A Simple Procedure for the Long-Term Cultivation of Chicken Embryos[1]

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A method is described which permits the growth of chicken embryos in petri dishes from the third to the 20th day of incubation. The procedure is relatively simple and has the advantage of providing ready access to the embryo and its membranes for tissue grafting, for introduction of teratogenic agents, and for microscopic observation of morphogenesis and growth.

INTRODUCTION

While the culture of explanted chicken embryos has long been an elegant method for the study of early development (see New, 1966, for references), techniques for the long-term cultivation of later-stage embryos have been less than satisfactory. The use of polyethylene bags has permitted development of the embryo for 10–12 days (Schlesinger, 1966; Elliott and Bennett, 1971) and modifications involving specially constructed glass dishes in combination with such polyethylene bags have permitted some survival up to 16 days (Corner and Richter, 1973). Special incubators have also been constructed to improve the success of embryo explants (Ramsey and Boone, 1972).

We were interested in analyzing the vascular responses of the chorioallantoic membrane (CAM) to implanted tumor grafts (cf. Folkman, 1974; Knighton *et al.*, 1974). For this purpose we found it essential to develop methods that would permit rapid and ready observation of large numbers of eggs under conditions facilitating examination with transmitted light, permitting time-lapse photography, and encouraging routine access to the grafted tissue. The procedures we describe in this report have now been used by us for growing several thousand eggs during the past several months; we report them here because we feel they can find wide application in the field of developmental biology.

Fertile eggs are first incubated for 3–4 days in standard fashion. At this time they are washed with a dilute solution of formaldehyde (1:4000), permitted to air-dry, and wiped with a solution of povidone–iodine (Betadine) to minimize contamination from the shell surface. The egg is left in a horizontal position for several minutes to assure that the embryo is properly positioned. The egg contents are then placed in the bottom of a 20 × 100-mm plastic Petri dish (Falcon No. 3003) by cracking the underside against the edge of that dish. Five to 10 ml of lactated Ringer's solution or tissue culture medium (we have used Eagle's basal medium and Fischer's medium for leukemic cells) containing 100–200 units/ml of gentamycin and mycostatin are added, and the dish is placed inside a larger, 25 × 150-mm plastic Petri dish (Falcon No. 1013) containing a thin layer of water for humidification, covered, and incubated at 36–37°C in a humidified atmosphere of 1–2% CO_2. Rapid circulation of the air–CO_2 mixture in the incubator is essential.

The addition of water to the outside dish

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call for frequent or prolonged observations at room temperature. While we have found the addition of medium or lactated Ringer's useful, this does not appear to be essential except when embryos are to be kept for long periods of time. Carbon dioxide is not essential for the first 10 days of development but appears to favor survival in later stages. Our experiments were carried out on White Leghorn embryos obtained from SPAFAS, Norwich, CT. We use a New Brunswick Forced Air CO_2 incubator.

Our survival data are roughly as follows.

(1) *Initial setup.* About 8/10 eggs make it into the incubator. Any damage to the yolk prevents subsequent survival of the embryo; we therefore discard such eggs immediately.

(2) *Short-term survival–first 3 days following explantation.* We lose about 50% of the eggs during this period from a variety of causes, not all of which are apparent.

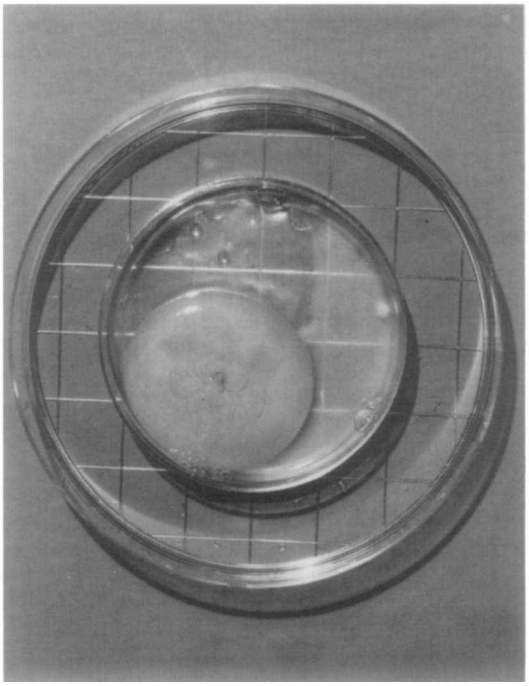

FIG. 1. Initial culture; 3.5 days of prior incubation. × 0.4.

detected initially, may show up at 24 hr. Occasionally an embryo is not upright in the dish. In others there appears to be contamination at the yolk–albumen interface. Still others do not continue development for unknown reasons. Our results parallel those reported by Corner and Richter (1973). Since we were primarily interested in chorioallantoic grafting, however, loss of embryos during this period of development preceded initiation of experimental protocols and hence was more of a nuisance than a disaster; for other types of experiments the 50% loss may prove to be a severe limitation.

(3) *Long-term survival—from Days 7 to 20 of development.* Following the losses of the first 3 days of culture, survival data are excellent. In general about 80% of surviving eggs continue for at least another week, and by selecting the "better" eggs, i.e., those in which the membrane is spreading smoothly over the yolk and albumen surface, we achieve almost 100% survival until Days 16–17 of development. During the subsequent three days, embryos appear sluggish and die. While some embryos have survived to Day 21, reaching Hamburger–Hamilton stage 44, most embryos die at stages 42–43.

While our primary purpose was to study the onset and progression of tumor-induced angiogenesis (Auerbach *et al.*, 1974; Knighton *et al.*, 1974), we have also explored the use of the egg culture method for a variety of other developmental questions. For example, irradiation *in ovo* on Days 0–4 (200 and 400 R) was used to produce teratological effects which were then monitored in petri dish cultures; malformations such as limb defects, beak abnormalities, circulatory aberrations, and growth defects were readily apparent and their progress could be documented photographically. We have studied the pattern of development of irradiated grafts; we have used lead shielding to permit the production of

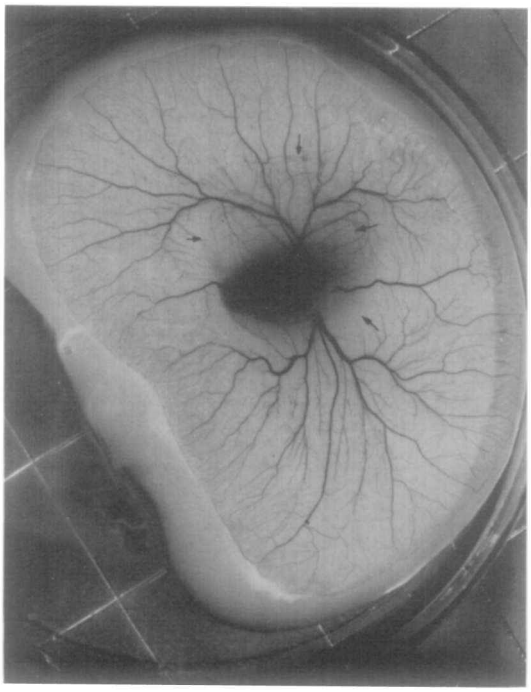

FIG. 2. Seven-and-half day embryos; arrows denote edge of developing chorioallantoic membrane. × 0.9.

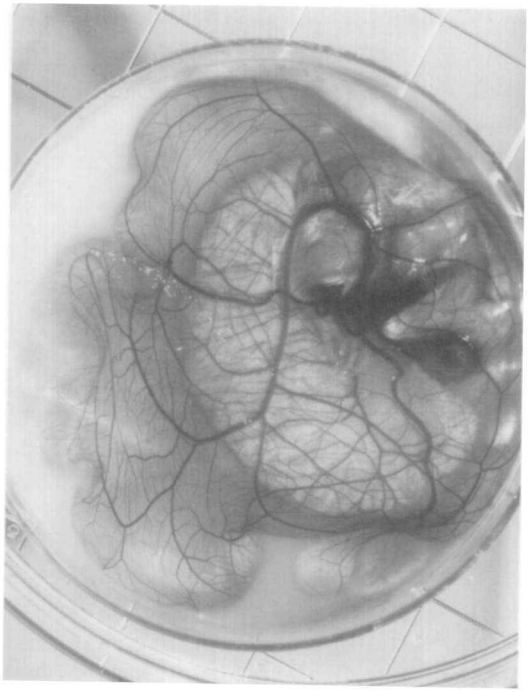

FIG. 4. Fourteen-and-half day embryos; note reduction in yolk volume facilitating transillumination of chorioallantoic membrane. × 0.8.

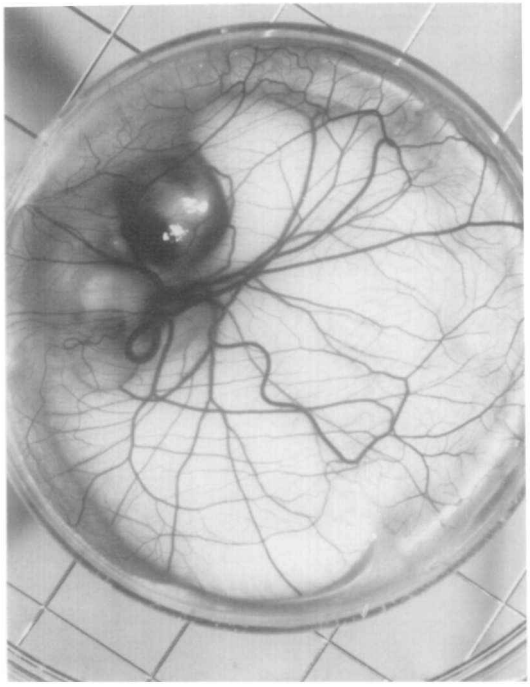

FIG. 3. Eleven-and-half day embryos; chorioallantoic membrane now covers entire petri dish surface. × 0.8.

local lesions in the CAM for study of vascular repair. We have administered cyclophosphamide both intravenously and by injection into the extraembryonic cavity to suppress immune reactivity. Primarily, however, we have found the method invaluable for the continued monitoring of a large variety of embryonic, adult, and tumor grafts during the establishment of vascular connections, induction of neovascular responses, and growth and differentiation of the transplanted tissues.

The method lends itself to preparation of large numbers of eggs for use not only for research purposes but for the teaching of embryology as well. For the latter, the fact that the total embryo is visible and remains so throughout its development from Day 3 to almost hatching is particularly attractive, for it permits the observation of embryonic behavior as well as of the relationship between the embryo and its extraembryonic membranes. The use of trans-

mitted light, moreover, facilitates the identification of embryonic structures and circulatory patterns. For chorioallantoic grafting in the classroom many grafts can be made on each egg and repeated observations are possible without having the chronic problem of trying to "find" the graft through a window of restricted dimensions. Surgical manipulations of various types are also much simpler to perform. These advantages must be balanced, however, against the somewhat reduced survival of cultured eggs and the increased expense of using plastic dishes; more incubator space is also needed for our procedure.

Why eggs put into petri dishes prior to 3 days of incubation do not survive is not entirely clear (cf. New, 1966 for discussion). If it is due to the surface configuration of the dish, then the temporary insertion of a retaining ring of a diameter smaller than that of the petri dish might permit survival during this early phase of embryogenesis. We also do not know why embryos die 2–4 days before hatching but believe that slight changes in humidity, increased supply of oxygen, and calcium supplementation might permit the final steps of embryonic development to be completed.

REFERENCES

AUERBACH, R., ARENSMAN, R., KUBAI, L., and FOLKMAN, J. (1974). Tumor angiogenesis: Lack of inhibition by radiation (submitted for publication).

CORNER, M. A., and RICHTER, A. P. J. (1973). Extended survival of the chick embryo *in vitro*. *Experientia* **29,** 467–468.

ELLIOTT, J. H., and BENNETT, J. (1971). Growth of chick embryos in polyethylene bags. *Poultry Sci.* **50,** 974–975.

FOLKMAN, J. (1974). Tumor angiogenesis. *Advan. Cancer Res.* (in press).

KNIGHTON, D., AUSPRUNK, D. TAPPER, D., and FOLKMAN, J. (1974). Study of the avascular and vascular phases of tumor growth in the chick embryo. (in preparation).

NEW, D. A. T. (1966). "The Culture of Vertebrate Embryos," 245 pp. Logos Press, London.

RAMSEY, J. B., and BOONE, M. A. (1972). Incubator for growing chick embryos *in vitro* and *in ovo*. *Poultry Sci.* **51,** 701–709.

SCHLESINGER, A. B. (1966). Plastic bag culture method for chick embryos. *CUEBS News* **2(3),** 10–11.

SPRATT, N. T., JR. (1947). A simple method for explanting and cultivating early chick embryos *in vitro*. *Science* **106,** 452.

WESSELLS, N. K., and WILT, F. H. (1967). "Methods in Developmental Biology, 813 pp. Crowell, New York.