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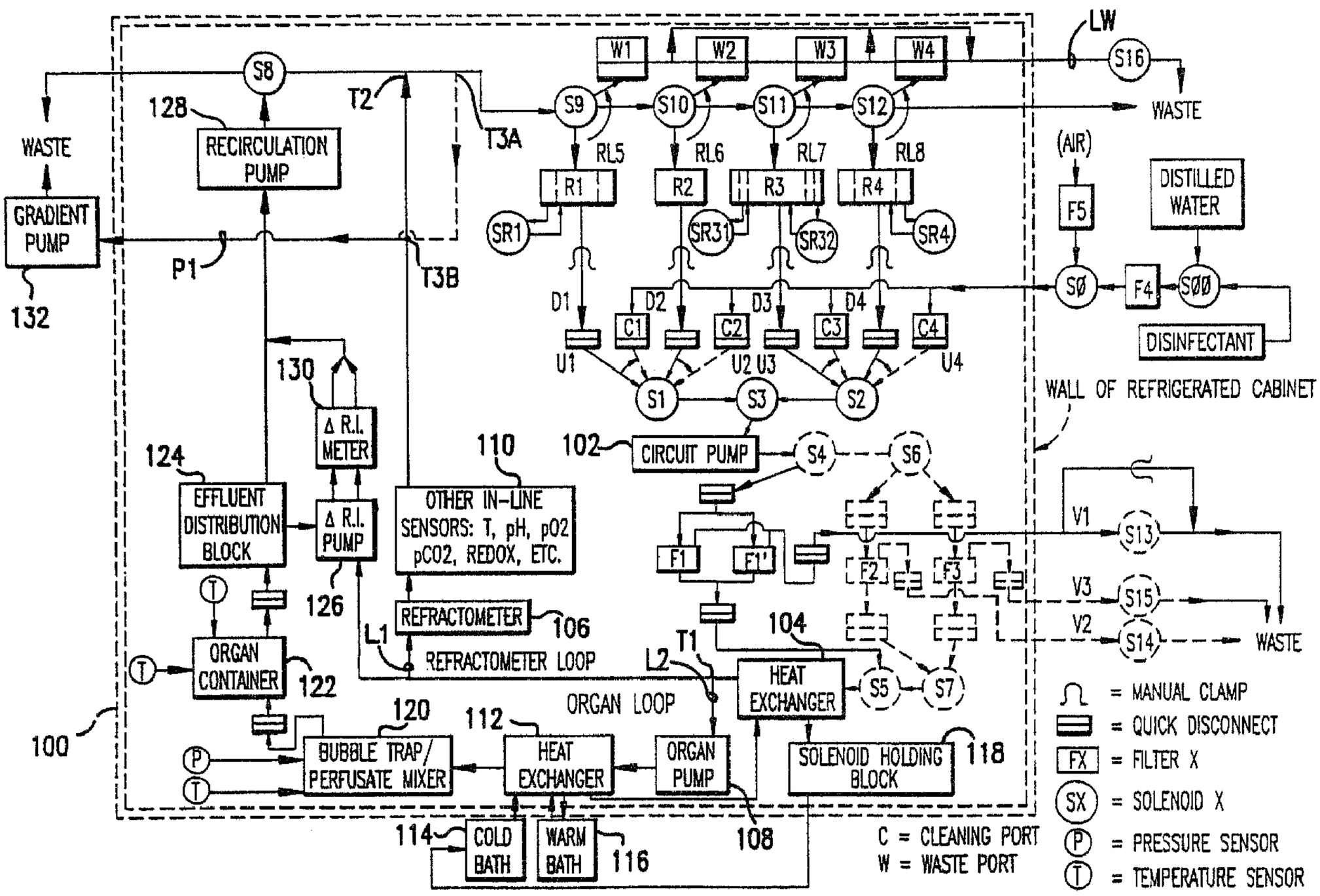
(54) Titre : APPAREIL ET METHODE DE PERFUSION DE CRYOPROTECTEUR ASSISTEE PAR ORDINATEUR
(54) Title: COMPUTER CONTROLLED CRYOPROTECTANT PERFUSION APPARATUS AND METHOD



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(54) Title: COMPUTER CONTROLLED CRYOPROTECTANT PERFUSION APPARATUS AND METHOD

(57) Abstract

A computer-controlled apparatus and method for perfusing a biological organ, such as a heart, kidney, liver, etc. The apparatus comprises a plurality of fluid reservoirs and an organ container for holding the biological organ. A first fluid flow path is defined as a loop from the plurality of reservoirs to necessary sensors and temperature conditioning means and back to the plurality of reservoirs. The reservoirs are selectively connectable to the first fluid flow path. Pump means are interposed in the first fluid flow path for pumping fluid from the first fluid flow path to a second fluid flow path. The organ container is located in this second fluid flow path. Pump means may also be included in the second fluid flow path for pumping fluid from the organ container to one or more of the reservoirs or to waste. One or more sensors are interposed in the fluid flow paths for sensing at least one of the concentration, temperature, pH, and pressure of the fluid flowing in the first and second fluid flow paths. Measuring means are interposed in the first and second fluid flow paths for measuring concentration and temperature differences between the upstream and downstream sides, in the fluid flow direction, of the organ container. The sensor(s) and the measuring means are connected to a programmable computer for providing a continuous information stream from the sensor(s) to the computer. The computer is coupled to the selection means and the pump means to continuously selectively control (a) the flow of fluid from each of the reservoirs individually to the fluid flow paths, and (b) at least one of the concentration, temperature, pressure and pH of the fluid flowing in the second fluid flow path, in accordance with a predetermined computer program without operator intervention.

COMPUTER CONTROLLED CRYOPROTECTANT PERFUSION APPARATUS AND METHOD

Field of the Invention

This invention relates to the field of organ perfusion. More particularly, it relates to a computer controlled apparatus and method for perfusing isolated animal, and more specifically, human, organs. Still more particularly, this invention relates to an apparatus and method for introducing and removing vitrifiable concentrations of cryoprotective agents into and from isolated organs or tissues for preservation and subsequent use.

Background of the Invention

Cryopreservation (that is, preservation at very low temperatures) of organs would allow organ banks to be established for transplant surgeons in much the same way blood banks serve the medical community today. The main difficulty with cryopreservation is that it requires the perfusion of organs with high concentrations of cryoprotective agents (water soluble organic molecules that minimize or prevent freezing injury during cooling to very low temperatures). No fully suitable equipment or process has been developed to date for carrying out this perfusion process. This has prevented the establishment of viable organ banks that could potentially save lives.

Devices and methods for perfusing organs with cryoprotectant have been described in the literature since the early 1970's. See, Pegg, D.E., Banking of Cells, Tissues, and Organs at Low Temperatures, *Current*

Trends in Cryobiology, A.U. Smith, Editor, Plenum Press, New York, 1970: pp. 153-180, but particularly pages 175-177; and Pegg, D.E., Perfusion of Rabbit Kidneys with Cryoprotective Agents, *Cryobiology 9*:411-419, 1972).

In the apparatus initially described by Pegg, two perfusion circuits operated simultaneously, one with and one without cryoprotectant. Cryoprotectant was introduced and removed by abruptly switching from the cryoprotectant-free circuit to the cryoprotectant-containing circuit, then back again. The pressure was controlled by undescribed techniques, and data was fed into a data logger which provided a paper tape output which was processed by a programmable desk-top Wang calculator. The experimental results were poor. The equipment and technique described were considered inadequate by Pegg and his colleagues, who later modified them considerably.

In 1973, G.J. Sherwood and J.R. Flower, in: *Organ Preservation* (D.E. Pegg, editor, Churchill Livingstone, London, 1973, pp. 152-174), described four potential perfusion systems, none of which are known to have been built. The first system consisted of a family of reservoirs connected directly to the organ via a multiway valve, changes being made in steps simply by switching from one reservoir to another.

The second system created changes in concentration by metering flow from a diluent reservoir and from a cryoprotectant concentrate reservoir into a mixing chamber and then to the kidney. No separate pump for controlling flow to the kidney was included. Total flow was controlled by the output of the metering pumps used for mixing. A heat exchanger was used before rather than after the filter, and there was an absence of any arterial sensing. As will become readily apparent below, the only similarity between this system and the present invention was the use of two concentration sensors, one in the arterial line and one in the venous line of the kidney. Organ flow rate was forced to vary in order to minimize A-V concentration differences. The sensing of concentration before and after the kidney in the circuit is analogous to but substantially

inferior to the use of a refractometer and a differential refractometer in the present invention. The present inventors' experience has shown that the use of a differential refractometer is necessary for its greater sensitivity. The concept of controlling organ A-V gradient by controlling organ flow is distinctly inferior to the system of the present invention.

The third system described by Sherwood *et al.* also lacked a kidney perfusion pump, relying on a "backpressure control valve" to recirculate perfusate from the filter in such a way as to maintain the desired perfusion pressure to the kidney. As with the second Sherwood system, the heat exchanger is proximal to the filter and no bubble trap is present. The perfusate reservoir's concentration is controlled by metered addition of cryoprotectant or diluent as in the second Sherwood system, and if flow from the organ is not recirculated, major problems arise in maintaining and controlling perfusate volume and concentration. None of these features is desirable.

The fourth system was noted by Pegg in an appendix to the main paper. In this system, perfusate is drained by gravity directly from the mixing reservoir to the kidney through a heat exchanger, re-entering the reservoir after passing through the kidney. Concentration is sensed also by directly and separately pumping liquid from the reservoir to the refractometer and back.

Modifications and additional details were reported in 1977 (Pegg, D.E., and Wusteman, M.T., Perfusion of Rabbit Kidneys with Glycerol Solutions at 5°C). The apparatus used one mixing reservoir and one reservoir for adding glycerol concentrate or glycerol-free perfusate to the mixing reservoir to control concentration. The volume of the mixing reservoir was held constant during perfusion, necessitating an exponentially increasing rate of diluent addition during cryoprotectant washout to maintain a linear rate of concentration change. The constant mixing reservoir volume and the presence of only a single delivery reservoir also made it impossible to abruptly change perfusate

concentration. All components of the circuit other than the kidney and a pre-kidney heat exchanger were located on a lab bench at ambient temperature, with the reservoir being thermostatted at a constant 30°C. The kidney and the heat exchanger were located in a styrofoam box whose internal temperature was not controlled. Despite this lack of control of the air temperature surrounding the kidney, only the arterial temperature but not the venous temperature or even the kidney surface temperature was measured. The use of a styrofoam box also did not allow for perfusion under sterile conditions. The only possible way of measuring organ flow rate was by switching off the effluent recirculation pump and manually recording the time required for a given volume of fluid to accumulate in the effluent reservoir, since there was no perfusion pump which specifically supplied the organ, unlike the present invention. Pressure was controlled, not on the basis of kidney resistance, but on the basis of the combined resistance of the kidney and a manually adjustable bypass valve used to allow rapid circulation of perfusate through the heat exchanger and back to the mixing reservoir. The pressure sensor was located at the arterial cannula, creating a fluid dead space requiring manual cleaning and potentially introducing undesired addition of unmixed dead space fluid into the arterial cannula. Pressure control was achieved by means of a specially-fabricated pressure control unit whose electrical circuit was described in an earlier paper (D.E. Pegg and C.J. Green, Renal Preservation by Hypothermic Perfusion. 1. The importance of pressure-control, *Cryobiology 10*:56-66, 1973). Arterial concentration but not venous concentration was measured. No computer control or monitoring was used. Concentration was controlled by feeding the output of the recording refractometer into a "process controller" for comparison to the output of a linear voltage ramp generator and appropriate adjustment of concentrate or diluent flow rate. Glycerol concentrations were measured manually at 5 minute intervals at both the mixing reservoir and the arterial sample port: evidently, the refractometer was not used to

send a measurable signal to a recording device. Temperature and flow were recorded manually at 5 minute intervals. Arterial pressure and kidney weight were recorded as pen traces on a strip chart recorder. None of these features is desirable.

Further refinements were reported by Jacobsen, I.A., Pegg, D.E., Wusteman, M.C., and Robinson, S.M., Transplantation of Rabbit Kidneys Perfused with Glycerol Solutions at 10°C, *Cryobiology 15*:18-26, 1978. A bubble trap was added, the sample port on the kidney bypass was eliminated (concentration was measured at the distal end of the bypass line instead), and temperature was recorded as a trace on a strip chart recorder rather than manually every 5 minutes. Additionally, these authors reported that bypass concentration lagged reservoir concentration by 5 min (v. 3 min or less for arterial concentration in the present invention) and that terminal cryoprotectant concentration could not be brought to less than 70 mM after adding 5 liters of diluent to the mixing reservoir (v. near-zero terminal concentrations in the present invention using less than 3 liters of diluent and using peak cryoprotectant concentrations approximately twice those of Jacobsen et al.).

A variation on the system was also reported the same year by Jacobsen (Jacobsen, I.A., Distribution and Removal of Glycerol by Vascular Albumin Perfusion in Rabbit Kidneys, *Cryobiology 15*:302-311, 1978). Jacobsen measured but did not report air temperatures surrounding the kidney during perfusion. He reduced the mixing reservoir volume to 70 ml, which was a small fraction of the 400 ml total volume of the circuit. No electronic-output refractometer appears to have been used to directly sense glycerol concentration and control addition and washout. Instead, the calculated values of concentrate or diluent flow rate were drawn on paper with India ink and read by a Leeds and Northrup Trendtrak Programmer which then controlled the concentrate/diluent pump. Despite the low circuit volume, the minimum concentration of cryoprotectant which could be achieved was about 100 mM.

Additional alterations of the same system were reported by Armitage et al. in 1981 (W.J. Armitage, G. Matthes, and D.E. Pegg, Seleno-dl-methionine Reduces Freezing Injury in Hearts Protected with Ethanediol, *Cryobiology 18*:370-377, 1981). Essentially, the entire perfusion circuit previously used was placed into a refrigerated cabinet. Instead of a voltage ramp controller, a cam-follower was used. Again, however, it was necessary to calculate the required rates of addition of glycerol or diluent using theoretical equations in order to cut the cam properly, an approach which may introduce errors in the actual achievement of the desired concentration-time histories. Finally, a modification was made in which an additional reservoir was added to the circuit. This reservoir was apparently accessed by manual stopcocks (the mode of switching to and from this reservoir was not clearly explained), and use of the new reservoir was at the expense of being able to filter the perfusate or send it through a bubble trap. The new reservoir was not used to change cryoprotectant concentration; rather, it was used to change the ionic composition of the medium after the cryoprotectant had been added. The volume of the mixing reservoir was set at 500 ml, allowing a final cryoprotectant concentration of 40 mM to be achieved.

The circuits described above represent the current state of the art of cryoprotectant perfusion by others known to the present inventors.

An approach to organ preservation at cryogenic temperatures previously described by the present inventors involved vitrifying rather than freezing organs during cooling. **Vitrification,** or solidification without freezing, can be brought about in living systems by replacing large fractions of water in these systems with cryoprotectant agents (also known as **cryoprotectants**) whose presence inhibits crystallization. In known techniques, however, it has never been possible to use sufficiently high cryoprotectant concentrations without killing the organ. Vitrification typically requires concentrations greater than 6 molar cryoprotectant,

whereas the limiting concentration for organ survival is typically about 4 molar.

One type of damage potentially caused by cryoprotectants is osmotic damage. Cryobiologists learned of the osmotic effects of cryoprotectants in the 1950's and of the necessity of controlling these effects so as to prevent unnecessary damage during the addition and removal of cryoprotectants to isolated cells and tissues. Similar lessons were learned when cryobiologists moved on to studies of whole organ perfusion with cryoprotectants. Attention to the principles of osmosis were essential to inducing tolerance to cryoprotectant addition to organs. Yet despite efforts to control deleterious osmotic effects of cryoprotectants, limits of tolerance to cryoprotectants are still observed. There appear to be genuine, inherent toxic effects of cryoprotectants that are independent of the transient osmotic effects of these chemical agents.

Studies by the present inventors and others have examined methods of controlling the non-osmotic, inherent toxicity of cryoprotective agents. The results indicate that several techniques can be effective alone and in combination. These include (a) exposure to the highest concentrations at reduced temperatures; (b) the use of specific combinations of cryoprotectants whose effects cancel each other's toxicities; (c) exposure to cryoprotectants in carrier solutions that are optimized for those particular cryoprotectants; (d) the use of non-penetrating agents that can substitute for a portion of the penetrating agent otherwise needed, thus sparing the cellular interior from exposure to additional intracellular agent; and (e) minimization of the time spent within the concentration range of rapid time-dependent toxicity. Means by which these principles could be applied to whole organs so as to permit them to be treated with vitrifiable solutions without perishing, however, have not been clear or available.

Some of these techniques are in potential conflict with the need to control osmotic forces. For example, reduced temperatures also reduce

the influx and efflux rate of cryoprotectants, thereby prolonging and intensifying their osmotic effects. Similarly, minimizing exposure time to cryoprotectants maximizes their potential osmotic effects. Thus, there must be a balance reached between the control of osmotic damage and the control of toxicity. Adequate means for obtaining this balance have not been described in the literature. It is also true that, in some cases, intensifying osmotic effects of cryoprotectants by minimizing exposure times to these agents can be beneficial and complementary to the reduced toxicity that results, but safe means for achieving this in whole organs have not been described.

Organ preservation at cryogenic temperatures would permit wastage of valuable human organs to be considerably reduced and would facilitate better matching of donor and recipient, a factor which continues to be important despite the many recent advances in controlling rejection. See, Takiff, H., *et al.*, *Transplantation 47*:102-105 (1989); Gilks, W.R., *et al.*, *Transplantation 43*:669-674 (1987). A recent approach to the induction of tolerance to transplanted organs requires 10-200 days for the host immune system to be "re-educated" to accept the graft as "self", a time that can only be attained by being able to cryopreserve the cadaver organ See, Posselt, A.M., *et al.*, *Science 249*:1293-1295 (1990); Remuzzi, G., *et al.*, *The Lancet 337*:750-752 (1991).

One major limitation in organ cryopreservation studies has been the lack of suitable equipment for controlling perfusion parameters such as cryoprotectant concentration-time history, pressure, and temperature. Previously described standard perfusion machines are not designed for this application and are unable to meet the requirements addressed here. Patented techniques heretofore known are described in:

U.S. Patent No. 3,753,865 to Belzer *et al.*

U.S. Patent No. 3,772,153 to De Roissart *et al.*

U.S. Patent No. 3,843,455 to Bier

U.S. Patent No. 3,892,628 to Thorne, G.H., *et al.*

U.S. Patent No. 3,914,954 to Doerig, R.K.

U.S. Patent No. 3,995,444 to Clark *et al.*

U.S. Patent No. 4,629,686 to Gruenberg, M.L.

U.S. Patent No. 4,837,390 to Reneau, R.P.

Equipment described for cryopreservation applications in the past have permitted only relatively simple experimental protocols to be carried out, and have often been awkward to use. Only Adem and Harness have reported using a computer for organ perfusion with cryoprotectant See, Adem, C.G., *et al., J. Biomed. Engineering 3*:134-139, 1981. However, their specific design has several major flaws that limits its utility.

The present invention overcomes substantially all of the deficiencies of known apparatus and methods.

Summary of the Invention

In its most basic form, the present invention is directed to a computer-controlled apparatus and method for perfusing a biological organ, such as a heart, kidney, liver, etc. The apparatus of the invention comprises a plurality of fluid reservoirs and an organ container for holding the biological organ. A first fluid flow path is defined as a loop from the plurality of reservoirs to necessary sensors and temperature conditioning means and back to the plurality of reservoirs. The reservoirs are selectively connectable to the first fluid flow path. Pump means are interposed in the first fluid flow path for pumping fluid from the first fluid flow path to a second fluid flow path. The organ container is located in this second fluid flow path. Pump means may also be included in the second fluid flow path for pumping fluid from the organ container to one or more of the reservoirs or to waste. One or more sensors are interposed in the fluid flow paths for sensing at least one of the concentration, temperature, pH, and pressure of the fluid flowing in the first and second fluid flow paths. Measuring means are interposed in the

first and second fluid flow paths for measuring concentration and temperature differences between the upstream and downstream sides, in the fluid flow direction, of the organ container. The sensor(s) and the measuring means are connected to a programmable computer for providing a continuous information stream from the sensor(s) to the computer. Finally, the computer is coupled to the selection means and the pump means to continuously selectively control (a) the flow of fluid from each of the reservoirs individually to the fluid flow paths, and (b) at least one of the concentration, temperature, pressure and pH of the fluid flowing in the second fluid flow path, in accordance with a predetermined computer program without operator intervention.

Additional features of the invention may include a heat exchanger interposed in the first fluid flow path for conditioning the temperature of fluid flowing from this fluid flow path. A second heat exchanger may be interposed in the second fluid flow path for conditioning the temperature of fluid flowing in the second fluid flow path. A third fluid flow path may be defined between the organ container and the plurality of reservoirs. A third pump may be interposed in the third fluid flow path for pumping fluid from the organ container to one or more of the reservoirs.

Features and Advantages of the Invention

This invention has a multitude of features and advantages, among the most important of which are:

1. It permits control of the concentration of cryoprotectant or any other fluid or drug in the perfusate of an organ according to a wide variety of predetermined concentration-time histories, more or less independently of the flow rate of perfusate through the organ, with provision for simultaneously varying the concentrations of other drugs or osmotic agents. Step changes in concentration are possible, and it is possible to bring concentrations effectively to zero.

2. It provides for in-line sensing of concentration, pH, perfusate temperature, and other parameters so as to avoid the need for sensors in the perfusate reservoirs and for manual measurements.

3. It permits minimizing differences between the concentration of cryoprotectant monitored and the concentration of cryoprotectant in the perfusate reservoirs by minimizing the time required for perfusate to travel from the reservoirs to the perfusate sensors and back to the reservoirs.

4. It permits minimizing differences between the concentration of cryoprotectant monitored and the concentration of cryoprotectant actually perfused into the organ by minimizing the time required for perfusate to travel from the main fluid circuit to the perfused organ (or superfused tissue).

5. It permits monitoring of the arterio-venous difference in cryoprotectant concentration across the organ as an index of the degree of, or opportunity for, organ equilibration with cryoprotectant.

6. It permits control of the temperature of the organ essentially independently of flow through the organ, and permits varying this temperature at will.

7. It permits control of the perfusion pressure, either keeping it fixed or changing it as desired, and if desired minimizing pulsation.

8. It protects against perfusion of unmixed solution and air (bubbles) into the organ.

9. It interface with a computer to control the perfusions, to provide real-time monitoring, display, processing, and recording of the data, to calibrate the sensors and pumps, and to direct the cleaning, disinfection, and priming of the perfusion circuit and to instruct and alert the operator when necessary.

10. It is capable of perfusing and cryoprotecting organs of widely varying size, e.g., anything from a rat heart to a human liver, and is capable of tissue superfusion as well.

According to a broad aspect of the present invention there is provided a method for preparing organs or tissues for long-term preservation through the introduction of a vitrifiable concentration of cryoprotectant and subsequently preparing the organs or tissues for transplantation by the removal of the cryoprotectant. The method comprises the following steps:

initially perfusing the organ or superfusing the tissue without cryoprotectant;

adding a cryoprotectant solution to the organ or tissue and gradually elevating the cryoprotectant concentration to a first predetermined level and reducing the temperature of the organ or tissue;

delaying an increase in the concentration of said cryoprotectant for a time sufficient to permit approximate osmotic equilibrium of the organ or tissue to occur;

elevating the cryoprotectant concentration of said solution to a level, greater than said first predetermined level, required for vitrification and maintaining the solution at the elevated concentration for a time sufficient to permit approximate osmotic equilibrium of the organ or tissue to occur;

perfusing the organ or superfusing the tissue with a reduced, non-vitrifiable concentration of cryoprotectant in combination with a nonpenetrating osmotic buffering agent to a first buffering agent concentration level for a time period sufficient to permit approximate osmotic equilibrium of the organ or tissue to occur;

washing out substantially all of the cryoprotectant while decreasing the concentration of the osmotic buffering agent to a second, nonzero level substantially below said first buffering agent concentration level and increasing the temperature of the organ or tissue; and

perfusing the organ or superfusing the tissue to remove the osmotic buffering agent sufficiently to render the organ or tissue suitable for transplantation.

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According to a further broad aspect of the present invention there is provided a computer-controlled apparatus for perfusing a biological organ, such as a heart, kidney, liver etc. The apparatus comprises a plurality of fluid reservoirs; an organ container for holding the biological organ; means defining a first fluid flow path between said plurality of reservoirs and said organ container; selection means interposed in said first fluid flow path for selectively connecting said reservoirs to said organ container; pump means interposed in said first fluid flow path for pumping fluid from one or more of said reservoirs to said organ container and for pumping fluid from said organ container to one or more of said reservoirs; means defining a second fluid flow path between the output side of said pump means and said reservoirs and bypassing said organ container; sensor means interposed in said fluid flow paths for sensing at least one of the concentration, temperature, pH, and pressure of the fluid flowing in said first and second fluid flow paths; a programmable computer; means coupling said sensor means to said computer for providing a continuous information stream from said sensor means to said computer; and means coupling said computer to said selection means and said pump means to continuously selectively control (a) the flow of fluid from each of said reservoirs individually to said first fluid flow path, and (b) at least one of the pressure and pH of the fluid flowing in said first fluid flow path, in accordance with a predetermined computer program without operator intervention.

Brief Description of the Drawing

Figure 1 shows the overall fluidic circuit diagram of this invention.

Figures 2A-C show side, top and bottom views, respectively of a two-chamber gradient former employed as reservoir **R1** in this invention.

Figures 3A-C show side, top and bottom views, respectively, of a three-chamber gradient former used as reservoir **R3** in this invention.

Figures 4A-C show front, side and rear views, respectively, of the HBM used in this invention: Figure 4D shows the basic mixing unit area of the HBM; and Figure 4E shows a top view of the base of the HBM.

Figure 5 shows the appearance of a typical protocol for introducing and removing cryoprotectant as viewed on the computer monitor during a perfusion.

Figures 6A-E comprise a flow chart of activities for organ cryoprotectant perfusion.

Figures 7A-B comprise a flow chart of the procedure for non-cryoprotectant perfusions.

Figure 8 shows the function (control of serum creatinine) of rabbit kidneys transplanted after perfusion with VS4.

Detailed Description of the Preferred Embodiment and Best Mode

In a preferred embodiment, the apparatus incorporating the principles and features of this invention is contained in a refrigerated cabinet **100** (shown by double dashed lines in Figure 1). The refrigerated cabinet contains two sides, the reservoir/solenoid side and the organ/refractometer side. The cabinet is faced with double paned transparent doors each containing approximately 1 inch of insulating air (which can be reduced in pressure and/or humidity if necessary) between the panes to avoid condensation of moisture on the doors and to minimize heat leak to the cabinet. The organ-side door is split to form a "Dutch door". This allows the upper portion of the organ-side door to be opened and closed to place the organ in the system and to remove the organ without changing the temperature below the upper portion of the door, where the organ container and most other equipment is located. The cabinet may also employ a "Dutch door" on the reservoir side of the cabinet to enable the operator to make any needed adjustments (e.g., fluid addition to the reservoirs, transfer of upper fluid lines, etc.) without disturbing cabinet temperature to an unnecessary degree.

The primary features of the invention and its mode of operation are shown in the fluidic logic schematic of Figure 1. All fluid available for circulation through the system is drawn into the main circuit by a circuit pump **102** through fluid uptake lines **U1**, **U2**, **U3**, or **U4** depending upon the computer-controlled actuation pattern of three-way solenoid valves **S1**, **S2**, and **S3**. Uptake lines **U1-U4** connect either to fluid delivery lines **D1-D4** leading from reservoirs **R1-R4**, respectively, or to cleaning ports **C1-C4**, through standard tubing quick disconnects. By clamping **D1-D4** and unplugging them from uptake lines **U1-U4**, lines **U1-U4** can be plugged into cleaning ports **C1-C4**, as indicated by the curved arrows. While this is presently done manually, it will be appreciated by those skilled in the

relevant arts that appropriate valves, tubing and controls could be added to handle this task automatically.

In the embodiment of the invention as presently constructed, the reservoirs **R1-R4** are supported on a thick transparent plastic shelf from which four magnetic stir tables hang which stir the four reservoirs. Thorough stirring of **R1**, **R3**, and **R4** is necessary for proper generation of the desired concentration-time histories. The on/off states and stir rates of the stir tables are independently controlled.

Ports **C1-C4** lead to sources of sterile (distilled) water, air, and disinfectant. Solenoid valves **S0** and **S00** are interposed in the delivery lines for these sources and are arranged to ensure that traces of disinfectant do not enter the perfusion system by accident. Solenoid **S0** controls whether air or fluid will enter the perfusion circuit for cleaning, while solenoid **S00** determines whether the fluid selected will be water or disinfectant. The breakup of the main cleaning line into four independent channels outside of the cabinet rather than just before reaching **C1-C4** ensures that each channel is independent of the others, i.e., not subject to any meaningful cross-contamination resulting from diffusion of unpurged solution backwards from the fluid uptake lines **U1-U4** into the cleaning lines leading to cleaning ports **C1-C4**.

Distilled water and disinfectant are drawn into the system through a sterilizing filter **F4**, while air is drawn into the system through an air filter **F5**. The disinfectant of choice at present is a clinically accepted dialysis machine cold sterilant such as Actril. The cleaning procedure is to wash the perfusate out of the system with water and then to displace the water with sterilant. Prior to the next perfusion, the sterilant is washed out of the system with water and the water is then washed out of the system with air. The system is then primed by displacing the air with appropriate perfusate. The air flush is used to avoid the persistence of any lingering traces of sterilant dissolved in the rinse water, and to avoid any possible dilution of the priming fluid with water (i.e., to reduce the

amount of priming fluid needed for displacing water from the system), to allow a visual check of the completeness of priming, and to reduce spillage of water in the cabinet when the reservoirs, filters, and organ cassette are placed into the system after cleaning but before priming. The air purge can, however, be omitted if desired. The air filter is used to prevent contamination from pathogens in the air, if necessary.

Solenoid valves **S9-S12** normally direct fluid to reservoirs **R1-R4** or to waste. Reservoirs **R1-R4** can also be detached from the system by removing recirculation lines **RL5-RL8** from reservoirs **R1-R4** and plugging them into waste ports **W1-W4**, respectively (as indicated by curved arrows), allowing reservoirs **R1-R4** to be removed from the system for cleaning, sterilizing, and refilling. When reservoirs **R1-R4** are removed, valves **S9-S12** direct fluid to waste ports **W1-W4**. The four waste lines corresponding to waste ports **W1-W4** converge to a single common waste line **LW**. A two-way solenoid valve **S16** is located on the common waste line. When the waste ports are not in use, the common waste drainage line is blocked by closing valve **S16** to prevent any possible backflow of waste or pathogens into the sterile cabinet.

The use of this system of uptake lines **U1-U4**, which are plugged alternately into reservoir delivery lines **D1-D4** or cleaning ports **C1-C4**, in combination with recirculation lines **RL5-RL8**, which are plugged alternately into the reservoir internal return lines (not shown in the figure) or into waste ports **W1-W4**, allows complete sterilization of the perfusion circuit. The blunt ends of the uptake lines **U1-U4**, delivery lines **D1-D4**, cleaning ports **C1-C4** and waste ports **W1-W4** may be sterilized by swabbing with disinfectant when the tubing is being transferred from one alternative position to the other. The tubing transfer is accomplished while applying digital pressure to the tubing so as to occlude it while making the transfer to prevent fluid leaks and further reduce the risk of contamination.

The fluid withdrawn from reservoirs **R1-R4** or from ports **C1-C4** is delivered through one of several filters **F1**, **F2**, and **F3**, depending upon the state of actuation of solenoid valves **S4** through **S7**. These actuation patterns will be described in more detail below. Experience has shown, however, that a single filter **F1** or two filters **F1, F1'** in parallel will be adequate for most studies (rendering valves **S4-S7** optional, as indicated by broken lines) since virtual step changes in concentration can be imposed even when only one filter or two filters in parallel are present in the circuit.

It is desirable to minimize the distance between the circuit pump **102** head and the solenoids **S1-S7** to minimize circuit dead space and dead time and minimize the effects of perfusate viscosity.

The filters are capable of sterilizing the perfusate and are autoclavable. All filter holders can be removed from the system for cleaning and sterilization by means of the quick disconnects shown in Figure 1. Vent lines **V1-V3** lead to solenoid valves **S13-S15**, located outside of the refrigerated portion of the cabinet **100**. These vent lines are opened and closed under computer control during priming and cleaning of the system to permit air to escape and thereby prevent the filters from becoming blocked or damaged. A manual bypass (shown only for the **S13** bypass) is provided for **V1-V3** for emergency purging of air from the circuit. Obviously, air purges of the system beyond filters **F1-F3** are not possible if filters **F1-F3** are present in the circuit; hence filters **F1-F3** must be removed before beginning the washout of sterilant if an air purge is to be included in that procedure.

In the presently preferred embodiment, a 90 mm diameter filter of 0.22 micron pore size is located in each filter holder. This size filter is able to pass enough vitrification solution at 0°C to permit the successful perfusion of a rabbit kidney, with an overlying 1.2 micron filter and a coarse prefilter to prevent clogging. The standard configuration for the operative version employs two identical filters in parallel. This is

necessary to accommodate the flows required for human organs and provides a safety factor for any air which may be inadvertently introduced into the arterial fluid, as well as minimizing pressure building proximal to the filter. This continuous filter sterilization and resterilization of the perfusate during the perfusion can serve as a back up for pre-sterilized solutions in case of contamination for any reason during the perfusion.

Once the fluid from the selected reservoir has passed through the appropriate filter, it goes through some preliminary temperature conditioning in a heat exchanger **104** and then travels to a position as close to the organ as possible, at which point it encounters a "T" type tubing connector **T1**. The bulk of the flow passively takes the path **L1** ("refractometer loop") that leads to a flow-through process control refractometer **106** that measures the index of refraction of the liquid and hence the cryoprotectant concentration. The remainder of the flow is directed through an organ loop **L2** by means of an organ pump **108**. The organ pump speed is controlled by the computer so as to maintain the desired organ perfusion pressure despite wide variations in the organ's vascular resistance. By changing the organ pump head and the diameter of the tubing going through it, a wide range of flows can be generated sufficient to perfuse organs of a wide range of sizes: organs as small as rat hearts and as large as human kidneys have been successfully perfused.

The flow rate delivered by the circuit pump **102**, which supplies both the refractometer loop **L1** and the organ loop **L2**, must be high enough to both exceed the flow rate through the organ at all times and to ensure that sufficient flow is available for the refractometer **106** and other in-line sensors, generally designated **110**, for measuring temperature, pH, and other desired parameters of the perfusate to permit accurate measurements. The flow must also be high enough to minimize the "dead time" between changes in reservoir concentration and changes in the sensed concentration and other sensed parameters in the refractometer loop as well as to minimize the "dead time" between the reservoir and the

organ. The circuit pump flow is limited by the need to prevent fluid from being delivered to the filters at a rate in excess of what these filters or the tubing leading to them can pass without failing, as well as by constraints of heat output and wear and tear on the circuit pump tubing. The speed of the circuit pump is usually not varied during an experiment and does not therefore usually require computer control, though computer control is available as an option.

After passing through the organ pump **108**, the perfusate passes through a second heat exchanger **112** that finalizes perfusate temperature conditioning. This is done by adjusting the flow of both cold and warm liquid from cold and warm baths **114**, **116**, respectively, using computer-controlled pumps (not shown) between heat exchanger **112** and baths **114**, **116**.

The computer is able to vary flow through both the cold path and the warm path so as to adjust perfusate temperature in the arterial line and therefore also in the effluent of the organ. The arterial and effluent temperatures provide an indication of the actual organ temperature. By controlling the flow rate of cold and warm bath fluid, organ temperature can be adjusted independently of organ flow, provided flow is not close to zero. Experience has shown that arterial and venous temperatures at least as cold as -6°C and at least as high as 25°C can be achieved with this invention. Generalized cabinet cooling is not an alternative to the heat exchange system for subzero perfusions because cooling of the cabinets to subzero temperatures will cause freezing of the more dilute solutions in the tubing lines. Specific jacketing and cooling of the organ container is of theoretical value, however, and may optionally be included.

The temperature-conditioned perfusate is then debubbled and mixed in a bubble trap/mixer **120** just before entering an organ container **122**. Arterial and venous temperature probes, generally designated "T" in Figure 1, penetrate the wall of organ container **122** through simple holes. Pressure and, optionally, temperature is sensed in the bubble trap.

Although shown separately in the drawing for ease of understanding, the bubble trap and mixer **120** are in fact an integral part of the heat exchanger **112**, so heat exchange continues to be controlled while debubbling and mixing are accomplished. Experience has shown that mixing is important due to the tendency for layering of dilute solutions on more concentrated, denser solutions. Details as to the specific construction of the heat exchanger/bubble trap/mixer (**HBM**) are described below.

Under normal circumstances, the cooling fluid effluent from this second heat exchanger **112** is used to cool the perfusate passing through the preliminary heat exchanger **104**. This cooling fluid then travels to a solenoid holding block **118** physically containing solenoids **S1-S12**, so as to draw off waste heat from these solenoids before returning to the cold bath.

The holding block **118** is equipped with an internal fluid path for drawing off waste heat from the solenoids and may be either metal or plastic. The solenoids are preferably 3-7 watt (or less) piston type 3-way solenoids of minimal internal fluid volume having orifices on the order of 0.156 inches or more and Cv values > 0.16 or more (e.g., NR (Neptune Research) Model 648T033 fitted with RC dropping circuits and 3-watt coils) while resisting pressures of up to 500 mmHg or so. Solenoids having 1/16 inch orifices and Cv values of 0.01 to 0.03 (e.g., Valcor's Model 20-2-3) are not fully satisfactory due to the high viscosity of the solutions used for cryopreservation (causing difficulty aspirating viscous fluid through **S1-S3**), the high flows desired for controlling dead times and for perfusing larger organs, the possibility of clogging, and the buildup of pressure between the circuit pump and **S8-S12**. The detailed actuation pattern and tubing arrangement of the solenoids is described below. The internal solenoids not held in the solenoid block, **SR1**, **SR31** and **SR32**, are described in more detail below.

A stopcock (not shown) in one of the coolant lines permits the in-line heat exchanger to be bypassed if desired. When the cooling function of the solenoid holding block **118** is in use, the effluent is directed to the solenoid holding block cooling system before returning to the cold bath.

An effluent distribution block (EDB) **124** (Figure 1) is connected to the output side of the organ container **122**. The EDB is designed so that a small amount of effluent is always present at the bottom of the block. This effluent or residual fluid is withdrawn by the two-channel "delta R.I. pump" **126** and sent to the differential refractometer ("delta R.I. meter") **130** where its refractive index is compared to that of the arterial perfusate from refractometer loop **L1** (pumped at the same rate as the venous effluent sample) and a difference signal generated. EDB **124** is drained also by the effluent recirculation pump **128**. The EDB **124** therefore allows effluent to be recirculated with or without first being delivered by the delta R.I. pump **126** to a differential refractometer **130**. The differential refractometer **130** sends a signal to the computer which provides a measurement of the difference in concentration between the fluid in the refractometer loop **L1** and the organ effluent in the organ loop **L2**. The nonlinear baseline resulting from this unorthodox use of the differential refractometer is accounted for in the software for running the perfusion program. Since the fluid in the refractometer loop will approximate the concentration of the fluid entering the artery of the organ, the delta R.I. output provides an estimate of the arterio-venous concentration gradient across the organ. When this gradient is large (in either the positive or negative direction), the organ is far from equilibrium. When the gradient is zero, the organ is at least largely in osmotic equilibrium with the perfusate.

All effluent from the organ (together with the arterial fluid sampled by the delta R.I. pump) is ultimately collected by the recirculation pump **128** and sent to solenoid **S8**, which controls whether the effluent is recirculated to the reservoirs or discarded to waste.

Effluent to be returned to a reservoir is combined with the fluid flowing through the refractometer loop **L1** at a T connection **T2**. As noted above, return to the correct reservoir is then controlled by the actuation of solenoids **S9** through **S12**.

The recirculation pump **128**, like the circuit pump **102**, need not require flow adjustment. It is normally set to a rate sufficient to exceed the maximum steady flow through the organ pump **108**. Since the output of the recirculation pump exceeds that of the organ pump, air is continually introduced into the tubing leading to solenoid **S8** and usually to the reservoirs **R1-R4**. Provisions to prevent excessive bubbling of the reservoirs as a result of this are described below.

Although the delta R.I. pump speed can be changed, it is usually kept constant throughout an experiment. In the presently operative version, it is not under computer control, but computer control would be a desirable option in some cases. The delta R.I. pump employs very small diameter tubing to reduce delays in fluid transit time. This small tubing is particularly important because the flow rate through the delta R.I. circuit is limited by the lowest flow rate through the organ, which may be small, and by the limited size of the fluid paths in commercially available differential refractometers.

The return of the differential refractometer output to the organ effluent line is proximal to the effluent recirculation pump. This placement rather than placement distal to the pump ensures a steady flow though the differential refractometer, whereas distal placement may prevent or alter differential refractometer flow by virtue of a higher exit pressure.

The present operative version of the embodiment of the invention uses silastic tubing of 1/8 inch diameter throughout the system, which is sufficient to accommodate the needed flows and is preferred. Silastic is compatible with Actril cold sterilant, is translucent (important for visualizing flow to detect problems and for observing any signs of

microbial growth), is impervious to common cryoprotective agents such as dimethyl sulfoxide, and is soft enough to be easily manipulated. However, silastic should not be used in circuits coming into contact with silicone cooling fluids, which swell and weaken silastic tubing.

Reservoir **R1** is constructed as a gradient former (Figure 2). Essentially the gradient former consists of two concentric cylinders, an outer cylinder **200** and an inner cylinder **201**. A fluid path **205** allows fluid to flow from the outer cylinder **200** to the inner cylinder **201** under the influence of gravity in response to a reduction of volume in the inner cylinder. The concentric orientation of the fluid compartments is very space efficient. The fluid delivery line **204** corresponds to the line **D1** of Figure 1. The unit shown is a modification of a commercially available gradient former. The necessary modifications for use with this invention are as follows.

1) The stopcock normally used to control flow from the outer cylinder to the inner cylinder in the commercial device is replaced by a pinch-type two-way (on/off) solenoid valve **202** (currently, a Bio-Chem Valve Corp. model 100P2WNC). The pinch-type valve is preferable for this application to a piston-type valve because of the small pressure difference available to drive fluid flow and the consequent need for a large working diameter fluid path. It is also preferable for easy removal from its tubing when the reservoir is to be removed from the cabinet for cleaning, leaving the solenoid behind. The base of the gradient former has been modified, at **203**, to make room for the solenoid and to support it on a platform so as to keep the solenoid oriented correctly. The solenoid is located a sufficient distance from the reservoir to avoid excessive heating of the reservoir fluids.

2) The diameter of the fluid path **205** from the outer cylinder **200** to the inner cylinder **201** has been enlarged to permit flow at an adequate rate of the viscous solutions required for organ cryopreservation. An inner diameter of 1/8 to 3/16 inch is adequate.

3) A lid 206 has been provided. The lid has an outer overhang 207 that prevents the lid from moving from side to side after it is placed on the cylinder. The lid has built-in outer and inner filling funnels 208a and 208b and a recirculation port 209.

4) Funnels 208a and 208b extend into respective internal fill tubes 210a and 210b. The internal fill tubes are preferably rigid hollow rods located next to the wall of the inner and outer cylinders and perforated at 1-2 cm intervals with holes 211a and 211b, respectively, which are approximately 3 mm in diameter. The function of the fill tubes is to reduce the creation of bubbles as recirculating fluid impacts the surface of the liquid in the reservoir. The purpose of the perforations is to enable air to escape from the tube through the perforations so as not to force air to the bottom of the reservoir to form bubbles. These functions are particularly important in perfusates containing protein, which tend to stabilize bubbles.

5) A fill mark has been provided to enable the reservoir to be filled reproducibly to the same, predetermined volume. The operator can establish his/her own fill mark depending upon the details of the application. The gradient formers have approximate graduations (horizontal lines on both the inner and outer cylinders, aligned so as to permit avoidance of parallax error in reading the liquid level in either cylinder) spaced approximately 0.5 cm apart for a 2 liter gradient former. These graduations are also important for establishing slight, deliberate mismatches in liquid level between inner and outer cylinders, which are necessary to prevent premature mixing of solutions of widely differing densities, such as cryoprotectant-free perfusate and vitrification solution. They also permit a rough quantitative check by the operator on the progress of the gradient as represented on the computer screen.

6) The plastic composition of commercially available gradient formers may create problems for certain types of cryoprotectant, which could conceivably attack the plastic. It is therefore preferred to use

reservoirs made of transparent material (e.g., glass, plexiglass or the like) that is compatible with the cryoprotectant chemicals or use reservoirs whose surfaces have been siliconized or otherwise treated to prevent the attack. In the inventors' experience, acrylic has been found to be an acceptable material.

7) The reservoir **R1** contains a stir bar **212**. The stir bar is housed in a jacket **213** attached to a freely spinning vertical pin **214** extending to the stir bar from the lid of the reservoir to prevent the jacket, and hence the stir bar, from moving laterally. This change is necessary to make sure chattering, and therefore poor mixing, does not occur while the perfusion machine is unattended. Support from above rather than below prevents unnecessary perfusate frictional heating and avoids drainage/cleaning problems.

Reservoir **R3** is also constructed as a gradient former. The details of reservoir **R3** are shown in Figure 3. In the drawing, those elements that are substantially the same as in reservoir **R1** are designated with the same reference number, except that the first digit has been changed from a "2" to a "3". Reservoir **R3** contains an outer compartment **315** ($R3_3$), an inner compartment **318** ($R3_1$), and a third intermediate compartment **316** ($R3_2$). Intermediate compartment **316** is connected to inner compartment **318** through a fluid conduit **320** controlled by a solenoid **317** ($SR3_1$). Compartment **316** also connects to outer compartment **315** by a fluid conduit **321** controlled by a solenoid **319** ($SR3_2$). The use of an outer compartment is necessary when concentration is being reduced to zero or nearly zero, for reasons noted below in the discussion of the function of the gradient pump and the action of the gradient formers. The outer compartment is necessary in preference to a larger volume of fluid in the middle compartment because increasing the volume of fluid in the middle compartment will cause the concentration profile of fluid flowing from the gradient former to waste in response to a constant efflux rate of inner cylinder fluid to become non-linear, therefore making control of

concentration-time history more complicated. More importantly, an excessive amount of fluid in the middle compartment would be required to approach a zero concentration in the circuit compared to the amount of fluid required in the outer compartment after virtual emptying of the inner and middle compartments.

Automated use of reservoir **R3** poses some problems which are successfully addressed in part by software and in part by the specific construction of **R3**. Specifically, actuation of solenoid $SR3_2$ allows fluid in the outer compartment ($R3_3$) to flow first into the middle compartment ($R3_2$) and from this compartment to the inner cylinder ($R3_1$). This is because the pressure head present between $R3_3$ and $R3_2$ is large when $R3_1$ and $R3_2$ are nearly empty, which occurs when $SR3_2$ is activated. At this point, $R3_3$ is still full. This large pressure head causes fluid to flow too rapidly into $R3_1$ if $R3_3$ is connected directly to $R3_1$ rather than using $R3_2$ as a buffer between $R3_3$ and $R3_1$. By adjusting the level of $R3_3$, the flow can also be partially controlled. But even with these two precautions, further control of flow is required by using an appropriate duty cycle for $SR3_2$. The flow to $R3_1$ should be slow at first and more and more rapid as the concentration is brought closer and closer to zero, whereas passive flow under the influence of gravity will always be fastest at first and slowest at the end unless the flow is metered by the sort of tailored duty cycle currently being imposed on $SR3_2$.

The other modifications to **R3** resemble those of **R1**.

Reservoir **R4** is a gradient former constructed in the same manner as **R1**.

An important element of the fluidic circuit is the gradient pump **132** connected to the circuit by a line **P1** (Figure 1). The function of the gradient pump is to allow for gradual changes in concentration within the appropriate reservoirs within the cabinet. The method by which this is accomplished will be described below. The placement of the line **P1** to the gradient pump at **T3A**, just after the point of joining of the

refractometer loop **L1** and the organ loop **L2**, presents one option for ensures removal of some of the air introduced by the organ effluent recirculation pump **128** and therefore helps reduce bubbling of the reservoir fluid.

A better option, however, and the one presently in use, is to draw no air into line **P1**. This is accomplished by connecting **P1** at point **T3B** and results in fully controlled concentration-time histories. The bubbling problem is then overcome by continuously regulating the speed of the recirculation pump **128** to be just slightly in excess of the combined flows of the organ pump **108** and the delta R.I. pump **126** so as to introduce little air. Attaching the recirculation output of **S8** directly to **P1** without regulating the speed of pump **128** results in degraded concentration history and is not recommended.

The purpose of the gradient pump **132** is to remove some of the recirculating fluid from the circuit. This removal of fluid causes the flow rate of fluid back to the reservoir of origin to be less than the flow rate of fluid from this reservoir to the circuit. This causes the level in the inner cylinder of the reservoir (**R1**, **R3**, or **R4**) to go down. This lowering of inner cylinder fluid level in turn causes the fluid in the outer or middle compartments to flow into the inner cylinder to keep the two levels similar. Thus the two dissimilar concentrations in the two cylinders are mixed in the inner cylinder, generating the concentration gradient which is then sent to the rest of the circuit. This is the manner in which the gradient pump effects the desired gradual changes in concentration which reach the organ and the refractometers. Any necessary adjustments to the gradient pump speed are made by the computer.

The principle involved is that of an ordinary linear gradient former in which the portion of the circuit external to the gradient former can be regarded, to a first approximation, as extra volume in the inner cylinder. Withdrawal and discard of fluid from the inner cylinder at a constant rate will result in a linear molar concentration increase with time despite the

presence of the rest of the circuit and the recirculation of fluid back to the reservoir. However, unlike an ordinary gradient former, the concentration of fluid leaving the gradient former at the moment the volume in the gradient former becomes zero will not be equal to the concentration of fluid in the outer (or middle) cylinder of the gradient former. Therefore, in order to approach a concentration of zero during cryoprotectant washout using an ordinary two-compartment gradient former, it is necessary to add additional fluid to the outer cylinder while continuing to discard fluid from the inner cylinder normally. This is why **R3** has been modified to have a third compartment: the extra fluid required to continue cryoprotectant washout can be added from this third compartment by the computer without operator intervention which could compromise temperature control and introduce inaccuracies. During introduction of cryoprotectant, on the other hand, the desired final concentration can always be reached by using a concentration in the outer compartment which significantly exceeds the final concentration desired in the circuit at the end of the gradient.

The **HBM** heat exchange system is shown in detail in Figures 4A-E.

Perfusate enters the **HBM** through an entry port **403**, travels through a central channel **400**, and leaves the **HBM** via an outlet port **406**. On either side of the central perfusate path are separate chambers for regulating temperature. The two innermost temperature control chambers **401** (one on each side of the perfusate path) are used for the circulation of coolant, while the outer chambers **402** are a pathway for the flow of room temperature fluid for offsetting the coolant. (For specialized applications involving, for example, normothermic perfusion, these pathways can be reversed.)

The direction of cold fluid flow is optional. Adequate temperature control has been found when all fluids (perfusate, coolant, and warming fluid) flow in the same direction (uphill) despite the lack of

countercurrent heat exchange. This mode allows the avoidance of air or carbon dioxide accumulation in the outer chambers.

Perfusate enters the bottom of the HBM unit through inlet 403 and travels upward in a zig zag pattern. It emerges into a small upper reservoir which has an air space above: this is the bubble trap area 404. Perfusate then travels beneath the bubble trap and goes through a perfusate mixing area 405 before finally traveling onward to the arterial outlet.

The inlets for cold 407 and warm 408 fluid are each split into two channels within the base of the unit. The outlets 410, 411 for warm and cold fluid, respectively, each receive fluid collected from two channels such that each channel of the same kind (i.e., each cold channel or each warm channel) is the same length and nominally experiences the same pressure difference from start to finish, so that flow rate through each like channel should be approximately equal.

All of the cold and warm fluid pathways include a length of flexible tubing 412 at the rear of the unit. These tubing segments serve two purposes. First, by introducing an air gap between the four channels, heat exchange between them is minimized. This is particularly desirable when all of the cold and warm fluid is flowing in the direction opposite to that of perfusate flow (i.e., in orthograde direction) and has not already undergone heat exchange with the perfusate. Second, each tube can be clamped. In this way, if by chance one cold channel or one warm channel should take all of the cold or warm fluid delivered while the other channel "airlocks", this situation can be corrected by clamping the channel receiving all of the flow and purging the air out of the inactive channel, bringing each channel into full function and equal flow.

Because in orthograde mode the temperature conditioning fluid enters the heat exchanging portion of the unit at the top and exits at the bottom, it is necessary upon installation to run the cold and hot pumps in retrograde direction in order to purge all air out of the cold and warm

channels. This is best accomplished if the cold and warm tubing leading to and from the bath is no more than about 1/8 inch in internal diameter, since at this diameter fluid flow will displace air from the tubing rather than allowing it to flow uphill in a direction opposite to the direction of fluid flow or otherwise to remain unpurged in various parts of the tubing. Thus, when the pump direction is reversed again from retrograde to orthograde, no air will be present in the tubing and none will be trapped in the heat exchange chambers of the unit.

In addition to serving a heat exchange function, the zig zag pattern is also designed to force mixing of previously perfused dense perfusate or, when perfusate density is rising rather than falling, to purge the less dense perfusate from the perfusate path.

As the perfusate emerges from the zig zag heat exchange area, it enters the bubble trap **404** at trap entry area **418**. Perfusate exits the bubble trap through exit region **419**. The zig zag pattern, in fact, is also designed to allow any air bubbles to exit the heat exchange area and to emerge into the bubble trap area. The bubble trap area is designed to have the following features.

1. Its volume is sufficiently large to reduce the pulsatile action of the perfusion pump to a minimum by distributing the shock of each stroke over a relatively large air volume. This simplifies pressure control and measurement and may be less damaging to the organ.

2. Its volume is sufficiently low to minimize the liquid volume present in the trap and thereby to minimize the dead time and dead volume between the organ pump and the organ itself. A minimal volume is also desirable to minimize layering of more dilute perfusate over more dense perfusate.

3. A pressure sensing port **413** is provided. Port **413** has no fluid connection to the perfusate, thus eliminating a "blind alley" in which fluid cannot be mixed properly or in which disinfectant might fail to penetrate or might be trapped.

4. The lid **414** of the trap is removable for cleaning.

5. A vent port **416** is provided which is used to adjust fluid level in the trap so as to make it the minimum required to serve the bubble trap function and to maximize pressure wave damping. The tubing from this vent leads to the outside of the cabinet, permitting adjustments to be made without opening the cabinet door. The same port leads to the electronic pressure transducer as well.

6. A third port **417** is provided through the bubble trap lid to permit the injection of drugs, vascular labeling materials, fixative, etc.

7. The walls of the bubble trap are angled near the trap entry and exit points **418**, **419**, respectively, to produce a certain amount of mixing of the perfusate both as it enters and as it leaves the trap, and to break up and minimize the volume of layers of dilute perfusate overlying more dense perfusate.

8. The option exists of introducing probes, such as a temperature probe via one of the ports in the trap lid to make measurements in the perfusate without permanent embedding of the sensor: the port consists of flexible tubing attached to a plastic threaded fitting. A probe can be freely admitted or withdrawn and the tubing clamped with hemostats or an equivalent clamp to effect a pressure-tight seal. This simplifies removal and reinstallation of the **HBM** when it must be cleaned and allows flexibility in probe selection and the opportunity of using the probe for other measurements elsewhere.

After leaving the bubble trap, the perfusate descends to a mixing area **405** (see Figure 4D). The basic unit of the 3-unit mixing path is a narrow horizontal entry area **HE** emerging into a "wide" basal area **BA** which rises to an area of flow restriction **FR** and ends in a descent **D** to the next horizontal entry area. Fluid entering **HE** is forced through an opening too small to support much layering of low density fluid on top of high density fluid, especially considering the right angle turn required just before **HE**. Fluid flowing into **BA** may, if less dense, rise immediately

upward toward **FR**. If more dense, it may be driven into the wall **W** and rise upward along this wall. Upon encountering **FR**, however, the denser liquid will be accelerated toward the less dense liquid rising directly from **HE**, creating turbulence and mixing. If **BA** fills with dense perfusate, the speed of the fluid emerging at **FR** directly upwards toward **D** should cause the dense liquid to mix with any low density fluid layered above **FR**. Furthermore, the narrow descending path **D** should draw layered liquid down the angle along with denser liquid, again preventing stagnant layers from persisting. In practice, three such mixing units aligned in series as shown in Figure 4B are sufficient to mix initially very poorly mixed perfusate, which is encountered frequently in the course of abruptly raising or lowering cryoprotectant concentration. One final function of the mixing units is to serve as a trap for any small bubbles which for any reason are not removed in the bubble trap area. (Bubbles in the mixing area are, however, easily purged by the operator prior to initiation of organ perfusion.)

After leaving the mixing region, the perfusate descends to an outlet port **406** leading directly to the organ. The path from the final mixing unit to port **406** is deliberately created at an angle to the horizontal in order to provide one last chance of stopping any bubbles from reaching the organ, since in order to reach the organ a bubble in this pathway would have to flow downhill, contrary to its tendency to flow uphill.

The mixing area and subsequent areas are purged of air by occluding the outlet tubing affixed to port **406** with the vent open until approximately 1/2 inch of fluid has accumulated in the bubble trap. The vent is then closed until the pressure has reached about 60-120 mmHg. Finally fluid is once again allowed to flow freely through port **406**. The jet of fluid through the mixing area and out port **406** sweeps all air out of the fluid path from the bubble trap to port **406**. If some air persists, it can be removed by repeating the process. After air has been purged, the vent is opened to allow unnecessary fluid in the bubble trap to exit the

trap under the influence of gravity, reaching a final depth of about 1/8 inch. A final depth of 1/8 inch cannot be set before purging the line of air because insufficient volume exists to avoid refilling the mixing area with air from the bubble trap during the purging process.

The **HBM** is designed to require removal for cleaning only infrequently. Disinfection and removal of disinfectant from the bubble trap area is effected automatically but does require some operator attention afterwards to ensure that all uppermost exposed surfaces are disinfected and later washed free of disinfectant without contaminating the outlet tubes.

After the perfusate exits the **HBM** unit through port **406**, it enters the organ in the organ container **122**. In the preferred embodiment, the organ container comprises a rectangular box with a hinged lid, lid stop, lid handle, sloped floor, specially sloped feet, arterial and venous thermocouple inlets, perfusate inlet, and effluent outlet in the foot opposite the inlet. The slope of the floor is downward in both the right to left and the back to front directions to ensure that all fluid runs to the foot outlet with very little fluid accumulation anywhere in the container. One needle probe is inserted directly through the wall of the arterial line. A second probe is placed directly in the stream of fluid emerging from the organ. In typical results, the arterial and venous temperatures differ by only tenths of a degree, but both are useful for quality control. The organ container may employ a soft mesh support for the organ similar to that used in the Waters organ cassette or the organ can be placed directly on the floor of the organ container or on a specially designed independent and removable support.

The organ container **122** and the organ pump **108** are placed in maximum proximity to reduce dead times and dead volumes between the two, and the tubing leading from the organ pump to the organ container is chosen to be as small in inner diameter as possible for the same reason.

Most perfusate does not go through the organ loop **L2** as described above but travels instead from the filters to the in-line analogue refractometer **106**. The presently preferred embodiment of the invention uses a modified commercially available refractometer from Anacon corporation. In particular, small diameter tubing inlet and outlets are used rather than the very large standard Anacon pipe fittings.

The modification of the refractometer sensing head appropriate for the final invention could contain the following changes from the ordinarily available Anacon unit.

1. The internal volume of the fluid path could be kept to a minimum.

2. Presently, it is necessary to purge the air space of the unit with a slow flow of dry nitrogen gas to prevent condensation of moisture due to the low temperatures and high humidities prevailing in the cabinet. In a modified version, the electronics area of the sensing device could be hermetically sealed with some desiccant inside to eliminate the need for a nitrogen purge.

The invention allows the operator to access reservoirs in any sequence and to otherwise custom-design the process which may be of interest. The operator is even free to switch solenoid positions depending on what he may want to do. Nevertheless, the following nominal application illustrates the actuation patterns required to deliver fluid from and to each individual reservoir and filter. It also illustrates the "standard protocols" for organ cryoprotectant perfusion and for cleaning of the system which the system was designed primarily to carry out.

Solenoid **S1** admits fluid from **R1** when off, or from **R2** when activated. Solenoid **S2** is open to **R3** when not energized, or to **R4** when energized. The output of **S1** and **S2** is to **S3**, which accepts fluid from **S1** (that is, from **R1** or **R2**) when in the resting state and which accepts fluid from **S2** (i.e., from **R3** or **R4**) when activated. The common outlet for **S3**

(always open) leads to the circuit pump **102**, which then withdraws fluid from the solenoid-selected reservoir.

If differential filters are to be included, then the output of the circuit pump **102** is to **S4**'s common port (always open). When **S4** is not energized, its output is directed to filter **F1**. The return from filter **F1** returns to the normally open port of **S5** and exits through the **S5** common outlet to the refractometer loop **L1** and the organ loop **L2**. If, on the other hand, **S4** is energized, then its output is directed to the common inlet port of **S6**. When **S6** is in the resting state, its output is directed to filter **F2**, and the return from filter **F2** enters **S7** through its normally open port. The output from **S7** travels to the normally closed port of **S5**, which must be energized to accept this output. Once fluid has entered **S5**, it flows out the **S5** common outlet to the refractometer loop and the organ loop. Finally if **S4** is energized and **S6** is also energized, fluid will be directed through both of these valves and will reach filter **F3**. The return from filter **F3** occurs via the energized **S7** and the energized **S5** solenoids and goes to the two loops **L1** and **L2** as above. As noted earlier, the use of filters **F2** and **F3** and therefore of solenoids **S4**, **S5**, **S6**, and **S7** is optional and will be useful primarily when very abrupt changes from one solution to another are required, or when particularly heavy particulate contaminates must be removed.

Effluent from the organ eventually returns to **S8**. If **S8** is activated, the fluid is discarded. If **S8** is not activated, the fluid is directed from **S8** to combine with fluid from the refractometer loop and returned to a desired reservoir.

Fluid traveling through the refractometer loop travels successively to solenoids **S9** **S10**, **S11**, and **S12** and then to waste if none of these solenoids are energized. Energizing **S9** diverts flow to the **R1** recirculation line. **S10**'s activation (in the absence of activation of **S9**) diverts flow to **R2**. Similarly, selective activation of **S11** or **S12** will, respectively, recirculate fluid to **R3** or **R4**.

There are two basic processes of solenoid-actuated fluid control, one for actual perfusions and one for system cleaning and priming. The perfusion process typically proceeds from **R1** through **R4** whereas priming must occur in the reverse order to load the fluid uptake and fluid recirculation lines for reservoirs **R2-R4** and, optionally filters **F2** and **F3** and their associated lines) while leaving the circuit primed with fluid from (typically) **R1** (or **C1**) at the end of the priming (or cleaning) process. The typical sequence of solenoid activations required to prime the system (or to clean it) is as follows.

When only **F1** (not **F2**) is present, priming (and cleaning) may proceed in any order of reservoirs, provided, in the case of priming, that the final reservoir corresponds to the first reservoir used for the subsequent perfusion.

SOLENOID CONTROL SEQUENCE for STANDARDIZED RINSING/PRIMING

(Uses: remove perfusate with filter-sterilized H_2O at end of experiment; replace cleaning H_2O with chemical sterilant solvent between perfusions; remove disinfectant using filter-sterilized distilled H_2O; remove water using air; remove air using reservoir fluid, i.e., prime the system.)

	Sub-Task Accomplished	SOLENOID # (+=ENERGIZED)												
	00*0*	1	2	3	4	5	6	7	8	9	10	11	12	13
1.	Deliver fluid from **R4** through **F1**	-	+	+	-	-	-	-	-	-	-	-	-	**
2.	Perfuse **R4** recirculation tubing	-	+	+	-	-	-	-	-	-	-	-	+	-
3.	Deliver from **R3** through **F3**	-	-	+	+	+	+	+	-	-	-	-	-	**
4.	Perfuse **R3** recirculation tubing	-	-	+	+	+	+	+	-	-	-	+	-	-
5.	**R2, F2**	+	-	-	+	+	-	-	-	-	-	-	-	**
6.	**R2** recirculation tubing	+	-	-	+	+	-	-	-	-	+	-	-	-
7.	**R1, F1**	-	-	-	-	-	-	-	-	-	-	-	-	**
8.	**R1** recirculation tubing	-	-	-	-	-	-	-	-	+	-	-	-	-
9	Organ loop discard tubing ***	-	-	-	-	-	-	-	+	+	-	-	-	-

* If the sequence above is to be done with reservoir fluid, **S0** and **SS00** will be off. **S0** and **S00** will also be off if the sequence above is to be done with water, and the cleaning ports **C1-C4** will be connected to uptake lines **U1-U4**. If the sequence above is to be done with disinfectant, **S0** will be off and **S00** will be on. If the sequence is to be done with air, **S0** will be on and **S00** will be off.

** **S13** (and, optionally, **S14** and **S15**), the filter vent solenoid(s), will be on for a portion of this step and off for the remainder of this step: it will be on just long enough to purge air from the line (usually 60 sec. on step 1 and 30 sec on each of the remaining steps for which the ** notation is used). This can be programmed not to happen if the filters are not present in the system.

*** this step is omitted when priming the system.

Note: Water control solenoid **S16** is on (waste tube open for disposal of fluid to waste) for steps 2, 4, 6, 8, and 9 and off for all other steps.

The standard process of solenoid actuation for withdrawing fluid from **R1-R4** and for creating gradients for a normal perfusion is as follows (assuming (1) use of optional filters **F2** and **F3**, (2) straightforward or typical use of the gradient-controlling solenoids, and (3) the existence of a gradient former as **R2**). The staged completion of a closed circuit upon going from one reservoir to another is to avoid recirculating solution of undesired composition to the new reservoir before its contents have displaced the previous solution from the circuit. If there is no problem with recirculating the previous solution, the precaution of delayed recirculation can be dropped.

SOLENOID CONTROL SEQUENCE for STANDARDIZED PERFUSION

	Sub-Task Accomplished	SOLENOID # (+=ENERGIZED)											
	00*0*	1	2	3	4	5	6	7	8	9	10	11	12
1.	Initial recirculation to **R1**	-	-	-	-	-	-	-	-	+	-	-	-
2.	**R1** gradient	Same as 1, but activate **SR1**											
3.	From **R2** to **F1**, no recirculation	+	-	-	-	-	-	-	+	-	-	-	-
4.	Deliver **R2** solution through **F2**, no	+	-	-	+	+	-	-	+	-	-	-	-
5.	Recirculate **R2** solution except	+	-	-	+	+	-	-	+	-	+	-	-
6.	Recirculate all **R2** solution	+	-	-	+	+	-	-	-	-	+	-	-
7.	Run a gradient from reservoir **R2**	Same as 6, but activate **SR2**											
8.	Perfuse from **R3** just to **S6/F2****	-	-	+	+	+	-	-	+	-	-	-	-
9.	Perfuse from **R3** to **F3**, circuit	-	-	+	+	+	+	+	+	-	-	-	-
10.	Recirculate to **R3** except from	-	-	+	+	+	+	+	+	-	-	+	-
11.	Recirculate all **R3** fluid	-	-	+	+	+	+	+	-	-	-	+	-
12.	Run first part of **R3** gradient***	Same as 11, but activate **SR31**											
13.	Run second part **R3** gradient	Same as 11, plus **SR31** and **SR32**											
14.	Open circuit, perfuse from **R4**	-	+	+	+	+	+	+	+	-	-	-	-
15.	Recirculate to **R4** except from	-	+	+	+	+	+	+	+	-	-	-	+
16.	Recirculate from both loops to **R4**	-	+	+	+	+	+	+	-	-	-	-	+
17.	Run **R4** gradient	Same as 16, but activate **SR4**											

* For normal perfusions, solenoids **S0**, **S00**, and **S13-S16** will always be non-actuated.

** This step prevents fluid from the previous reservoir, initially present in the line between the new reservoir and the filter previously equilibrated with fluid from the new reservoir, from contaminating the previously equilibrated (new) filter.

*** As noted in discussion, **SR32** activation must follow a duty cycle initially, ending in permanent activation of **SR32** until end of use of **R3**. The duty cycle involves switching back and forth between solenoid patterns 12 and 13 as dictated by the duty cycle requirements.

The number of reservoirs could be less than or greater than the number specified here, with corresponding changes in solenoid number. Furthermore, the number of layers of **R1-R4** need not conform to the descriptions given above. The limits would be one reservoir at the least and perhaps eight reservoirs at the maximum, in which any reservoir could have from one to four compartments. The upper limits are based partly on volume and crowding constraints and partly on the difficulty of imagining any procedure complex enough to require more reservoirs for its control.

Another variation would be to employ different capacity reservoirs at different positions (e.g., instead of the herein preferred embodiment, one might have a 2-liter reservoir followed by a one-liter reservoir followed by a 3-liter reservoir followed by a one-liter reservoir, and so on).

In principle, the use of individual reservoirs could be abandoned in favor of one multicompartment reservoir consisting of perhaps four to twenty concentric cylinders each activated by solenoids or even by manual levers external to the temperature-controlled area, all stirred by a single central stir table. Abrupt or step changes in concentration could still be accommodated if the stepped change is not delivered via the stirred central area. The relative positions of the reservoirs could also change.

The arterial concentration sensor could be located proximal to rather than distal to the origin of the organ loop in the circuit, but should not be located proximal to the filters.

A pressure sensor to sense pressure developing on the circuit pump side of the filters could be incorporated as a warning device.

Description of the Method

The complete cryopreservation method using the above-described apparatus comprises four parts. The first part consists of the pretreatment of the organ prior to its removal. The second part is the choice of cryoprotective agents. The third part is the actual protocol for introducing and removing the cryoprotectant. And the final part is treatment of the organ and the recipient upon organ transplantation.

Part I: Organ Pretreatment with Cytoprotective Drugs In vivo and Organ Procurement

The donor is pretreated in the normal manner except for the infusion of iloprost, which is a relatively long-lived analogue of PGI_2. Iloprost has been found by the present inventors to be effective in blocking the toxicity of subsequently-encountered cryoprotectant after either intravenous infusion to the systemic circulation or when given directly into the artery (or portal vein) of specific organs of interest. The best mode dose of iloprost appears to be 25 micrograms/kg given by either route, although direct intra-arterial infusion is presently preferred to maximize organ exposure to the agent while minimizing iloprost-mediated systemic hypotension. 15 μg/kg is also effective, but appears less effective than 25 μg/kg. Acceptable limits of iloprost concentration for this application are 5-75 μg/kg, depending on species, infusion rate, duration of operation, etc. Iloprost is infused over the course of 20 min; acceptable infusion duration limits are 1-60 min for cadaveric organ donors. In the latter, for example, an acceptable variation would be to infuse iloprost briefly to protect the organ from the warm ischemia of organ procurement and then to compensate for brief exposure by perfusing with iloprost-containing solution at elevated or cold temperatures for a sufficient time (5-40 min). The second variation is to

infuse iloprost at relatively low concentration over a relatively long time (20-60 min) so as to minimize hypotension; donor infusions for longer than 60 min are impractical.

After iloprost pre-treatment in vivo, organs of interest are flushed in situ with cold Euro Collins solution, UW solution or a comparably effective solution either simultaneously or in a phased manner so as to stabilize all organs quickly and thereby avoid conflicts in organ procurement. (Should normothermic preservation techniques supersede hypothermic preservation for hearts, the heart can be flushed with warm rather than cold solution.) The flushing solution(s) should initially contain iloprost (1 μg/ml in best mode, acceptable limits being 0-10 μg/ml), anticoagulants (e.g., heparin, 10,000 units/liter in the present embodiment, acceptable variations being 1,000-20,000 units/liter), vasodilators (e.g., papaverine, 40-90 mg/liter in best mode, 0-80 mg/liter as acceptable limits) and other desired agents, but a second flushing solution should be used to wash out all of these agents as cooling and blood washout is completed. The excised organ (except for organs that are best maintained by normothermic perfusion) should be transferred to an iced bath of flush solution and transported to a perfusion machine capable of introducing and removing cryoprotectants in the fashion to be described.

Part 2: Cryoprotective Agents: Formulae of the Vitrification Solutions VS4, VS41A, VS5, and VS51A

All experiments have been carried out using solutions known as VS4 or VS41A. VS4 is composed of dimethyl sulfoxide (D), formamide (F), and 1,2-propanediol (P) such that the mole ratio of D to F is 1:1, the total mass of D+F+P per liter is 490 grams, and the total mass of P per liter is 150 grams. Thus, per liter, D + F = 340 grams, F = 124.33 grams, and D = 215.67 grams. This mixture of cryoprotectants is preferred based on the results described below. Acceptable variations for

the proportions of D, F, and P are: D:F weight ratio can be as low as 1.4 and as high as 3.5; for the former, the proportion of P:(D + F) should be elevated to 18:34 and/or total concentration raised to 50-51% w/v (grams/deciliter) by addition of extra P.

At low cooling rates (5-l0°C/min) VS4 will vitrify at 1,000 atm of hydrostatic applied pressure but not at ordinary ambient pressures. The formula known as VS4lA is required for use at ambient pressures ("lA" refers to 1 atmosphere). VS41A is prepared by multiplying all VS4 constituent masses by 55/49: thus, the total concentration of solutes in VS41A is 550 grams/liter vs the 490 grams/liter of VS4.

VS4 and VS41A appear to be particularly beneficial due to the exceptional ability of formamide to penetrate kidney tissue, the ability of dimethyl sulfoxide to block the toxicity of formamide, the beneficial balance between the three ingredients (maximizing vitrification tendency while minimizing both toxicity and total solute concentration), the lack of a colloid (typical colloid concentrations of about 4-7% w/v elevate viscosity unacceptably), the extraordinarily slow rate of devitrification of these solutions at appropriate pressures (1,000 atm and 1 atm, respectively), and the good stability of VS4lA at -135°C during at least 6 months of storage.

The cryoprotectants used for organ perfusion are to be adjusted between the limits represented by VS4 and VS41A, depending upon the organ's tolerance to high pressures and the organ's tolerance to high cryoprotectant concentrations, so as to optimize the tradeoff between pressure and concentration required to maintain vitrifiability. For example, an organ that cannot tolerate 1,000 atm but that can tolerate 500 atm should be perfused with a solution intermediate between VS4 and VS41A (i.e., total grams of D + F + P per liter = 520), with the relative proportions of D, F, and P remaining unchanged. Very large organs that require extremely slow cooling rates at ambient pressure should be perfused with concentrations in excess of 550 grams/liter, to a maximum

of about 600 grams/liter, to ensure vitrifiability at these very low cooling rates at ambient pressure. At elevated pressures, similar proportional increases in solute concentration will be required as cooling rate is lowered.

Recent experiments (see results below) with kidney slices indicate that a formula identical to that of VS4 but with 2,3-butanediol replacing l,2-propanediol, wherein 2,3-butanediol consists of a mixture of the dextro- and levo-rotatory forms with minimal meso form present (<5% w/w), provides viability identical to the viability obtained with VS4. This formula, known as VS5, may have greater stability than VS4. Similarly, VS51A is composed as per the above description of VS41A, except for the replacement of 1,2-propanediol by dextrose and levorotatory isomers of 2,3-butanediol (<5% w/w meso form). Variations between VS5 and VS51A are to be used as per the descriptions above for VS4 to VS41A.

All cryoprotectant solutions must contain, in addition to the cryoprotectants themselves, slowly-penetrating solutes comprising the "carrier" or "vehicle" solution for the cryoprotectants. Typical examples would be UW solution, Euro Collins solution, or Renal Preservation Solution 2 (RPS-2). Euro Collins and possibly RPS-2 are believed to be superior to UW as carrier solutions for kidneys, whereas the opposite is likely to be true for livers, and hearts may do best with none of these particular carriers. The best mode process uses Euro Collins as the carrier solution of choice.

Part 3: Protocol for Cryoprotectant Introduction and Removal

A typical protocol for cryoprotectant introduction and removal presently in use in the present inventors' laboratory and yielding reliable, high-quality survival of rabbit kidneys after cryoprotectant washout, transplantation, and long-term functional and histological follow-up, is

shown in Figure 5 and described in more detail in the flow charts of Figures 6A-E.

Perfusion pressure. The organ is perfused at pressures sufficient to overcome the organ's critical closing pressure but otherwise low enough to avoid needless damage to the vascular tree. The best mode perfusion pressure is 40 mmHg, without significant pulsation. A desirable range of acceptable pressures has been found to be 20-70 mmHg for different species, including man.

Initial perfusion. In the best mode protocol, perfusion is first carried out for 15 min to establish baseline values for vascular resistance, to establish calibrations (for pressure and refractive index), to ensure complete blood washout, and to thermally equilibrate the organ. Clinically, the initial perfusion time is arbitrary, and can be adjusted (from zero minutes to 1-2 days or more) to meet the requirements of the organ procurement and transportation process. In the inventors' laboratory, the perfusate in this period is Euro Collins solution. However, this initial perfusate could also be UW solution or other stabilizing solution in a clinical setting depending upon the needs of the hospital or procurement team.

Initial temperature. Initial perfusion temperature required for organ procurement and transportation need not be identical to the perfusion temperature established just before introduction of cryoprotectant. For example, most organs may be shipped while surrounded by crushed ice at 0°C while other organs may be shipped while being perfused at normothermia (37°C). When organs are ready for cryoprotectant administration, however, a preselected, standardized perfusion temperature is established. In the best mode process, initial perfusion temperature is 3.5-4°C, and the acceptable limits are 0-15°C. The inventors consider that organs requiring normothermic perfusion for best long-term maintenance can nevertheless be cooled to within this same temperature range and treated in a manner similar to that of

hypothermically-preserved organs without damage within the relatively short times required for this process.

First elevation of cryoprotectant concentration. Following the initial baseline perfusion, cryoprotectant concentration is elevated at a constant rate until a first plateau of concentration is established. When using a VS4-type mixture of cryoprotectants, the proportions of different cryoprotectants in the mixture are held constant while total concentration is allowed to change. The rate of increase in total concentration for VS4-type solutions is set to 51 mM/min (3.06 M/hr) in the best mode process, acceptable variations being 35-75 mM/min. These rates are considerably in excess of the 30 mM/min rates used by known techniques for glycerol and propylene glycol which are considered to be unnecessarily and undesirably slow for vitrification solution solutes. Linear elevation of concentration promotes equilibration without creating unnecessarily large osmotic stresses.

First concentration plateau. The first plateau is set in the best mode process at 25% w/v total cryoprotectant (250 grams/liter, or about 3.8 molar), acceptable variations being 20-32% w/v or w/w. The first plateau should be set to a level that is close to half the concentration of the final vitrification solution: lower first plateau levels will increase osmotic stress upon subsequent perfusion with vitrification solution, whereas substantially higher first plateau levels will produce increased toxicity due to longer exposure times to concentrated cryoprotectant. The duration of the first plateau is set to 10 min in the best mode procedure, acceptable variations being 5-30 min, depending on perfusion pressure (and thus organ flow rate), vascular resistance, and organ permeability to cryoprotectant. The duration should be great enough to allow the organ to osmotically equilibrate with the arterial perfusate, as indicated by a zero (or virtually zero) arteriovenous concentration difference, to minimize unnecessary osmotic stress during the subsequent jump to vitrification solution.

Temperature reduction during first concentration rise. During concentration elevation, temperature is simultaneously lowered to protect the organ from chemical toxicity of the cryoprotectant. In the best mode process, temperature reduction begins as the arterial cryoprotectant concentration reaches 1.3 molar; acceptable limits are 0.5 molar to 3.5 molar. Temperature descent is terminated as the arterial concentration reaches the first concentration plateau (as noted above, 25% (3.8M) in the best mode procedure and 20-32%, or about 3-4.9 M for VS4 solutes, within the process limits). The concentration change during cooling is thus about 2.5 M in the best mode process and may vary from about 1 M to 4.4M.

As noted above, pre-cooling temperature should fall within the limits of 0-15°C. The temperature after cooling should fall within the range of -13°C to +5°C. Cooling should not continue to below the freezing point of the organ. In the best mode process, final temperature is presently -1.5°C, representing a fall of 5°C from the initial temperature and a cooling rate of about 0.25°C/min. The maximum cooling rate possible within the above limits is about 2.1°C/min, which should be slow enough to avoid possible thermal shock to the organ. Minimum temperature drop during cooling is 2°C, maximum temperature drop is 28°C.

Perfusion with vitrification solution, second concentration plateau. A step change in concentration from the first concentration plateau to the vitrification solution is necessary to control exposure time to highly concentrated cryoprotectant. In the best mode procedure, the concentration of vitrification solution is, as noted above, 490-550 grams/liter or about 7.5-8.4 molar for VS4-type solutions (extending as high as 600 grams/liter for hard-to-vitrify materials such as livers). For VS5-type solutions, the final concentrations may be reduced slightly, to about 480-540 grams/liter. For non-VS4-type vitrification solutions, the concentration limits for the present process are 40%-60% w/v cryoprotectant. Concentration is held steady at the vitrifiable

concentration for 20 min in the best mode procedure, acceptable variations being 10-50 min. Concentration must be held steady sufficiently long for the removal of non-vitrifiable water from the cells and from the interstitial spaces, i.e., long enough for the organ to closely approach osmotic equilibrium with the perfusate.

Temperature during perfusion with vitrification solution. In the best mode procedure, temperature is held constant at 0 to -5°C (the temperature of choice presently being -1.5°C) as concentration rises to vitrifiable levels. Temperature constancy rather than renewed temperature descent is desirable to control viscosity: as viscosity rises with temperature reduction, effective organ resistance must also increase, reducing organ osmotic equilibration rates, necessitating increased organ exposure times to the cryoprotectant, and possibly exerting greater damage to the vascular endothelium. Lower temperatures also increase the likelihood of "chilling injury". However, an acceptable variation would be to further lower temperature as the jump to vitrification solution commences or shortly thereafter, particularly for organs perfused at or near the high temperature limits up to this point and particularly for concentrations above 49% w/v and organs that are particularly susceptible to cryoprotectant toxicity and require lower temperatures to suppress this toxicity. Although VS41A and VS5lA have freezing points close to -40°C, perfusion to temperatures this low are not included in the present process, since temperatures this low appear unnecessary, cumbersome, and most likely counterproductive. The low-temperature limit of the process is therefore set during vitrification solution perfusion at only -20°C and +5°C is retained as the upper limit, permitting limited additional cooling during vitrification solution perfusion.

The next step of any practical vitrification procedure will be to remove the organ from the perfusion machine and cool it to cryogenic temperatures, with or without prior pressurization. After the organ is warmed, however, it will have to be placed back into the perfusion

machine to resume the type of perfusion protocol shown in Figure 5 at the beginning of the third concentration plateau.

First concentration reduction: third concentration plateau. The choice of concentration for the third concentration plateau in the best mode protocol is 30% w/v (300 grams/liter; 4.6 M) VS4 solutes (D, F, and P in the usual proportions), acceptable variations being 20-35% (w/v or w/w) cryoprotectant (roughly 3 to 5.5 M). The concentration at this stage should not be less than 40% (2/5) of the concentration of the vitrification solution in order to avoid osmotic damage; in the best mode process, the concentration at the third plateau is over 3/5 of the concentration at the second plateau.

An "osmotic buffering agent" (non-penetrating extracellular low-molecular-weight solutes that counteract the osmotic effect of greater intracellular vs extracellular concentrations of cryoprotectant during the cryoprotectant efflux process) is present in the third plateau perfusate (although not shown in Figure 5). Preferred osmotic buffering agents are raffinose or sucrose. Although mannitol has been used successfully in virtually all of the inventors' experiments, mannitol has been found to penetrate renal cells with resulting detrimental effects. Mannitol and even sucrose will not be workable for the liver, either, since its cells are much more permeable to both solutes than are most mammalian organs' cells.

Osmotic buffer concentration in the best mode 30% washout plateau solution is 250 mM. In protocol variations employing lower third plateau concentrations (e.g., 20% w/v cryoprotectant), more osmotic buffer is required (to an upper limit of 1,000 mM). In variations employing higher third plateau concentrations (e.g., 35% w/v cryoprotectant), less osmotic buffer is required (to a lower limit of about 150 mM). The presence of osmotic buffer within these limits is required to counteract the otherwise-fatal osmotic effects of a large stepwise drop in penetrating cryoprotectant concentration. The duration of the third concentration plateau is 16 min

in the best mode process (acceptable limits = 5-40 min), which is just enough time for osmotic equilibration of the organ with the washout perfusate.

Temperature during the third concentration plateau. The choice of perfusion temperature during the third plateau depends on the previous thermal history. In the best mode process, perfusion temperature is retained at -l.5°C. The only cases in which the temperature will be different during the second and third concentration plateaus is in variations in which temperature is reduced during second plateau perfusion to values below or near the freezing point of the third plateau perfusate or when chilling injury requires additional warming to minimize overall damage. In these variations, the temperature of the third plateau perfusate is set to the minimum value consistent with minimizing damage, and the organ is warmed to this temperature before being perfused with perfusate at the concentration of the third plateau.

Gradual concentration reduction to zero: The next stage in the process is the gradual reduction of cryoprotectant concentration to zero or virtually zero. In the best mode process, this is carried out at a constant rate of about -43 mM/min (acceptable variations being -31 to -65mM/min). Non-constant declining concentration schedules (rapid fall at high concentrations, slower fall at lower concentrations) are also an acceptable variation, e.g., a linear fall at 1.5 times the average linear rate for the first third of the washout followed by a linear fall at 0.86 times the average linear rate for the second two-thirds of the washout.

As penetrating cryoprotective agent concentrations fall, the concentration of osmotic buffer also falls in proportion, reaching a final nonzero concentration of osmotic buffer when penetrating cryoprotectant concentration reaches zero. This final nonzero concentration of osmotic buffer is 50 mM in the best mode process and may acceptably vary from 25 mM to 500 mM. During reduction of cryoprotectant concentration, absolute transmembrane osmotic forces attributable to the cryoprotectant

transmembrane concentration gradient become reduced, thus reducing the requirement for osmotic buffering. Reducing osmotic buffer concentration during cryoprotectant washout is therefore designed to minimize osmotic damage from the osmotic buffer both during cryoprotectant washout and thereafter and is further designed to reduce potential cellular uptake of nominally non-penetrating osmotic buffering agent. No previous perfusion technique of cryoprotectant washout has ever made use of the "declining osmotic buffer principle".

Temperature control during gradual cryoprotectant washout. During cryoprotectant washout, temperature is elevated to facilitate washout, reduce osmotic forces, and restore a perfusion temperature appropriate for an organ containing no cryoprotectant. In the best mode process, temperature elevation begins as concentration falls to 4.7 molar and continues linearly with concentration drop until the initial perfusion temperature is reached when arterial concentration reaches 1.3 to 0.8 M (1°C per 0.68 to 0.78 M rise in concentration; 3.4-3.9M concentration change during warming). Acceptable variations for the concentration at which temperature initially rises are 2.5-5.5 M and for the concentration at which temperature rise is completed are 0.5M-4.5M.

Osmotic buffer washout. The final step in the process is to wash out the osmotic buffer. In the current best mode process, 50 mM sucrose is attained at the end of cryoprotectant washout. Although it is acceptable to leave such low concentrations of osmotic buffer in the organ during short holding times before transplantation, interstitial osmotic buffer (OB) is expected to cause osmotic expansion of the interstitial space during blood reflow, with consequent temporary reduction in organ perfusion in vivo. This effect will become unacceptable at higher OB concentrations (>100 mM) and will necessitate OB washout before transplantation. A further problem with leaving OB in the organ for extended times before transplantation is potential leakage of OB into organ cells with consequent cellular swelling and reduced perfusion upon transplantation.

The inventors have typically washed out 50 mM mannitol over the course of 30 min with complete success upon transplantation. Higher concentrations of OB (up to 500 mM) may be washed out over more extended times (30-90 min) that depend on perfusion resistance response to OB dilution. For clinical purposes, the duration of the post-washout perfusion period, comprising the osmotic buffer washout plus subsequent perfusion with no osmotic agent, is adjustable to fit the logistic requirements of organ transportation and transplantation.

Part 4: Treatment of the Organ and the Recipient at the Time of Transplantation and Thereafter

It is essential that the recipient receive aspirin (acetylsalicylate, 1-3 mg/kg) and heparin (100-250 units/kg) shortly before release of the vascular clamps, both higher and lower concentrations resulting in vascular obstruction and failure. The best mode concentrations are 2 mg/kg and 200 units/kg, respectively. It may also be helpful to gradually re-infuse iloprost (5-40 μg/kg, IV) beginning 5 min before clamp release and continuing for at least an additional 15 min, to obviate reperfusion injuries such as damage resulting from temporary hypoxia and inflammatory responses. The best mode method involves the infusion of 7-10 μg/kg of iloprost IV beginning 5 min before revascularization and continuing until 15 min after revascularization. No benefit has been observed from the use of calcium channel blockers.

Process for Control (Non-Cryoprotectant) Perfusions.

The equipment described here is capable of creating, besides organ cryoprotection protocols, a wide varitey of protocols for conventional organ hypothermic and normothermic preservation. In addition, a wide variety of normothermic pharmacological, physiological, and pathophysiological protocols are possible. The present inventors exeplify

many of these possibilities by describing the steps required to carry out many of these protocols in Figures 7A-7B, which are self-explanatory.

Results

Figure 8 shows post-operative serum creatinines of rabbits receiving kidneys previously perfused with VS4 in Euro-Collins solution. Prior to procurement, organs were treated in vivo with zero, 15, and 25 μg/kg of iloprost given by systemic intravenous infusion over a 20-minute period. Kidneys in these three groups were exposed to VS4 at +2, 0-2, and -1 to -6°C, respectively. Initial and final perfusion temperatures were 2°C in all cases. Rabbit survivals in these three groups were 5/16 (31%), 6/10 (60%), and 10/10 (100%), respectively. Only data for rabbits surviving the first night after surgery are included. Rabbit survivals depended entirely on the function of the kidney previously perfused with VS4: a contralateral nephrectomy was performed at the time of transplantation, and no support by dialysis was attempted. Histology at long-term follow-up in these rabbits was poor without iloprost, marginal with the lower dose of iloprost, and normal with the higher dose of iloprost and the lowest perfusion temperatures. The results of control (no cryoprotectant) perfusions with Euro Collins are included in Figure 8 as well (bottom curve). Although damage in the best VS4 group is greater than in the controls, all damage appears to be fully reversible within a short time postoperatively.

Table I: Viability of Kidney slices Treated with VS4 vs. VS5

Treatment	K/Na ratio of tissue (mean +/- SEM)
VS4	3.43 +/- 0.07
VS5	3.27 +/- 0.12
$p > 0.05$	

K/Na ratio measured after washing out the cryoprotectants and incubating the cortical slices at 25°C for 90 minutes to permit active transport of K^+ and Na^+.

While various embodiments of the present invention have been described above, it should be understood that they have been presented by way of example, and not limitation. Thus the breadth and scope of the present invention should not be limited by any of the above described exemplary embodiments, but should be defined only in accordance with the following claims and their equivalents. It will be understood by those skilled in the art that various changes in form and detail may be made therein without departing from the spirit and scope of the invention.

CLAIMS:

1. A method for preparing organs or tissues for long-term preservation through the introduction of a vitrifiable concentration of cryoprotectant and subsequently preparing the organs or tissues for transplantation by the removal of the cryoprotectant, comprising the steps of:

(a) initially perfusing the organ or superfusing the tissue without cryoprotectant;

(b) adding a cryoprotectant solution to the organ or tissue and gradually elevating the cryoprotectant concentration to a first predetermined level and reducing the temperature of the organ or tissue;

(c) delaying an increase in the concentration of said cryoprotectant for a time sufficient to permit approximate osmotic equilibrium of the organ or tissue to occur;

(d) elevating the cryoprotectant concentration of said solution to a level, greater than said first predetermined level, required for vitrification and maintaining the solution at the elevated concentration for a time sufficient to permit approximate osmotic equilibrium of the organ or tissue to occur;

(e) perfusing the organ or superfusing the tissue with a reduced, non-vitrifiable concentration of cryoprotectant in combination with a nonpenetrating osmotic buffering agent to a first buffering agent concentration level for a time period sufficient to permit approximate osmotic equilibrium of the organ or tissue to occur;

(f) washing out substantially all of the cryoprotectant while decreasing the concentration of the osmotic buffering agent to a second, nonzero level substantially below said first buffering agent concentration level and increasing the temperature of the organ or tissue; and

(g) perfusing the organ or superfusing the tissue to remove the osmotic buffering agent sufficiently to render the organ or tissue suitable for transplantation.

2. A method according to claim 1, wherein said cryoprotectant comprises a solution consisting essentially of dimethyl sulfoxide, formamide, and 1,2-propanediol.

3. A method according to claim 1, wherein said cryoprotectant comprises a solution consisting essentially of dimethyl sulfoxide, formamide, and 2,3-butanediol.

4. A method according to claim 1, further comprising the steps of:

first, medicating the organ or tissue in vivo with a cryoprotective agent; and

second, removing the organ or tissue from a donor.

5. Use of a method according to claim 1 to prepare an organ or tissue for transplantation by a method comprising the step of:

medicating a recipient for a first predetermined period of time prior to transplanting the organ or tissue into the recipient with an anti-inflammatory agent, an antiplatelet agent, and an anticoagulant; and

transplanting the organ or tissue into the recipient.

6. The method of claim 1, wherein a tissue is prepared.

7. The method of claim 1, wherein an organ is prepared.

8. The method of claim 7, wherein the organ is a kidney.

9. A computer-controlled apparatus for perfusing a biological organ, comprising:

a plurality of fluid reservoirs;

an organ container for holding the biological organ;

means defining a first fluid flow path between said plurality of reservoirs and said organ container;

selection means interposed in said first fluid flow path for selectively connecting said reservoirs to said organ container;

pump means interposed in said first fluid flow path for pumping fluid from one or more of said reservoirs to said organ container and for pumping fluid from said organ container to one or more of said reservoirs;

means defining a second fluid flow path between the output side of said pump means and said reservoirs and bypassing said organ container;

sensor means interposed in said fluid flow paths for sensing at least one of the concentration, temperature, pH, and pressure of the fluid flowing in said first and second fluid flow paths;

a programmable computer;

means coupling said sensor means to said computer for providing a continuous information stream from said sensor means to said computer;

means coupling said computer to said selection means and said pump means to continuously selectively control (a) the flow of fluid from each of said reservoirs individually to said first fluid flow path, and (b) at least one of the pressure and pH of the fluid flowing in

said first fluid flow path, in accordance with a predetermined computer program without operator intervention;

temperature conditioning means interposed in said first fluid flow path for conditioning the temperature of fluid flowing from said reservoirs to said organ container; and

measuring means interposed in said first and second fluid flow paths and coupled to said computer for measuring concentration and temperature differences between the upstream and downstream sides, in the fluid flow direction, of said organ container and for providing information corresponding to the measured differences to said computer.

10. A computer-controlled perfusion apparatus according to claim 9, wherein said pump means comprises:

a first pump interposed in said first fluid flow path for pumping fluid from one or more of said reservoirs to said organ container; and a second pump interposed in said second fluid flow path for pumping fluid from said organ container to one or more of said reservoirs.

11. A computer-controlled perfusion apparatus according to claim 9, further comprising:

means defining a third fluid flow path between the output side of said pump means and said reservoirs and bypassing said organ container;

a second temperature conditioning means interposed in said third fluid flow path for conditioning the temperature of fluid flowing in said third fluid flow path; and

measuring means interposed in said second and third fluid flow paths and coupled to said computer for measuring concentration and temperature differences between the upstream and downstream sides, in the fluid flow direction, of said organ container and for providing information corresponding to the measured differences to said computer.

12. A computer-controlled perfusion apparatus according to claim 9, wherein said biological organ is a heart, kidney or liver.

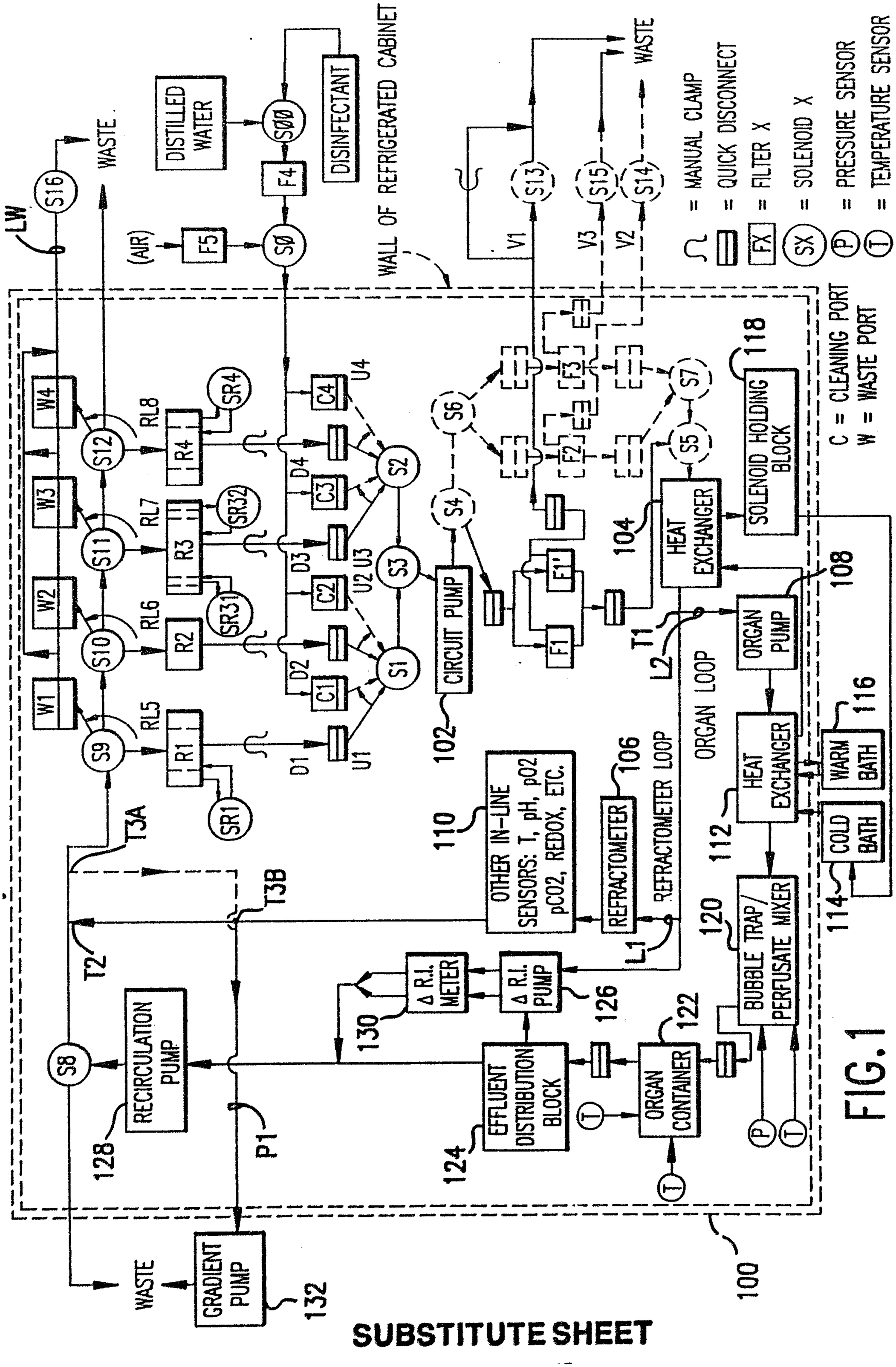

FIG. 1

SUBSTITUTE SHEET

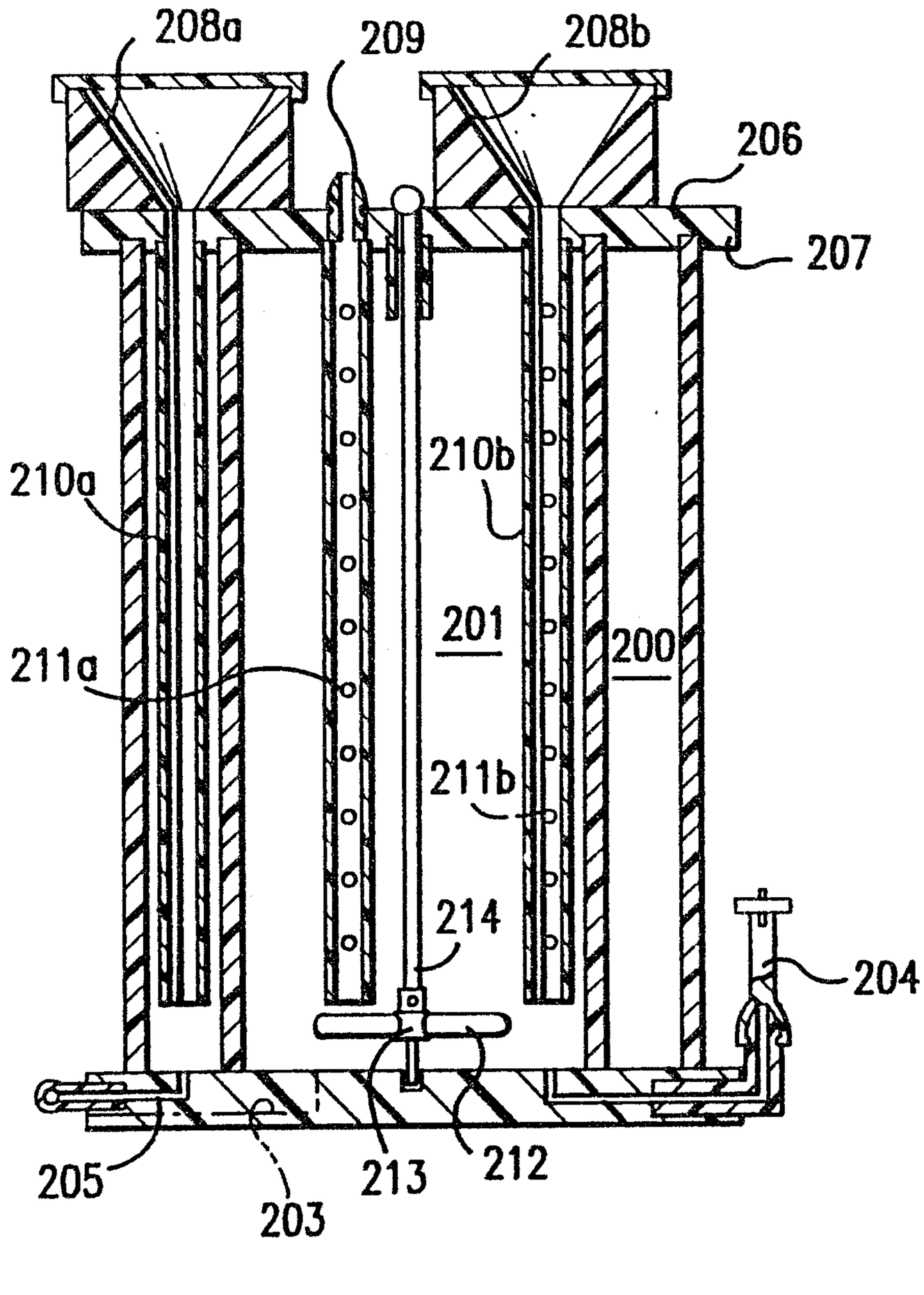
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FIG.2A

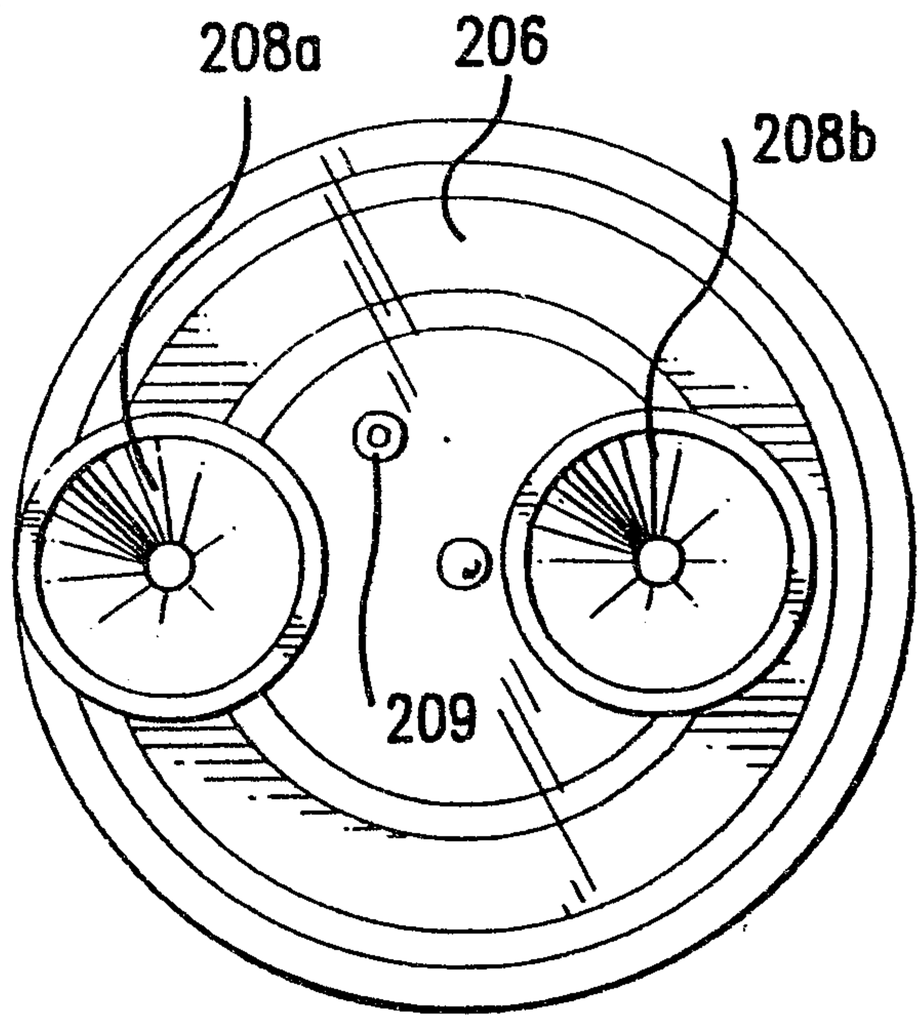

FIG.2B

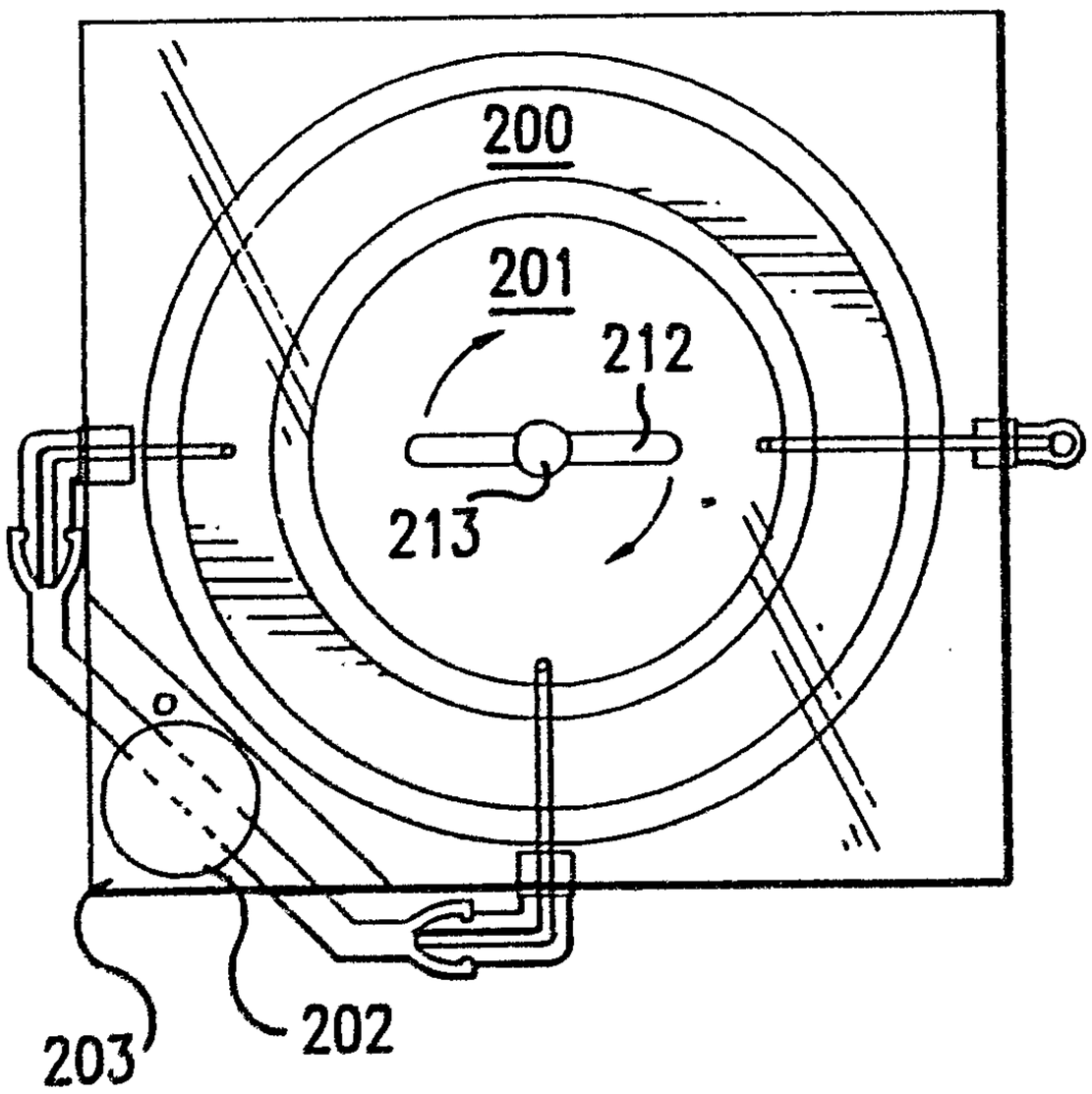

FIG.2C

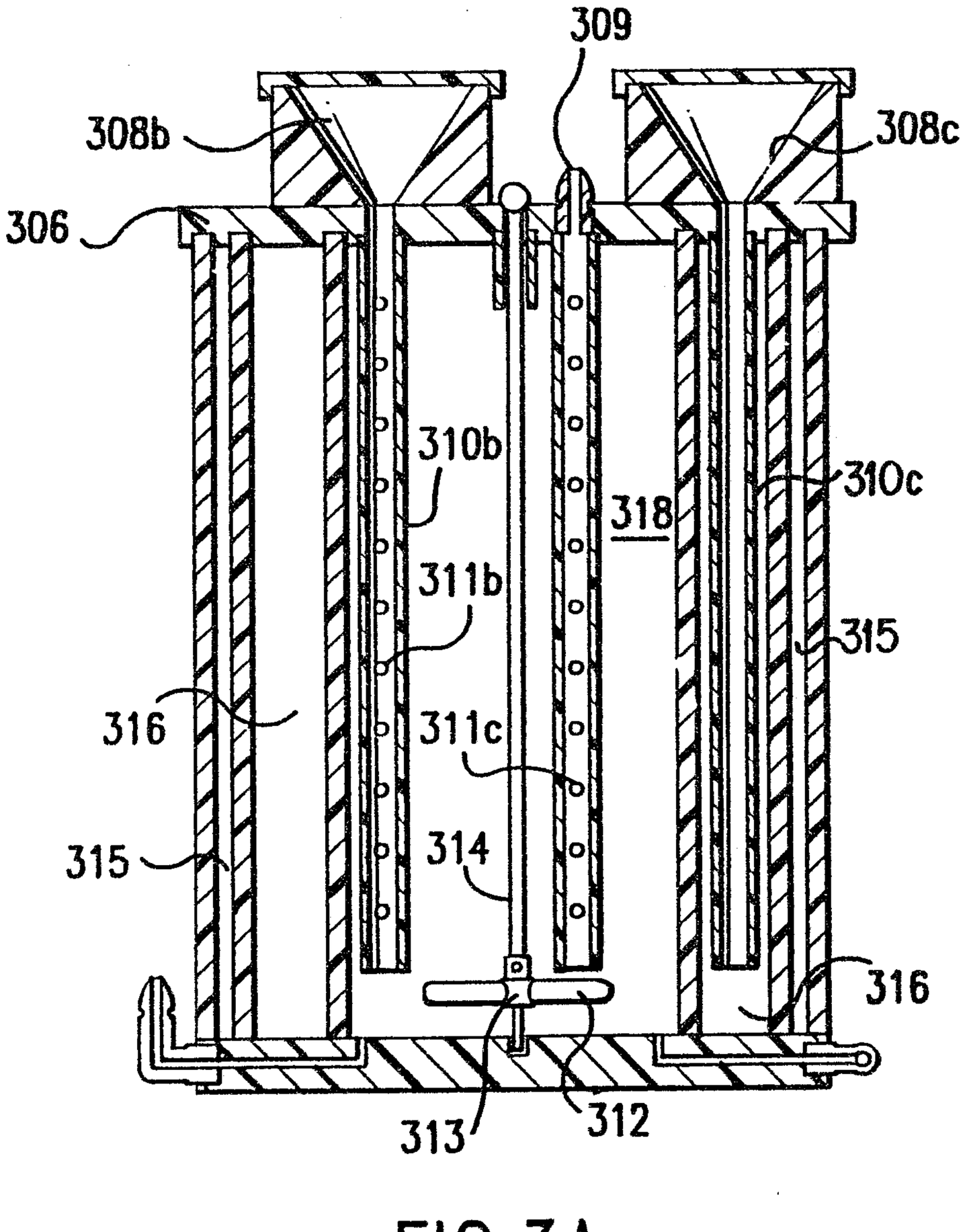
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FIG.3A

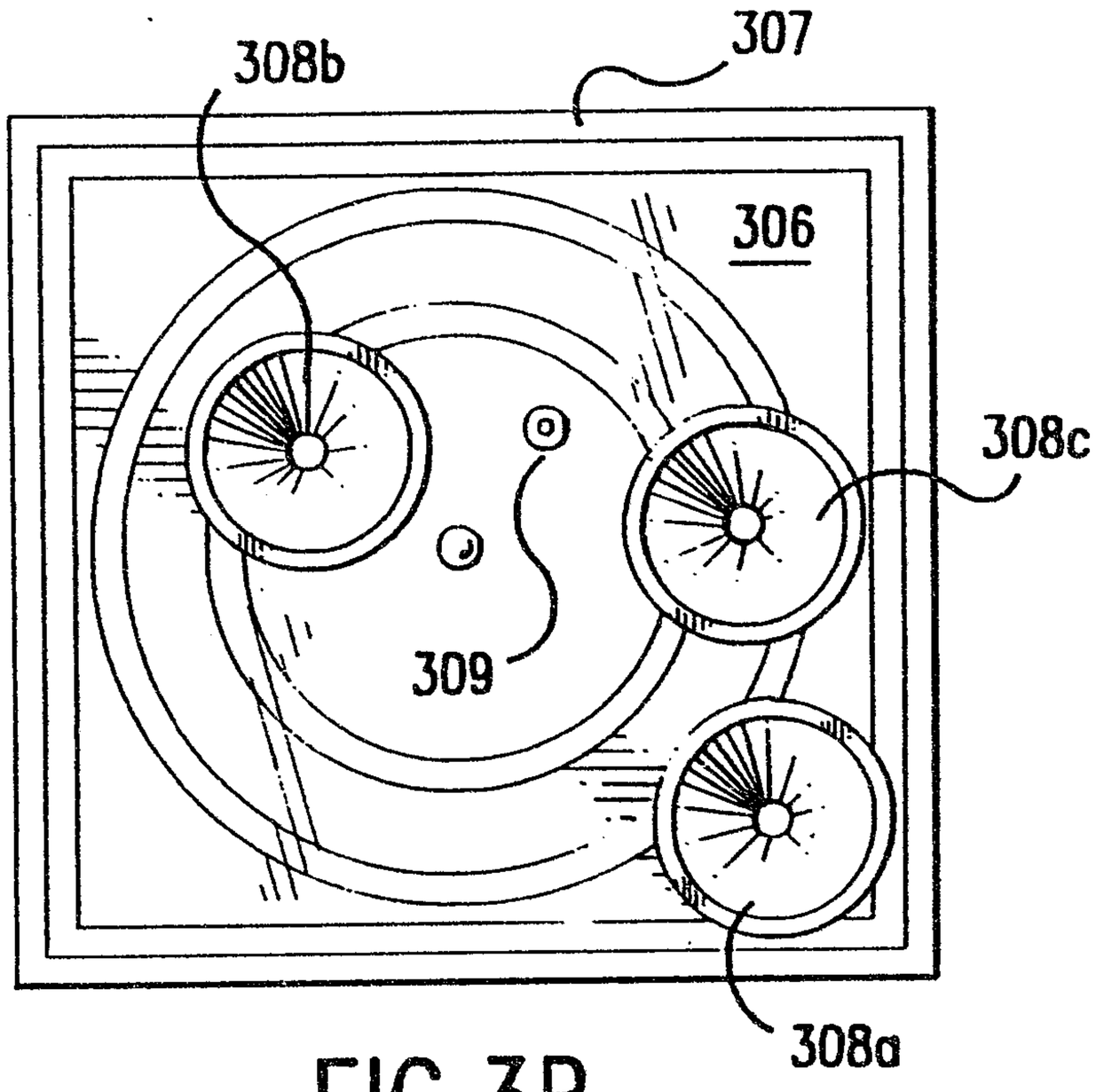
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FIG.3B

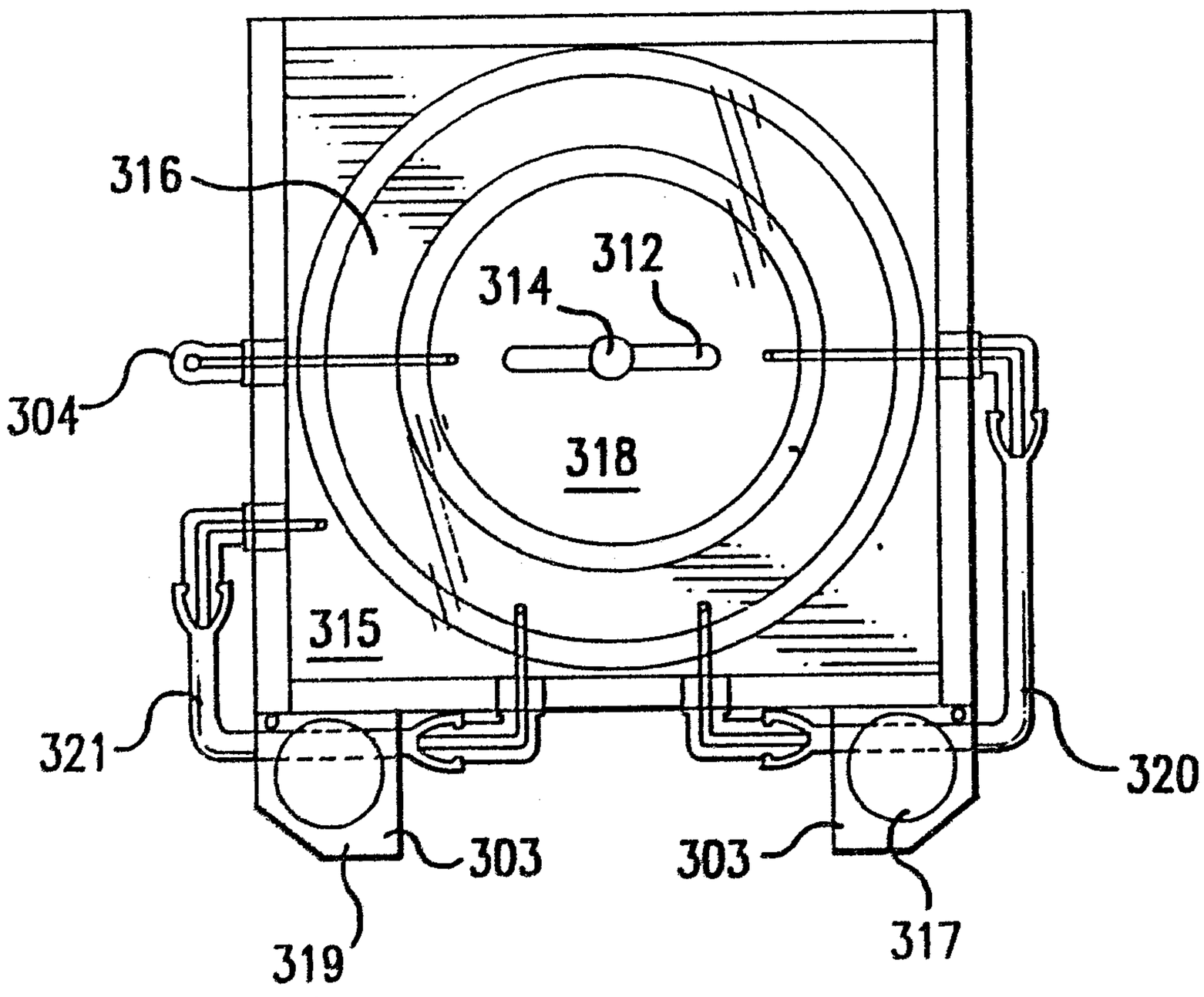
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FIG.3C

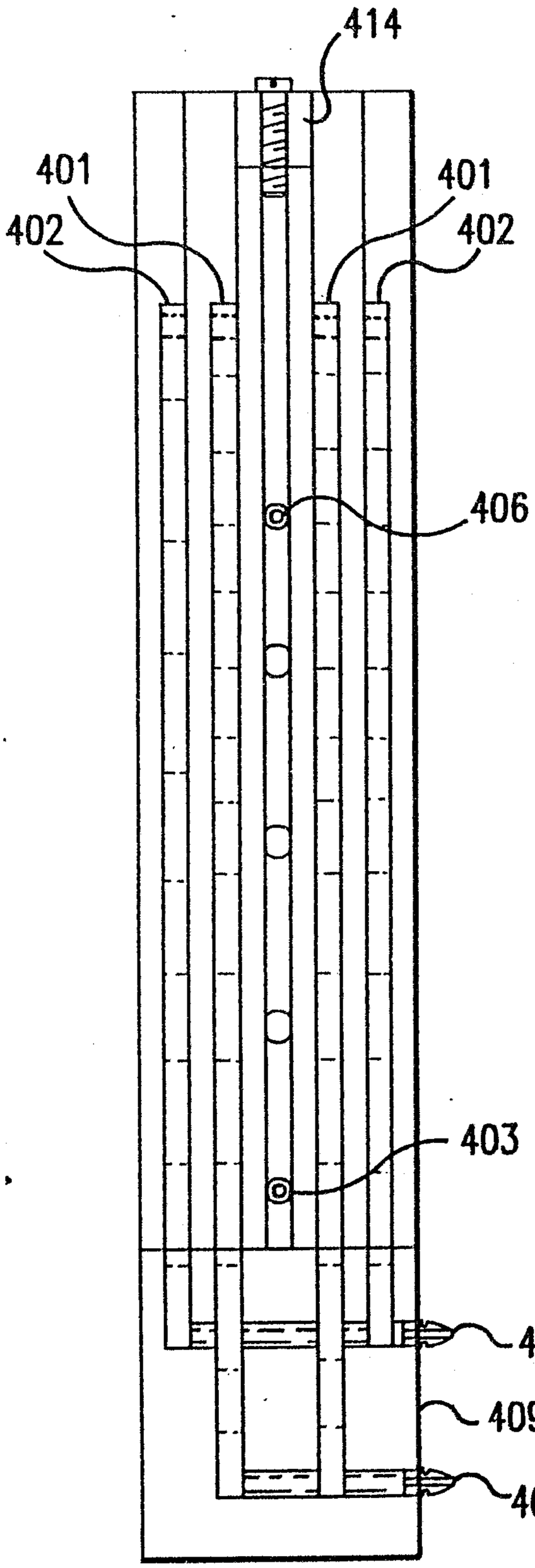
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FIG.4A

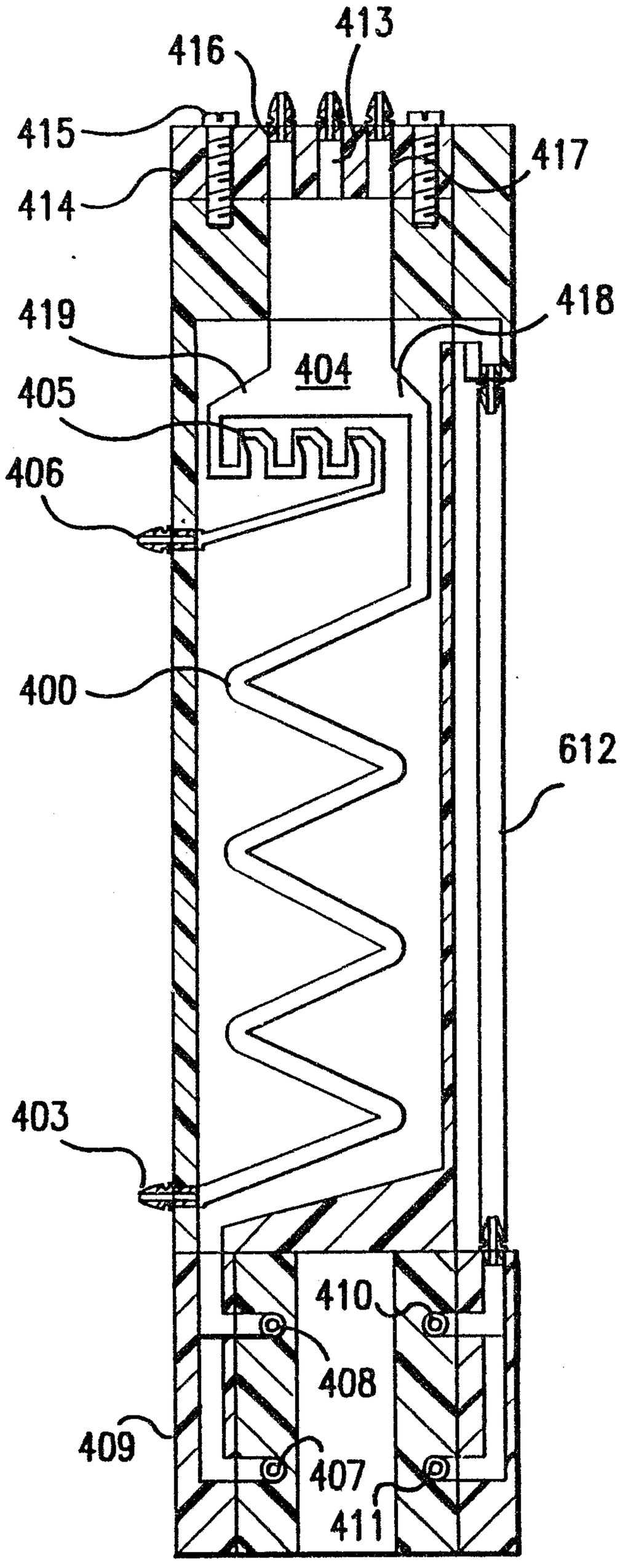
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FIG.4B

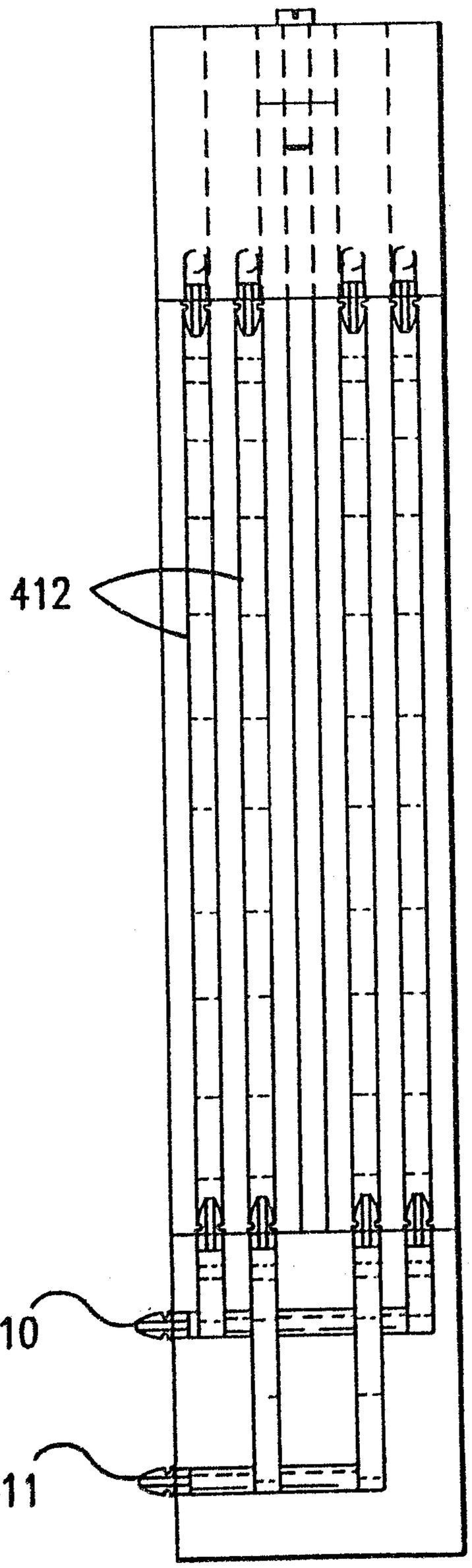


FIG.4C

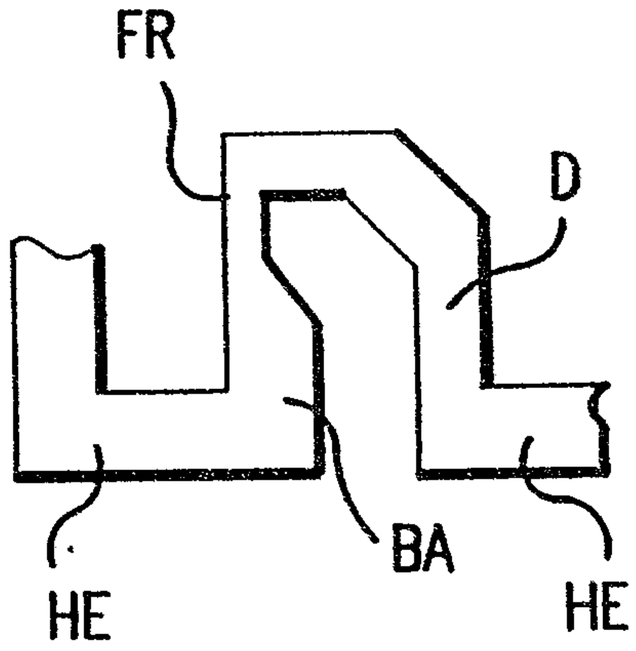


FIG.4D

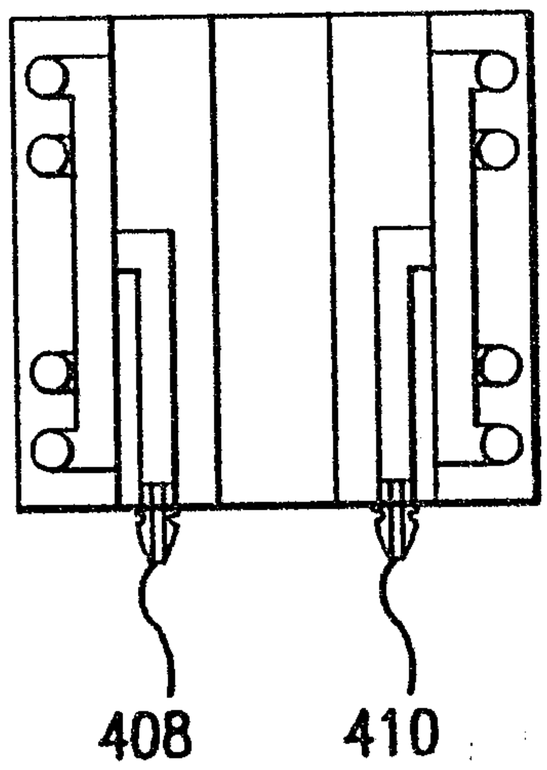
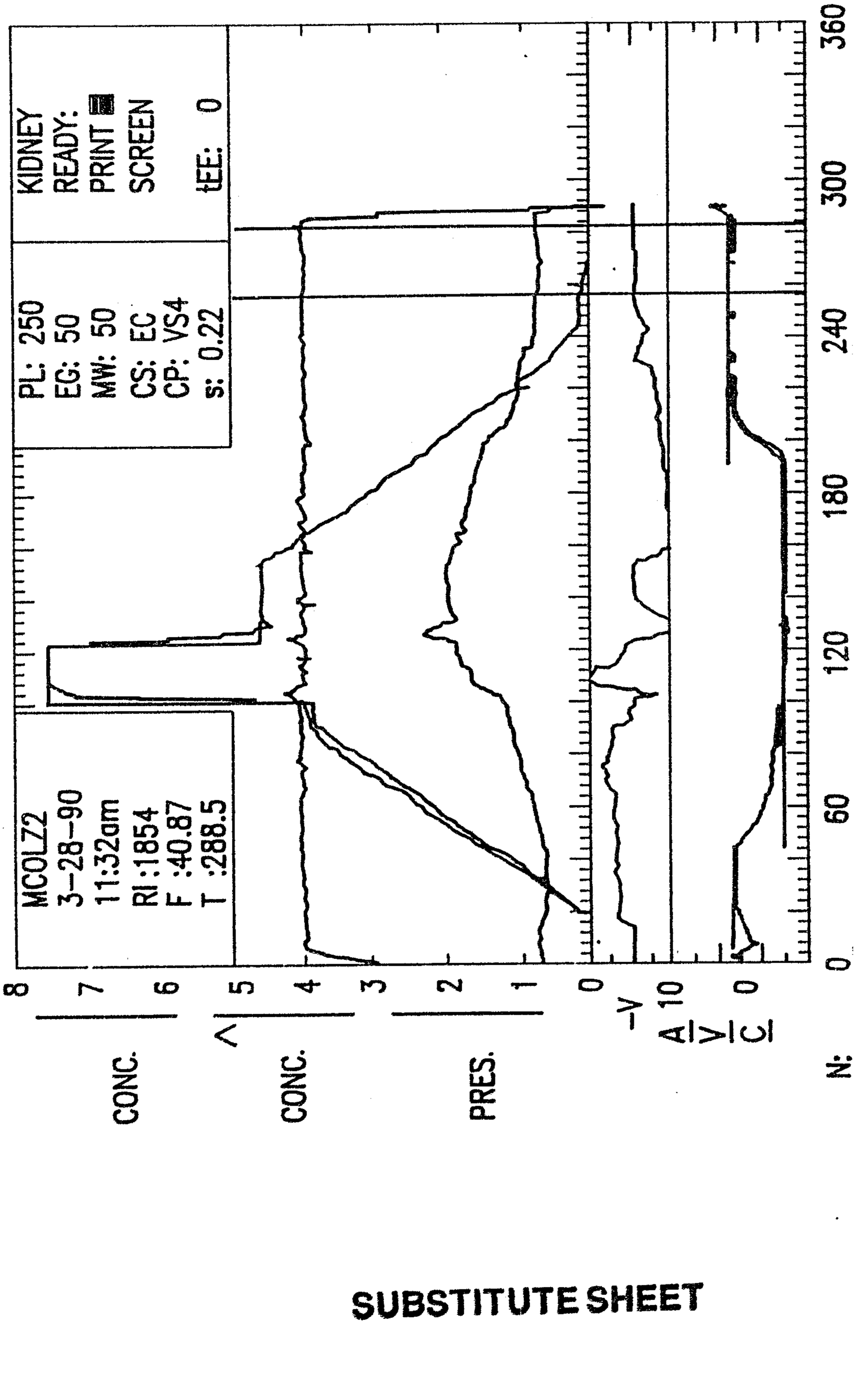


FIG.4E


KIDNEY
READY:
PRINT
SCREEN
tEE: 0
PL: 250
EG: 50
MW: 50
CS: EC
CP: VS4
s: 0.22
MCOLZ2
3-28-90
11:32am
RI:1854
F :40.87
T :288.5
360
300
240
180
120
60
0
8
7
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3
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CONC.
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FIG.5

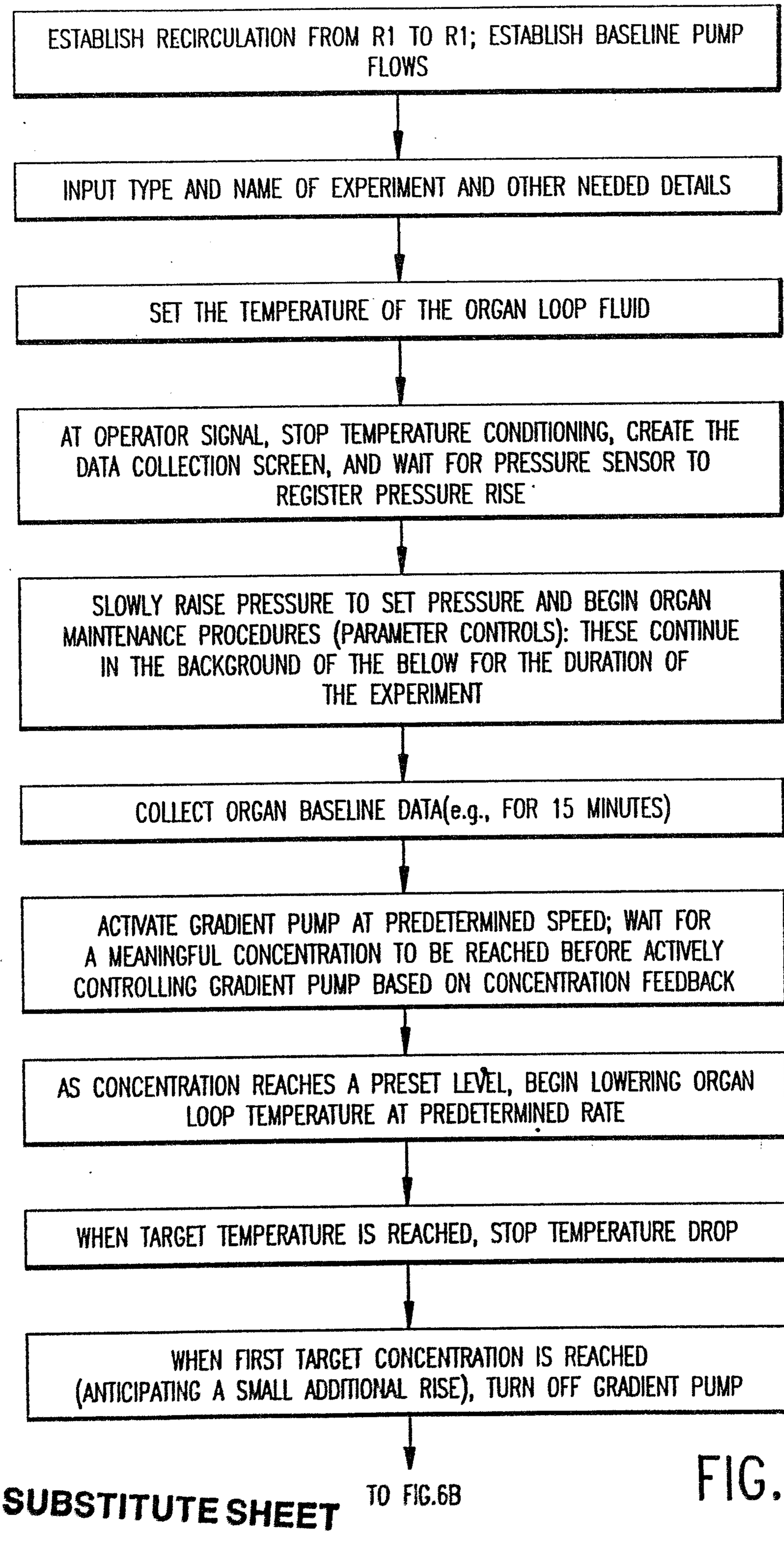

ESTABLISH RECIRCULATION FROM R1 TO R1; ESTABLISH BASELINE PUMP FLOWS
INPUT TYPE AND NAME OF EXPERIMENT AND OTHER NEEDED DETAILS
SET THE TEMPERATURE OF THE ORGAN LOOP FLUID
AT OPERATOR SIGNAL, STOP TEMPERATURE CONDITIONING, CREATE THE DATA COLLECTION SCREEN, AND WAIT FOR PRESSURE SENSOR TO REGISTER PRESSURE RISE
SLOWLY RAISE PRESSURE TO SET PRESSURE AND BEGIN ORGAN MAINTENANCE PROCEDURES (PARAMETER CONTROLS): THESE CONTINUE IN THE BACKGROUND OF THE BELOW FOR THE DURATION OF THE EXPERIMENT
COLLECT ORGAN BASELINE DATA(e.g., FOR 15 MINUTES)
ACTIVATE GRADIENT PUMP AT PREDETERMINED SPEED; WAIT FOR A MEANINGFUL CONCENTRATION TO BE REACHED BEFORE ACTIVELY CONTROLLING GRADIENT PUMP BASED ON CONCENTRATION FEEDBACK
AS CONCENTRATION REACHES A PRESET LEVEL, BEGIN LOWERING ORGAN LOOP TEMPERATURE AT PREDETERMINED RATE
WHEN TARGET TEMPERATURE IS REACHED, STOP TEMPERATURE DROP
WHEN FIRST TARGET CONCENTRATION IS REACHED (ANTICIPATING A SMALL ADDITIONAL RISE), TURN OFF GRADIENT PUMP
TO FIG.6B

FIG.6A

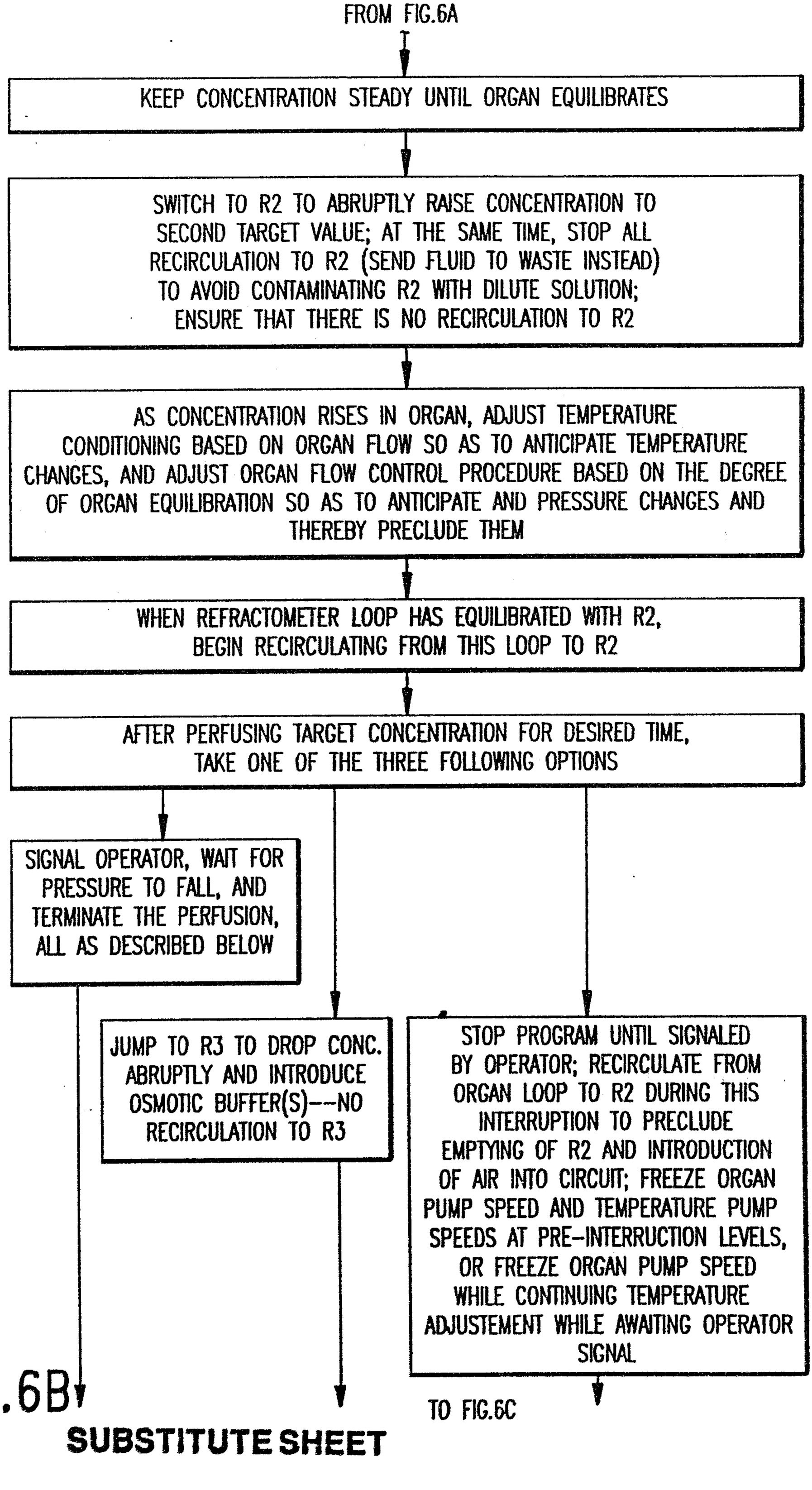

FROM FIG.6A
KEEP CONCENTRATION STEADY UNTIL ORGAN EQUILIBRATES
SWITCH TO R2 TO ABRUPTLY RAISE CONCENTRATION TO SECOND TARGET VALUE; AT THE SAME TIME, STOP ALL RECIRCULATION TO R2 (SEND FLUID TO WASTE INSTEAD) TO AVOID CONTAMINATING R2 WITH DILUTE SOLUTION; ENSURE THAT THERE IS NO RECIRCULATION TO R2
AS CONCENTRATION RISES IN ORGAN, ADJUST TEMPERATURE CONDITIONING BASED ON ORGAN FLOW SO AS TO ANTICIPATE TEMPERATURE CHANGES, AND ADJUST ORGAN FLOW CONTROL PROCEDURE BASED ON THE DEGREE OF ORGAN EQUILIBRATION SO AS TO ANTICIPATE AND PRESSURE CHANGES AND THEREBY PRECLUDE THEM
WHEN REFRACTOMETER LOOP HAS EQUILIBRATED WITH R2, BEGIN RECIRCULATING FROM THIS LOOP TO R2
AFTER PERFUSING TARGET CONCENTRATION FOR DESIRED TIME, TAKE ONE OF THE THREE FOLLOWING OPTIONS
SIGNAL OPERATOR, WAIT FOR PRESSURE TO FALL, AND TERMINATE THE PERFUSION, ALL AS DESCRIBED BELOW
JUMP TO R3 TO DROP CONC. ABRUPTLY AND INTRODUCE OSMOTIC BUFFER(S)--NO RECIRCULATION TO R3
STOP PROGRAM UNTIL SIGNALED BY OPERATOR; RECIRCULATE FROM ORGAN LOOP TO R2 DURING THIS INTERRUPTION TO PRECLUDE EMPTYING OF R2 AND INTRODUCTION OF AIR INTO CIRCUIT; FREEZE ORGAN PUMP SPEED AND TEMPERATURE PUMP SPEEDS AT PRE-INTERRUCTION LEVELS, OR FREEZE ORGAN PUMP SPEED WHILE CONTINUING TEMPERATURE ADJUSTEMENT WHILE AWAITING OPERATOR SIGNAL
TO FIG.6C

FIG.6B

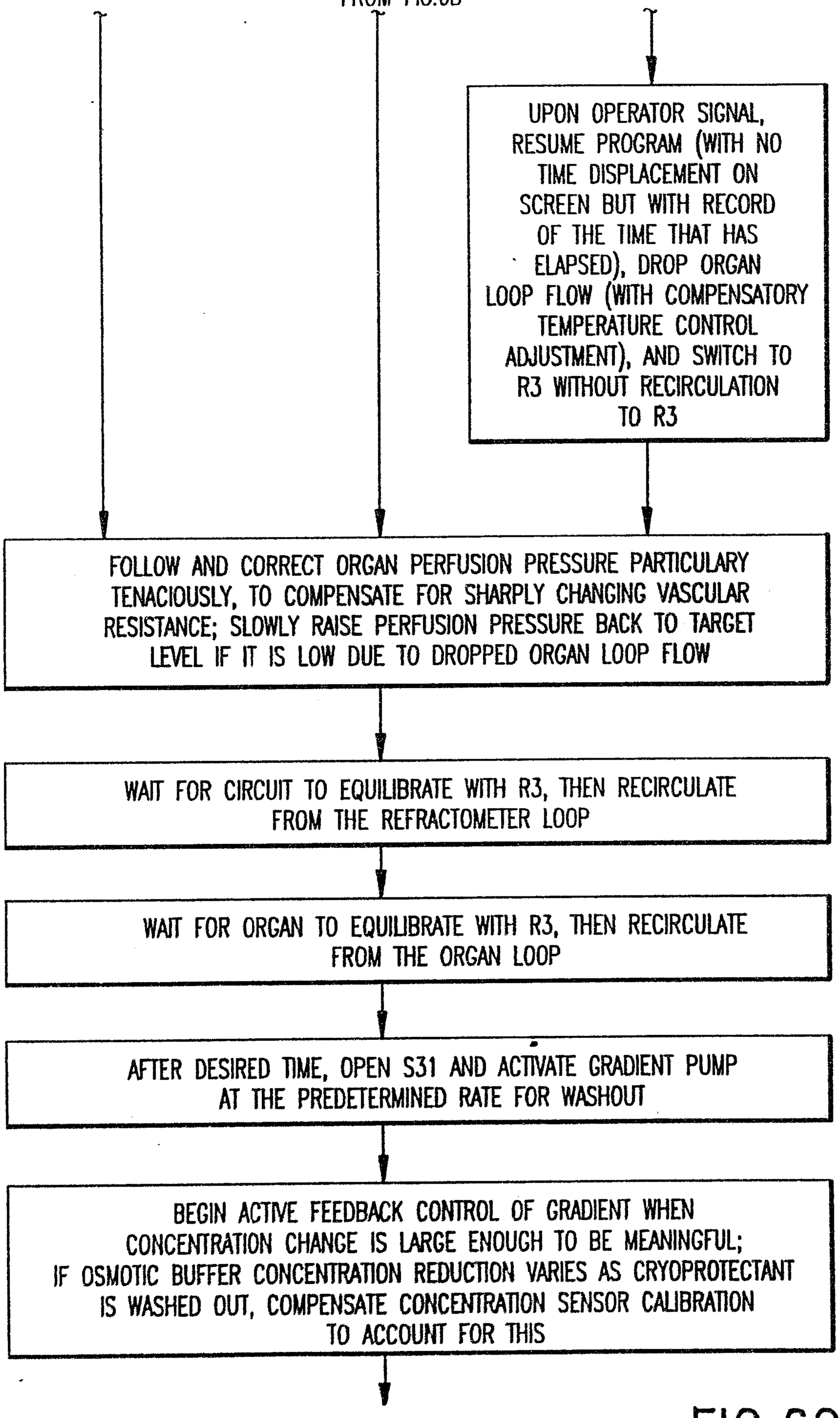

FROM FIG.6B
UPON OPERATOR SIGNAL, RESUME PROGRAM (WITH NO TIME DISPLACEMENT ON SCREEN BUT WITH RECORD OF THE TIME THAT HAS ELAPSED), DROP ORGAN LOOP FLOW (WITH COMPENSATORY TEMPERATURE CONTROL ADJUSTMENT), AND SWITCH TO R3 WITHOUT RECIRCULATION TO R3
FOLLOW AND CORRECT ORGAN PERFUSION PRESSURE PARTICULARY TENACIOUSLY, TO COMPENSATE FOR SHARPLY CHANGING VASCULAR RESISTANCE; SLOWLY RAISE PERFUSION PRESSURE BACK TO TARGET LEVEL IF IT IS LOW DUE TO DROPPED ORGAN LOOP FLOW
WAIT FOR CIRCUIT TO EQUILIBRATE WITH R3, THEN RECIRCULATE FROM THE REFRACTOMETER LOOP
WAIT FOR ORGAN TO EQUILIBRATE WITH R3, THEN RECIRCULATE FROM THE ORGAN LOOP
AFTER DESIRED TIME, OPEN S31 AND ACTIVATE GRADIENT PUMP AT THE PREDETERMINED RATE FOR WASHOUT
BEGIN ACTIVE FEEDBACK CONTROL OF GRADIENT WHEN CONCENTRATION CHANGE IS LARGE ENOUGH TO BE MEANINGFUL; IF OSMOTIC BUFFER CONCENTRATION REDUCTION VARIES AS CRYOPROTECTANT IS WASHED OUT, COMPENSATE CONCENTRATION SENSOR CALIBRATION TO ACCOUNT FOR THIS
TO FIG.6D

FIG.6C

FROM FIG.6C

AT PREDETERMINED POINTS, BEGIN AND FINALLY END TEMPERATURE RISE AS CONCENTRATION FALLS

UPON REACHING A PREDETERMINED THRESHOLD CONCENTRATION THAT OCCURS NEAR THE TIME R3's INNER CHAMBER WOULD OTHERWISE BECOME EMPTY, TURN OFF OR SLOW DOWN GRADIENT PUMP

ACTIVATE CUSTOMIZED S32 DUTY CYCLE SO AS TO INTRODUCE FLUID FROM R3's OUTER CHAMBER TO R3's INNER CHAMBER SLOWLY AT FIRST AND THEN MORE AND MORE RAPIDLY SO AS TO REDUCE CONCENTRATION IN R3's INNER CHAMBER SLOWLY IN ACCORDANCE WITH PREDETERMINED SCHEDULE; IN TANDEM WITH THIS, PHASE GRADIENT PUMP BACK IN AND/OR CEASE FULL RECIRCULATION TO R3 IN ORDER TO REDUCE RETURN OF CRYOPROTECTANT TO R3 FROM THE CIRCUIT AND ORGAN

WHEN CONCENTRATION IS SUFFICIENTLY CLOSE TO OR EQUAL TO ZERO, JUMP TO R4 (WHICH CONTAINS NO CRYOPROTECTANT AND LITTLE OSMOTIC BUFFER; IF APPROPRIATE, PHASE IN RECIRCULATION TO R4 AS WAS DONE FOR R2 AND R3 ABOVE

IF APPROPRIATE, AFTER DESIRED PERIOD, ACTIVATE R4's SOLENOID VALVE AND WASHOUT OSMOTIC BUFFER TO DESIRED DEGREE; ADJUST CALIBRATION TO DISPLAY THE WASH OUT OF THE OSMOTIC BUFFER

SIGNAL OPERATOR THAT ORGAN IS READY FOR REMOVAL FROM THE PERFUSION MACHINE AND NOW CAN BE TRANSPORTED OR TRANSPLANTED; CONTINUE TO MAINTAIN ORGAN PERFUSION PARAMETERS UNTIL LARGE DROP IN PRESSURE IS SENSED AS THE ORGAN IS REMOVED FROM THE CIRCUIT

TO FIG.6E

FIG.6D

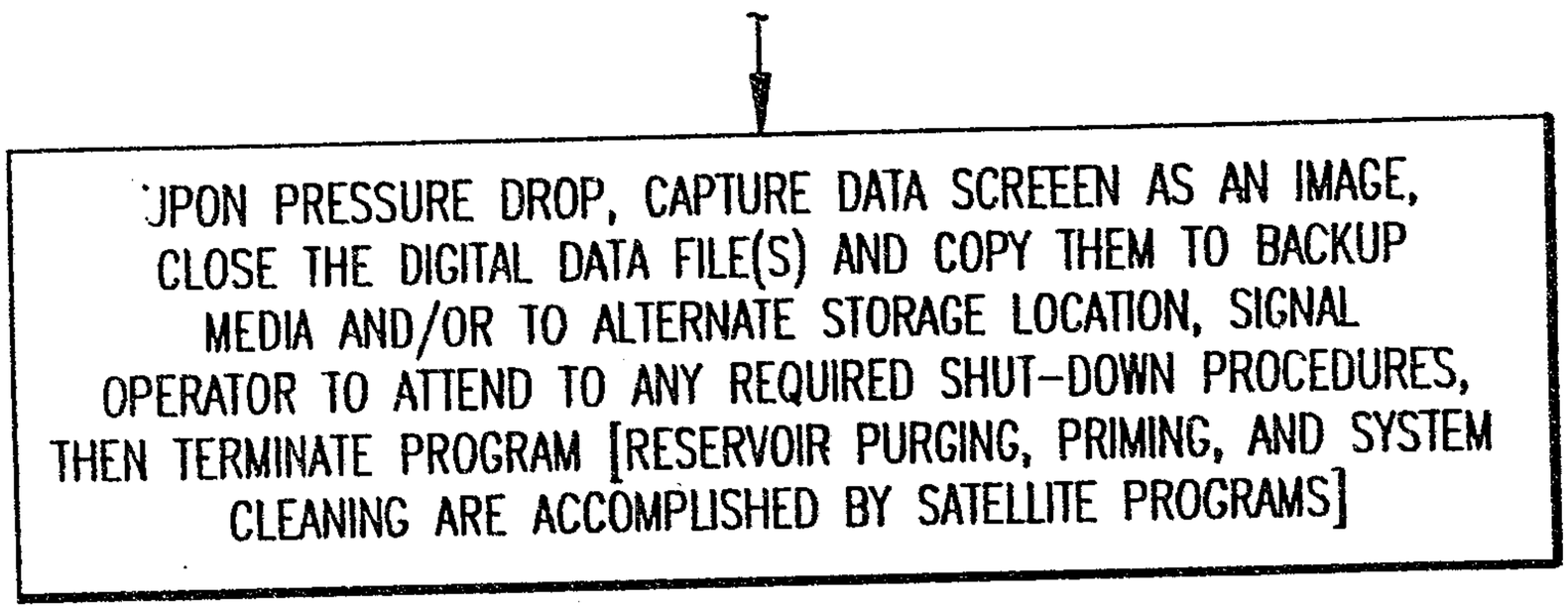

FROM FIG.6D
UPON PRESSURE DROP, CAPTURE DATA SCREEEN AS AN IMAGE, CLOSE THE DIGITAL DATA FILE(S) AND COPY THEM TO BACKUP MEDIA AND/OR TO ALTERNATE STORAGE LOCATION, SIGNAL OPERATOR TO ATTEND TO ANY REQUIRED SHUT-DOWN PROCEDURES, THEN TERMINATE PROGRAM [RESERVOIR PURGING, PRIMING, AND SYSTEM CLEANING ARE ACCOMPLISHED BY SATELLITE PROGRAMS]

FIG.6E

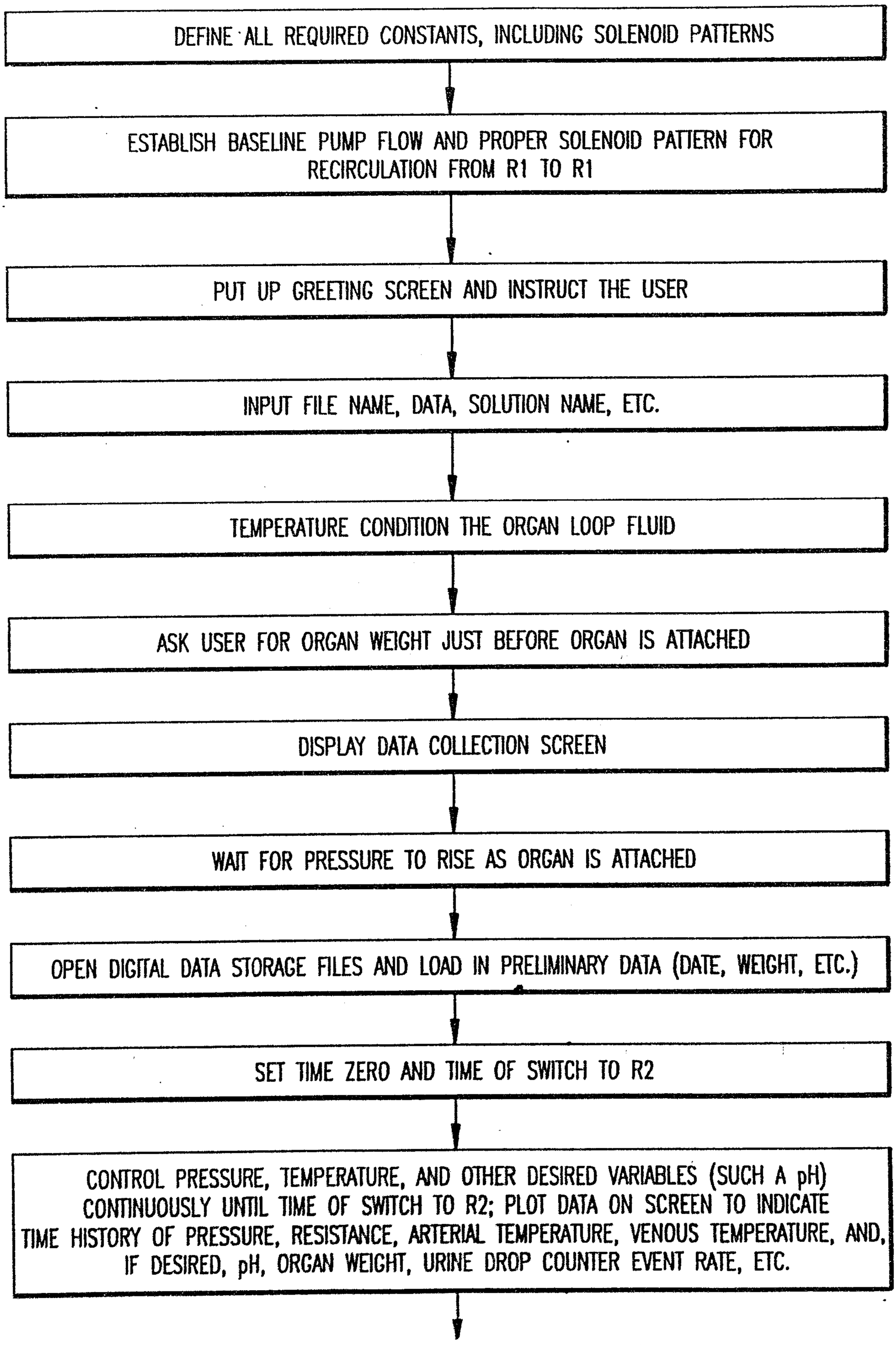

DEFINE ALL REQUIRED CONSTANTS, INCLUDING SOLENOID PATTERNS
ESTABLISH BASELINE PUMP FLOW AND PROPER SOLENOID PATTERN FOR RECIRCULATION FROM R1 TO R1
PUT UP GREETING SCREEN AND INSTRUCT THE USER
INPUT FILE NAME, DATA, SOLUTION NAME, ETC.
TEMPERATURE CONDITION THE ORGAN LOOP FLUID
ASK USER FOR ORGAN WEIGHT JUST BEFORE ORGAN IS ATTACHED
DISPLAY DATA COLLECTION SCREEN
WAIT FOR PRESSURE TO RISE AS ORGAN IS ATTACHED
OPEN DIGITAL DATA STORAGE FILES AND LOAD IN PRELIMINARY DATA (DATE, WEIGHT, ETC.)
SET TIME ZERO AND TIME OF SWITCH TO R2
CONTROL PRESSURE, TEMPERATURE, AND OTHER DESIRED VARIABLES (SUCH A pH) CONTINUOUSLY UNTIL TIME OF SWITCH TO R2; PLOT DATA ON SCREEN TO INDICATE TIME HISTORY OF PRESSURE, RESISTANCE, ARTERIAL TEMPERATURE, VENOUS TEMPERATURE, AND, IF DESIRED, pH, ORGAN WEIGHT, URINE DROP COUNTER EVENT RATE, ETC.
TO FIG.7B

FIG.7A

FROM FIG.7A

MEANWHILE, IF GRADUAL DRUG ADDITION IS TO BE ACCOMPLISHED USING R1, ACTIVATE GRADIENT FORMER TO APPROPRIATE RATE; RECORD DRUG CONCENTRATION AND/OR MEDIATOR RELEASE FROM ORGAN IN RESPONSE TO DRUG IF DESIRED AND POSSIBLE

OCCASIONALLY RECHECK PRESSURE TRANSDUCER ACCURACY AND UPDATE ITS CALIBRATION ACCORDINGLY

SWITCH TO R2; RECIRCULATION TO R2 CAN CEASE IF A STEP CHANGE IN CALCIUM, DRUG, AUTOCOID, BLOOD, OR SERUM CONCENTRATION FROM ZERO OR BASELINE IS BEING EFFECTED; IF R2's ROLE IS TO STABILIZE A PREVIOUSLY-ESTABLISHED CONCENTRATION, FLUID RECIRCULATION TO R2 NEED NOT BE INTERRUPTED; IF PROTOCOL INVOLVES TEMPERATURE CHANGES, SUCH AS PERFUSION AT NORMOTHERMIA WITH BLOOD, TEMPERATURE CHANGE IS ALSO IMPOSED AT ABOUT THE SAME TIME [FOR BLOOD PERFUSION, AN AUXILIARY HEAT EXCHANGER IS USED TO WARM THE BLOOD PRIOR TO ITS ENTRY INTO THE CIRCUIT PUMP HEAD, AND THE AUXILIARY FILTER ACCESSED BY S4 AND S5 IS A BLOOD FILTER]

IF APPROPRIATE, RESTORE RECIRCULATION (TO R2) AFTER CONTAMINATING SOLUTION FROM R1 HAS BEEN ELIMINATED FROM THE REST OF THE CIRCUIT; THIS CAN BE DONE FIRST FROM THE REFRACTOMETER LOOP AND SECOND FROM THE ORGAN LOOP TO MINIMIZE BOTH FLUID WASTE AND CONTAMINATION

IF APPROPRIATE, SET TIME OF SWITCH TO R3 AND PROCEED SIMILARY THROUGH R3 AND, IF DESIRED, R4, DEPENDING ON THE DESIGN OF THE EXPERIMENT; FOR SOME EXPERIMENTS, R4 MAY CONTAIN FIXATIVE.

AFTER CYCLING THROUGH ALL DESIRED RESERVOIRS THE DESIRED NUMBER OF TIMES, INFORM THE OPERATOR THAT THE PERFUSION IS COMPLETED AND THE ORGAN IS READY TO REMOVE

WHEN PRESSURE FALLS IN RESPONSE TO ORGAN REMOVAL, CLOSE DATA FILES, CAPTURE SCREEN AS AN IMAGE, DUMP SCREEN TO HARD COPY IF DESIRED BY OPERATOR, SET ALL PUMP SPEEDS TO ZERO, PROVIDE ANY FINAL INSTRUCTIONS TO USER, AND TERMINATE PROGRAM

FIG.7B

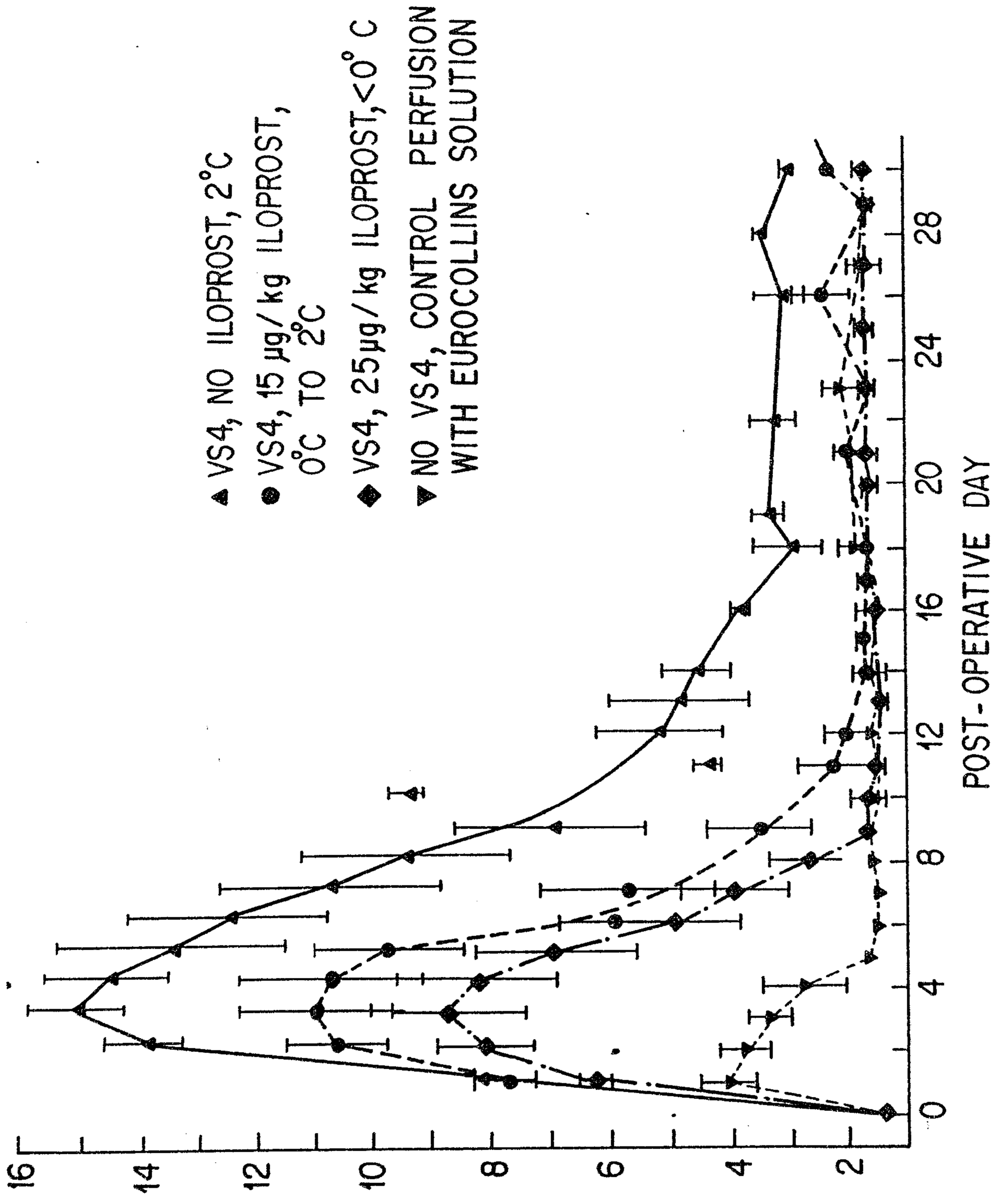
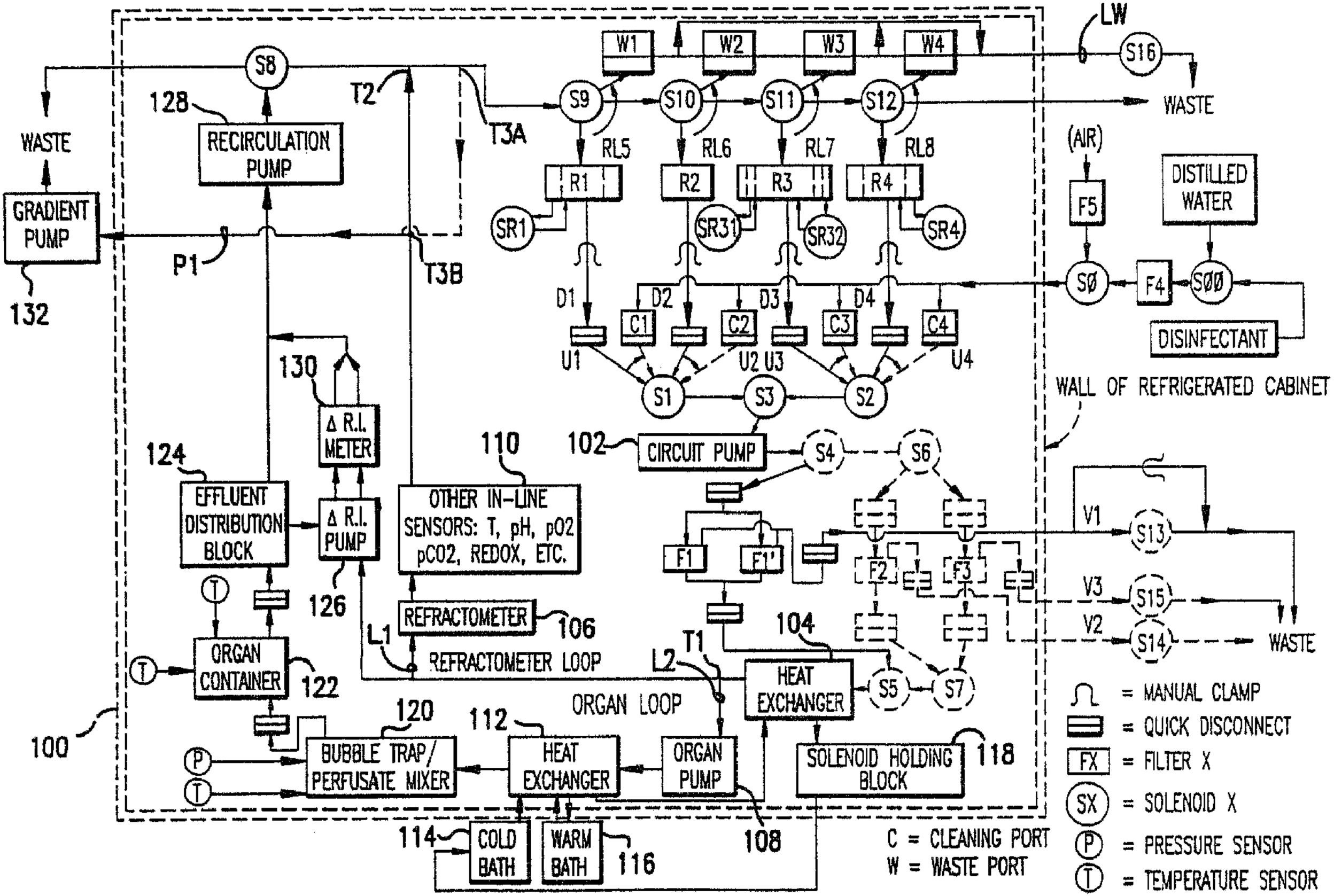

▲ VS4, NO ILOPROST, 2°C
● VS4, 15 µg/kg ILOPROST, 0°C TO 2°C
◆ VS4, 25µg/kg ILOPROST, 0°C TO 2°C
▼ NO VS4, CONTROL PERFUSION WITH EUROCOLLINS SOLUTION
POST-OPERATIVE DAY
0
4
8
12
16
20
24
28
CREATININE MG/DL
2
4
6
8
10
12
14
16

FIG.8


LW
S16
WASTE
W1
W2
W3
W4
S8
S9
S10
S11
S12
T2
T3A
128
RECIRCULATION PUMP
WASTE
GRADIENT PUMP
132
P1
T3B
RL5
RL6
RL7
RL8
R1
R2
R3
R4
SR1
SR31
SR32
SR4
(AIR)
F5
DISTILLED WATER
SØ
F4
SØØ
DISINFECTANT
D1
D2
D3
D4
C1
C2
C3
C4
U1
U2 U3
U4
S1
S3
S2
WALL OF REFRIGERATED CABINET
130
Δ R.I. METER
110
102
CIRCUIT PUMP
S4
S6
124
EFFLUENT DISTRIBUTION BLOCK
Δ R.I. PUMP
OTHER IN-LINE SENSORS: T, pH, pO2 pCO2, REDOX, ETC.
F1
F1'
F2
F3
V1
S13
V3
S15
V2
S14
WASTE
126
REFRACTOMETER
106
L1
REFRACTOMETER LOOP
104
T1
L2
ORGAN CONTAINER
122
HEAT EXCHANGER
S5
S7
ORGAN LOOP
120
112
100
BUBBLE TRAP/ PERFUSATE MIXER
HEAT EXCHANGER
ORGAN PUMP
SOLENOID HOLDING BLOCK
118
114
COLD BATH
WARM BATH
116
108
C = CLEANING PORT
W = WASTE PORT
= MANUAL CLAMP
= QUICK DISCONNECT
FX = FILTER X
SX = SOLENOID X
P = PRESSURE SENSOR
T = TEMPERATURE SENSOR