correct bridge voltage. This deflection is determined by the preliminary calibration. After the voltage check, the thermistor is connected to the bridge, and the instrument is ready for use.

Conclusions

Several months' use of this instrument in field work under extreme conditions has proved its ruggedness and ease of handling. It has filled a definite need in the Edgewood work and should be quite useful wherever an accurate, simple, and fast thermometer is necessary in physiological studies. The introduction of another matched temperature-sensitive element in the opposite bridge arm would make this instrument applicable to the measurement of temperature differences.

The Cultivation of Mammalian Liver Cells in Large Numbers[1]

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The cultivation *in vitro* of cells of ectodermal origin may become important in certain investigations, particularly in the application of tissue culture to virus studies, biochemicar analyses, and studies of malignancy. However, the cultivation of such cells presents difficulties because of the greatel facility of growth of the connective tissue elements present in such explants.

The technic to be described was developed in the course of experiments on the attempted cultivation of the agent of infectious hepatitis. This method made it possible to produce cultures containing large numbers of liver cells with few fibroblasts, without employing a very elaborate technic. The method is based on the roller-tube technic of Gey (*2*), with the following modifications: the random distribution of many pieces of explanted tissue in roller tubes, and the use of a medium which selectively favors the growth of epithelial cells.

Materials

For a typical experiment involving 24 tubes the following sterile materials were required: 24 acid-free and grease-free test tubes, 18 x 150 mm.; a few capillary pipettes about 3 mm. in diameter at the tip; a few capillary pipettes, 200 mm. long, with the usual diameter at the tip (about 1 mm.) and bent at a 45° angle at a point 2–3 mm. from the tip; 24 solid rubber stoppers to fit the culture tubes; 3–50-cc. pointed centrifuge tubes; 1:600 heparin solution; 3–20-cc. bulb pipettes; a Petri plate, 100 mm. in diameter, containing a watch glass; a syringe and needle suitable for withdrawing 20 cc. of heart's blood from a rabbit; and a few dissecting instruments, including one fine, small, curved scissors, preferably strabismus scissors. A small amount of ice and rubber bulbs or tubing for manipulation of the pipettes are also needed.

Assembling the Culture

The explant material was obtained from a rabbit 7–10 days old, lightly anesthetized and exsanguinated. A portion of the liver was removed aseptically and placed in the watch glass contained in the Petri plate. The tissue was minced with strabismus scissors until the bits appeared to be about 1 mm. in diameter. The minced tissue was then suspended in Gey's solution (*2*) in the watch glass and transferred to a 50-cc. centrifuge tube. The top of the Petri plate was used as a shield to prevent contamination from the air. About 20 cc. of Gey's solution were then added to the tissue suspension in the tube. The latter was rotated until the tissue was distributed and was then allowed to stand. After a few minutes all the particles of about 1 mm. had settled below a well-defined plane, leaving a supernate containing erythrocytes and minute fragments. This supernate was removed by suction, and the washing of tissue fragments in Gey's solution was repeated twice more. After the supernate was removed for the third time a wide capillary pipette with rubber bulb was used to distribute small amounts of the sediment into the cotton-plugged culture tubes. The volume of the rather watery sediment which was picked up each time for placing in the culture tubes was such as to contain about 60 pieces of tissue in about 0.3 cc. of the saline solution.

The tissue was distributed by means of the capillary pipettes with bent tips. Throughout the procedure the culture tube was kept horizontal to avoid contamination from the air. The technic of distribution was as follows: The pipette was used to spread the small amount of fluid over the lower half of the tube until the entire surface was moistened, and the clump of fragments was distributed in a rough ring around the tube. Then the point of the bent tip was turned toward the surface of the tube and run up and down between the bottom of the tube and approximately halfway to the open end, while the left hand slowly rotated the tube. Bits of tissue were thus distributed over the entire lower half of the tube. Clumps remaining after this procedure were pushed apart by the tip of the capillary pipette, and any noted gross unevenness of distribution was corrected. Quite often many pairs of tissue fragments were left in contact by this procedure, but since the purpose of the method was to obtain a maximum total circumference of explants in as little time of manipulation as possible, this was not regarded as objectionable. Thus, 60 fragments which included 10 contiguous pairs would reproduce the effect of 50 entirely separate pieces. After the tissue fragments had been placed in all the tubes they were fixed in position by adding to each tube 0.25 cc. of heparinized normal rabbit plasma, which was distributed by rotating the tubes in groups of six in pipette rests until clotting was observed. After nutrient medium had been added to each tube, they were stoppered and placed in a roller-tube mechanism (*1*) rotating at 6 r.p.h.

The plasma was obtained as follows: Sterile 1:600 heparin solution (H.W.D.) was placed in a chilled tube in an ice bath, one-hundredth of the volume of blood to be drawn. Blood was then drawn from a rabbit's heart and quickly placed in the tube. Centrifugation was carried out in ice at 2,000 r.p.m. for 6 minutes, the plasma then being drawn off into another chilled centrifuge tube. This tube, if kept in ice, would not show clotting of the plasma for several hours. Within a few minutes of drawing off, however, the plasma was used to coat the tubes. The heparin concentration was such that the change of temperature from that of the ice bath to room

[1] This investigation was conducted under the Commission on Measles and Mumps, Army Epidemiological Board, Preventive Medicine Service, Office of the Surgeon General, U. S. Army.

temperature was sufficient to cause clotting. It should be noted that heparinized chicken plasma, where available, may be more convenient, since it remains liquid in the refrigerator for many days and can be coagulated by the addition of hemostatic globulin.

The setting up of such a set of 24 tubes required two hours at most, from the anesthetizing of the rabbit to the placing of the tubes in the roller. This involved the work of two people and included the collection and separation of heart's blood for plasma. Bacteriologic aseptic technic was rigorously followed throughout. The rate of contamination was less than one tube per 24.

The Medium

The development of a satisfactory medium for the selective stimulation of epithelial cells was the major problem of this study. After exhaustive comparisons of salt solutions, relative amounts of serum, effect of embryonic extract, and optimum frequency of refeeding of cultures, the following medium was found to give the best results:

Balanced salt solution was made up according to Gey (*2*) except for the addition of aspartic acid, which Parshley and Sims (*3*) had found stimulating to epithelial cells of skin and thyroid when used in conjunction with Sims' salt solution. Since the addition of aspartic acid and its subsequent neutralization increased the total concentration of salt in the medium, a corresponding adjustment in the amount of NaCl was decided on by calculation. The final salt solution, identical with Gey's except for the amounts of NaOH and NaCl and the addition of aspartic acid, was made up by weighing out and dissolving (in the order shown) in double-distilled water to a final volume of 1 l. the following salts: NaCl, 8.0 grams; KCl, 0.375 gram; $CaCl_2$, 0.275 gram; $NaHCO_3$, 0.25 gram; $MgCl_2 \cdot 6\ H_2O$, 0.21 gram; Na_2HPO_4, 0.12 gram; KH_2PO_4, 0.025 gram; dextrose, 1.0 gram; and aspartic acid, 3.0 grams. Carbon dioxide was then bubbled through the solution for several minutes. NaOH was added in N/10 concentration until the pH was 7.0. The solution was sterilized by filtration through a Seitz filter. The medium as used consisted of 40 per cent of normal rabbit serum and 60 per cent of the salt solution. It was placed in the 18 x 150-mm. culture tubes in amounts of 1.6 cc. per tube, and the roller was sufficiently elevated at one end to prevent contact of the medium with the rubber stopper. Twice a week the medium was withdrawn and replaced with a similar amount of fresh medium. Once a week, between withdrawal of nutrient fluid and an addition of fresh medium, the clotting procedure was repeated in order to patch the plasma coat.

The Growth of Liver Cells

In the case of the great majority of rabbits used, the first outgrowths of liver cells from the explants could be observed in 48 hours in many fragments. In three days there were partial collars of liver cells about most of the fragments. The cells grew out radially in a rather regular form involving columns two cells wide; these would frequently curve and give off branching columns in a Y-configuration. After a few days the growth was usually such that the identity of the columns of cells was lost, and by mutual pressure the growth would form a single, continuous sheet of polygonal cells. Since the explants were often only a few millimeters apart, there was frequent contact between outgrowing sheets of cells arising from different explants. At such points of contact, growth would stop.

In more isolated explants, growth would continue until, after 10–12 days, the 1-mm. explant would be surrounded, in many cases, by collars of outgrowth 3–4 mm. wide, so that the entire sheet of cells formed an approximate circle 7–8 mm. in diameter. Thereafter, growth became markedly slower, and after the 14th day almost no extension in growth was noted. No increase in granularity of cytoplasm was observed until this time, even in the cells nearest the explant. Cultures were not maintained after the 21st day. The cells were large and polygonal, forming a continuous sheet. The nuclei were large, oval, and optically empty in the unstained preparation, with sharp borders except during mitosis. When the preparations were fixed and stained in situ, the nuclei and nucleoli were deeply stained with hematoxylin and the cytoplasm with eosin.

Not all explants, even in the best cultures, showed growth. However, so many fragments of tissue were seeded that the total area of outgrowth was considerable. Taking 3 mm. as an average of the width of the collar of outgrowth around the 1-mm. explant, we find that the sheet of liver cells from one such explant exceeded 30 mm.^{2} in area. Twenty-five such explants would provide 7 cm.^{2} of liver cells, and 33, about 10 cm.^{2} The majority of the successful tubes ranged between these two estimates. This qualification is made advisedly, because at irregular intervals either a liver would fail to grow out at all or the growth would be quite limited and short lived. For this reason, when infectious material was being studied, the cultures were originally fed with the medium described, and the infectious material was added at the time of the second feeding, after three days, at which time it was possible to predict rather accurately the course of that set of cultures. In the case of rabbits whose livers did not demonstrate good growth at that time, the infectious material was stored in the frozen state until a successful culture reached its first refeeding.

Fibroblasts were found in small numbers in almost all tubes. They occurred singly, or in small groups, between explants of liver cells. In all the tubes in which the liver cells multiplied satisfactorily the fibroblasts were very few in comparison. They were more in evidence in those in which some manipulation of the medium had interfered with the outgrowth of liver cells. In such cases there was an increase in the absolute number of those cells.

In a few tubes no fibroblasts could be found. This cannot be taken to mean that a pure culture of liver cells existed in such tubes, but the appearance of the solid sheets of liver cells, often with no other cell type in the vicinity, makes it seem possible that a pure liver culture might be derived from such explants in daughter tubes.

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