THE CIRCUMFUSION SYSTEM FOR MULTIPURPOSE CULTURE CHAMBERS

I. Introduction to the Mechanics, Techniques, and Basic Results of a 12-Chamber (In Vitro) Closed Circulatory System

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ABSTRACT

A self-contained mechanical system for circulating nutrient fluid through 12 tissue culture chambers is described in detail. This system utilizes nonperforated cellophane membranes in the chambers which separate the circulating nutrient from the tissue culture environments. The nutrient, therefore, is dialyzed through the cellophane of each chamber; some cell products are retained in the microenvironment between the closely apposed cellophane and cover slip, whereas the other cell products move from chamber to chamber in the circulating nutrient. The resultant environmental conditions directed by the circumfusion systems are highly favorable for maintaining the differentiation of chick embryo tissues over protracted periods; a number of micrographs are shown.

INTRODUCTION

In the discipline of tissue culture, the word perfusion has been used to denote systems in which a fluid nutrient flows from a supply source, either continuously for a single passage through one or more culture vessels (1–21) or in a circulating pattern for repeated passages through a culture vessel (17, 22, 23). The word implies that the fluid nutrient "flows through" the cultivated tissues, but all the proposed and extant perfusion systems except one (24) have directed the fluid nutrient to "flow around" the tissues in cultivation. A true perfusion system, then, would be a device in which the moving fluid nutrient would be injected directly into the vessels of the cultivated tissues. Although such a mechanism has been reported (24), subsequent investigators found this procedure impractical and even questionable (25). However, a recent report (48) confirmed the feasibility of this procedure. The word "circumfusion" was used by Trowell (26), who rocked his organ culture vessels, and has been suggested by Moscona et al. (25) as a more appropriate term for tissue culture systems in which the fluid nutrient only flows around the cultivated tissues rather than through them.

The circumfusion system for multipurpose culture chambers (27–30) described in this report is a closed circulatory system for 12 chambers arranged in parallel; i.e., each chamber has a pseudoartery and pseudovein made of spaghetti-sized Teflon tubing. At one end the 12 arteries are plugged into the 12 culture chambers through one side of the chambers' rubber gaskets, and at the other end they pass through a rubber gasket

in the top of a positive pressure polycarbonate supply bottle. Similarly, the 12 veins are plugged into the contralateral side of the 12 rubber gaskets of the chambers, and their opposite extremities are inserted through the rubber gasket at the top of a negative pressure polycarbonate receiving bottle. The 400 ml of fluid nutrient utilized for each 12-chamber circumfusion system are moved from the negative pressure bottle to the positive pressure bottle through two polyvinyl chloride tubes which are "milked" of their content by a double peristaltic pump. The positive pressure in the bottle forces the fluid nutrient up through the 12 arterial tubes to the 12 chambers, and from the chambers the fluid nutrient passes through the 12 venous tubes back to the negative pressure bottle, thus completing the cycle.

Through this system the fluid nutrient comes into direct contact with the polyvinyl chloride and Teflon tubes, polycarbonate in the two bottles, glass cover slips, dialysis cellophane membranes, and pure gum rubber gaskets of the culture chambers. None of these materials has proved to be toxic to the cultivated tissues. The moving fluid nutrient is separated from the tissue explants by a cellophane membrane, and therefore makes only an indirect contact with the culture environment through dialysis. The entire system, including the gassing apparatus, is self-contained in a small Plexiglass incubator and operates on 110-v alternating current. The heat from the pump motor is more than adequate to supply the incubation. A continuously blowing fan circulates the heated air, and the excess heat is withdrawn by an intermittent exhaust fan controlled by a thermostat.

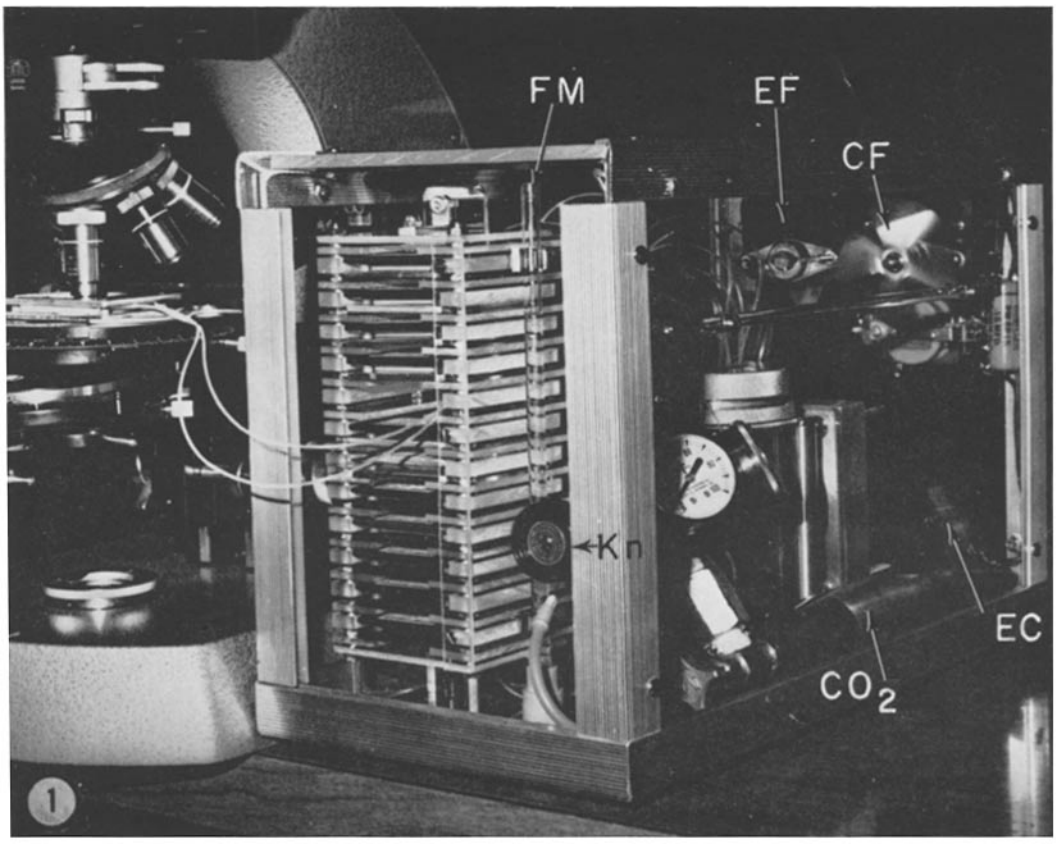
The units described herein have been in the developmental stage for 3 yr (30) and have been operational in the presently described form for the past year. They appear to fulfill the intended purpose for their development; that is, they provide a useful and practical system for the enhancement of tissue and organ differentiation by the creation of an in vitro environment which, in principle, more closely resembles the in vivo environment than individual culturing devices. This has been accomplished by (*a*) providing multiple interconnected units of cell cultures with a circulating and rapidly moving source of dialyzed basic and vital nutrients; (*b*) retaining some of the cellular secretions and products in the nutrient and microenvironments; (*c*) the use of a reliable respiratory system; (*d*) the maintenance of a constant physiological pH; and (*e*) the containment of all components in a temperature-controlled air-circulating incubator. This circumfusion system is an extension of the cellophane technics used in multipurpose culture chambers (29, 30); unconnected chambers utilizing the same cultivation procedures as the circumfused chambers were used for controls in evaluating the system.

MECHANICS OF CIRCUMFUSION SYSTEM

A circumfusion system for 12 multipurpose culture chambers is shown in Fig. 1 alongside a microscope with one of the chambers on the microscope stage. The flow of the fluid nutrient through the tubing to the chambers and bottles via the pump, as well as the specifications of these components, are shown by diagram in Fig. 2. The nutrient flow in this diagram is counterclockwise and was laid out in a circular form for convenience of interpretation. For simplicity, only one of the 12 chambers with its two Teflon leads is shown.

The dual polyvinly chloride tubing used in the pump has a wall thickness of $\frac{1}{16}$ inch and an inside diameter of $\frac{1}{8}$ inch. Larger tubings would require greater torque of the pump motor to compress them completely. To utilize the $\frac{1}{8}$-inch polyvinyl tubings with a $\frac{1}{16}$-inch wall diameter and still inject a sufficient amount of nutrient into the positive pressure bottle (shown on the left side of the diagram) to adequately insure uniform circulation required a double set of tubes, hence the dual pump. One polyvinyl tube was sufficient for smaller-sized spaghetti Teflon tubes (0.015 inch) leading to the chambers, but tests favored the larger spaghetti tubing with an inside diameter of 0.034 inch. This larger size obviated the possibility of intratubular obstructions produced by gas bubbles and solid aggregates from the nutrient but, to be most effective, required the double pumping system.

At first, clock motor-pump units with latex tubing were used for the peristaltic pumping system, but this was undesirable as the latex was short-lived and crumbled in a few days, depending upon the rpm used. The polyvinyl tubes, on the other hand, will last several months and, when silicone grease is applied to their external surfaces, they appear to be virtually indestructible (five months with first pump at the time of the writing). If a leak develops in the polyvinyl from excessive wear in the pump, sterile replacements are kept available to be plugged into the tops of the bottles without risk of contamination. Since there are two tubes in the pump, a leak in one tube will not stop the pumping action, although it will slow it until replacements have been inserted. Additionally, leaks are from minute holes, and the high pressure in the tubing permits only a small loss of nutrient and prohibits the entrance of contaminants.



Key to Symbols

A, rubber tubing and No. 23 syringe needle adapter between pressure regulator and CO_2 coil
Ac, acinar cells
C, colloid, PAS (saliva digestion) positive material in thyroid
CC, CO_2 coil (8 ft of 0.022-inch Teflon spaghetti tubing)
CF, circulating fan
CO_2, CO_2 cylinder (150 ml capacity)
Cp, capsule
EC electrical connector
EF, exhaust fan
ex, explant
F, fans
Fl, follicle of thyroid gland
FM, Flowmeter
GR, gathering rings of 0.095-inch Teflon spaghetti tubing to hold the arterial and venous Teflon leads together
H, Hürthle cell in thyroid gland (?)
Kn, knob of pressure regulator
L, lasso around 24 pieces of 0.034-inch Teflon spaghetti tubing
Mc, macrophages
P, pull cord for lasso
PR, pressure regulator
PS, plastic shield to separate Teflon tubes from motor and pump
Py, pulley for lasso
SP, springs for ejecting chambers from stacking rack
SR, stacking rack for 12 chambers
St, stromal capsule
T, thermometer
Tr, tubule-like tract in liver epithelium
Ts, thermostat
(+), positive pressure polycarbonate bottle
(−), negative pressure polycarbonate bottle

FIGURE 1 A circumfusion unit with the front panel completely removed and a chamber placed on the microscope stage. The Teflon leads for each chamber pass through the slot from which the chamber is removed. The flowmeter (*FM*) and knob (*Kn*) for controlling the pressure regulator are shown to the right of the stacking rack. The circulating fan (*CF*) operates continuously as shown in the photograph, and with the operation of the motor for the pump there is a continuous supply of heat so that opening the system for microscope observation of the various chambers reduces the temperature by only a few degrees.

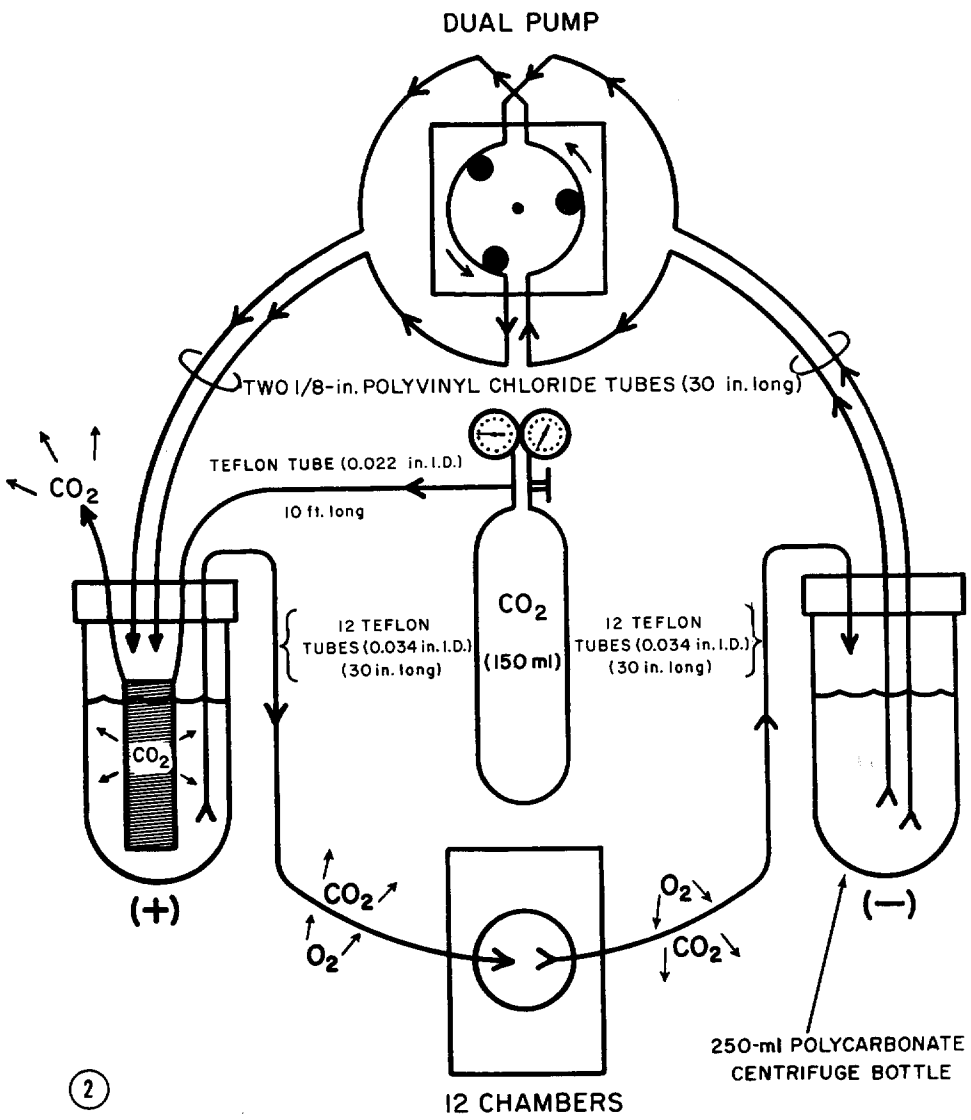


FIGURE 2 Diagram of the circumfusion system with specifications for the various tubings used. See text for full description.

Pumps with two gear motors have been evaluated—one, a $\frac{1}{250}$ hp motor with 21-inch pounds of torque, turned the pump at $4\frac{3}{4}$ rpm; the other, a $\frac{1}{70}$ hp motor with 17-inch pounds of torque, turned the pump at 18 rpm (R. J. Matthias & Assocs., Houston, Texas). The heat produced by either motor was harnessed to the incubator by the continuously circulating fan shown in the upper right portion of Figs. 1 and 3. As the fluid was withdrawn from the negative pressure polycarbonate bottle on the right (Fig. 2) to the positive polycarbonate bottle on the left, a flow was established through the 12 chambers via the spaghetti Teflon tubes. Each length of polyvinyl tubing eliminated 1.5 ml per revolution, so the pump, turning at $4\frac{3}{4}$ rpm, moved $2 \times 1.5 \times 4\frac{3}{4} = 14.25$ ml/min or, at 18 rpm, moved $2 \times 1.5 \times 18 = 54$ ml/min. Since there are 12 chambers, fluid moves through each chamber with the slow pump at a rate of about 1.2 ml/min, or each chamber has a fluid exchange of 1710 ml per 24 hr. Each pumping unit utilized 400 ml of nutrient, so the equivalent of the entire available nutrient passes through each chamber 4.3 times per 24 hr. With the faster pump, the figures are increased by a factor of 3.8.

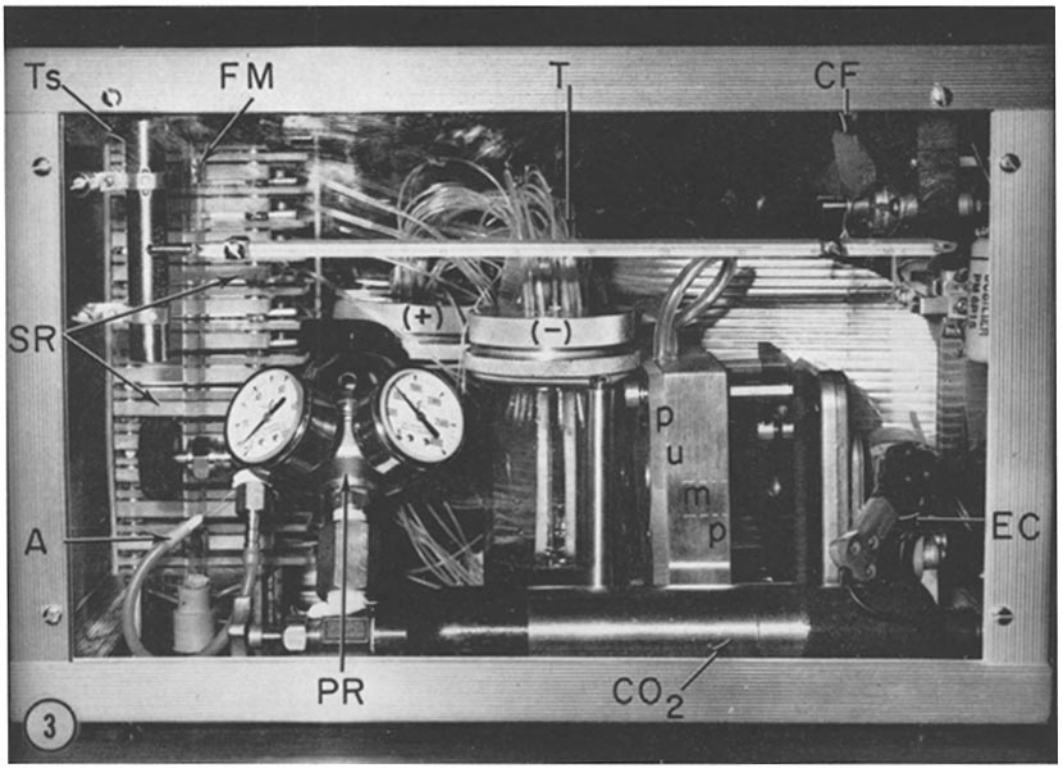


FIGURE 3 A circumfusion system unit viewed from the right side. The circulating fan (*CF*) is shown at the top right. The thermometer (*T*) is anchored on brackets against the Plexiglass side wall. The CO_2 gas cylinder (CO_2), pressure regulator (*PR*), and adapter (*A*) for the Teflon CO_2 coil in the positive pressure (+) polycarbonate bottle are indicated. The flowmeter (*FM*) through which the CO_2 gas bubble rises is shown against the stacking (chamber) rack (*SR*). The thermostat (*Ts*) is also affixed with metal brackets to the Plexiglass wall. An electrical connector (*EC*), shown at the lower right, is used to separate the top of the incubator from the electrical supply when removed during loading and nutrient exchanges (see also Fig. 5).

If the pumps are turned off for approximately 30 min, the Teflon spaghetti tubes will appear fuschia in color because of a loss of CO_2 by diffusion through their walls and the resultant color changes of the phenol red pH indicator (0.001–0.002%) in the nutrient. If the unit is established without a provision for a replacement of the CO_2 lost by diffusion, the alkalinity of the entire system rises considerably the first day. To overcome this CO_2 loss, 100% CO_2 (Coleman grade) is dispensed from a small 150-ml cylinder through a 10-ft-long coil of 0.022-inch Teflon (Fig. 2) immersed in the nutrient of the positive pressure polycarbonate bottle (Fig. 4). The other end of this tube is inserted into a water-filled glass tube (CO_2 flowmeter, Figs. 1, 3, and 5), and the pressure regulator (Fig. 3) is adjusted to 3–5 pounds. A flow rate of one bubble per 5–15 sec has been found sufficient to maintain a stable pH (7.2–7.4). The knob regulating the flow is to the left in Fig. 3, so that adjustments may be made when the front panel of the incubator is elevated. This front panel is shown removed in Fig. 1, with 11 of the 12 chambers in a horizontal position one over another in the vertical stacking rack. The rack is spring-loaded, and a catch holds the chamber in its place. A fingertip movement releases this latching device and the spring ejects the chamber forward. One of the chambers had been removed and is shown on the stage of the microscope. Since the pump motor and fan are operating constantly, removal of the panel for microscope observation lowers the incubator temperature only a few degrees, and the 37°C temperature returns rapidly after replacement of the panel.

In operation, the pump-motor unit is turned on its side (Fig. 3), so that the heat rises in the back of the incubator, and some of it escapes through the exhaust

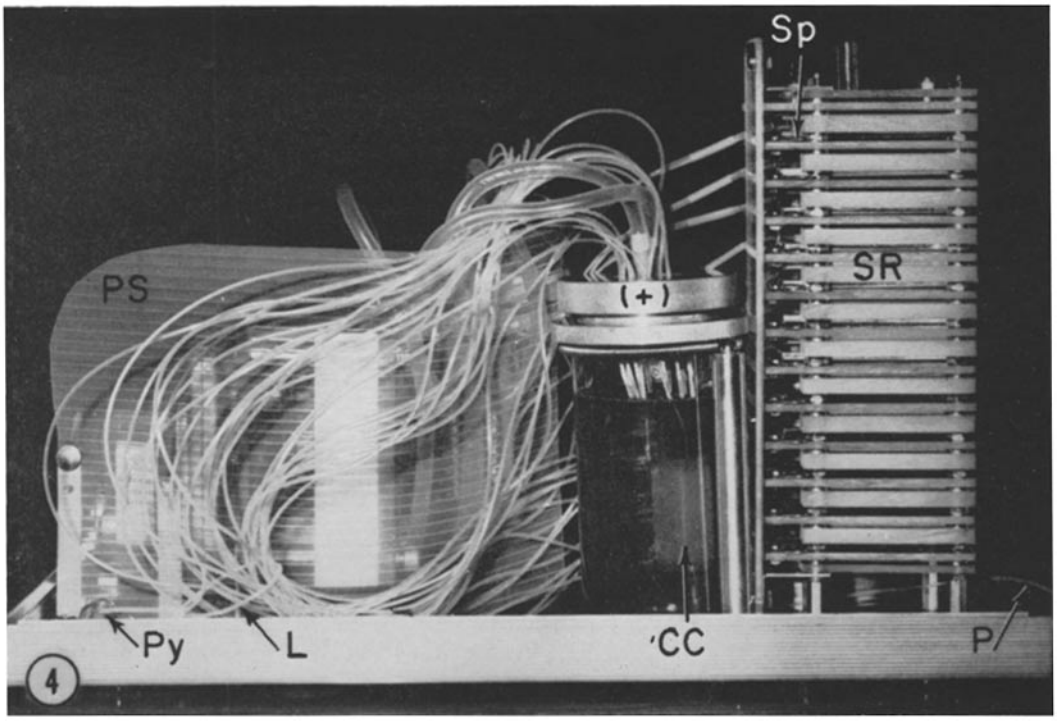


FIGURE 4 Left side of circumfusion system unit shown with the top Plexiglass section removed. The stacking (chamber) rack (*SR*) is shown to the right, and the location of springs (*SP*) for ejecting the chambers when appropriately tripped from the front are shown. The positive pressure (+) polycarbonate bottle is shown with the CO_2 coil (*CC*) submerged in the fluid nutrient. The Teflon arterial and venous tubes leading from the two bottles toward the rear of the unit are shown gathered by a lasso (*L*) of Teflon spaghetti tubing in the lower left portion of the photograph. This lasso is fed through a pulley (*Py*), and the lasso pull cord (*P*) passes forward in the bottom of the unit to the front portion where it may be activated by raising the front panel. The plastic shield (*PS*) prevents the Teflon tubes from being snagged by the angular edges of the motor and pump.

fan portal (Fig. 1). A 2-inch hole was cut in the Plexiglass floor under the motor to facilitate this flow, whereas the circulating fan is on continuously and blends the outside air, which is normally flowing through these two portals, with the inside heated incubator air. The thermostat is located in the front of the incubator as shown in Fig. 3 and is regulated with a screw-driver through a hole in the top of the incubator. When the desired temperature (37°C) has been attained, a relay intermittently actuates the exhaust fan.

The incubator (R. J. Matthias & Assocs., Houston, Texas) was made of extruded aluminum framing material and ¼-inch Plexiglass. The incubator is composed of a base (7¾ × 15½ inches) to which the motor, stacking (chamber) rack, and bottle holders are attached. The top of the incubator composed of the sides (10½ × 15½ inches), roof (7¾ × 15½ inches), and front and back panels (7¾ × 15½ inches) is a detachable unit to which the thermometer, thermostat, relay, and fans are fastened.

The top of the incubator is shown lifted off the base, and in a vertical position (Fig. 5). An electrical plug (Figs. 1, 3, and 5) was used to disconnect the power from the blower system, so that the top could be moved some distance for easier accessibility when setting up the unit.

In Fig. 4, the Teflon spaghetti tubes from the bottles are shown folded toward the back and then looped around to the front to each of the chambers. A lasso of Teflon tubing (0.034 inch) is fed through a plastic loop in the back of the incubator with a long lead brought forward underneath the stacking rack. As a chamber is withdrawn from its rack to the microscope, this lasso is pulled forward so the mass of tubes also moves forward. When a chamber is returned to the stacking rack, the lead on this lasso is pulled tightly; and this, in turn, pulls all the spaghetti

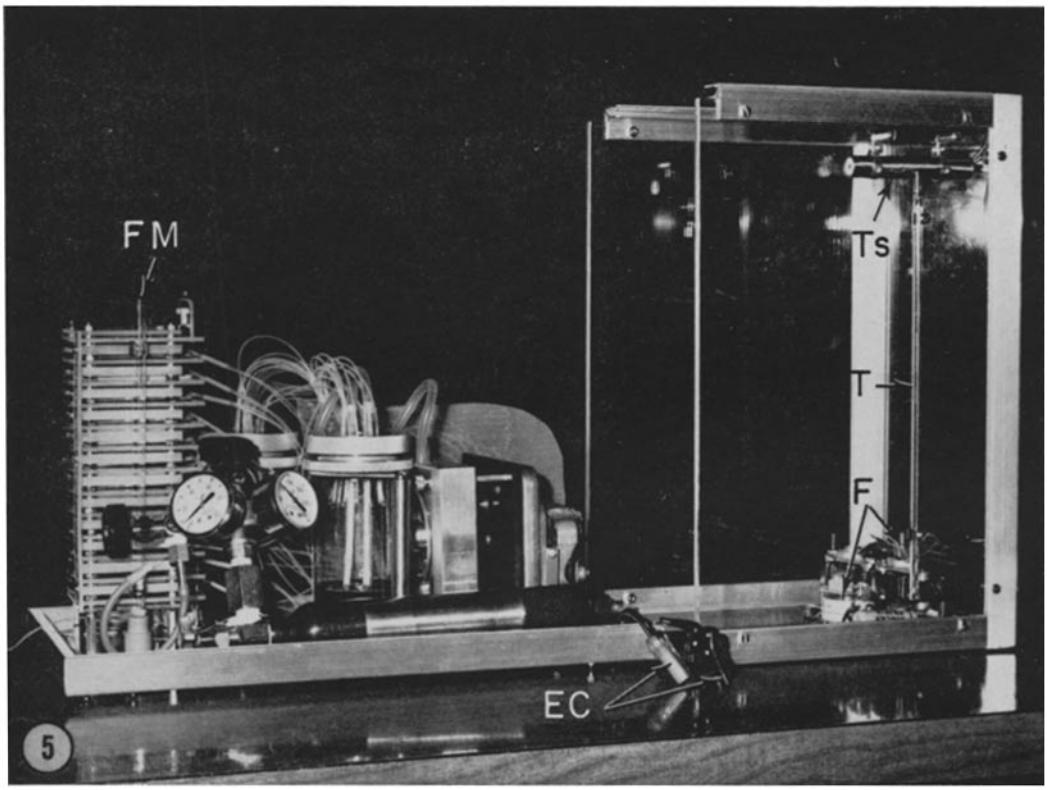


FIGURE 5 View of the circumfusion unit after the complete removal of the top of the incubator and detachment of the electrical connector (*EC*). Attached to this top section are the thermostat (*Ts*), thermometer (*T*), fans (*F*), and relay. The flowmeter (*FM*) is denoted on the right side of the stacking rack.

tubes toward the back of the incubator, facilitating the replacement of the chamber into the rack by preventing curling or kinking of the spaghetti tubing. The plastic shield (Fig. 4) separates the Teflon leads from the motor and facilitates their movements.

Although the polycarbonate bottles are marked positive or negative, the negative pressure bottle does not remain negative. When initially actuated, the pump flow does induce a positive and negative pressure in the two tightly sealed bottles. Escaping CO_2 accumulates in the negative pressure bottle so that the negative pressure diminishes to atmospheric values, although it remains negative relative to the higher pressure in the positive pressure bottle, and the flow is not impeded. In contrast to the escaping of the CO_2 through the Teflon tubes, the higher oxygen tension of the atmosphere induces a flow of O_2 through the Teflon to the fluid nutrient. Using a physiological gas analyzer (Beckman-Spinco Model 160, Palo Alto, California), this O_2 tension of the fluid coming into the chambers has been determined to range between 120 and 140 mm O_2.

TECHNIQUES OF THE CIRCUMFUSION SYSTEM

Preparation of the Autoclavable Unit (Culture Harness)

The culture harness shown in Fig. 6 has been prepared for autoclaving. This consists of two polycarbonate bottles (The Nalge Co., Inc., Rochester, New York) with screw-on metal caps (R. J. Matthias & Assocs., Houston, Texas) connected to each other by 2 ($\frac{1}{8}$ inch I.D. × $\frac{1}{16}$ inch wall) flexible polyvinyl chloride tubes (Tygon, The U. S. Stoneware Co., Akron, Ohio; 30 inches long) and by 24 spaghetti-sized (0.034 inch I.D.) Teflon tubes (E. I. du Pont de Nemours & Company, Wilmington, Delaware; 30 inches long) attached to the 12 pure gum rubber gaskets (Acme-Hamilton Mfg. Corp., Trenton, New Jersey; No. 107 floating stock). The gaskets are shown wrapped for autoclaving in Fig. 6 and unwrapped in Fig. 7. Each culture harness is fabricated with new tubing and gaskets; the only reusable components are

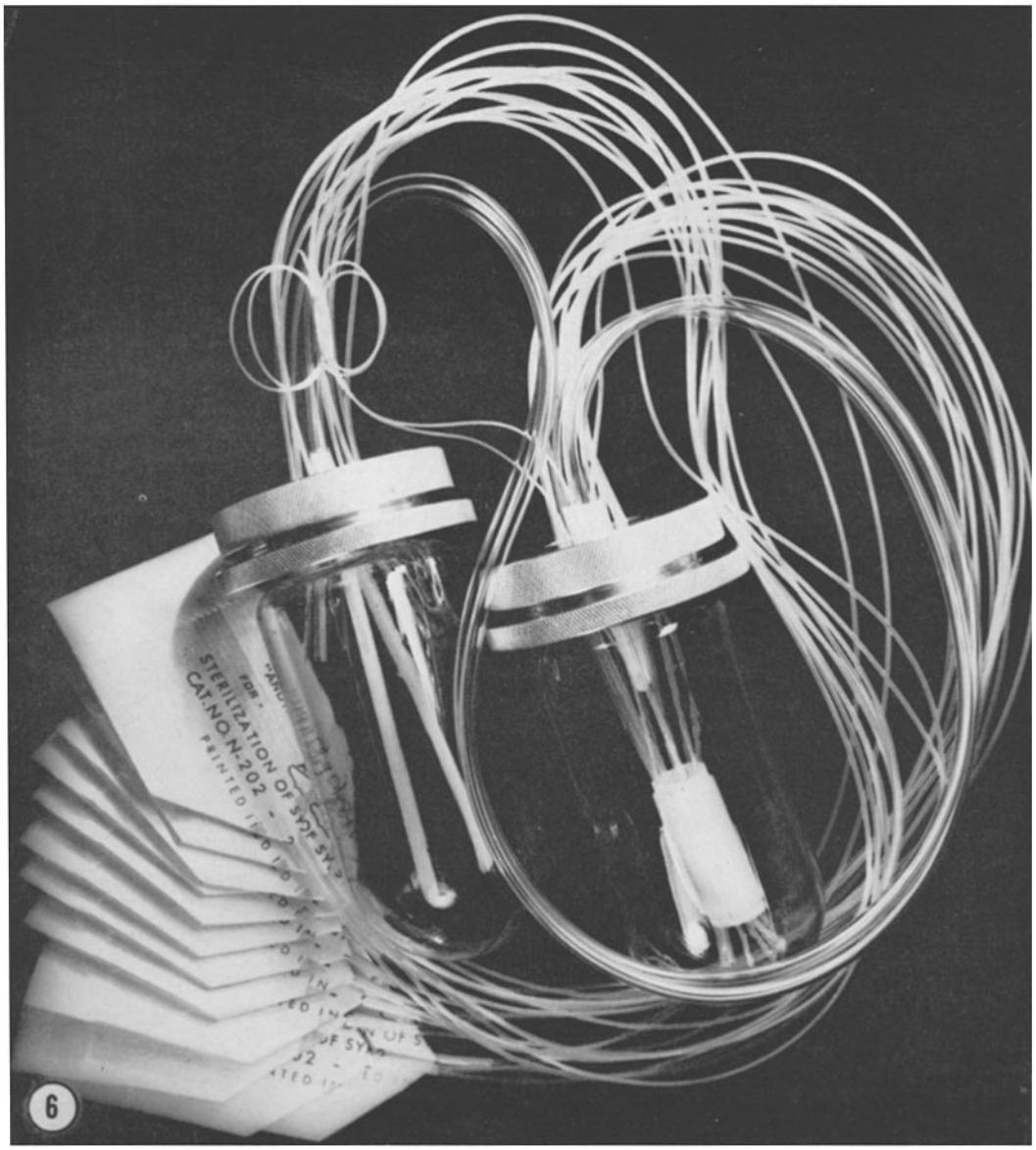


FIGURE 6 The culture harness (autoclavable unit) shown prepared for autoclaving. The 12 rubber gaskets have been individually wrapped in paper bags. This unit is then placed in a plastic tray and wrapped with cotton cloth for autoclaving.

the polycarbonate bottles and the two-piece, specially devised metal screw caps which fit on their tops. These caps hold circular pure gum rubber gaskets (Acme-Hamilton Mfg. Corp., Trenton, New Jersey; 1⅝ inch O.D. × ¼ inch thickness) through which the 12 spaghetti-sized Teflon arteries or veins and the 2⅛ inch (diameter) Teflon tubes for the polyvinyl chloride tubes pass.

Insertion of the spaghetti Teflon tubes through the pure gum rubber chamber gaskets (1¾ × 2 inches × ⅛ inch with 1⅛ inch center hole) is done in the following way: (*a*) a gasket is placed between two pieces of Plexiglass of the same dimensions as the gasket, and the three pieces are tightened together in a small vise; (*b*) a 14-gauge, thin-wall needle with a stylus is inserted through one edge of the gasket;

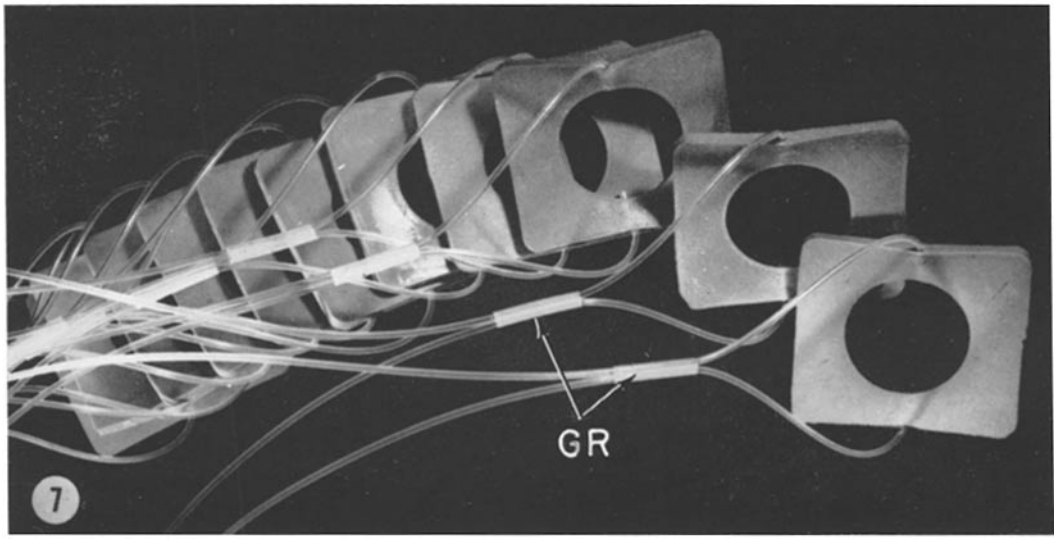


FIGURE 7 Gaskets for the chambers prepared with arterial and venous Teflon tubing prior to being wrapped for autoclaving. The gathering rings (*GR*) are indicated. These gathering rings assist in the replacement of the leads after examination of a chamber by keeping this portion of the pairs of Teflon more rigid.

(*c*) the stylus is pulled out; (*d*) the Teflon tube is run through the needle; and (*e*) the needle is withdrawn and pulled off the 30-inch piece of Teflon. Another Teflon tube is similarly placed in the opposite side of the gasket. After 12 gaskets are completed, a ½-inch length of Teflon tubing (0.095 inch I.D.) is used as a gathering ring and is pulled down each pair of tubes toward the gasket (Fig. 7). This ring keeps the Teflon tubes in better alignment and prevents them from kinking when a chamber is inserted into the stacking rack. The other ends of the spaghetti-sized Teflon tubes are run through the gaskets at the tops of the polycarbonate bottles by inserting the 14-gauge needle through the bottom of the gasket and running the Teflon tube down from the top. Following this procedure, the needle is withdrawn free from the Teflon tube rather than being left on it.

Into each end of the two polyvinyl chloride tubes (30 × ⅛ inches I.D. × 1/16 inch wall thickness) are inserted 2-inch and 5-inch lengths of Teflon tubing (⅛ inch I.D.; Fig. 6). Short lengths of amber latex rubber tubing are cut and placed around these polyvinyl-Teflon unions to act as safety locks, particularly during autoclaving when the polyvinyl becomes quite soft. Tubing spreaders facilitate both of these applications. The 2-inch lengths of Teflon are then plugged through two small holes drilled into the gasket at the top of the positive pressure polycarbonate bottle (Fig. 8), and the 5-inch lengths of Teflon are plugged into the gasket at the top of the negative pressure polycarbonate bottle (Fig. 9). In operation, the fluid is sucked up through the long lengths of Teflon in the negative pressure bottle and dripped into the positive pressure bottle through the short lengths of Teflon. Conversely, the 12 Teflon spaghetti-sized tubes (arteries) extend approximately 5 inches into the positive pressure bottle (Figs. 4 and 8) through which the fluid will pass as it is pushed by the increased air pressure to the chambers, whereas the negative pressure bottle (Figs. 3 and 9) receives the 12 1½-inch lengths of spaghetti Teflon tubes (veins) from which the fluid drips as it comes from the chambers. Therefore, a glance at the set of tubes in the negative pressure bottle (Fig. 3) will indicate the flow condition of each chamber.

The CO_2 coil is composed of 10 ft of Teflon spaghetti tubing (0.022 inch I.D.) wound on a Teflon tube 1½ inches in length by ⅝ inch in diameter. The coil is placed in the positive pressure bottle (Figs. 4 and 8), and the two ends of the spaghetti tubing are inserted through the gasket at the top of the bottle. One end is adapted to the CO_2 gas cylinder (Fig. 3) and the other end to the glass tube filled with water to act as a flowmeter (Figs. 1, 3, and 5).

After the autoclavable unit has been assembled, the entire unit is rinsed in ethanol (95%) to remove finger marks and dust particles and allowed to dry. The gaskets then are wrapped in paper bags (Fig. 6) with the Teflon tubes protruding through the open end of the bag. The paper bags are standard hospital

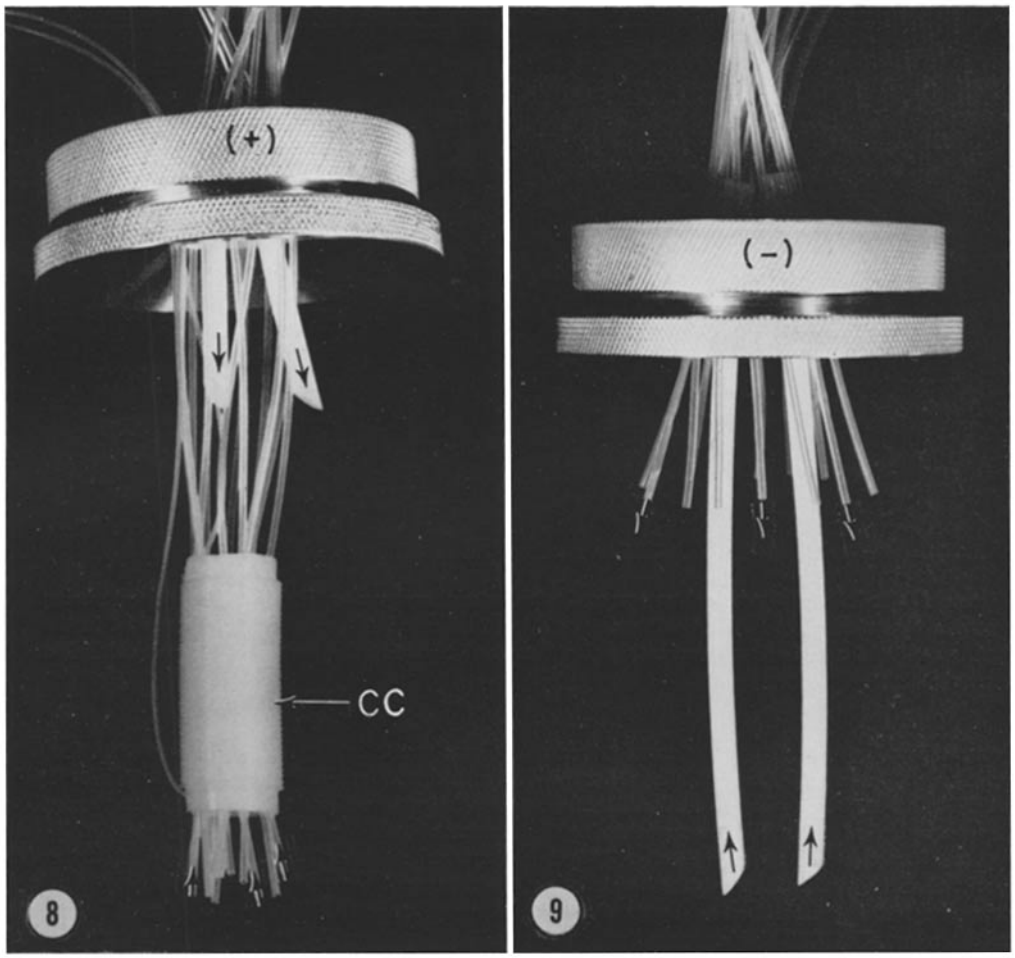


FIGURE 8 View of the internal components of the positive pressure (+) polycarbonate bottle. The 2-inch-long (⅛ inch I.D.) Teflon tubes through which the fluid nutrient flows into the bottle and the 12 5-inch (0.034 inch I.D.) Teflon arterial leads through which the nutrient flows from the bottles to the chambers are shown surrounded by the CO_2 coil (*CC*). This is composed of about 8 ft of 0.022-inch I.D. Teflon spaghetti tubing through which the CO_2 passes slowly and diffuses through the Teflon walls into the fluid nutrient.

FIGURE 9 The internal components of the negative pressure (−) polycarbonate bottle showing the 2 5-inch-long (⅛ inch I.D.) Teflon tubes through which the fluid moves up in its passage to the peristaltic pump and the 12 1½-inch-long leads of 0.034-inch I.D. Teflon sphaghetti tubing through which the fluid enters the bottle in its passage from the chambers.

sacks used for autoclaving syringes. The metal caps with the rubber gaskets are screwed loosely on the polycarbonate bottles, and the entire unit is carefully positioned in a basket and wrapped with several layers of muslin for autoclaving. Particular attention must be paid to the polyvinyl tubings so that their distortion during the autoclaving is obviated, viz. by wrapping them so that there are no sharp bends, no direct contact with the metal caps, or no excessive weight upon them.

Cultivation Procedures With Culture Harness

Since the individual chamber gaskets are wrapped separately in paper bags, the culture harness may be

unwrapped after autoclaving without contamination. At this time, the polycarbonate bottle caps are tightened. In fabricating multipurpose culture chambers, usually four chambers are established at one time, but with the circumfusion system it is best to fabricate only one chamber at a time, inasmuch as the springlike rigidity of the Teflon tubes may move and contaminate the unwrapped gasket before the chamber is completed. Therefore, as each chamber is put together, a weight is placed on the Teflon tubes near the gasket to prevent movement. The culture chambers are fabricated by the usual cellophane procedures outlined in detail in other publications (29, 30). These procedures establish the explants in a thin culture environment between the closely apposed dialysis cellophane membrane and one of the cover slips or in the alternate cellophane sandwich type of cultivation in which the explants are between two sheets of cellophane (46). Therefore, the nutrient flowing through the circumfusion system affects the cellular environment by an indirect route, viz., through the cellophane membrane. A mica cover slip is placed on the outside surface of the closing glass cover slip for protection against accidental breakage which would contaminate the entire system. Although the culture cover slip is not protected by mica, should it be broken the cellophane sheet inside would protect the integrity of the system. Although this culture would be lost, the other 11 chambers would be unaffected. The nutrient is not injected into the chambers by the syringe-needle techniques formerly described for multipurpose culture chambers, but rather the chambers are filled from the positive pressure bottle at a later time by the action of the pump.

Installation of Culture Harness into Pumping System

After the 12 chambers have been fabricated, they are inserted one at a time through the rear of the stacking rack. They are locked into their separate spring-loaded slots of the stacking rack only after the pumping unit is full of nutrient and is operational. The pump is rotated 90° to a horizontal position, and the two polyvinyl chloride tubes are carefully placed in it to allow adequate freedom after closing the top Plexiglass plate of the pump. The pump is then repositioned into the vertical alignment, which permits the heat from the motor to rise toward the air-circulating fan without overheating the polyvinyl tubing. In an earlier model (30) the pump was positioned horizontally over the motor, and the heat was found to be unfavorable to the nutrient.

Filling the System with Fluid Nutrient

The best method of filling the unit with the fluid nutrient is to have two extra sterile polycarbonate bottles available with each pump; 250 ml of nutrient are poured into one, and the bottle of the positive pressure assembly is removed and replaced with the nutrient-filled bottle. Similarly, the other spare bottle is filled with 150 ml of nutrient, and this is used to replace the bottle of the negative pressure assembly. The pump then may be started immediately. The fluid at once rises through the 12 pseudoarteries and flows into the chambers. Each chamber is tilted so that the air bubble returns through the pseudovein back to the negative pressure bottle. In this way, the chambers are filled with nutrient and the circulation is established. Air-bubble locks may occur in the tubes in the initial stage of starting the pump and for 1–2 hr thereafter, but these may be dislodged easily by thumping the tubes with the fingers.

Controlling pH with the CO_2 Core

After the circulation flow has been well established and all 12 pseudoveins are dripping uniformly, the CO_2 system (150-ml cylinder supplied at 1200–1500 psi by The Matheson Company, Inc., East Rutherford, New Jersey) is actuated in the following way: the pressure regulator (No. 36, The Matheson Company, Inc., East Rutherford, New Jersey) is set at approximately 5 pounds, and an adapter is made by honing smooth a No. 23 needle and inserting it into one of the Teflon leads of the CO_2 coil. This needle is then adapted to the pressure regulator with a short rubber tubing, and the other end of the coil is attached to a bubble flowmeter. This flowmeter is made by cutting a glass tube to the height of the stacking rack and inserting a rubber serum-bottle top into the bottom end. Using a 14-gauge needle, the Teflon tube from the CO_2 coil is run through this rubber bottle top and into the bottom of the tube which is filled with water (Figs. 1, 3, and 5). The control valve of the regulator (Figs. 1, 3, and 5) is then turned on so that one bubble per 5–15 sec will appear, rising in the flowmeter. At this flow rate, pH is uniformly maintained (the bubble rate does not alter the pH of the chambers significantly). The slowly moving CO_2 in the coil (about 8 ft are submerged) adequately balances the CO_2 lost from the 24 Teflon leads. Within the limits of this flow rate, a relatively constant amount of CO_2 diffuses into the fluid. Properly controlled, the gas cylinder will last several months.

Incubation

The top of the incubator is positioned and the front panel slide is closed. As the pump continues to turn, the motor warms the air which is circulated by the fan. The temperature is controlled by the thermostat which actuates the exhaust fan periodically through a relay.

Maintenance of Circumfusion System

There have been several ways exploited for maintaining the continuity of the circumfusion system. Nutrients in varying proportions have been used, removed, and replaced at varying intervals. Obviously, the correct nutrient program to be used for the most efficacious results would be dependent upon the tissues being cultivated. However, it has been established that a high serum content in this system, if left for several weeks without an exchange, is undesirable. For instance, when 20% calf serum and 5% whole egg ultrafiltrate (Microbiological Associates, Inc., Bethesda, Maryland) were used as supplements for Fischer's V-614 nutrient (Difco Laboratories, Detroit, Michigan) with chick embryo systems, a heavy precipitation of calcium phosphate occurred on the cellophane surface, which impaired visualization in about 3 wk. Control chambers (unattached to the circumfusion system) using the same nutrient with semiweekly changes were free of this visual obstruction, although after several months a similar precipitation sometimes occurred. Chick embryo tissues have been under surveillance for a quarter of a year (13 wk) in Fischer's V-614 supplemented with only 7½% calf serum and 5% whole egg ultrafiltrate. Exchanging 50–100 ml/wk of this nutrient prevented the precipitation in the cellophane. However, this proved to be an inadequate nutrient, as some tissues slowly waned in numbers of cells and eventually succumbed. The male and female gonadal tissues were resistant to such a demise. When the 250 ml of nutrient with 20% calf serum were exchanged semiweekly, the time before the first appearance of the precipitate was extended from 2–8 wk. Semiweekly exchanges of the nutrient (250 ml) were made by unscrewing the cap on the positive pressure bottle and replacing the bottle with another one containing 250 ml of fresh nutrient. This was the standard procedure used and the results shown were obtained in systems so regulated. It was also found that if the calf serum were reduced by 5% and replaced by bovine ultrafiltrate every 3 wk until the nutrient contained calf serum (5%) and bovine ultrafiltrate (15%), the opacity would not appear in 105 days (time of writing).

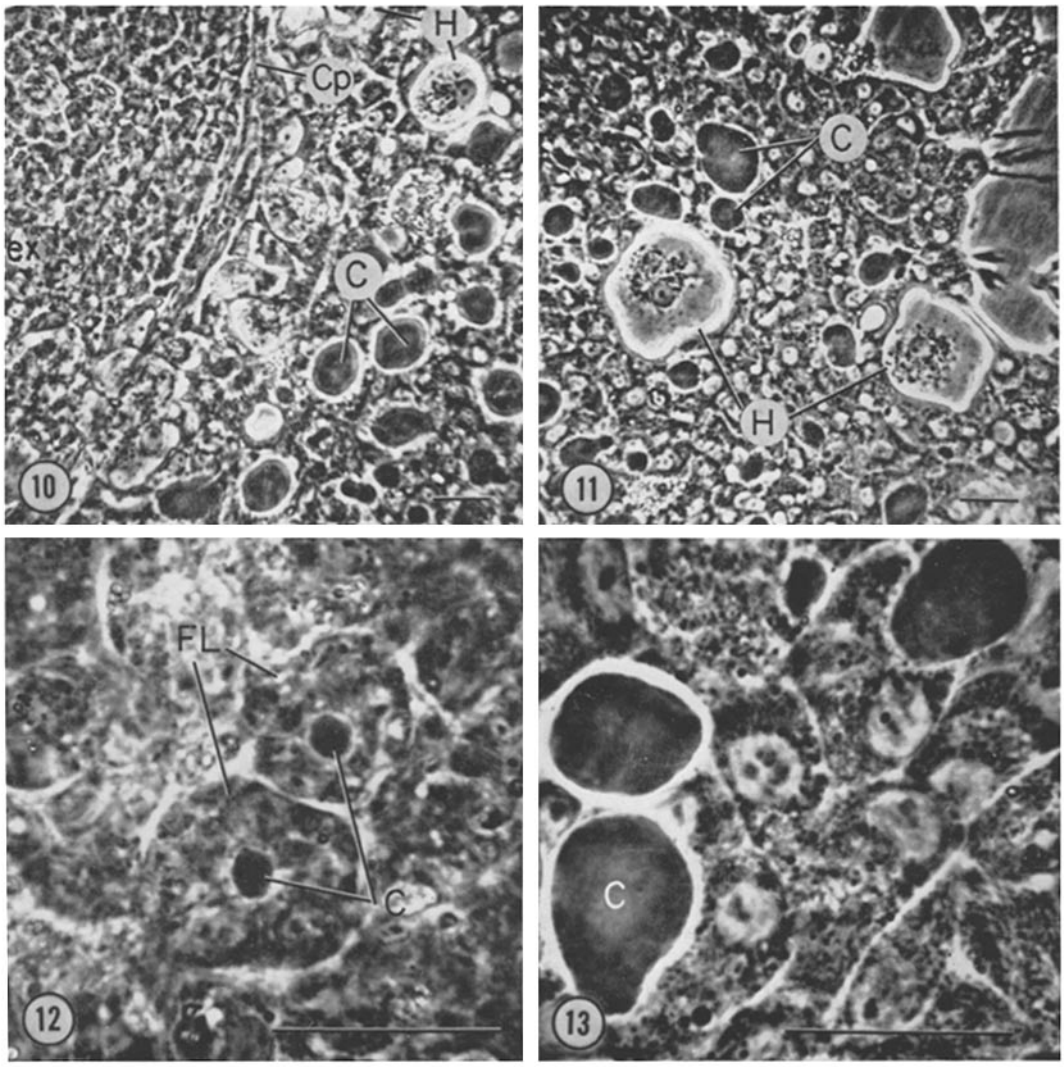
There are two other types of aggregations which occur, regardless of the nutrient used, but these are not quite so objectionable. The arterial lead produces two eddy whirls where it enters the chamber. On the dependent cover slip, a crystalline accumulation soon appears in these eddy areas. Similarly, on the uppermost coverslip, an accumulation of fatty globules occurs in the whirling eddies. Both of these opacities may be dislodged by inverting the chamber. This inversion induces each accumulation to reverse its position. They are washed out temporarily in the process, although they will recur overnight. Since these aggregations are at the periphery of the culture cover glass, they generally are not objectionable, and their occurrence may serve to maintain some clarity of the nutrient. Water loss from the Teflon may contribute to the crystalline precipitation on the dependent cover slips. The drying effect of the incubation system, particularly with the air circulation enhanced by the exhaust fan, produces an arid climate conducive to a greater water loss through the Teflon.

RESULTS

The circumfusion systems were evaluated with 14-day-old chick embryo tissues and organs explanted in multipurpose culture chambers under full sheets of dialysis cellophane. With each evaluation, control chambers unattached to a system were established for comparative analyses. In every instance, the tissue differentiation was greatly enhanced by the circumfusion. A series of micrographs of some of the organs studied in various fast and slow circulating systems are presented here along with brief descriptions. These studies demonstrate the maintenance of differentiation which occurred over a protracted number of days in the original explanted tissue and, in some cases (thyroid, liver and adrenal), a remarkable state of differentiation in emigrating cells, as well. Both the fast and slow pumps produced excellent environments, but an unequivocal decision on their comparative value toward this end has not been established at this time. The fast pumps, however, were advantageous in that the filling of the chambers and the dislodging of air bubbles were done with considerably more ease over a shorter period of time.

Thyroid Gland

In Fig. 10, a portion of a chick embryo thyroid gland is shown after 71 days of cultivation. The original gland tissue is on the left side of the micrograph and is sharply demarcated from the emigrating cells on the right by its thin-walled capsule of spindle-shaped cells. The emigrating sheet of epithelial cells had a conspicuous interspersion of large phase-black amorphous globules. In this explant, these globules were found all the way to the periphery of the epithelial sheet (Fig. 11), as well as in the follicular groupings of cells in the original explant (Fig. 12). The amorphous globules in the epithelial outgrowth are shown at higher power (Fig. 13) to be approximately one to three times as large as the surrounding cells. This culture was fixed in formaldehyde and stained



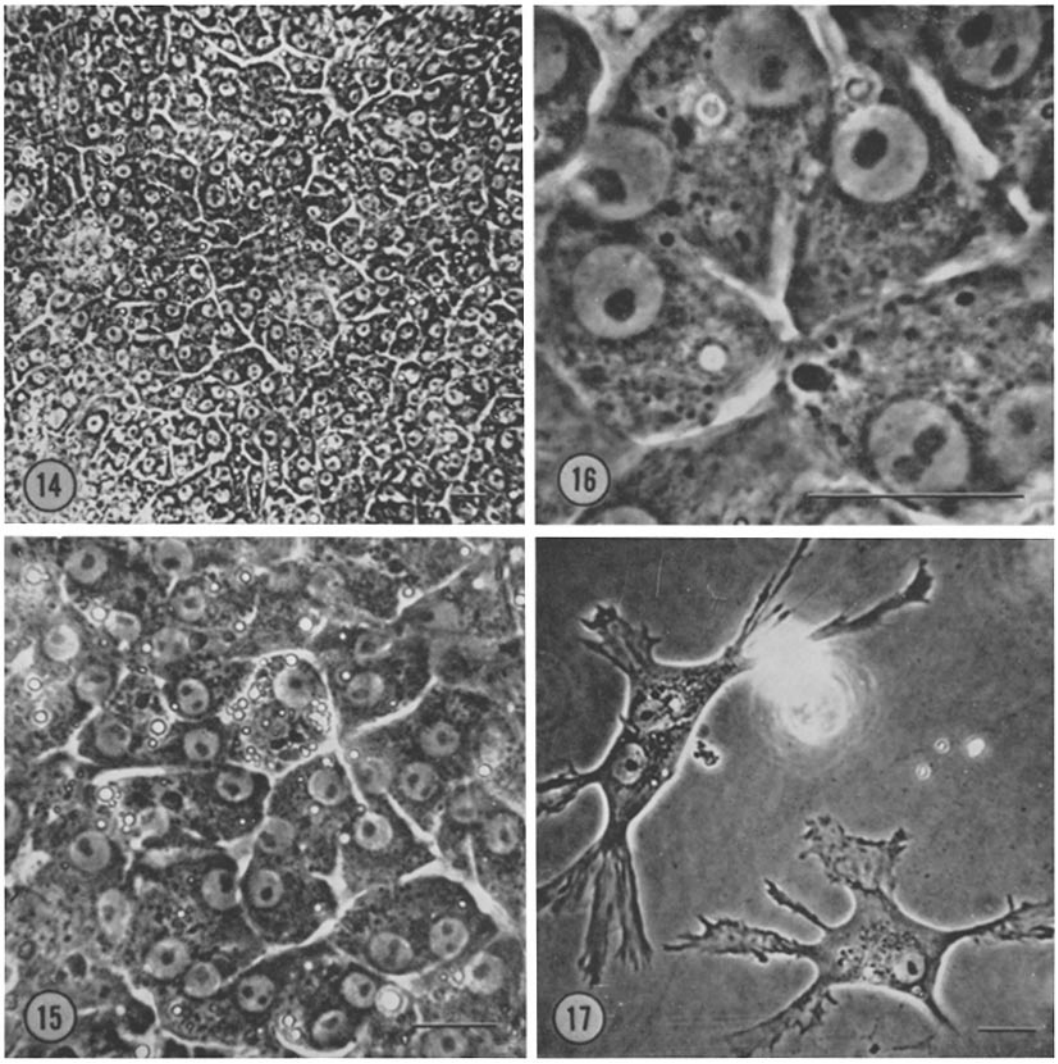
FIGURES 10 through 13 Thyroid gland of 14-day chick embryo cultured for 71 days in a circumfusion system. Scale mark = 20 μ.

FIGURE 10 Low-power view showing the explant (*ex*) on the left enclosed by a thin capsule (*Cp*) of stromal cells. The emigrating epithelium to the right of the capsule contained large black globules of PAS-positive colloid (*C*) and isolated cells with an isolated cytoplasm presumably Hürthle (*H*) cells. × 400.

FIGURE 11 The periphery of the epithelial outgrowth showing the colloid (*C*) and Hürthle cells (*H*). × 400.

FIGURE 12 A high-power view of the follicles (*Fl*) in the original explant surrounding colloid (*C*). × 1500.

FIGURE 13 A high-power view of the emigrating epithelium and the colloid (*C*) accumulation. × 1500.



FIGURES 14 through 17 Hepatocytes emigrating from a liver explant of a 14-day chick embryo after 13 days of cultivation in a circumfusion system. Scale mark = 20 μ.

FIGURE 14 A low-power view of the epithelium of hepatocytes, showing their typical round nuclei and angular architecture. The light areas indicate the position of Kupffer cells lying on the cellophane below the epithelium. × 250.

FIGURE 15 Medium-power view of the epithelium of hepatocytes. × 550.

FIGURE 16 High-power view of the epithelium of the hepatocytes. × 1450.

FIGURE 17 Medium-power view of hepatocytes which had wandered distal to the periphery of the epithelium. Their ruffled membranous extensions characterized hepatocytes dissociated from the main sheet of cells. × 375.

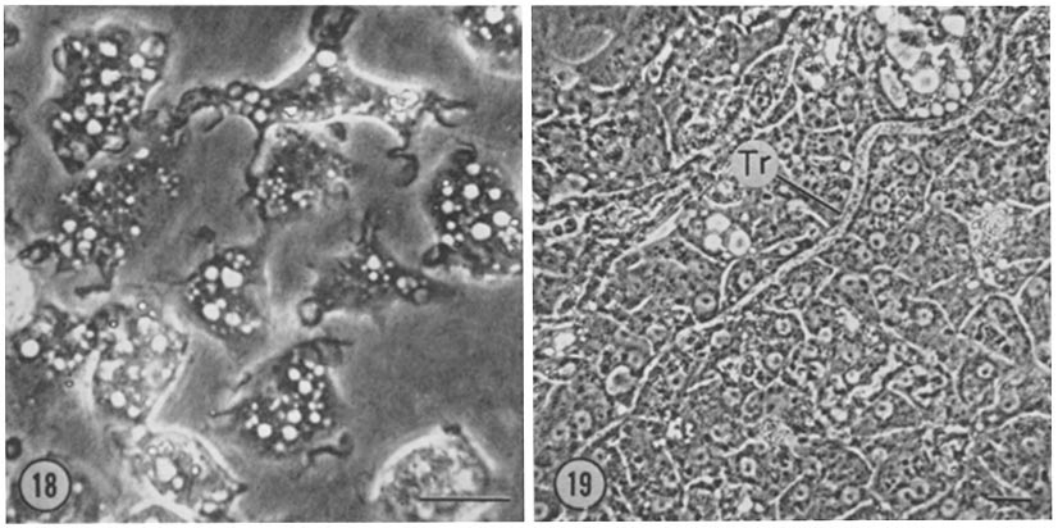


FIGURE 18 Medium-power view of Kupffer cells from a liver explant of a 14-day chick embryo cultivated 20 days in a circumfusion system. These cells accumulated on the cellophane below the hepatocytes on the glass cover slip. × 600. Scale mark = 20 μ.

FIGURE 19 Epithelium of hepatocytes after a 20-day period of cultivation in a cellophane sandwich of the circumfusion system. A tubule-like (*Tr*) structure is shown coursing through the epithelium. × 275. Scale mark = 20 μ.

after digestion with saliva (1 hr at 37°C) by the PAS method for mucopolysaccharides. The globules were found to stain a brilliant red, indicative, in this culture, of thyroid colloid. Conspicuous in the thyroid gland (Fig. 10) and emigrating cells (Fig. 11) were occasional cells which appeared to have undergone a solation of the cytoplasm. These cells were swollen with a clear cytoplasm so that the nucleus and juxtanuclear complex of granules were sharply evident. They appear to be somewhat analogous to Hürthle cells.

Liver

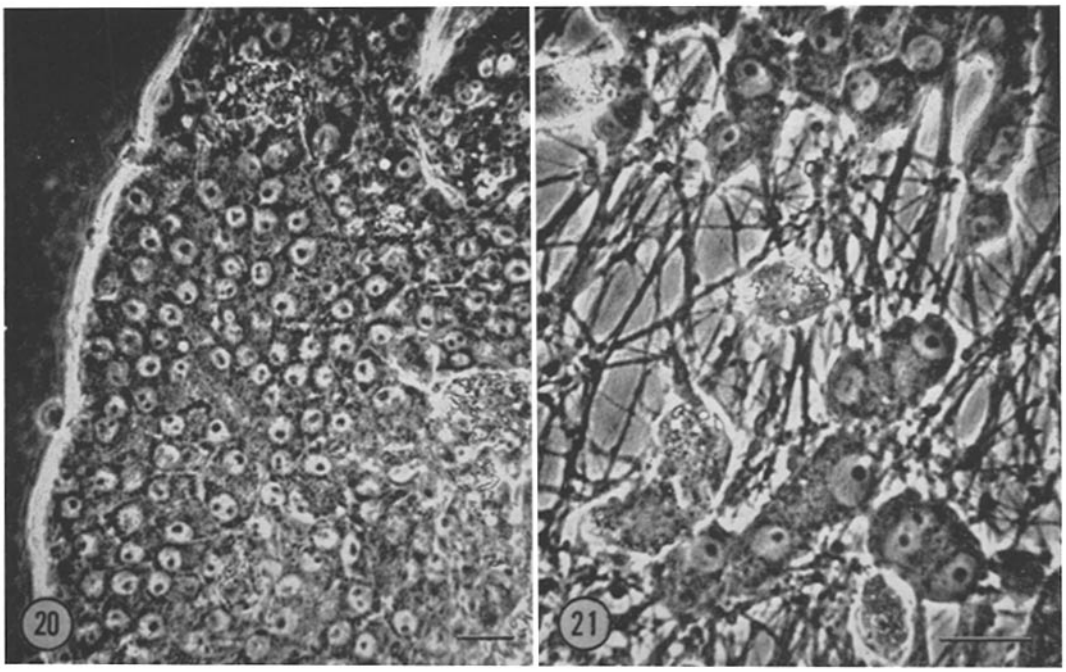
Hepatocytes emigrating from a fragment of liver after 13 days of cultivation are shown in Figs. 14 through 17. The liver explant is in the lower left corner of Fig. 14, and the irregular angular architecture of the emigrating liver cells with their characteristically round nuclei is shown at all magnifications. These liver cells grew out on the glass cover slip, but immediately below them and lying on the cellophane membrane was an abundance of Kupffer cells. The light areas in Fig. 14 indicate the position of the Kupffer cells on the cellophane below the epithelium. Escaping hepatocytes at the periphery of the epithelial sheets were characterized by long ruffled membrane extensions as were the two cells shown in Fig. 17. A view of the Kupffer cells lying on the cellophane is shown in Fig. 18. Their ruffled and curled membranes and phase-white droplets indicated a remarkable macrophagic activity of pinocytosis and phagocytosis. In Fig. 19, a sheet of hepatocytes cultivated for 20 days in a cellophane sandwich had a tubule-like structure coursing through it. Other areas of outgrowth had definite groupings or lobules of hepatocytes.

Adrenal Gland

The adrenal gland typically had compact aggregations of small cells with round nuclei and did not tend to flatten on the surface of the glass cover slip (Fig. 20), as did the liver cells. In some areas of the adrenal, fibers of the nervous system often extended between explants, and the adrenocytes frequently were found intermingled with them (Fig. 21).

Pancreas

The pancreas tissue is soft and assumed the shape of a flat, round mass under the cellophane membrane. Usually, the entire explant looked



FIGURES 20 and 21 The adrenal gland of a 14-day chick embryo after 71 days of cultivation in a circumfusion system. Scale mark = 20 μ.

FIGURE 20 Typical aggregates of adrenocytes compactly organized with characteristic round nuclei. × 375.

FIGURE 21 Adrenocytes intermingled with elements of the autonomic nervous system. × 600.

very much like a section through the original tissue (Fig. 22). This explant had been in cultivation for 47 days in a cellophane sandwich. In this type of preparation there was little emigration, and the peripheries of the explants were sharply demarcated by a thin encirclement of stromal cells. In Fig. 22, the border peripheral to the stromal cells was lined with macrophages, though in some explants globular masses of acinar cells were found external to the stromal cells. The glandular activity was characterized by large droplet accumulations from the acinar clusters (Fig. 23).

Kidney

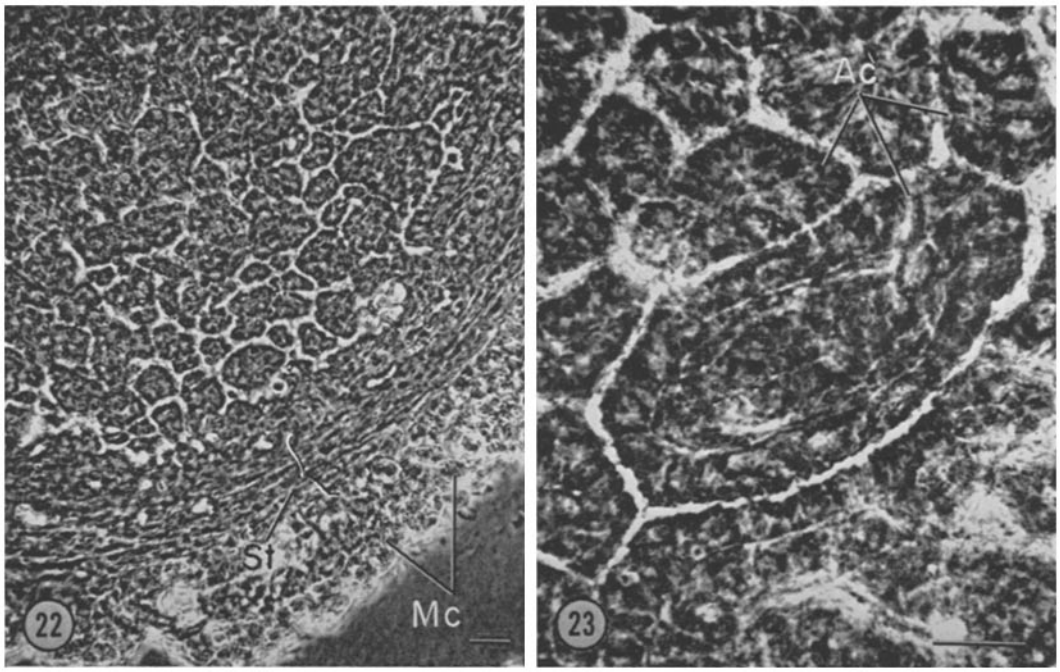
Kidney explants retained their tubules in the original explanted tissue. Frequently, these tubules contained secretory accumulations, and the tubular form changed very litle over a prolonged period of time. In Fig. 24, a portion of a kidney explant is shown after 12 days of cultivation and in Fig. 25 at higher power after 71 days of cultivation. Kidney explants in cellophane sandwiches differed in that frequently they became surrounded by an epithelium of columnar cells.

Male Gonad

The tissue of the male gonad occasionally had cystlike or tubular structures similar to those of the kidney and female gonad, as shown in Fig. 26. Typically, the cultivations were characterized by an interweaving network of epithelium in straplike groupings which persisted for a long time. In Fig. 27, such a portion of a male gonad explant is shown after 51 days of cultivation.

Female Gonad

The female gonad tissue differed from the male gonad in that the explants contained cystlike groupings (Fig. 28), as well as accumulations of round cells similar to those shown for the adrenal tissue in Fig. 20.



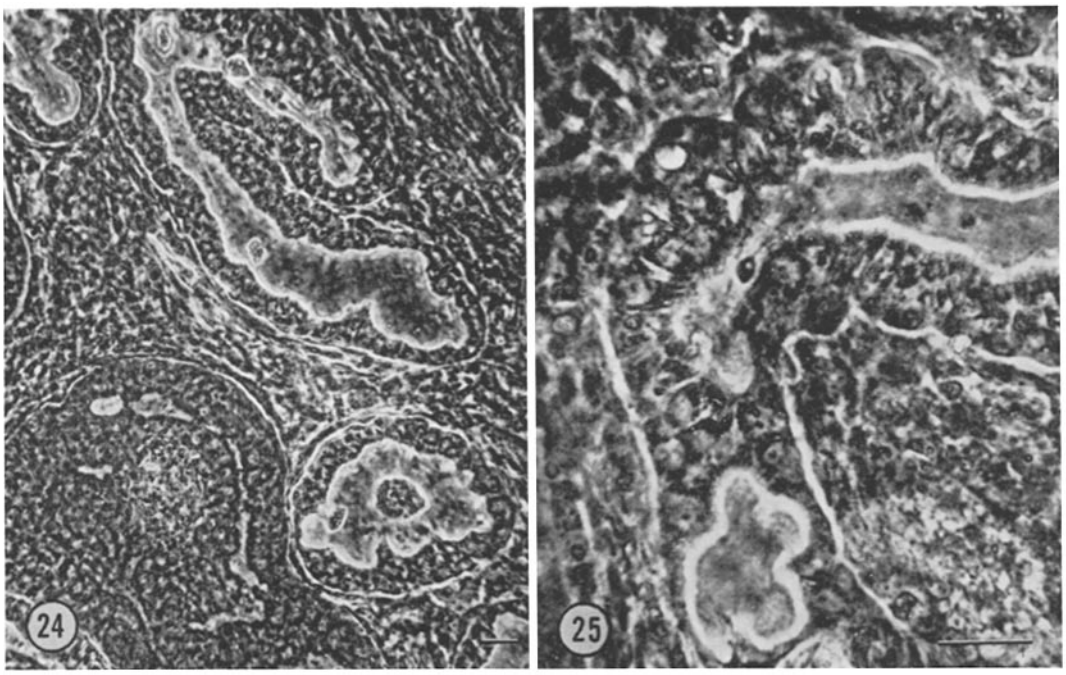
FIGURES 22 and 23 Pancreas tissue from 14-day chick embryos after 42 and 47 days of cultivation in cellophane sandwiches of a circumfusion system. Scale mark = 20 μ.

FIGURE 22 Low-power view showing approximately $\frac{1}{10}$th of an entire explant of the pancreas after 42 days of cultivation. Typically, the tissue was circumscribed by a thin-walled stromal capsule (*St*), and the acinar cells were arranged in lobules separated from each other by their secretory accumulations. External to the stromal capsules macrophages (*Mc*) often accumulated. × 275.

FIGURE 23 Medium-power view of acinar cell aggregates (*Ac*) after 47 days of cultivation. × 625.

DISCUSSION

More than half a century has elapsed since Burrows in 1912 (1) reported the first perfusion system for cultivated cells. His unit consisted of one culture chamber, two reservoirs for supplying and receiving the nutrient, pressurized air for moving the fluid, and paraffin for sealing the glass parts together. A teased cotton wick was used in the chamber to assist the fluid flow, and the entire unit was mounted on a rack so that the chamber could be observed with the microscope. 25 yr later, De Haan (2) published a report on the latest form of his perfusion method. In essence, it also consisted of two reservoirs and a culture chamber, as did Burrows' apparatus. De Haan, of course, had refined and expanded the flexibility of the components and had constructed a special incubator for the housing of three units. In 1951, Pomerat (3, 4) reduced the perfusion system to a compact single unit that would fit on the stage of the microscope for time-lapse motion picture studies. As in the earlier models, this system consisted of only one chamber but one that was very thin, so that the newer technique of phase-contrast microscopy could be used with it. Pomerat's chamber actually was composed of two glass cover slips separated by a thin wall of paraffin, and contained two special hand-drawn capillary tubes, the supply reservoir being simply a piece of glass tubing with one end pulled thin and bent to a right angle. The capillary portions were paraffined between the two glass cover slips, and the supply tube was vertical to contain a few milliliters of nutrient which slowly flowed through the chamber. The assembly was mounted with paraffin onto an aluminum metal rack which was placed on the stage of the microscope. This was a very practical system for short-term observations of cells with phase-contrast



FIGURES 24 and 25 Kidney tissue from 14-day chick embryos after 12 and 71 days of cultivation in circumfusion systems. Scale mark = 20 μ.

FIGURE 24 Low-power view showing tubule-like structures in the kidney explants after 12 days of cultivation. × 225.

FIGURE 25 High-power view showing tubule-like structures in a kidney explant after 71 days of cultivation. × 625.

microscopy, but no provision was made for the maintenance of sterility, so cultures seldom survived more than 24 to 36 hr.

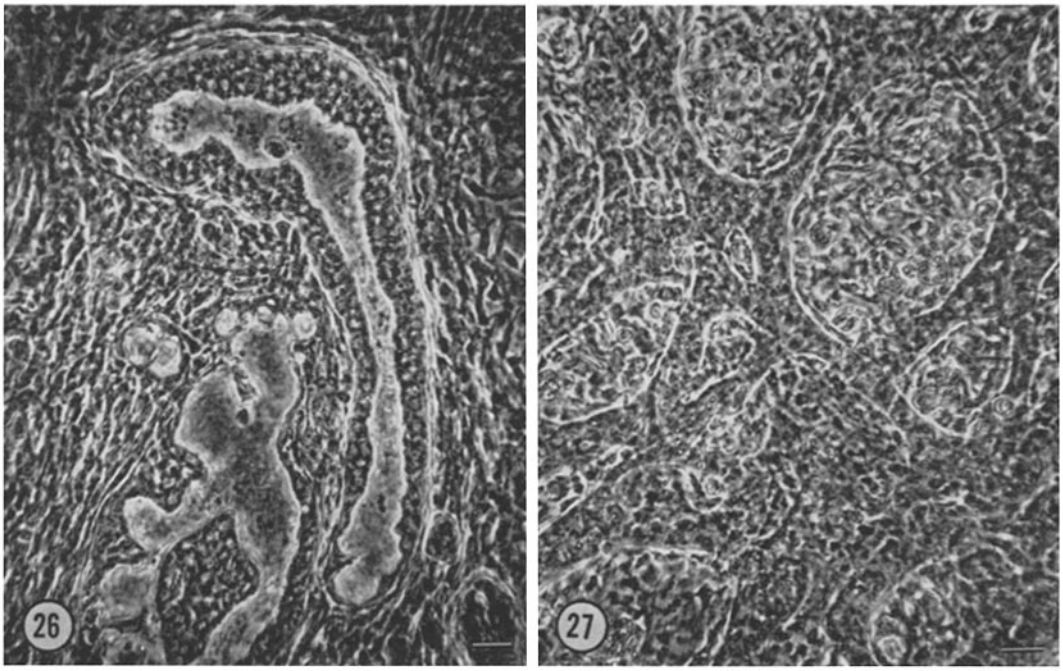
Fortified by Pomerat's success, a number of special chambers amenable to phase-contrast microscopy but considerably more stable and with the advantage of sterile maintenance were constructed. In 1953, Christiansen et al. (5) milled a ¼-inch acrylic plastic sheet, drilled holes in the side, and used two cover glasses spaced approximately 1 mm apart, so that the phase-contrast microscope could be used. They were able to cultivate cells for as long as 3 days with this device and studied the effects of cyanide and other enzyme inhibitors. In 1954, Buchsbaum and Kuntz (6) demonstrated a remarkable advance in chamber, gas control, and perfusion techniques. Here again, the mechanism of the system was unidirectional, in that the perfusate flowed from a supply to a receiving reservoir. In 1955, Dick (7) modified the acrylic chamber design of Christiansen et al. and used a technique of syphoning or sucking rather than forcing the fluid through the chamber. His work was designed primarily for studies of only a few hours, as he did not make provisions for sterility.

Richter and Woodward (8) produced a perfusion chamber designed to receive 12 × 50 mm cover slips containing explants which had been started in roller tubes. In principle, this was similar to the multipurpose culture chamber, except that a long slot was used in place of the circular hole in the gasket. They used a continuous perfusion by an electrically driven peristaltic pump action at a flow rate of 0.2 ml/hr to 8 ml/min. Other simple perfusion chambers based on predecessors were reported by Leinfelder and Danes (9), Officer (10), Paul (11), Toy and Bardawil (12), Constable and Moffat (13), and Cruickshank et al. (14). Probably the most sophisticated of all the

single-flow perfusion systems was the one reported by Schwartz (15). This was an elaborate system, which used a chamber similar to Buchsbaum's, with a special support for the lens of the eye, as used earlier by Bakker (31). This continuous and unidirectional flow system was highly controlled by properly gassing the nutrient and metering the perfusate before and after its passage through the chamber. pH meters, Redox meters, and temperature meters with ingenious switching devices were used. Also, a pressure transducer was attached to the lens chamber by a sidearm, and pressure could be controlled by a needle valve placed in the outflow side.

Morgan and Dawe (16) adapted a multipurpose culture chamber in a unidirectional-flow perfusion device. They used polyethylene tubing and metallic needles inserted through the gasket of the chamber. They also used a large gas bubble in the chamber's vault and attempted to control this by a communication with a gas bottle. They maintained HeLa cells continuously for 5 months in such a chamber, and salivary gland rudiments of 13- to 14-day mouse embryos up to 30 days.

More recently, Kruse et al. (18) reported on replicate culture flask techniques; i.e., a series of stoppered T-flasks were fitted with influent and affluent gas and nutrient solution lines. Gas tensions and composition were controlled with a flow rate meter and a system of pinch clamps. The gravity flow of the culture medium was regulated by a motorized clamp, and proliferation curves for 8 days of cultivation of Jensen sarcoma cells of the rat were calculated. Using the same perfusion system for T-60 flasks and 8- or 9-day perfusion periods, Kruse and Miedema (19) showed different patterns of glutamic acid, proline, and glycine utilization in dense versus dilute cell populations.



FIGURES 26 and 27 Male gonad tissue from 14-day chick embryos after 26 and 51 days of cultivation in circumfusion systems. Scale mark = 20 μ.

FIGURE 26 Low-power view of cystlike and tubule-like structures found in a male gonadal tissue explant after 26 days of cultivation. × 250.

FIGURE 27 Low-power view of typical straplike and interdigitating cords of epithelium in a 51-day cultivation of male gonadal tissue. × 250.

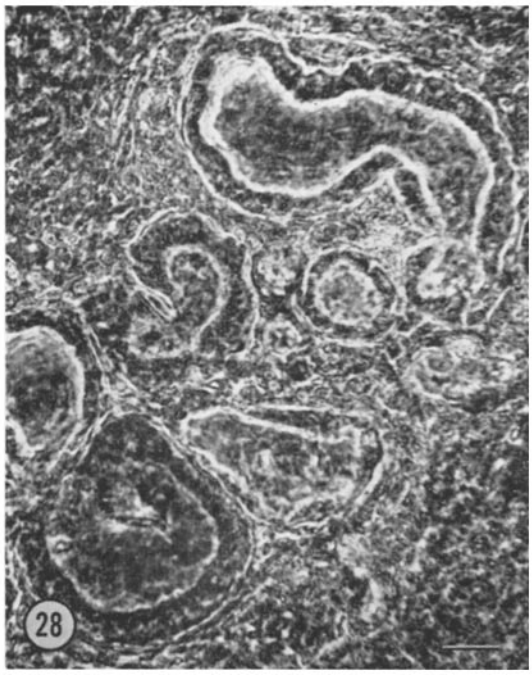


FIGURE 28 Low-power view of female gonadal tissue from a 14-day chick embryo after 75 days of cultivation in a circumfusion unit. Typical cystlike groupings of epithelial cells are shown. × 375. Scale mark = 20 μ.

They also indicated that the role of contact inhibition was diminished in perfusion system environments.

Finally, White (20) and Thomas and Cramer (21) demonstrated at a recent Tissue Culture Association Meeting (1965) still other techniques for perfusion.

The preceding review has covered most of the work with tissue culture perfusion since Burrows' conception up through the 1965 Tissue Culture Association Meeting, with respect to systems which contain supply and receiving reservoirs or their equivalents, and in which the movement of fluid through a chamber or multiple units of flasks is in one direction for one passage across the cell populations. All these systems permitted some visual observation either with bright-field or phase-contrast microscopy.

In the literature survey, only 3 units were found which involved a recycling of the nutrient or a circulatory system. The first of these, introduced by De Haan in 1928 (22), was an apparatus which made possible the circulation of large volumes of liquid, but this apparently was quite complicated, as the apparatus never appeared again. In 1939, Lindberg (23) published a technique on the circulation of large quantities of fluid through a two-chamber culture flask. This was an ingenious device, utilizing gas pressure as the driving force, and the nutrient fluid was moved from one vault to the next and back again, depending upon the gas pressure and the fluid levels used. The rate was governed somewhat by the size of the grains of sand in the glass tube connecting the two flasks. By this method, however, the cells could not be observed under the microscope. McCoy et al. (17) developed what they called a glass helix perfusion system for the massive growth of cells in vitro and they provided an alternate path for the fluid which permitted its recycling. Their method, like Lindberg's, was for massive cultivation and did not provide a means for microscope observation of cells.

Of all the perfusion systems, the most unique is found in a book by Carrel and Lindberg published in 1938 (24). Whole organs were cultured in glass vessels and the fluid nutrient was injected directly into the arteries of the tissues. Of course, the growth of the cells was in the organs, so that microscope observations could not be made, but sectioned material revealed remarkable preservation of the organ architecture. This technique has been revived as recently reported by Folkman et al. (48).

The technique for multipurpose culture chambers using nonperforated cellophane over the explants as a means of cultivation with an increased preservation of tissue and organ differentiation was reported in 1958 (29). The in vivo systems from which these tissues and organs were obtained possessed circulatory, respiratory, and pH control. The cellophane technique for multipurpose culture chambers had only intermittent fluid exchanges and respiratory refreshment, and the pH stability was limited to the buffers in the nutrient. The advantages with nonperforated cellophane in these chambers appeared to reside in the prevention of (*a*) the washing-out of cell products (particularly proteins) from the culture environment, and (*b*) the entrance of large molecules (serum proteins) of the nutrient into the culture environment. Additionally, and perhaps of equal importance, was the compression of the tissues effected by the closely apposing cellophane.

The basic idea for extending that which the cellophane technique had already provided was to include the three systems they omitted, viz., circulatory, respiratory, and pH control. The circum-

fusion system detailed in this report was built around these refinements for the cellophane chamber technique and, as demonstrated by the results shown, has proved to be effective in achieving a more perfect environment for the enhancement and longevity of chick embryo tissue and organ differentiation. In developing the circumfusion system for the multipurpose culture chambers, then, the following four objectives conjointly became a common goal: (*a*) to find mechanically usable materials that would not themselves interfere with the cultivation process, i.e., be toxic to the in vitro system; (*b*) to find an arrangement for an all inclusive multichamber unit that was both compact and functionally superior to unconnected culture chambers with respect to tissue performance (differentiation); (*c*) to develop a reliable instrument that could be used continuously for many months, without the possibility that one system failure would seriously affect the circumfusion unit; and (*d*) to develop a unit that would be practical and useful to other workers and, especially, that would be widely applicable as a complementary technique for the many existing types of investigations of cells in vitro. The present instrument approaches a fulfillment of these objectives, although it is expected that refinements will be suggested, developed, and incorporated into the units from time to time. It is no secret that the circumfusion system is made from many nonbiological components, which, although they may appear to be nontoxic, may have insidious, though profound, effects on the living tissues and organs they support. Efforts will, therefore, always be made to upgrade the performance of this instrument.

In this report, emphasis has been placed on the mechanics and techniques of the circumfusion system, and only a brief summary of tissue performance was given. There are a great many tissues now being evaluated. However, at this time, it was thought most important to prove the system by a full disclosure of its mechanics and only a synopsis of how the original cellophane techniques for multipurpose culture chambers have been extended by an enhancement of the culture environment. Future reports will deal in greater detail with the results and responses of the tissues and organs cultivated in this system.

Perfusion by all of the previous techniques cited has involved moving fluids across, around, and against the cultivated cells and tissues. Trowell (26), working with mature organ cultures, found that neither circulation of the medium nor a more complete medium including serum and embryonic extract improved the length of survival time of his cultures. In fact, he found it necessary to use a protein-free fluid nutrient as a means of discouraging growth, dedifferentiation, and cell emigration. Trowell (32) also reported that for a 9-day period better results were obtained by reconditioning the medium every 3 days than by changing it. The circumfusion system reported herein coincides with Trowell's observations, in that the protein is screened from the culture environment by the cellophane membrane, and the fluid nutrient is only partially exchanged (therefore reconditioned) semiweekly.

In the introduction to his recent 3-volume treatise, *Cells and Tissues in Culture* (33), Willmer stated that all perfusion systems have had an inherent defect, "namely that of leaching out important constituents from the tissues being perfused." Wilde (34) also has pointed out the loss of differentiation by cells of long-term passage and has attributed it to the washing away of slowly synthesized extracellular material (ECM) each time the nutrient is changed. The cellophane obviously inhibits this defect to a great extent, and the enhanced appearance and performance of the tissues and organs obtained by this technique supports the "leaching out" hypothesis. Moreover, the cellophane's compressive effect, as stated before, has an unquestionable effect on differentiation. Grobstein (35) has said that ". . . general culture environments which preserve the integrity of relatively complex populations of cells have favored differentiation, whereas environments and factors which disperse and simplify the relations within such populations have tended to inhibit differentiation." Wild's report (34) also makes it clear that cellular differentiation in his studies was enhanced in situations where cells were contiguous masses. In other words, the cellophane keeps the tissue and organ explants compactly confined and enhances the homotypic factors. However, if the cellophane has a wrinkle and the explant is lodged in this relaxed area rather than in a compressed area, differentiation is not nearly so well defined. In such areas, the explant becomes a ball-like structure. A few fibroblasts may wander out onto the glass from it, but ultimately it shrinks in size, presumably because of a contraction of the encircling fibroblasts and/or their produced collagen. Therefore, it is thought

that the compressive effect of cellophane produced on tissues washed in protein-free nutrient or balanced salt solution and fed a serum (20%) supplemented nutrient on the opposite side of the cellophane is in itself a measurable contribution to differentiation. The restriction to the microenvironment by the cellophane and the compression of the cellophane imposed by the osmotic effect of the serum in the nutrient appear to be additive, perhaps even synergistic. Additionally, the circumfusion system with its multiple units effectively establishes a favorable means for an interchange of heterotypic factors which unquestionably affect tissue and organ differentiation (35) as well.

The most important additions of the circumfusion system to the conditions existing in unattached chambers are, of course, the circulatory, respiratory, and pH control mechanisms. Although none of these can be singled out as being more important than the others, the respiratory and pH control are largely dependent on the circulatory control. As noted earlier, the atmospheric oxygen diffuses through the Teflon tubes, rendering a high and uniformly dissolved oxygen concentration in the rapidly moving nutrient coming into the chambers. In unattached chambers, the pH in the immediate explant environment may fall to lethal levels, particularly in the center of the explant. This leaves a pH gradient in the control chambers on the shelves in the incubator, whereas in attached chambers of the circumfusion system, the pH is stabilized throughout each chamber by the rapidly moving fluid nutrient. Additionally, the circulatory system provides a more even distribution of the basic nutrients and maintains a smoother temperature control. Unquestionably, these three additions conjointly favor tissue differentiation, as characterized by the retention of organotypic morphology and function observed in the cultures.

Many possibilities for experimentation of differentiated tissue now exist, but it is hoped that the circumfusion system for multipurpose culture chambers will permit new depth to the old procedures and provide an increased breadth to the newer ones. Effects of nutrient-soluble components, such as drugs, hormones, vitamins, and amino acids, as well as gas requirements and pH, can now be tested not only for cell strains but for multiple units of confronting differentiated cell populations as well. Cytological phenomena, such as those formerly studied in the unattached chambers (pinocytosis, references 36, 37; zeiosis, references 38–41; nuclear rotation, references 30, 42; intranuclear inclusion formation references 42–44; nucleolar secretions and changes, references 30, 42; and activities of the Golgi complex, references 39, 40, 42, 45), will be reviewed by time-lapse techniques and supplemented with analyses of the cellular fine structures in the enhanced environments of the circumfusion system. Moreover, this procedure should offer a greater possibility for eliciting a redifferentiation response by neoplasms and cell strains in vitro, if there is one (30, 39, 46, 47), and interactions between neoplastic and nonneoplastic cell populations.

The development of this circumfusion system has not been a one-man project but has involved the technical assistance and thought of all members of these laboratories. Therefore, I should like to acknowledge the valuable assistance of my coworkers, M. K. Peterson, Y. Matthias, B. J. Trammell, and S. Muckleroy. Special appreciation is due R. J. Matthias for his engineering skill and willingness to overcome the many obstacles that have been encountered along the way. Encouragement for a useful system of this type was first initiated at least ten years ago by my teacher, C. M. Pomerat. Although the present fulfillment of this idea is late for him, I am grafeful for his prodding and lingering suggestion. Lastly, I would like to acknowledge the interest and stimulation given me by my friend and pathologist, Dr. John Shively, of this Institution. He, in particular, has stressed the urgent need for in vitro hematopoietic systems as they may be related to clinical studies.

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