulating bottles. Approximately five bubbles per minute are sufficient from a pressure-regulating bottle tube with an outlet diameter of 4 mm. Faster bubbling has no effect on the operation of the apparatus, but simply wastes control gas. The apparatus will operate properly as long as there is any bubbling through the pressure-regulating bottles, no matter how slow it is. However, it is not advisable to set the bubbling rate too slow, in case the drop in pressure of the control gas causes the bubbling to cease altogether. After the rate of bubbling has been adjusted, the apparatus is ready for the installation of the circulating flasks.

Preparation of the Circulating Flasks

The flasks are first rinsed with tap water, then filled with cleaning fluid, and allowed to stand for at least one hour. They are then thoroughly rinsed with tap water followed by distilled water. After the distilled water has been poured out, they are rinsed with alcohol and left to dry in a warm oven.

After the flasks are thoroughly dry, very fine silica sand[5] is poured into the reservoir chamber, and shaken down into the middle glass tube until the top of the sand is ap-

[5] The silica sand must be very carefully washed and dried before it is inserted in the circulating flask. It is first poured into a deep glass cylinder, filled with tap water. As soon as the sand has settled to the bottom, the water above the sand, containing dust and small particles of sand, is poured off. Fresh water is added, the sand thoroughly stirred, and the water on top again poured off. This process is repeated until the water in the glass cylinder remains clear after the sand has been stirred. The water is then poured off, and the sand covered with cleaning fluid. It is again thoroughly stirred, and allowed to stand overnight. The cleaning fluid is then poured off and the sand washed with water, which has been passed through a stone filter, until all traces of acidity are gone. The final washing should be done with distilled water. The sand is then spread on the bottom of a flat dish and dried in a warming oven.

proximately even with the bottom of the reservoir chamber. Some of the sand will pass out through the holes at the bottom of the middle glass tube, into the outer glass tube. The sand level in the outer glass tube should be approximately 1 cm. above the holes at the bottom of the middle glass tube. After the sand has been inserted, the flasks are ready for sterilization. They should be sterilized in an upright position. The rubber stoppers are not inserted until after sterilization.

Method of Connection

After the fluid medium has been inserted, and the openings of the culture and reservoir chambers have been closed with their rubber stoppers, the flask is ready for connection with the other portion of the apparatus. This is accomplished by attaching a gum rubber tube, from one of the capillary glass tubes, to the cotton filter bulb which leads to the culture chamber of the flask.

After the flasks have been installed, and the rate of flow of the control gas has been adjusted, they should operate for an indefinite period without further attention.

SUMMARY

An apparatus has been developed which permits the cultivation of a large number of tissue fragments in a large quantity of culture medium. The tissue fragments are cultivated in a thin layer of well oxygenated and constantly circulating medium.