(c) *ibid.*, p. 1384; (d) **54,** 82 (1965); (e) *ibid.*, p. 388; (f) *ibid.*, p. 770; (g) *ibid.*, p. 1061; (h) *ibid.*, p. 1303; (i) *ibid.*, p. 1606; (j) **55,** 91 (1966).

[2] The evidence presented above is absolute. Nevertheless, in order to remove even the shadow of a doubt, an extensive series of experiments was done in September and October 1964, using a chamber in which the central pool and the vaseline gaps were 1 mm wide. In all cases node $N_1$ was placed near the center of the central pool, and with the amplifier across the second gap action potentials were initiated by direct electrical stimulation with rectangular pulses of current delivered across the first gap. A second stimulating device placed across the first gap was used to apply continuous anodal polarization to the treated segment of fiber. The experimental results were perfectly consistent. In all cases the sodium-free 114-mM solution of potassium ions caused depolarization at a remarkably low rate. Since for obvious reasons when the treated segment is 1 mm long the flow of demarcation current is less effective in maintaining the membrane potential of the treated segment than when this segment is only 270 $\mu$ long, as a rule after a few minutes the treated segment became unable to produce action potentials. However, in all cases during periods of moderate, continuous anodal polarization the treated segment became able to produce action potentials, having the characteristic large and long-lasting negative after-potential, for as long as the observations were continued (0.5–3 hr). Since loss of internal sodium to the external sodium-free medium decreases the height of the maximal action potential, in several experiments the action potential of the treated segment was made to undergo successive increases and decreases by using alternatively potassium Ringer's and normal sodium Ringer's to which 110 mM potassium chloride had been added.

[3] The conclusion reached in ref. 5 from work done with nerve trunks, that the resting membrane potential is not a potassium potential, was confirmed by R. Stämpfli [*Ann. N.Y. Acad. Sci.*, **81,** 265 (1959)] using single isolated fibers.

[4] (a) Honrubia, V., and R. Lorente de Nó, these PROCEEDINGS, **48,** 2065 (1962); (b) *ibid.*, **49,** 40 (1963). See ref. 1*g*, footnote 3.

[5] Lorente de Nó, R., *A Study of Nerve Physiology* (Rockefeller Institute, 1947), vols. 131, 132.

[6] Lorente de Nó, R., *J. Cell. Comp. Physiol.*, **33,** Suppl. (1949).

---

## *CONNECTIONS BETWEEN CELLS OF THE DEVELOPING SQUID AS REVEALED BY ELECTROPHYSIOLOGICAL METHODS**

BY D. D. POTTER, E. J. FURSHPAN, AND E. S. LENNOX

NEUROPHYSIOLOGICAL LABORATORY, DEPARTMENT OF PHARMACOLOGY, HARVARD MEDICAL SCHOOL, BOSTON, MASSACHUSETTS, AND THE SALK INSTITUTE FOR BIOLOGICAL STUDIES, LA JOLLA, CALIFORNIA

*Communicated by Stephen W. Kuffler, December 20, 1965*

During the past 6 years many examples have been discovered of specialized connections ("electrical synapses") between excitable cells (e.g., certain nerve cells, cardiac and visceral muscle). As revealed by electrophysiological methods, these junctions provide pathways for the flow of ionic currents between the interiors of the coupled cells (e.g., refs. 1–4). They thereby provide for rapid coordination of electrical activities. In all the cases cited, electron microscopy has shown "tight junctions" at which the extracellular space is partially or completely obliterated by apposition of the membrane elements.[5–7]

With the same electrical methods, similar connections have also been discovered in glial tissue[8, 9] and in certain epithelia (e.g., toad urinary bladder[10]) which do not generate electrical signals. In these tissues too, "tight junctions" have been seen.[11, 12]

The widespread occurrence in animal tissue of "tight junctions"[13] and in plants of what may be a similar structure, the plasmodesmata, requires that functions in addition to providing for electrical communication must be sought for these connections, e.g., a means of coordinating cell activity.

One situation in which cell intercommunication must be extensive is in embryonic development. This led to experiments seeking connections among the cells of embryos using intracellular microelectrodes. The embryo of the squid *Loligo pealii* was chosen for the present study.

*Materials and Methods.*—Thirty developmental stages have been distinguished for this species by Arnold[14] whose numbering system will be used.

Cleavage is meroblastic. Cells with diameter of 20–50 $\mu$ form a cap, the blastodisc, on the yolk cell which retains most of the mass of the original ovum (see stages 9 and 13, Fig. 1). The blastodisc envelops the yolk cell almost entirely by stage 17; the developing organs then first appear as thickenings of the blastodisc. Subsequent differentiation of the external structures is illustrated in Figure 1, stages 21, 23, 25, 27. In these later stages only the outer yolk mass can be seen in the drawings; however, a stalk of the yolk cell extends internally along the embryo making extensive contact with embryonic cells. The embryo is transparent even at late stages, permitting deep tissues to be seen.

The chorion (shown surrounding stage 9, Fig. 1) was removed with fine forceps. The exposed embryo was bathed in sea water cooled to 15–17°C. In experiments on later stages (22–27) urethan was added to a final concentration of 0.5–0.8% to reduce movement.

The techniques for electrical measurements were conventional. Current was supplied to the interior of a cell by applying a potential between an intracellular microelectrode and an Ag-AgCl electrode in the bath. Resulting potential changes were recorded between another microelectrode and the same bath electrode. In many experiments a second recording microelectrode was also used.

The electrodes were filled either with 3 *M* KCl or with a dye solution which allowed marking the

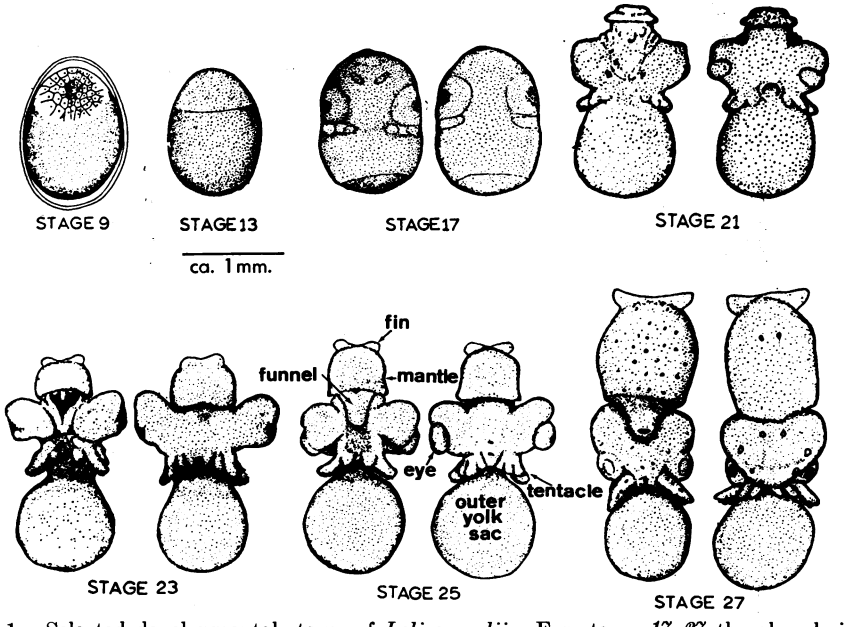


FIG. 1.—Selected developmental stages of *Loligo pealii*. For stages *17–27* the aboral side is shown on the left of each pair. Drawings kindly supplied by Dr. John Arnold.[14] Reproduced with permission of *Biol. Bull.*

cell impaled during electrical measurements.[15] The dye, Niagara Sky Blue 6B, was used in a 3.5–4% aqueous solution.

Impalement of an embryonic cell was accomplished by bringing the microelectrode into contact with it and then passing a pulse of inward current (about $10^{-8}$ A; 1 sec duration). Entry was signaled by appearance of the resting potential, and with dye-filled electrodes the same current pulse usually ejected enough dye to stain the impaled cell. If the pulse failed to cause entry, the dye diffused away in extracellular spaces leaving no mark. To verify the identification of the marked cells, experimental embryos were fixed overnight at 0°C in a fixative[16] acidified to retain the dye marks: glutaraldehyde (25% solution), 2.5 ml; acrolein, 0.2 ml; 0.1 *M* acetate buffer, pH 4.0, 5 ml; 2 × artificial sulfate-free sea water, 2.5 ml. After dehydration in ethanol they were cleared in propylene oxide and embedded in epon. The hardness of the epon was adjusted so that serial sections could be cut at 5 $\mu$ with a steel knife.[15]

*Results.*—In the basic experiment two cells of the embryo were impaled with microelectrodes; current was supplied to the interior of one cell, and the internal potential of the other recorded to test for current flow between the cells. In most cases (106 out of a total of 121 embryos) interaction was tested between the yolk cell and some cell of the embryo proper. The earliest embryos studied were about one day old (stage 10) and already had many cells. From this time until about the eighth day of development there is widespread tight electrical coupling between the yolk cell and cells of the embryo proper. Coupling was observed across many intervening cells. Dye with a molecular weight of about 1000 injected into one cell was sometimes observed to move to its close neighbors. The strong electrical coupling of embryonic cells to the yolk cell disappears or becomes greatly diminished roughly 4 days before hatching in all of the tissues studied, with the exception of the external epithelium of the outer yolk sac.

An experiment is illustrated in Figure 2. In (*A*) is shown the experimental embryo (stage 22) after fixation and embedding in epon. Facsimiles of the microelectrodes drawn on the photograph show their positions during the experiment. First, electrodes *1* and *3* were inserted deep into the yolk cell, one near each end, and recorded resting potentials of about 50 mV each. Then electrode *3* was used to pass a rectangular current pulse (bottom trace, Fig. 2*B*), while electrode *1* registered the resulting potential change within the yolk cell (middle trace). The slow rise and fall of the potential at electrode *1* reflects the charging time of the membrane capacity. Microelectrode *2* filled with dye was just outside an epithelial cell of the developing tentacle and recorded (top trace) the small potential (2 mV) of the extracellular field at that point. When it was made to enter the epithelial cell of the tentacle, by passing a pulse of inward current, three concomitant events were observed: (*a*) the epithelial cell became stained by dye ejected during the current pulse (Fig. 2*C*). In this photograph made just after withdrawal, the microelectrode can be seen near the marked cell. (*b*) The base line for the entering electrode shifted by the resting potential of the cell, in this case 47 mV (Fig. 2*D*). (*c*) When a pulse of current was passed once more through electrode *3*, a substantial potential change was now recorded with electrode *2*, indicating a very effective spread of current from the yolk cell to the impaled epithelial cell (compare trace *2* in *B* and *D*). These characteristic large, slow potential changes could only be recorded when microelectrode *2* was within a cell.[17] Electrode *2* was next thrust deep into the tentacle without, however, entering any cell (Fig. 2*E*). In this case, only a small fast voltage change was recorded during the current pulse applied to the yolk cell (Fig. 2*F*, trace *2*). This potential shift is smaller by a factor of

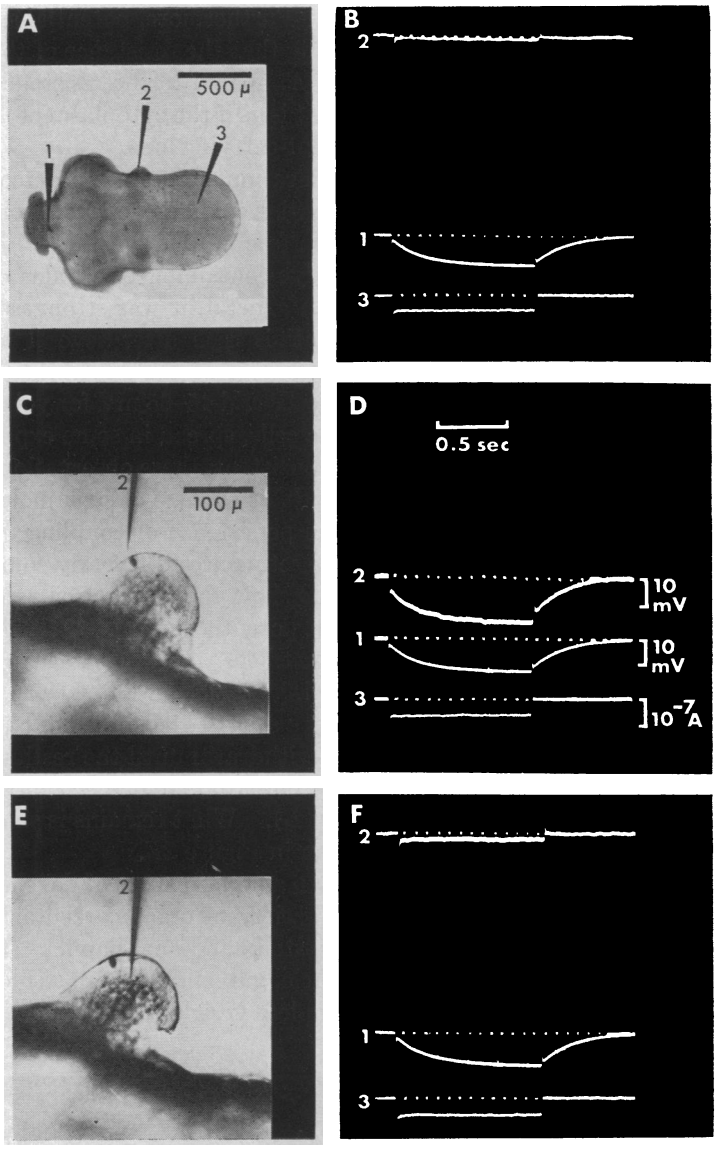


FIG. 2.—Electrical coupling between a tentacle epithelial cell and the yolk. The photographs in *C* and *E* were taken during the course of the experiment. Each group of three oscillograph traces in *B*, *D*, and *F* consists of recordings (from top to bottom) of potential recorded with electrode *2*, potential recorded with electrode *1*, and current supplied through electrode *3*. The calibrations in *D* apply to *B*, *D*, and *F;* the base lines are shown dotted.

eight than the one recorded intracellularly, but somewhat greater than that seen when the electrode tip was just outside the embryo (Fig. 2*B*), indicating an appreciable resistance of the pathways from the interstitial spaces to the external environment (see Fig. 5).

This basic experiment has been repeated with similar results on a variety of embryonic cells (a total of about 450). In all cases at stage 25 or earlier, tight

electrical coupling to the yolk cell was found. Furthermore, potential spread from cell to cell with very little decrement, indicating that the resistance of the pathway from the interior of one cell to another is low compared to the resistance from the interior to the external medium (see Fig. 5). It is as if the cytoplasm of the embryo forms a continuous compartment for the current-carrying ions.

There was no indication of electrical rectification in the cell interconnections, that is, the magnitude of the resulting potential changes depended only on the intensity of the current and not on its direction (see ref. 1).

The coupling was demonstrable whether dye-containing electrodes or conventional KCl-filled micropipettes were used. In general, it was strongest when the resting potentials were high. Resting potentials seen after several hours were usually as large as those recorded at the beginning of the experiment and were usually in the range 40–50 mV for embryonic cells and 45–65 mV for yolk cells.

The dye (mol wt 993), used for marking the cells, spread in some experiments to three or four neighboring cells. Although inconsistent, these observations raise the possibility that the intercellular connections permit passage of such molecules.

The eye provides a striking example of the persistence of coupling until a late stage of development. In stage 24 cells of the retina are still clearly coupled to the yolk. The bottom traces in Figure 3*A* and *B* show the pulse of current supplied inside the yolk cell. The resulting change in potential, within the yolk cell near the base of the eye stalk, is shown on the middle traces. The top trace in Figure 3*A* shows the potential change within a retinal cell. It is somewhat slower, but nearly as large as that seen in the yolk cell, indicating a substantial spread of current from yolk to retinal cell. When the electrode was withdrawn until the resting potential (45 mV) was no longer recorded, most of the potential change accompanying the current pulse also disappeared (top trace, part *B*). What remains is similar to the potential change recorded deep within the arm in Figure 2*F*.

The electrical coupling is widespread. Up to a certain stage of development the epidermis was coupled to the yolk cell in a variety of organs: tentacle, mantle, fin, funnel, gill, outer yolk sac membrane, eye-stalk, and anal papilla. Deep cells, presumably mesodermal in character, in tentacle, gill, and fin were also electrically connected to yolk. The otocyst, when a complete vesicle, retained its coupling to

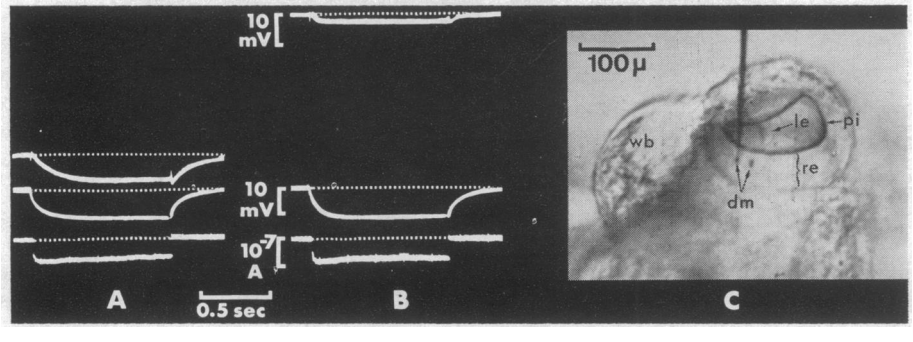


FIG. 3.—Electrical coupling between a retinal cell and the yolk (stage 24). (*C*) A photograph of the eye taken during the experiment: *dm*, dye marks within two retinal cells; *le*, lens; *pi*, pigment layer at outer surface of retina; *re*, retinal layer; *wb*, white body. For explanation of oscilloscope traces, see text.

yolk as did the outermost cell layer of the heart even after it had begun to beat (stage 24).

All of the tissues tested, with one exception, lost their electrical connection to the yolk cell during the period from late stage 25 through stage 26, about 4–5 days before hatching. This is illustrated for the retina in Figure 4. Part *C* shows the eye of an embryo late in stage 26 with the microelectrode tip within the marked retinal cell (*dm*). The recordings made with the electrode tip inside this cell and after withdrawing it were very similar (upper traces of *A* and *B*, respectively). The observed potential changes reflect the appreciable resistance between interstitial spaces and external medium.

In the few cases studied during the transition period (late stage 25 and early stage 26), the loss of coupling was found to be not uniform throughout the embryo. In one case, coupling to the yolk was undetectable in the retina, but still clearly present in tentacle epidermis. By late stage 26 all tissues tested (heart, otocyst, retina, and the epidermis of funnel, arm, and back) were apparently uncoupled from the yolk cell with the exception of the external epithelium of the outer yolk sac. This epithelium, although continuous with the skin of the embryo proper, has a different histological appearance. It seems to be separated from the yolk by at least one intermediate (endothelial) cell layer. The coupling, which was very tight, persisted to the latest stage studied (stage 28).

In preliminary experiments electrical coupling between the maturing ovum and the follicular epithelium has been examined in ovaries of the adult squid. Six follicles from two squid were tested and coupling found in each case.

*Discussion.*—The effective spread of current among cells of the squid embryo in experiments described above is most simply explained by assuming low-resistance intercellular contacts such as have been described in other tissues (see introduction). There are alternatives which, although unlikely, are discussed briefly here. The circuit shown in Figure 5 indicates that current would be channeled from cell to cell if the interstitial resistances (*de* and *ef*) had high values or a large resistance was interposed between point *f* and the bath (e.g., an external lamina around the embryo). Several observations argue against these alternatives. (*a*) It was observed repeatedly that only small potential changes were recorded with an electrode deep in the tissue until a cell was entered. (*b*) The amplitude of the potential change was not sensitive to distance of the recording electrode from the yolk cell where current was supplied, but depended mainly on whether the electrode was

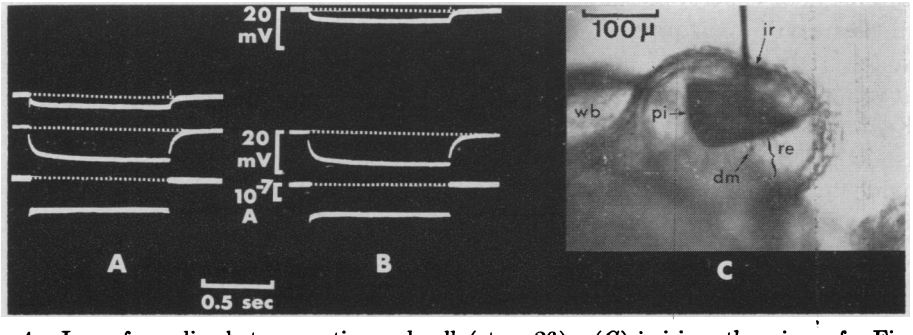


FIG. 4.—Loss of coupling between retina and yolk (stage 26). (*C*) *ir*, iris; otherwise as for Fig. 3.

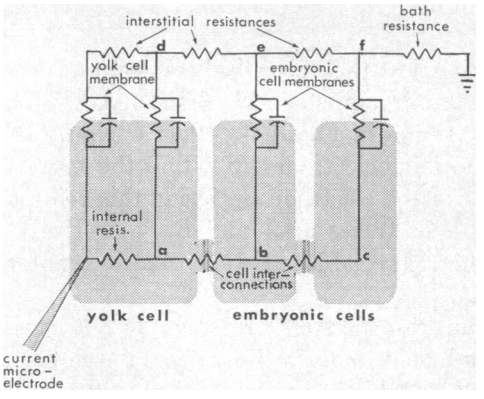


FIG. 5.—Suggested equivalent electrical circuit. This circuit would reproduce the various electrical recordings made on the squid embryo, provided the junctional resistances (*ab* and *bc*) and the interstitial resistances (*de* and *ef*) are low compared to the cell membrane resistances.

inside or outside a cell. (*c*) No barrier is seen surrounding the embryo in electron micrographs of stages which show strong coupling.[18] Moreover, it was observed that cells at the embryo surface may, when disturbed by the electrode, float free.[19]

The experiments reported here have revealed that most, and perhaps all, embryonic cells have an electrical connection to the yolk. Neither histology nor electron microscopy supports the idea that each cell sends a process directly to the yolk, so that in most cases this coupling must be by way of interconnected embryonic cells. A novel aspect of these experiments is the presence of coupling between cells of different character (e.g., yolk cell to retina by way of still other cells; cf. ref. 3). This raises the interesting possibility that ectoderm and mesoderm are directly coupled.

Electrical connections between embryonic cells are not peculiar to squid. Dr. J. Sheridan (Neurophysiology Laboratory, Harvard Medical School) is at present making similar experiments on developing chicks. He has established the presence of coupling between notochordal cells, between neural plate cells, and between notochord and neural plate.

What are possible functions of the intercellular connections? Their low electrical resistance suggests that they provide an effective means of sharing among the cells the buffering and pumping of small ions. Moreover, if the contacts pass larger substances, nutrients may be transported by this route from yolk to embryonic cells. But the most striking possibility is that control of embryogenesis is achieved, at least in part, by such means, either through alteration of membrane structure at the contacts or through passage of substances from one cytoplasm to the next. Arnold has provided evidence that the yolk in these embryos is important in specifying differentiation.[20]

Intercellular control mechanisms not only operate during embryogenesis but are doubtless also important for maintenance of the differentiated character of adult tissues. It is perhaps of interest that tight junctions are characteristic of tissues that retain the ability to divide.

Examples are known of nonselective intercellular pathways in the form of protoplasmic bridges, e.g., between spermatids of various mammals.[21] These appear to provide for synchronous division and equivlaent differentiation of all the cells of the group. However, in order for *differences* between cells to become established and be maintained, the communication between the cells must be more selective. The intercellular connections in the squid embryo may very well represent such selective pathways.

Certain cells, when isolated and explanted in culture, migrate and divide. These

activities cease on re-establishment of contact. This "contact inhibition" and its absence in cells transformed by certain viruses has received much attention (e.g., refs. 22–24). It is possible that contact inhibition is mediated by intercellular connections. It is known that tight junctions occur between fibroblasts in culture.[25] We have begun electrophysiological and electronmicroscopical studies of cell cultures with Dr. S. Palay. In preliminary experiments very effective electrical coupling has been observed between normal cells of established mouse and hamster lines 3T3 and BHK (kindly supplied by Drs. Howard Green and Renato Dulbecco). Cells of the same lines transformed by polyoma and SV40 viruses also showed effective coupling. Thus if transformed cells have defects in their intercellular contacts, they are not revealed by the electrical methods. Stoker[26] has shown that normal cells inhibit transformed cells on contact and has suggested that the defect is in the emission of a contact-promoted signal.

The authors wish to thank Dr. Renato Dulbecco for his helpful advice, and Miss Florence Hector and Miss Carola Pickering for their unfailing technical assistance.

* These experiments were carried out at the Marine Biological Laboratory, Woods Hole, Mass., and were supported by grants from the National Institutes of Health (5 TI-HE-5143-09, NB-02253-06, and NB-03273-05).

[1] Furshpan, E. J., and D. D. Potter, *J. Physiol.*, **145**, 289 (1959).

[2] Bennett, M. V. L., E. Aljure, Y. Nakajima, and G. D. Pappas, *Science*, **141**, 262 (1963).

[3] Furshpan, E. J., *Science*, **144**, 878 (1964).

[4] Burnstock, G., M. E. Holman, and C. L. Prosser, *Physiol. Rev.*, **43**, 482 (1963).

[5] Robertson, J. D., in *Cellular Membranes in Development*, ed. M. Locke (New York: Academic Press, 1964), p. 62.

[6] Karrer, H. E., *J. Biophys. Biochem. Cytol.*, **8**, 135 (1960).

[7] Dewey, M. D., and L. Barr, *J. Cell Biol.*, **23**, 553 (1964).

[8] Kuffler, S. W., and D. D. Potter, *J. Neurophysiol.*, **27**, 290 (1964).

[9] Kuffler, S. W., J. G. Nicholls, and R. Orkand, *J. Neurophysiol.*, in press.

[10] Loewenstein, W. R., S. J. Socolar, S. Higashino, Y. Kanno, and N. Davidson, *Science*, **149**, 295 (1965).

[11] Wolfe, D., in preparation.

[12] Peachey, L. D., and H. Rasmussen, *J. Biophys. Biochem. Cytol.*, **10**, 529 (1961).

[13] Farquhar, M. G., and G. E. Palade, *J. Cell Biol.*, **17**, 375 (1963).

[14] Arnold, J. M., *Biol. Bull.*, **128**, 24 (1965).

[15] Alvarez, J., and E. J. Furshpan, in preparation.

[16] Sandborn, E., P. F. Koen, J. D. McNabb, and G. Moore, *J. Ultrastruct. Res.*, **11**, 123 (1964).

[17] The potential change in the epithelial cell (trace *2*, Fig. 2*D*) was 16% greater than that recorded at the other end of the yolk cell (trace *1*). This was due to the drop in potential along the internal resistance of the yolk cell.

[18] Arnold, J. M., personal communication.

[19] Even though a cell previously had low-resistance connections with its neighbors, its loss from the embryo did not lead to any long-term short-circuiting of the remaining intact cells; normal resting potentials were recorded for hours despite the ejection of numerous surface cells. The cell interconnections may be labile, sealing off following injury. Related observations have been made in another tightly coupled tissue, vertebrate heart [Délèze, J., in *Electrophysiology of the Heart*, ed. B. Taccardi and G. Marchetti (New York: Pergamon Press, 1965), p. 147].

[20] Arnold, J. M., *Biol. Bull.*, **129**, 72 (1965).

[21] Fawcett, D. W., *Exptl. Cell Res.*, Suppl. 8, 174–187 (1961).

[22] Abercrombie, M., and E. J. Ambrose, *Cancer Res.*, **22**, 525 (1962).

[23] Dulbecco, R., *Science*, **142**, 932 (1963).

[24] Enders, J. F., in *The Harvey Lectures*, Series 59 (New York and London: Academic Press, 1965), p. 113.

[25] Devis, R., and D. W. James, *J. Anat.* (*London*), **98**, 63 (1964).
[26] Stoker, M., *Virology*, **24**, 165 (1964).

---

# *NUCLEIC ACID HOMOLOGY STUDIES OF ADENOVIRUS TYPE 7-SV40 INTERACTIONS*

BY PAUL R. REICH,* STEPHEN G. BAUM,† JAMES A. ROSE,* WALLACE P. ROWE,† AND SHERMAN M. WEISSMAN*

NATIONAL CANCER INSTITUTE AND NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

*Communicated by Robert J. Huebner, December 9, 1965*

Adenovirus infection of monkey kidney cells in tissue culture is abortive; the early T antigens are formed efficiently and typical adenovirus cytopathic changes occur, but only limited amounts of viral structural antigens, morphologic particles, and infectious virus are produced.[1-3] In cells infected with SV40 virus, this block is eliminated; the viral antigens and infectivity are formed with efficiency comparable to that in human cells.[1-3] This potentiation of adenovirus growth by SV40 genetic material is also observed with the adenovirus type 7 (Ad. 7) strain E46$^+$ which carries the SV40 information for production of SV40 T antigen.[4-10]

This report describes the application of the nucleic acid homology technique to determine whether SV40 enhancement acted at a step before or after Ad. 7 DNA synthesis, and whether SV40 DNA could be demonstrated in E46$^+$.

*Materials and Methods.—Tissue cultures:* Primary monolayer cultures of African green monkey kidney (AGMK) and human embryonic kidney (HEK) cells were obtained from the Division of Biologic Standards, NIH, and Microbiological Associates, Inc. They were maintained in Eagle's basal medium #2 (BME) containing 2% agammaglobulinic, heated (56°C for 30 min) calf serum. The medium contained glutamine, penicillin, and streptomycin. Cultures were incubated at 37°C in air.

*Viruses:* The origin of the E46$^+$ and E46$^-$ strains of Ad. 7 have been described.[9] E46$^-$ is the Ad. 7 line (obtained from E46$^+$) which does not induce SV40 T antigen, and which cannot be propagated serially in AGMK cells. SV40 virus, strain 777,[11] and the Pinckney strain[12] of Ad. 7 were used. Virus inocula for E46$^+$ and SV40 were prepared in AGMK cells, E46$^-$ in HEK cells, and Pinckney strain in KB cells. The Pinckney and E46$^+$ strains were known to be contaminated with the 22-m$\mu$ diameter "adenovirus-associated virus," [13-15] but the E46$^-$ strain was free of the contaminant.

*Virus infection of AGMK cells:* Two 32-oz flask cultures of AGMK (approximately $10^7$ cells) were used for each assay. The maintenance medium was decanted and 10 ml of virus suspension was added. Input multiplicity was about 2 plaque-forming units (PFU) per cell for SV40, and approximately 0.7 PFU per cell for E46$^+$ and E46$^-$. Cultures were held at 37°C for 4 hr; the fluid was then decanted and the cell sheet washed twice with 10 ml of BME. The cultures were fed with 40 ml of maintenance medium. Only the earliest changes characteristic of SV40 cytopathogenic effect were seen 72 hr after infection. At the time of harvesting, the cells were scraped into the fluid medium and centrifuged at 500 *g* for 15 min. The cell pack was resuspended in 2 ml of BME; a portion was taken for virus titration, and the cells were stored frozen (−20°C) prior to DNA extraction.

*Adenovirus titrations:* The cell packs were frozen and thawed twice for titration of adenovirus yield. Titrations were done in tube cultures of HEK cells in medium containing 1% SV40 rabbit antiserum. Cultures were observed for 21 days.