freely in a tube of an angle rotor is determined by the diameter of the centrifuge tube containing the capsule and the angle at which the tube is held (the "rotor angle"). A 45° rotor angle permits the greatest capsule length for a given tube diameter.

When high centrifugal fields ($> 20{,}000\ g$) are employed the possible collapse of the plastic centrifuge tubes is prevented by filling them completely, with mineral oil layered above the aqueous level. After centrifugation the capsule may conveniently be removed from the centrifuge tube by first lowering over it an open end of a closely fitting section of glass tubing. When the tube is withdrawn, the capsule and some salt solution come with the tube. The capsule is rinsed and blotted dry and then enveloped in a piece of transparent Scotch tape. It is then scored through the tape at a position slightly above the meniscus, and broken apart. Supernatant fluid can be readily removed by capillary siphoning, and the sediment resuspended in fresh solvent with the aid of glass probes.

References

1. ELFORD, W. J. *Brit. J. Exptl. Pathol.*, **17**, 399 (1936).
2. BRAKKE, M. K., BLACK, L. M., and WYCKOFF, R. W. G. *Am. J. Botany*, **38**, 332 (1951).
3. KAHLER, H., and LLOYD, B. J., JR. *J. Phys. & Colloid Chem.*, **55**, 1344 (1951).

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Establishing Long-Term Cultures of Mammalian Normal, Solid Tumor, and Ascites Tumor Cells on Glass

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By embedding his tissue fragments in a clot of lymph Harrison (*1*) was the first to succeed in growing or maintaining animal cells *in vitro*. Shortly thereafter, Burrows (*2*) suggested the use of a plasma clot as the solid phase of tissue culture media, and this technique has become very widely adopted by tissue culture workers.

The practice of embedding the tissue to be cultured in a clot, although originally the difference between success and failure, has greatly restricted the dimensions of tissue cultures and has placed serious limitations on experimental applications, especially in the field of metabolism. To cope with the problem, various investigators have tried replacing the plasma clot with simpler materials or else have sought means for separating the cells from the plasma. Outstanding among these have been Evans and Earle and their associates, who introduced perforated cellophane (*3*) as a solid substrate for cell growth, and later found that certain lines of cells, after growing on the cellophane, could be induced to grow directly on the glass surface of the culture vessel. Interest in obtaining animal cell cultures of the type described by Earle (strain L) led to the development of the techniques presented in this paper for establishing and maintaining the growth of several cell strains directly on glass.

A slight modification in the customary roller tube culture technique was employed for the original cultures. It is the general practice to coat the entire inner surface of the roller tube with clotted plasma; this was modified by using only a strip of plasma, about ¼ in. wide, streaked down one side of the tube with a capillary pipette. Three explants approximately $6 \times 2 \times 1$ mm were seated in this strip, and coagulation was induced by the addition of a drop of 50% chick embryo extract. One ml of medium of the following composition was added to each tube: 10% chick embryo extract (final concentration), 50% Earle's balanced salt solution, and 40% horse serum. The tubes were incubated at 37° C on a conventional roller tube apparatus. Fluid changes were made 3 times weekly unless the condition of the cultures indicated that a departure from this routine was desirable. Good growth was noted generally within 48 hr in the plasma around the explants, and later, as the area of growth increased, the cells were observed to migrate out of the plasma onto the glass. After about 2 months' incubation, growth on the glass usually extended completely around the tube, covering all, or nearly all, the surface of the lower half. Covering of the area was not always accomplished by a steadily advancing sheet of cells, but often through random scattering. Apparently cells or cell clumps became detached from areas of dense growth, floated free, and resettled in other open areas. These islets then started new growth centers and thus speeded up total population of the available surface. After this amount of growth had occurred, nearly all evidence of the original explants had disappeared and most of the narrow strip of plasma had been lysed or otherwise lost. Thus a dense culture of cells growing directly on glass had been achieved.

After obtaining growth of the cells on glass, the next and more perplexing problem was that of subculture by the scraping and pipetting technique. Most experiments fail at this point, apparently because the cells do not survive such harsh physical treatment or change of environment. To facilitate this step, two recommendations found in the literature of Sanford and Earle and their associates were followed. The first of these involved the use of "conditioned medium," as described for single cell experiments (*4*); i.e., the cells were scraped into the old medium, and this suspension was then transferred to the new vessel with no further addition of medium. In most instances the pH of this medium was quite low; hence adjustment was made to approximately pH 7.6 by adding a few drops of sodium bicarbonate.

The second step was based on Earle's (*5*) finding that a heavier cell suspension favors transplantability. In order to utilize this principle, the first subcultures were made to vessels with a smaller surface area than that of the parent culture flask, thereby concentrating

the cell population. The growth in the roller tube covered an area of at least 3000 mm², which was greater than three times the 950 mm² surface of Carrel D3.5 flasks (as supplied by Otto Hopf, Upper Black Eddy, Pa.) employed for the first daughter cultures. By taking advantage of this difference in the size of the culture areas, a dense population of cells was transferred from the roller tubes to the Carrel flasks, thereby favoring their survival and continued growth. After various intervals, ranging from 2 weeks to several months, the growth in the Carrel D3.5 flasks was scraped off and transferred to Carrel D5.0 flasks, which have a surface area of 1960 mm². Each of the latter cultures in turn was later transferred to two or more D5.0 flasks. Several of the cell strains thus obtained have been successfully transferred from the D5.0 flasks to Earle's large T-60 flasks, each having a floor area of 6000 mm², and have rapidly covered the entire floor area of the flasks. The cultures are being carried in these flasks as stock cultures.

Four cell strains have been established to date, using the above procedure for their isolation. These include the cells of the mouse tumor Sarcoma 180, the Walker rat carcinoma 256, and two apparently different cell types grown from normal embryonic mouse skin. The cells of the Walker carcinoma appear to be epithelial in character, and differ considerably in their morphological and growth properties from the connective tissue cells of the other cultures. Complete descriptions and characteristics of all these cell strains will be published at a later date.

Two additional cell strains have been isolated and established in the type of culture described above. One of these was grown from fragments of a mouse spontaneous tumor and was obtained more through good fortune than planned procedures. In this experiment the explants were embedded in thick plasma clots (approximately 2.0 mm) in Carrel D3.5 flasks. Following 3 months of growth, a hole 1.5–2.0 cm in diameter had developed in the plasma clot of one of the cultures; microscopic examination disclosed that the area of the glass exposed by the lysis of the clot was completely covered with a growth of very healthy cells. While the flask was held firmly to prevent surging of the medium, this area was scraped and the cells so loosened from the glass were drawn up into a capillary pipette along with about 0.5 ml of medium. The cells were transferred to another Carrel D3.5 flask, where they quickly settled onto the glass surface and continued to proliferate, completely covering the floor of the flask within 2½ weeks. This strain of cells is at present over a year old, has been transferred numerous times, still multiplies rapidly, and appears quite healthy in every respect. The designation LLC-M_1 has been applied to the cells and will be used henceforth.

Although it was originally thought that the LLC-M_1 strain was derived from a spontaneous tumor, subsequent histological examination of this growth revealed that it was more likely to be a hypertrophic lymph node. This conclusion was further supported by the fact that the mass occurred in the popliteal region of the leg. There also was considerable doubt that this swelling, or lymph node, had undergone malignant changes, and if such had occurred it was of the lymphatic elements, not of the fibroblastlike cell grown in the cultures. The cultured cells also have failed in several attempts to produce tumors following injection into mice. It is now thought, therefore, that strain LLC-M_1 is a normal adult connective tissue cell grown either out of the stroma of the node or from adjacent subcutaneous tissue.

The remaining strain of cells adapted to serial culture on glass is one started from the Ehrlich ascites tumor of mice.[1] This is a mammary carcinoma which has been induced to grow free in ascitic fluid in the peritoneal cavity of the mouse. These cells can be removed from the animal in the form of a cell suspension, thus minimizing the problems of culture. As much as 5–8 ml of fluid can be aspirated from the peritoneal cavity of a single mouse and upon centrifugation will yield up to 50% tumor cells. A variety of methods and materials has been used to start cultures of these cells, but all have been more or less modifications of the following general procedure: The fluid is removed from the mouse, treated with heparin to prevent coagulation, and spun in an angle centrifuge for about 10 min at approximately 1500 rpm. The supernatant is discarded, the cells are resuspended in 5–10 volumes of tissue culture medium, thoroughly mixed, and pipetted to Carrel D3.5 flasks in 0.5 ml amounts. These flasks are incubated for 2 hr to allow the cells to settle and adhere to the glass, after which an additional 0.5 ml of medium is added to bring the volume up to the customary 1.0 ml used on D3.5 flask cultures.

As many as 60 cultures of ascites tumor cells have been put on in half an hour, with generally 90–100% of them providing good cultures for experimental work. Some of these have been successfully subcultured by scraping, and one line at present has been maintained in serial culture for over 8 months.

Morphologically, in culture, these cells closely resemble those of the Walker rat carcinoma except that they are slightly smaller and somewhat more elongated. It seems quite possible that they are epithelial cells. All attempts to culture the cells in or on a plasma clot have failed completely, and growth on cellophane has been only slight and of short duration. These ascites tumor cells seem to prefer cultivation on glass and grow best in stationary or quiet cultures, as growth in roller tubes, even on glass, has been very poor.

The first-described technique for the establishment of animal cells in long-term culture on glass has worked successfully in this laboratory for cells from both normal and malignant mouse tissues. Further studies are in progress in which these procedures are

[1] This tumor was obtained through the courtesy of H. N. Christensen, Tufts College Medical School.

being employed in an endeavor to obtain similar strains of various cell types from human and other animal species. The publication of these results, as well as a more detailed report on the cell strains mentioned in this paper, is anticipated in the near future.

References

1. HARRISON, R. G. *Proc. Soc. Exptl. Biol. Med.*, **4**, 140 (1906–07).
2. BURROWS, M. T. *J. Am. Med. Assoc.*, **55**, 2057 (1910).
3. EVANS, V. J., and EARLE, W. R. *J. Natl. Cancer Inst.*, **8**, 103 (1947).
4. SANFORD, K. K., EARLE, W. R., and LIKELY, G. D. *Ibid.*, **9**, 229 (1948).
5. EARLE, W. R., *et al. Ibid.*, **12**, 133 (1951).

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Studies on the Antidiuretic Action of Morphine[1]

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The antidiuretic action of morphine is a well-documented phenomenon (*1*), both clinically and experimentally. The mechanism by which morphine influences renal function, however, has been a matter of some controversy. Recent evidence (*2, 3*) favors the view that morphine directly stimulates the supraoptico-posterior lobe system of the pituitary gland, causing release of the antidiuretic hormone. Despite this recurrent thesis, no efforts have yet been directed toward characterizing the urinary antidiuretic substance produced by the administration of morphine, in order to compare its properties with those of the antidiuretic hormone.

We have investigated this hypothesis of origin indirectly in normal and hypophysectomized rats receiving morphine. Experiments were conducted to demonstrate the presence of the antidiuretic substance in the urine of the test animals and to characterize it chemically. These characteristics were then compared with the properties reported for the antidiuretic hormone.

The method used for the assay of antidiuretic activity was a modification of a technique published by Dicker and Ginsburg (*4*). This consisted in administering subcutaneously 1 mg morphine sulfate/kg body weight to 6–9 adult male rats in metabolism cages, and in collecting the pooled urinary output for 24 hr. The pooled urine, either in its native state or after the various chemical and physical treatments described, was dialyzed (Visking cellulose casing, wall thickness 0.00072 in.) in running tap water for 10 hr and in distilled water for 1 hr. The dialyzed urine was then concentrated to its original volume and assayed directly. The assay animals consisted of two groups of 4 adult male rats (150–250 g), (*a*) the control group receiving 1 ml isotonic saline/100 g body weight injected subcutaneously, and (*b*) the test group receiving 1 ml of the prepared urine/100 g by the same route. These animals were hydrated with specific quantities of water (5% of their body weight), and their urine output recorded at 60, 90, and 120 min.

[1] This work was aided by the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

TABLE 1

Material assayed	Mean percentage water excreted at 120 min (saline control = 100%)
Urine from unmedicated rats	99*
Pitressin (1.5 milli-u/100 g)	40†
Urine from rats receiving morphine	
Expt. 1	60†
2	59†
3	71
4	59
Urine from hypophysectomized rats receiving morphine	
Expt. 1	98*
2	94*

* Analysis of variance revealed no significant difference between this urine and the saline control.
† Analysis of variance revealed a highly significant difference between this urine and the saline control.

The data from the experiments designed to demonstrate the presence of antidiuretic activity in the urine of rats receiving morphine are summarized in Table 1. The effect recorded is the percentage of the water given to hydrate the test rats, which was excreted within 2 hr, in relation to a comparable volume excreted by the control rats, adjusted to 100%. It is interesting to note that, although morphine consistently produced urine with distinct antidiuretic potency, neither Demerol (10 mg/kg given subcutaneously at 6-hr intervals for 1 day) nor methadone (0.5 mg/kg given subcutaneously at 6-hr intervals for 1 day) had a similar action. No attempt was made in

TABLE 2

Treatment of urine from rats receiving morphine sulfate	Mean percentage water excreted at 120 min (saline control = 100%)
Untreated urine (control)	62
Heat (constant volume)*	105
Heat (to dryness)†	109
Acid hydrolysis‡	112
Alkaline hydrolysis§	118
Sodium sulfite‖	
Expt. 1	101
2	85
3	85

* Refluxed for 10 hr at 60° C.
† Incubated in an open container for 10 hr at 60° C, then restored to original volume and filtered.
‡ Refluxed in 10% HCl (5 ml acid to 50 ml urine) for 3 hr at 100° C.
§ Refluxed in 2 *N* NaOH (2.5 ml base to 30 ml urine) for 30 min at 100° C.
‖ Treated at room temperature with *N*/10 sodium sulfite (5 ml of this solution to 30 ml urine) for 5 min.