floating cells are seen, probably due to overgrowth. Further overgrowth results in retraction and rolling up of the cell sheet. The majority of the cells have the diploid ($2n = 36$) number of chromosomes. They have been stored in 10% dimethyl sulphoxide in medium in liquid nitrogen (–196°C) and successfully resuscitated. Studies on the infection of the cells with arboviruses are in progress.

[8] We thank Professor D. S. BERTRAM, Director of the Department of Entomology at the School, for his continued interest; Mr. A. O. LANGI for technical assistance and Dr. L. HAMILTON, Middlesex Hospital Medical School, London, for supplying the *Xenopus* tadpoles. We are especially grateful to the Wellcome Research Laboratories, Beckenham, for supplying medium NCTC 109, and to Mr. D. J. RICHMOND of the Laboratories for responding to our request for medium promptly and willingly. The work is supported in part by a grant from the Medical Research Council of Great Britain.

Résumé. On décrit une méthode pour l'établissement d'une culture de cellules du crapaud a griffes sudafricain, *Xenopus laevis*, afin de l'infecter avec des arbovirus. Les cellules (XTC-2) ont subi une culture continue pendant 3 ans. Elles ont un nombre diploïde de chromosomes. Elles furent cultivées aux températures de 22°C et de 28°C dans un milieu de culture dilué de NCTC 109 avec 10% de sérum de veau foetal.

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Extended Survival of the Chick Embryo in vitro

Recent work has suggested that warm-blooded central nervous tissues, cultured in isolation under in vitro conditions, preserve many of the functional properties responsible for normal prenatal behavior patterns in situ[1]. It would be desirable to compare this development with that of similar preparations cultured in a more natural environment. Chorio-allantoic transplantation[2] offers optimal conditions, at least for avian tissues, but since its use in ovo would place severe restrictions upon physiological experimentation it was decided to adapt this method for use in vitro. Techniques for culturing chick embryos in vitro with preservation of the extra-embryonic circulation are in wide use now, using egg albumin as a source of nutrients, but survival has been for only a few days[3,4]. Because methods which utilize the yolk together with the albumin offer much improved survival[5,6] we chose these as the starting point for devising a suitable long-term culture system.

Fig. 1. Photograph of a cultured embryo, explanted after 2 days of incubation in ovo, which attained normal stage 41 after development for 15 days in vitro.

Methods. Fertilized white leghorn chicken eggs were incubated in ovo for 2 days at 38–39°C and about 65% relative humidity. This pre-incubation prior to transferring the embryos to in vitro conditions (using sterile precautions) improved the percentage of preparations attaining advanced stages of development. Specially designed dishes were used for this study, of such shape and size that the egg contents largely filled the vessel, with the yolk sinking down to occupy the bottom portion. The outward tapering walls of the dish ensured that the albumin formed a pool which submerged the germinal area, and that a large area ($\pm$ 8 cm in diameter) would be available for gas exchange at the upper surface. These geometric considerations are apparently critical for survival of these embryos within impermeable containers (R. DEHAAN, personal communication). The preparation was covered with a sterile plastic petri-dish and placed inside a polyethylene bag, which was then closed off tightly. All cultures were transferred to an incubator for premature babies, maintaining the same temperature and humidity as during the initial incubation in ovo, and in which a specially constructed rack enabled easy daily individual examination.

Results. A first wave of mortalities struck at stage 25 and lasted through stage 30[7], claiming almost 50% of the cultures. Of 37 cases followed thereafter, only 1 death occurred before stage 34, but 21 preparations were then lost between stages 35 and 38. The remaining 15 all attained embryonic stage 40, and 5 of these survived into stage 41 (Figure 1). The earlier fatalities were correlated with the degree of yolk leakage but the final wave of deaths affected many cases where the yolk sac appeared intact and the albumin transparent. The most clearly relevant differences from the natural in ovo situation

[1] M. A. CORNER and S. M. CRAIN, J. Neurobiol. *3*, 25 (1972).
[2] V. HAMBURGER, *A Manual of Experimental Embryology* (University of Chicago Press, Chicago 1960).
[3] D. A. T. NEW, *The Culture of Vertebrate Embryos* (Logos Press, London 1966).
[4] R. L. DEHAAN, in *Methods in Developmental Biology* (Eds. F. H. WILT and N. R. WESSELS; Crowell, New York 1967), p. 401.
[5] A. B. SCHLESINGER, C.U.E.B.S. News *2*, 10 (1966).
[6] H. VOLLMAR, Z. Zellforsch. mikrosk. Anat., *23*, 566 (1936).
[7] J. L. HAMILTON, *Lillie's Development of the Chick* (Holt, Rhinehart and Winston, New York 1952).

are: 1. lack of a calcium source, normally obtained from the eggshell[8]; 2. the much lower carbon dioxide concentration as compared with the normal egg, where the CO_2 is known to reach a very high level towards the end of incubation[9]; and 3. the altered geometry of the chorio-allantoic membrane.

Cultured embryos were motorically responsive to tactile stimulation, and also displayed 'spontaneous' motility which was quite similar in character to that described in ovo[10]. Most of the oldest preparations in fact were in almost continuous movement, as is normal for that stage of development[11].

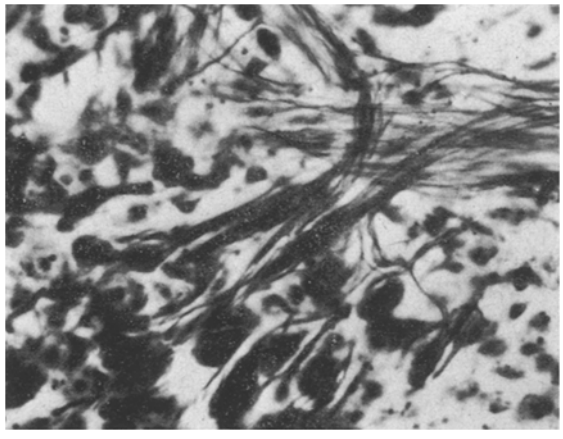

Fig. 2. An abundant, well-fasciculated, outgrowth of nerve fibres from a monolayer cluster of neurons and neuroblasts in various stages of development (lower left). Isolated at 6 days of incubation and cultured for 10 days on the chorio-allantoic membrane (background cells) in vitro. Bodian's protargol-silver staining method, magnified × 500.

The chorio-allantoic membrane was prepared for grafting of isolated neural tissues according to the standard procedure[2]. In most of the experiments to date, spinal cord segments of 1 or 2 somites in length were dissected out from donor embryos of about the same age as the host (4–7 days). The neural tissue was cleaned of adherring mesoderm and then placed onto the membrane. The grafts became quickly vascularized and usually survived for as long as did the host embryo. Explanted tissues were fixed in situ after varying periods of culture (up to 12 days) and then silver stained in toto using the Bodian method. Despite the absence of any readily recognizable histotypic organization in these flattened pieces of spinal cord, an extensive neuritic outgrowth was found to have taken place and nerve cells in various stages of differentiation could be identified (Figure 2).

Résumé. On décrit une technique pour obtenir la survie prolongée des embryons du poulet in vitro, et son emploie pour cultiver des tissus isolés du système nerveux centrale.

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[8] P. M. JOHNSTON and C. L. COMAR, Am. J. Physiol. *183*, 365 (1955).
[9] A. H. J. VISSCHEDIJK, Br. Poultry. Sci. *9*, 197 (1968).
[10] V. HAMBURGER, Q. Rev. Biol. *38*, 342 (1963).
[11] M. A. CORNER and A. P. C. BOT, Progr. Brain Res. *26*, 214 (1967).
[12] Acknowledgments. We are grateful to Prof. S. M. CRAIN, of the Albert Einstein Medical College in New York, for having first called our attention to the possibility of obtaining such prolonged survival of chick embryos in vitro. Our thanks are also due to Miss JOKE STROES of the Mobach pottery works in Utrecht, who made for us the ceramic dishes used in this study.

Cell Cycle Determination of Phytohemagglutinin-Stimulated Lymphocytes from the Opossum, *Didelphis virginiana*

Due to a number of remarkable cytogenetic characteristics, the opossum (*Didelphis virginiana*) has been utilized recently to investigate chromosomal morphology, nucleic acid metabolism, and sex chromatin physiology[1–4]. The lymphocyte culture technique was employed exclusively in these investigations due to the ease of obtaining large numbers of dividing cells in a very short period of time. Since investigations of nucleic acid synthetic patterns require a knowledge of the cell cycle and its component parts, a generation time (PLM) curve was constructed for opossum lymphocytes in vitro.

Materials and methods. Lymphocytes obtained from either thoracic duct lymph or cardiac blood of 8 opossums (7 males and 1 female) were cultured according to modifications of the technique of MOORHEAD et al.[5] (see SCHNEIDER and RIEKE[2], for details). Following 36 h of incubation, the cultures were pulse-labeled for 30 min with 1 μC/ml of H^3-thymidine (spec. act. 6.05 C/m*M*, New England Nuclear). The cells were subsequently washed and replaced in fresh media containing 120 times excess non-radioactive thymidine. Cultures were harvested at intervals from 0–28 h. Slides were coated with liquid Kodak NTB 2 emulsion, exposed 1–3 days at 4 °C, developed and stained. Between 100 and 600 mitotic figures were scored at each interval.

Results and discussion. The Figure is the PLM curve derived from opossum lymphocytes in tissue culture. Labeled mitotic figures first appeared 1.5 h after administration of H^3-thymidine. The ascending limb of the first peak approached 100% labeled mitotic figures at 7 h. The descending limb, which was skewed somewhat to the right, dipped only slightly below 50% labeled mitoses before immediately giving rise to a second similar, but much lower, peak. A third peak appeared at approximately 22 h after introduction of isotope, followed by a general trailing off of the curve to 28 h. Both thoracic duct and blood lymphocytes produced the same basic curve configuration.

The following cycle times may be derived from the curve: mean T_c (generation time) = 10.5 h, mean G_2 = 2.75 h and mean S = 9.5 h. T_m (mitotic time), calculated from the mitotic index, was approximately 30 min. It

[1] L. K. SCHNEIDER and W. O. RIEKE, J. Cell Biol. *33*, 497 (1967).
[2] L. K. SCHNEIDER and W. O. RIEKE, Cytogenetics 7, 1 (1968).
[3] A. K. SINHA, Experientia *23*, 889 (1967).
[4] L. K. SCHNEIDER, Experientia *26*, 914 (1970).
[5] P. S. MOORHEAD, P. C. NOWELL, W. J. MELLMAN, D. M. BATTIPS and D. A. HUNGERFORD, Expl Cell Res. *20*, 613 (1960).