

POLARIZED INTERCELLULAR BRIDGES IN OVARIAN FOLLICLES OF THE CECROPIA MOTH

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ABSTRACT

Fluorescein-labeled rabbit serum globulin was injected into vitellogenic oocytes of the cecropia moth. Though the label spread throughout the ooplasm in less than 30 min, it was unable even after 2 h to cross the complex of intercellular bridges connecting the oocyte to its seven nurse cells. After injection into a single nurse cell, fluorescence was detected in the oocyte adjacent to the bridge complex within 3 min and had spread throughout the ooplasm in 30 min. Here also, the cell bodies of the six uninjected nurse cells remained non-fluorescent. Four of the nurse cells are not bridged directly to the oocyte but only through the apical ends of their siblings. Unidirectional movement must therefore occur in the apical cytoplasm of the nurse cells, as well as in the intercellular bridges. The nurse cells of healthy follicles had an intracellular electrical potential -40 mV relative to blood or dissecting solution, while oocytes measured -30 mV. A mV difference was also detected by direct comparison between a ground electrode in one cell and a recording electrode in the other. Three conditions were found in which the 10 mV difference was reduced or reversed in polarity. In all three cases fluorescent globulin was able in some degree to cross the bridges from the oocyte to the nurse cells.

INTRODUCTION

Since the mid-19th century intercellular bridges wide enough to transmit cytoplasmic organelles have been recognized in the differentiating germ cells of animals. Perhaps the earliest indication of these structures was Huxley's finding in 1858 that a cytoplasmic strand connecting each oocyte with what would now be considered a chamber of nurse tissues can be seen in freshly dissected aphids. Platner (1886), Lee (1895), and others working with fixed and sectioned material observed an additional manifestation of intercellular bridges: deeply staining ligaments, which they considered to be spindle remnants connecting adjacent spermatocytes in many species of animals.

These and a variety of other 19th century observations were integrated by Giardina in 1901

into what is remarkably similar to present views on the origin and function of intercellular bridges. It was clear from Giardina's review that clusters of connected germ cells can occur in both male and female gonads, and that each bridge arises at mitosis as a consequence of incomplete cytokinesis. Giardina used as evidence for cytoplasmic continuity across the bridges the fact that, as in better known syncytia, the nuclei of the attached siblings proceed through subsequent mitoses in exact synchrony with each other.

Confirmation that the bridges are open channels of cytoplasm was first achieved at the fine structure level by Burgos and Fawcett (1955) working with cat testes. The configuration they observed has since been seen by many other electron microscopists in both male and female germ cells. In

most cases the bridge has a diameter of at least 1 μm and what appears to be a mechanically reinforced wall. It is frequently seen to contain cytoplasmic organelles, particularly mitochondria, endoplasmic reticulum, or ribosomes. The groups of animals in which this now well-defined structure has been found include mammals, birds, amphibians, tunicates, insects, arachnids, crustaceans, annelids, rotifers, and nematodes; the present rate at which new cases are being described implies a substantially wider distribution.

Synchronized development in the nuclei of the connected siblings has been confirmed in the recent literature for male germ cells (Fawcett et al., 1959; Dym and Fawcett, 1971; King and Akai, 1971) and for the prefollicular oögonia and oocytes of developing ovaries (Franchi and Mandel, 1962; Brown and King, 1964; Koch and King, 1966; Zamboni and Gondos, 1968; Ruby et al., 1969, 1970; Skalko et al., 1972). In contrast to these cases the period of synchrony is followed in some invertebrate ovaries by an asynchronous phase. The phenomenon has been particularly well studied in insects and in this connection, also, Giardina's 1901 paper was a landmark. In his experimental material, the ovary of the water beetle *Dytiscus*, the period of synchrony gives rise to a clone of 16 connected cells. At this point both mitosis and synchrony terminate; 1 cell becomes an oocyte, while the remaining 15 become nurse cells which develop large, endopolyploid nuclei rather than entering meiosis. Despite the loss of synchrony, the syncytial nature of the complex persists. The intercellular bridges, in fact, increase their diameter, and what Giardina interpreted as the movement of cytoplasm from the nurse cells into the growing oocyte ensues.

In other insects a mass movement of materials across the bridges is particularly obvious at the termination of nurse cell function when much or all of the residual cytoplasm of these cells empties into the oocyte (Bier, 1963; Pollack and Telfer, 1969; King, 1970). Before this event the nurse cells spend a period, often lasting several days or longer, in a state of vigorous RNA synthesis (Bier, 1963). Here also traffic across the intercellular bridges shows a net flux toward the oocyte. In the cecropia moth *Hyalophora*, oocyte RNA increases from less than 0.5 to 2.5 μg over a several day period, while the nurse cells which synthesize most of this RNA retain a constant 0.5 μg (Pollack and Telfer, 1969). At the termination of the

synthetic period the residual 0.5 μg also flows into the oocyte. An additional well-established case of one-way transport is the finding that the centrioles of the nurse cells migrate or are carried into the oocyte in *Drosophila* (Koch and King, 1969; Mahowald and Strassheim, 1970).

The successive functional states of the intercellular bridges in insect follicles can be thought of in terms of polarity. In synchronized cell clusters there is no apparent bias on the direction of exchange so that any bridge, along with the two cells it connects, constitutes an unpolarized system. In differentiated nurse cell-oocyte complexes, by contrast, the bridged cells form a polarized system, with a net flow of cytoplasmic components toward the oocyte.

Described here are experiments which explore the physiological bases of polarity in ovarian follicles of the cecropia moth. We are able to demonstrate that fluorescein-labeled proteins injected intracellularly can cross the bridges from the nurse cells to the oocyte, but not in the reverse direction. Evidence is also presented that an electrical potential gradient accompanies the polarity of protein movement.

MATERIALS AND METHODS

The observations were made on the ovaries of the saturniid moth, *Hyalophora cecropia*. Follicles undergoing yolk deposition in this species measure from 0.5 to 1.8 mm in length, with most of the volume being occupied by the oocyte and, until the follicle is 1.5 mm long, by the seven nurse cells. The cells are therefore large enough to be injected with relative ease or to be impaled with microelectrodes (Fig. 1). They survive in vitro incubation for several hours in either blood (Hausman, et al., 1971) or the dissecting solution that was developed by L. Anderson (1971) for studying the mechanism of vitellogenesis (40 mM KCl, 15 mM MgCl₂, 5 mM CaCl₂, 0.11 M Tris-HCl, pH 6.2). The follicles tested were dissected from animals on the 17th-19th days of the pupal-adult molt and measured 0.9-1.2 mm in length. Follicles of this size are young enough so that their bridges should have continued to function in the animal for one more day.

Electron Microscopy

For electron microscopy, the follicles were fixed in 2.5% glutaraldehyde buffered with Na-cacodylate, at pH 7.4. Isotonicity was maintained by adding 1.5 g of sucrose/100 ml of solution (Telfer and Smith, 1970). The tissue was postfixated with osmium tetroxide

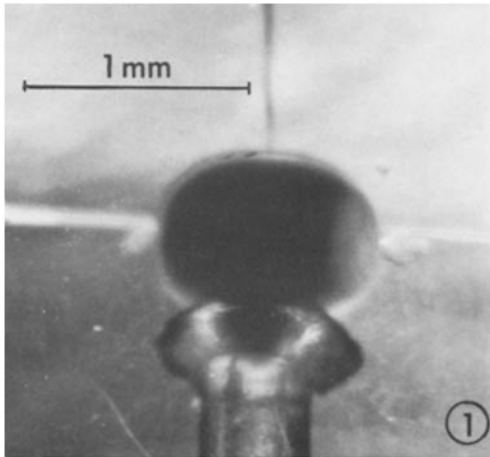


FIGURE 1 A vitellogenic follicle held by a suction clamp and impaled with three microelectrodes. The opaque mass is the yolk-filled oocyte; the nurse cells form a translucent hemisphere on the right; the follicular epithelium envelops the oocytes; broken epithelial connectives that attached the follicle to its neighbors can be seen on both right and left. $\times 30$.

and embedded in Araldite. Sections were stained with uranyl acetate and Reynolds' lead stain.

Microinjections

The protein used for injection was fluorescein-labeled rabbit serum globulin (FSG, Nutritional Biochemicals Corporation, Cleveland, Ohio). It was selected initially because histological methods were already in use for its detection in cecropia follicles (Hausman et al., 1971). Serum globulins are a mixture of proteins and the labeling process presumably increases their heterogeneity, so that precise statements about their physical characteristics are not possible. The commercial preparation was dialyzed against dissecting solution before injection.

During injection the follicle was held with a suction clamp (Fig. 1) consisting of a piece of small-bore polyethylene tubing melted at the end to form a concavity into which the follicle would fit (Cross and Brinster, 1969). Suction was applied by a 100 μ l syringe.

The injection was made through a Pyrex glass micropipette with a 3 μ m diameter tip. The needle was filled with distilled water, attached by a water-filled length of polyethylene tubing to a 10 μ l syringe, and inserted on a custom-built micromanipulator. Dialyzed FSG was then drawn into the needle. To ensure that the needle was not plugged, the plunger of the injection syringe was depressed enough to start a slight flow of FSG from the tip. The needle was then inserted into the cell and the plunger depressed to eject about 0.1 μ l from the syringe. Since the FSG

solution continued to flow from the tip of the needle after withdrawal from the cell, the volume injected was less than that pushed out of the syringe. To measure the volume more precisely, the FSG solution was mixed with [3 H]histidine (1,000 parts of dialyzed FSG:1 part H_2O containing the radioactive label) to yield a solution of known radioactivity and of viscosity comparable with that of the solution routinely used. Six oocytes were injected by the standard procedure, rinsed in dissecting solution, and dissolved in 5% sodium dodecyl sulfate and 3.5 mM dithioerythritol in preparation for scintillation counting. The injected follicles were shown in this way to contain an average of 0.015 μ l of the FSG solution, with the range being 0.01–0.02 μ l. The volume of oocytes at the stage injected is approximately 0.5 μ l, while that of each nurse cell is about 0.03 μ l. As will be seen below, fluorescence microscopy of follicles fixed immediately after injection revealed that the volume actually delivered to the cytoplasm was, in both cases, substantially less than the total volume of the cell.

The site of injection was near the center of the oocyte, approximately 250 μ m from the nurse cells (Fig. 10). Nurse cell injections could not be so precisely localized. During insertion of the needle it was possible to distinguish between nurse cells, but not between the cytoplasm and nucleus of each cell. The distribution of fluorescence in fixed follicles left no doubt as to which compartment had been injected, however (Fig. 13).

Fluorescence Microscopy

After injection, follicles were transferred to depression slides containing dissecting solution and incubated at room temperature for times ranging from 30 s to 3 h. They were then freeze substituted (Melius and Telfer, 1969), embedded in paraffin, and sectioned at 7 μ m. The sections were examined with a Zeiss fluorescence microscope (Carl Zeiss, Inc., New York) using an Osram HBO-200 mercury vapor lamp (Atlantic Instruments, Warminster, Pa.), no. 2 excitation filter and barrier filters 50 and 47.

Fluorescent tissue was photographed using Kodak Tri-X film exposed for 30–60 s. Fading occurs very rapidly upon illumination of sections prepared in this way, but can be sufficiently retarded to permit photographic recording if the paraffin is not removed. The resulting images are sharp at the low magnification needed for this report, though the inducing light diffracted by irregularities in the paraffin was sometimes recorded by the film.

Electrical Measurements

Potential differences were measured using 3 M KCl-filled glass capillary electrodes (Ling and Gerard, 1949). The tip diameters were less than 1 μ m each. They were coupled by KCl-agar bridges

to Ag-AgCl electrodes. The signals were amplified using a high input impedance, negative feedback, DC preamplifier, and they were recorded on a split-beam storage oscilloscope. Measurements were made within a Faraday cage, and care was taken to ground all instruments, as well as the operator, through a common ground. Follicles were held by the suction clamp discussed above. Three custom-built micromanipulators were then used to position the electrodes in or adjacent to the follicle (Fig. 1). Three different procedures were used to determine potential differences between the nurse cells and the oocyte (Fig. 2), all giving essentially the same result. It was possible to reverse the polarity of the oocyte and one of its nurse cells. This was accomplished by passing a current of 5×10^{-8} A between a stimulating electrode in the oocyte and a ground in the nurse cell (Fig. 3).

RESULTS

Morphology of the Intercellular Bridges

Basic to an understanding of the observations described in this report is the number and con-

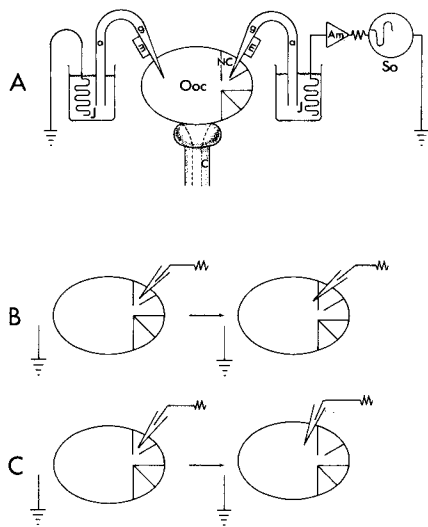


FIGURE 2 Three methods used to measure electric potential differences between nurse cells and oocytes and between these cells and the surrounding medium. Method A was the most frequently used and gave direct readings of the potential difference between two cells. Methods B and C involved taking the potential difference between one cell and the medium, then the other cell and the medium. *a*, KCl-agar bridge; *Am*, DC pre-amplifier; *C*, suction clamp; *g*, KCl microelectrodes; *J*, KCl, Ag-AgCl electrodes for liquid-metal junction; *m*, micromanipulators; *NC*, nurse cells; *Ooc*, oocyte; *So*, storage oscilloscope.

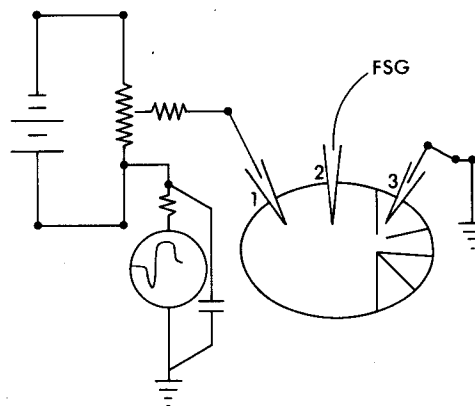


FIGURE 3 Method used to test the effect of electrically reversed polarity on FSG injected into an oocyte. Current was delivered through electrode 1. Current magnitude was monitored on a storage oscilloscope. Electrode 3 served as ground and was positioned in a nurse cell. Number 2 was the micropipette used for injection of FSG.

figuration of the intercellular bridges. King and Aggarwal (1965) showed that there are seven nurse cells in the cecropia follicle, that three intercellular bridges connect the oocyte to the nurse cells, and that there are additional bridges between nurse cells. These features fit exactly the bridge distribution found by Knaben (1934) in the moth *Tischeria*, and by Hirschler (1942) in the butterfly *Macrothylacia*. As the first important addition to Giardina's concepts of sibling cluster formation, Knaben and Hirschler emphasized that when a cell already connected to two or more bridges divides all preexisting bridges remain with one daughter cell. As a consequence of this behavior four of the eight cells in the lepidopteran sibling cluster are connected to single bridges which arise at the last set of synchronized mitoses; two cells each connect to two bridges produced at the last two mitoses, and two cells each connect to three bridges (Fig. 4). Knaben and Hirschler agreed that the oocyte is always formed by one of the two cells with three bridges, and this has been confirmed for cecropia by King and Aggarwal (1965).

The model has proven to be applicable to all insects with nurse cells, if allowance is made for species differences in the number of cell divisions involved. Hirschler (1945) was able to cite cases of two and four cell clusters in which the numbers and distribution of bridges fit the model, and King et al. have shown more recently that in the *Drosophila* follicle, the 8-cell, lepidopteran-type

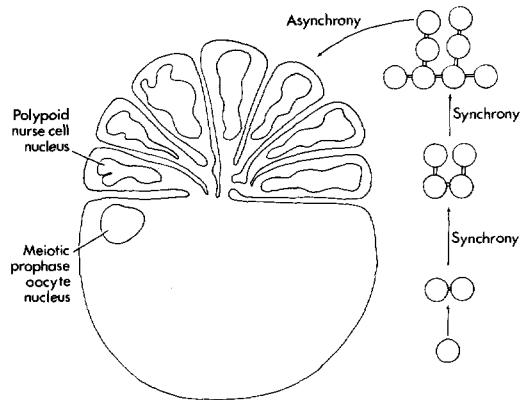


FIGURE 4 Diagram of the development and morphology of the lepidopteran follicle. Three sets of synchronous mitoses yield an eight cell cluster which differentiates into a first meiotic prophase oocyte and seven polyploid nurse cells. Three nurse cells are bridged directly to the oocyte. The other four are bridged only through the apical ends of their siblings.

cluster undergoes one more division to produce 16 cells and 15 bridges, 4 of which attach to the oocyte (Brown and King, 1964; Koch et al., 1967).

By the time that vitellogenesis has commenced, the cecropia follicle has grown in such a way that the seven nurse cells form a hemisphere in which the individual cell is shaped as a cone with its base at the surface of the follicle and its apex at the center (Figs. 5–7). The three intercellular bridges connecting the nurse cells to the oocyte, as well as the four between nurse cells, are gathered in a complex near the anterior pole of the oocyte. As will be shown below, protein injected into a nurse cell moved directly through the bridge complex and into the oocyte without detectably entering the expanded portion of any other nurse cell. This invariably occurred even though four of the nurse cells are not bridged directly to the oocyte. It is therefore necessary to view transport as occurring in this system not between pairs of connected cells, but through a polarized complex of bridges and apical cytoplasm.

Concerning the dimensions of the system at the stages analyzed, the nurse cell cluster has a diameter of about $800\ \mu\text{m}$, and the oocyte is nearly spherical with a diameter of $800\text{--}1000\ \mu\text{m}$. The exact dimensions vary with the stage of vitellogenesis. The intercellular bridges, measured by light microscopy of serial sections, have diameters of $30\text{--}35\ \mu\text{m}$. While they are thus exceptionally large relative to the $1\text{--}2\ \mu\text{m}$ bridges seen by

electron microscopy in nonpolar systems, they are still relatively narrow channels for such large cells. The bridge complex in the center of the follicle occupies an oval region about $60\ \mu\text{m}$ wide and up to $100\ \mu\text{m}$ long. The path length from nurse cell to oocyte could conceivably vary from as little as $3\ \mu\text{m}$ for the three nurse cells connected directly to the oocyte, to as much as $100\ \mu\text{m}$ for the four connected indirectly through the apices of their siblings.

Fluorescence microscopy of follicles incubated in female blood containing FSG permitted a particularly clear visualization of the spaces and bridges between nurse cells and the oocyte. In Fig. 5, which was prepared for purposes described in a later section, the intercellular spaces appear as bright areas due to the infusion of fluorescent blood protein. Two of the three cytoplasmic bridges connecting the nurse cells to the oocyte are demonstrated in this photomicrograph. Also visible in Figs. 5–7 is an area of oocyte cytoplasm adjacent to the bridges in which no yolk spheres are found. This is the area into which nurse cell products first move as they enter the oocyte (Pollock and Telfer, 1969).

Fine Structure of the Intercellular Bridges

Fundamental to an understanding of bridge polarity is the finding that the bridges are open channels of cytoplasm with no evidence of membrane barriers separating the oocyte and nurse cells. This relationship has been documented in many studies of *Drosophila* (e.g., Koch et al., 1967) as well as in cecropia (King and Aggarwal, 1965). Meyer (1961), by contrast, believed that a zone of homogeneous dense material sometimes occludes the lumen of the bridge in *Drosophila* ovaries, and Dym and Fawcett (1971) described stacks of flattened vesicles completely filling some of the bridges between spermatogonia of mammals. It was therefore necessary to verify the open structure of the bridges during the precise stages analyzed in cecropia.

An electron micrograph of an intercellular bridge between a nurse cell and an oocyte of a 1-mm long follicle is shown in Fig. 8. The membrane of the bridge is lined on the cytoplasmic side by a layer of densely staining material about $70\ \text{nm}$ thick. The layer is not confined to the bridge, but extends along the adjacent cell membranes of both the oocyte and the nurse cell for up

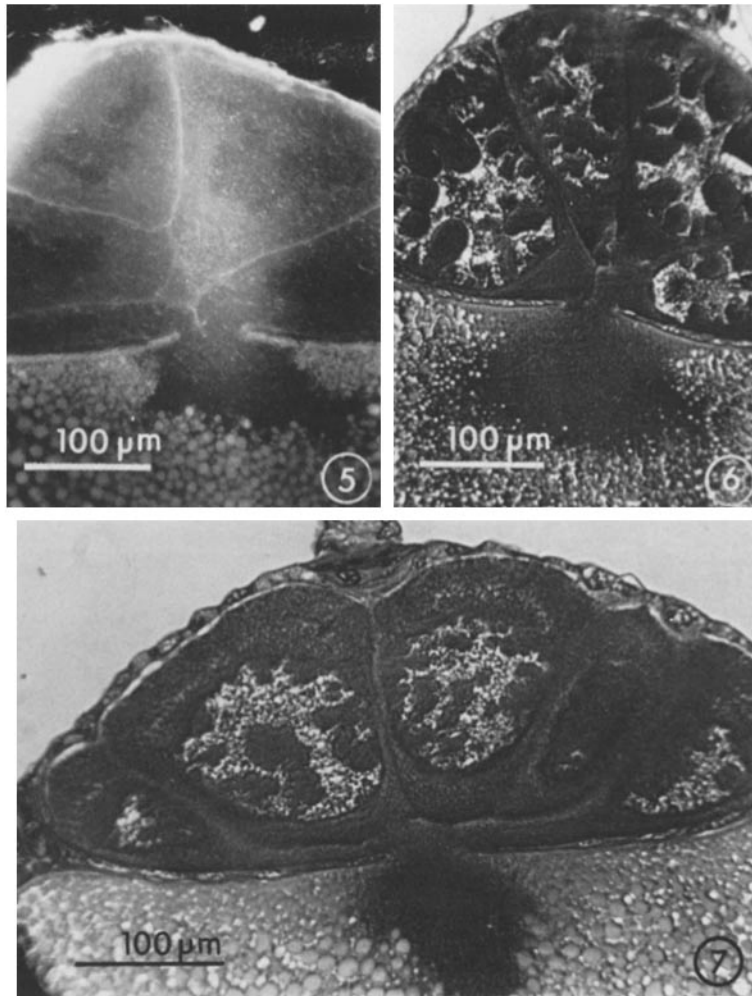


FIGURE 5 A vitellogenic follicle incubated in blood containing FSG. The blood has filled the spaces between nurse cells. Note the continuity of the nurse cell and oocyte cytoplasm. Yolk spheres in the ooplasm are visible due to autofluorescence. $\times 165$.

FIGURE 6 A longitudinal section through a vitellogenic follicle. This follicle was fixed in Carnoy's, stained with periodic acid-Schiff's stain and hematoxylin, and photographed using phase-contrast illumination. Nurse cells with irregularly shaped nuclei fill the top half of the figure. Two of the three bridges connecting them to the oocyte, below, are visible. $\times 165$.

FIGURE 7 A follicle slightly older than that seen in Fig. 6, and prepared in the same way. $\times 165$.

to $15\ \mu\text{m}$. This material, which was first described by Burgos and Fawcett (1955), is usually assumed to stabilize the bridge. Also conspicuous is the occurrence of cytoplasmic protuberances from both oocyte and nurse cells into the intercellular space adjacent to the bridge (King and Aggarwal, 1965). Some of the protuberances have greatly swollen tips. The dense stratum lining the cell

membrane of the bridge region extends into the neck of each protuberance, but not into the swollen tip.

The cytoplasm within the bridge and in the cells on either side contains mitochondria, a low density of ribosomes and microtubules, and numerous vesicles having the appearance of smooth endoplasmic reticulum. Since the bridge has at

this stage a diameter of 30–35 μm , only a small portion of its contents can be seen in any one figure. The cytoplasm in the center of the bridge, and for up to 50 μm into the cells on either side, invariably appeared like that shown in Fig. 8. In no case was there evidence of a continuous membrane separating the two cytoplasms, a result which is not surprising in view of the facility with which microinjected FSG moves from the nurse cells to the oocyte.

In some insects, the oocyte is connected to nurse cells by a greatly attenuated bridge known as a trophic cord. Concentrated arrays of microtubules, oriented lengthwise, have been demonstrated in the trophic cord (Hamon and Folliot, 1969; MacGregor and Stebbings, 1970; Huebner and Anderson, 1970; Brunt, 1970) and could conceivably function in polarized transport in those instances. Microtubules were present in the bridges of vitellogenic follicles in cecropia (Fig. 8), but were widely scattered by comparison. They were separated by sufficient numbers of mitochondria and membranous vesicles to make it unlikely that a microtubular apparatus occludes the bridges in the living state. Like King and Aggarwal (1965), therefore, we found no suggestion of a mechanical barrier to the transmission of organelles or molecules across the bridges of cecropia.

Microinjection of FSG

DEMONSTRATION OF FOLLICLE VIABILITY AFTER INJECTION: Interpretation of the extent and direction of movement of FSG injected into oocytes and nurse cells required an appraisal of the effects of the injection on the follicle. One index used for this purpose was the capacity of the follicle to continue forming yolk spheres after microinjection of dissecting solution, the FSG solvent used in later experiments. Hausman et al. (1971) showed that cecropia follicles would continue to incorporate environmental proteins into yolk spheres for at least 6 h *in vitro* if incubated in female blood samples containing vitellogenin, the primary yolk protein precursor in the blood. Yolk deposition can be monitored under these conditions by including FSG in the medium and appraising its incorporation by fluorescence microscopy of paraffin sections after freeze substitution.

After injection of the standard volume of cecropia saline into the oocyte, follicles were

incubated in depression slides with 0.3 ml of a mixture containing 1 part FSG solution in 9 parts of blood from an 18 day developing adult female. The depressions were protected with a cover slip and incubated at 25°C in a humidified Petri dish with shaking. After incubations of up to 4 h, the follicles were freeze substituted, embedded in paraffin, sectioned, and examined by fluorescence microscopy. Fig. 9 shows a section of an oocyte that had received an injection of saline, followed by 3 h of incubation in a mixture of FSG and cecropia female blood. The tear seen in the follicular epithelium identifies the point of entrance of the injection needle. Within the oocyte, fluorescence is localized in the outermost stratum of yolk spheres. A comparable stratum of fluorescent yolk spheres was produced by uninjected control follicles. Both compare favorably with the results of Hausman et al. (1971) for follicles incubated for this time period. At least one key function, yolk deposition, was therefore able to survive the injection procedures used.

It will be noted later that oocytes maintain a –30 mV difference in potential from cecropia dissecting solution when incubated for periods of several hours. It was repeatedly found that microinjection of FSG did not affect the magnitude or persistence of the potential difference for at least an hour.

FSG INJECTIONS INTO OOCYTES: Follicles were injected with the standard volume of FSG and incubated in dissecting solution for 30 s, 30 min, 60 min, or 180 min before freezing. In oocytes that had been incubated for only 30 s, the site of delivery showed in ultraviolet-illuminated paraffin sections as a brilliant yellow-green against the lower and darker green autofluorescence of the yolk spheres (Fig. 10). Yolk spheres adjacent to the site of injection were already surrounded by the FSG. By 30 min the FSG had diffused throughout the cytoplasm of the oocyte (Fig. 11), including that adjacent to the nurse cell-oocyte bridges. There was no discernible fluorescence in the nurse cells.

In 10 follicles fixed 60 min after FSG injection into the oocyte there was still no evidence of movement of the protein into the nurse cells, (Fig. 12). In 60 follicles incubated for 3 h fluorescence continued undetectable in the nurse cells above the level of autofluorescence. Despite its ability to spread throughout the oocyte, FSG was therefore unable to cross the intercellular bridge into the nurse cells.

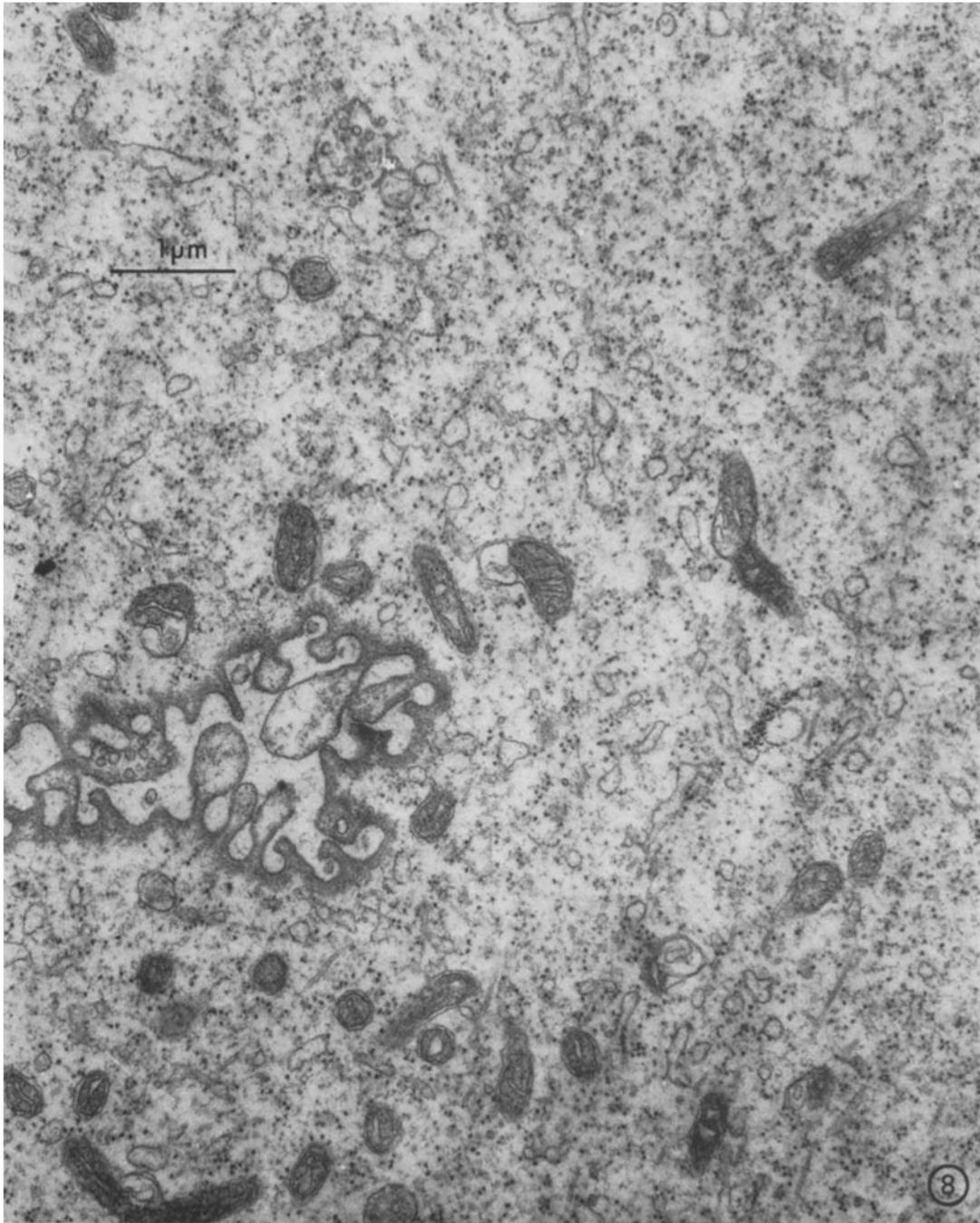


FIGURE 8 An electron micrograph showing one side of an intercellular bridge of a vitellogenic follicle. A nurse cell is at the top, and an oocyte at the bottom. Ribosomes, mitochondria, and a few microtubules and other organelles are present in the bridge as well as in the cytoplasm on either side. The cell membrane of the bridge and that of adjacent regions of the connected cells is irregularly shaped and lined by a dense deposit. $\times 18,500$.

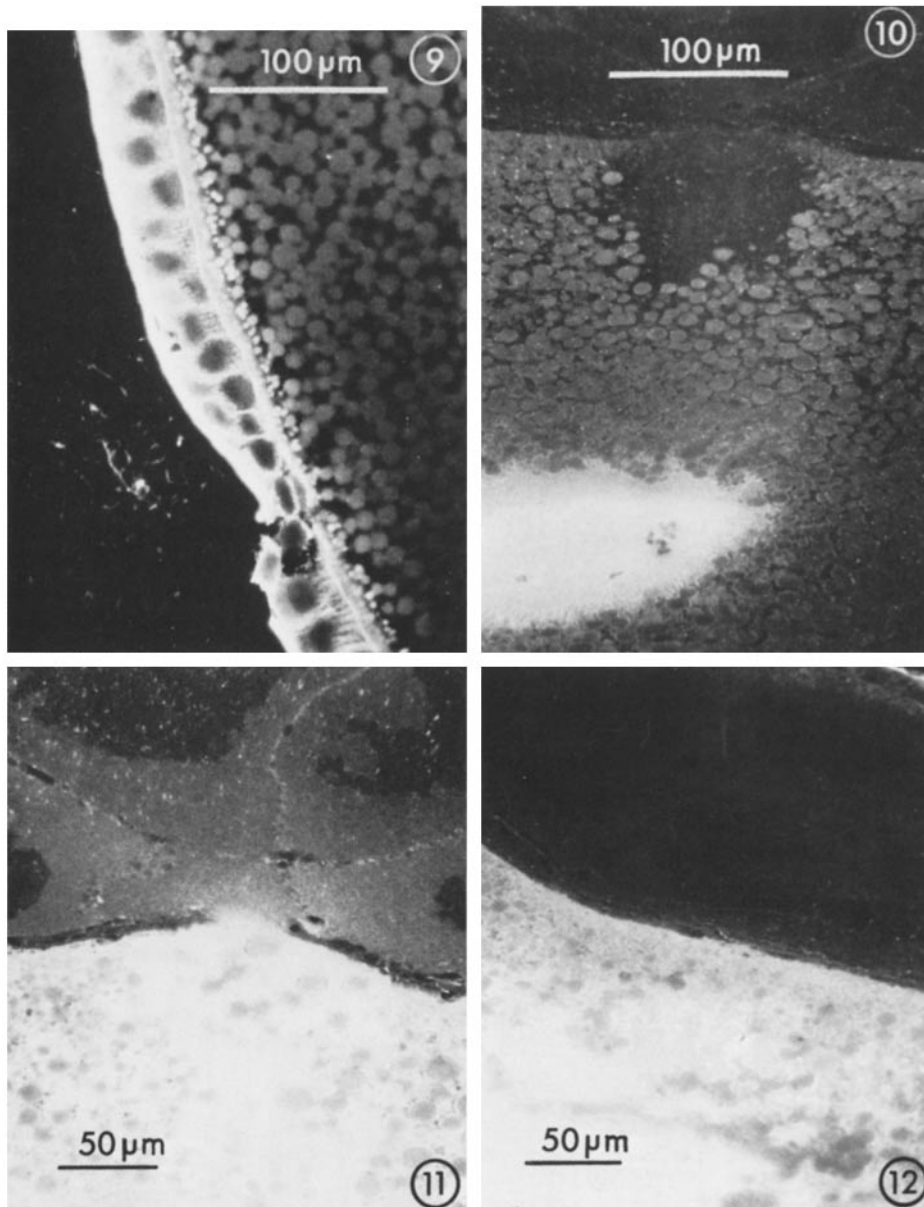


FIGURE 9 Fluorescence micrograph of a follicle incubated for 3 h in blood containing FSG. The follicle had been injected with $0.01 \mu\text{l}$ of cecropia saline. The tear in the epithelium is the point at which the needle entered the follicle. Fluorescence of the yolk spheres at the surface of the oocyte indicates that the injection did not prevent the continuation of yolk deposition. $\times 240$.

FIGURES 10-12 Fluorescence micrographs of follicles in which the oocytes had been injected with FSG. In each case the oocyte is at the bottom and the nurse cells at the top of the figure. Fig. 10: 30 s incubation. $\times 240$. Fig. 11: 30 min incubation. Some of the fluorescence in the nurse cell cytoplasm adjacent to the bridge is due to glare. $\times 260$. Fig. 12: 60 min incubation. $\times 260$.

INJECTION OF FSG INTO NURSE CELLS: The bridges were tested in the reverse direction by injecting nurse cells, rather than oocytes. Fig. 13 shows such a follicle fixed within 30 s after injection of the nurse cell cap. The entry point of the needle through the follicular epithelium can be clearly seen. The needle entered the nurse cells at an angle which apparently carried it through one cell and into another. Of importance in this photograph was the limitation of the fluorescent area to the injection site. The FSG delivered was sufficiently small in volume to remain localized in a discrete area; the pressure required to deliver the injection did not force the FSG across the cytoplasmic bridges into the oocyte. Also noteworthy was the fact that the needle had not penetrated the nucleus of either cell. Of 46 injections made into nurse cells at room temperature, only 7 showed nuclear fluorescence.

In follicles fixed only 3 min after nurse cell injection, fluorescence occurred in the ooplasm adjacent to the intercellular bridges (Fig. 14). Longer incubation times revealed increased movement of FSG in the oocyte. In the follicle seen in Fig. 15, which had been incubated for 20 min after injection, fluorescence was observed throughout the cytoplasm of the oocyte. The concept that FSG moves in only one direction across the cytoplasmic bridges was confirmed by the lack of fluorescence in all nurse cells except one, which was presumably the cell injected. Even after 1 h significant amounts of FSG could not be detected in the uninjected nurse cells though it was in all cases widely distributed in the oocyte (Fig. 16).

Electric Potential Measurements

Polarity of FSG movement across intercellular bridges that apparently contain unobstructed cytoplasm could be due to any of several causes. A fluid stream moving from the nurse cell to the oocyte or a difference in solvent or electrical characteristics between the two cells is among the obvious possibilities. While alternatives have not been ruled out, one factor that could profoundly affect the distribution of charged proteins such as those in FSG was clearly demonstrated. Observations described in this section show that an electrical potential difference of 10 mV exists between nurse cells and oocytes, the nurse cell being negative to the oocytes.

Measurements of intracellular electrical potential yielded average values of -39.5 mV for 73

nurse cells, and -29.0 mV for 78 oocytes (Table I) when the ground electrode was in dissecting solution. The inorganic cation composition of this medium is very similar to that measured in cecropia blood and, in fact, measurements of intracellular potentials relative to samples of blood from the animals that had yielded the ovarian follicles gave very similar results.

That the nurse cell potential averages 10–11 mV negative to the oocyte was confirmed by inserting a recording electrode in one cell and a ground electrode in the other. In 113 cases an average difference of 10.0 mV was obtained with the nurse cells invariably negative to the oocyte. Reversing the roles of the electrodes after implantation confirmed this result.

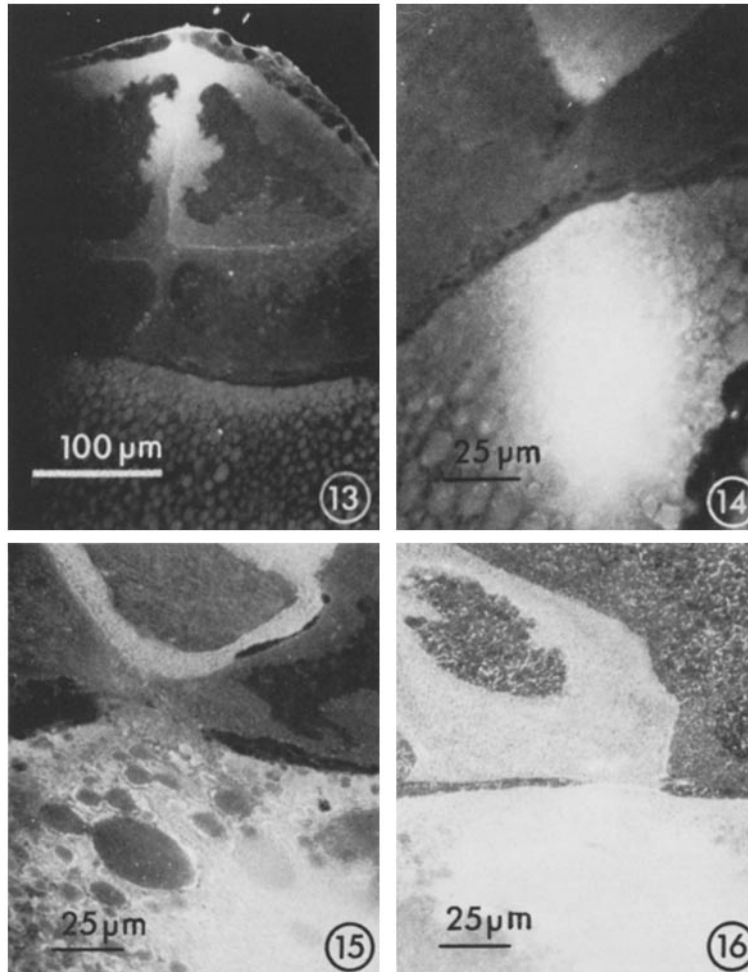
Moving one of the electrodes across the length of a nurse cell or oocyte did not detectably affect the 10 mV intercellular potential difference until the moving electrode had passed out of the cell. This lack of deviation suggests that the potential gradient between the two cells is concentrated primarily in the cytoplasmic bridge complex.

Electrical potential differences were twice monitored for as long as 1 h, during which time they showed less than a 50% decay. The potential difference was regularly monitored for 10-min periods and no decay was detected during this period.

Tests of the Theory That a Potential Gradient Polarizes FSG Movements

To test the possibility that polarized movement of FSG in the intercellular bridges results from a potential gradient, several attempts were made to abolish or reverse the 10 mV difference between the oocyte and nurse cells. Two methods, chilling and 2,4-dinitrophenol (DNP) inhibition, presumed that the potential gradient is supported by metabolic energy. As a third experimental approach, the potential gradient was reversed by an externally imposed current. Finally, a naturally occurring reversal in overaged animals with aborting follicles is described.

EFFECT OF LOW TEMPERATURE: Follicles were placed in dissecting solution in a water bath maintained at 5.5°C . 10 prechilled follicles were injected through their nurse cells with FSG; an additional 10 follicles were injected directly into the oocytes. All 20 remained for 2 h at 5.5°C and were then frozen in preparation for fluorescence microscopy.



FIGURES 13-16 Fluorescence micrographs of follicles in which nurse cells had been injected with FSG. Fig. 13: 30 s incubation. The needle had entered the follicle at top center and had passed into two nurse cells. $\times 170$. Fig. 14: 3 min incubation. The intercellular bridge connecting the fluorescent nurse cell, top center, with the oocyte at the bottom of the figure, is out of section. $\times 360$. Fig. 15: 20 min incubation. $\times 360$. Fig. 16: 60 min incubation. In no case did an unimpaled nurse cell become fluorescent. $\times 360$.

In all but two cases, both of which were oocyte-injected follicles, fluorescence was found at the site of injection. In no case had the fluorescence spread beyond this localized region. Due to an increase in either the viscosity or the absorptive ability of the cytoplasm, the FSG failed to reach the intercellular bridges within 2 h. A further indication of the increased viscosity of the cytoplasm was that in 80% of the injected nurse cells, the nucleus rather than the cytoplasm was the apparent site of injection. This result was obtained in less than 15% of the nurse cells injected at room temperature, and this suggests that the nucleus is

able to roll out of the path of the needle at the higher temperature. Whatever the cause, the failure of FSG to spread beyond the site of injection at low temperatures made this experimental approach useless for the analysis of bridge polarity.

EFFECT OF 2,4-DNP: Another approach was made to determine whether the polarity of FSG movement through the bridges was dependent on metabolic energy, this time using 2,4-DNP. In preliminary experiments, 5×10^{-4} M was found to be the lowest concentration of DNP affecting FSG localizations. While the structure of

TABLE I
*Intracellular Electrical Potentials of Nurse Cells
 and Oocytes in Vitellogenic Cecropia Follicles*

Electrode positions*		Number of obser- vations	Mean potential difference
Recording	Ground		
			<i>mV</i> ± <i>SE</i>
Nurse cell	Medium	73	-39.5 ± 1.3
Oocyte	Medium	78	-29.0 ± 1.1
Nurse cell or Oocyte	Oocyte or Nurse cell	113	-10. ± 0.6

* Electrical circuits were as shown in Fig. 2.

the follicle appeared normal for up to 60 min in this concentration of DNP, longer incubations resulted in fragility and disintegration during subsequent handling. Despite this disadvantage, electrical measurements indicate that the effects of DNP on the cell potential were reversible for over 45 min.

The effect of 5×10^{-4} M DNP in dissecting solution on cell potentials was tested in two ways. Both involved placing the follicle in a chamber to which two 10-ml syringes were attached by flexible polyvinyl chloride tubing. Using these it was possible to exchange one incubation medium for another medium, and then change back again.

The effect of DNP on the nurse cell potential was measured against an extrafollicular ground in six follicles. In all cases the 40 mV potential differences between the nurse cell and the surrounding medium began to fall within 1 min of DNP addition, dropping to 8 (± 1) mV, nurse cell negative, within 10 min. After replacement of the DNP solution with dissecting solution, the nurse cells recovered their normal potential within 10 min.

In a second set of observations, the effect of DNP on the potential difference between nurse cell and oocyte was tested by inserting a measuring electrode into one cell and a ground electrode into the other. After monitoring the follicle for 10 min to determine that the potential was steady, the incubation medium was replaced with dissecting solution containing 5×10^{-4} M DNP. The potential difference between nurse cell and oocyte began to diminish within 1 min (Fig. 20). Within 4-5 min the potential difference became steady. The inhibited value varied from follicle

to follicle from 1 to 4 mV. Dissecting solution was then reintroduced. Within 2 min, the potential difference began to return to normal and within 7-8 min the cells had recovered their original 10 mV potential difference. When DNP was again added, the change in potential difference occurred more rapidly than during the first exposure, presumably due to the cell having already depleted its ATP reserve.

Since the potential difference was significantly reduced by DNP, it was plausible to test whether the barrier to FSG movement from the oocyte to the nurse cell was also reduced. For this purpose, follicles were injected through a single nurse cell, and incubated in cecropia saline for 10 min. Sufficient DNP was then added to bring the concentration to 5×10^{-4} M, and the follicle was incubated for an additional period of time in this medium.

The follicle seen in Fig. 17 had been incubated in DNP for 10 min. Two intercellular bridges may be seen. One connects the injected nurse cell with the oocyte, both of which appear fluorescent. The second connects the oocyte to an uninjected nurse cell, and the FSG appears to have moved into this bridge. The distribution seen in Fig. 17 was observed in all of the 10 follicles that were incubated in DNP for 10 min after nurse cell injection, and was never seen in a control follicle. Whether the configuration resulted from the commencement of FSG diffusion into the bridge or from a movement en masse of oocyte cytoplasm could not be determined.

Longer incubations in DNP showed migration of FSG further into uninjected nurse cells, with small amounts of fluorescence distributed throughout an occasional nurse cell. Due to the decay of fluorescence during illumination, the level reached could not be recorded photographically. 10 follicles were incubated for 40 min and 6 for 60 min in DNP. All showed low levels of fluorescence in at least two of the uninjected nurse cells. The level of FSG varied from cell to cell in the same follicle, with some nurse cells in every case lacking detectable fluorescence. In no case was a level of fluorescence obtained comparable with that achieved by the oocyte after nurse cell injection. The results thus paralleled the DNP effects on the intercellular potential difference in that polarity seemed to have been reduced, but not abolished. If the potential difference contributes to the polarity of the intercellular bridges, the inhibited

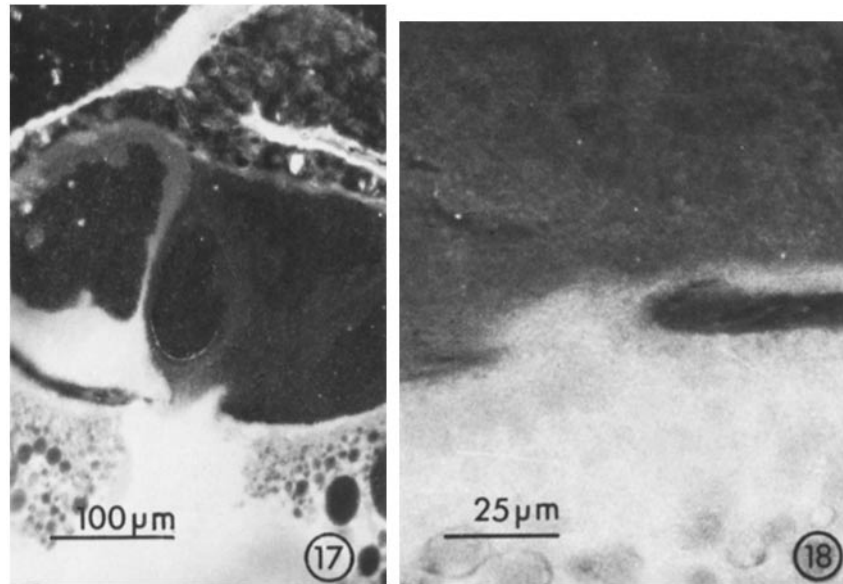


FIGURE 17 Fluorescence micrograph of follicle incubated 10 min in cecropia saline after FSG injection, and then in 5×10^{-4} DNP for 10 min. The injected nurse cell is on the left. Note the fluorescence leading into a nurse cell on the right. $\times 165$.

FIGURE 18 Effect of an experimentally reversed potential gradient on an FSG-injected follicle. After the oocyte had been injected, a current of 5×10^{-8} A was applied for 10 min in the manner shown in Fig. 4. Fluorescence, normally confined to the oocyte below, had crossed an intercellular bridge and entered the nurse cell containing a ground electrode. The configuration of this bridge and nurse cell is the same as that shown on the right side of Fig. 7. $\times 600$.

levels, 1–5 mV, were still enough to block gross movements of FSG from the oocytes to the nurse cells, though not as effectively as the usual 10 mV difference.

EFFECTS OF ELECTRICALLY REVERSED POLARITY: For a more direct test of the role of cell potentials in controlling the movement of FSG, an imposed current was used to reverse the normal polarity of the follicle. Three micromanipulators were required for this experiment (Fig. 3), one to carry the injection needle, and the other two to carry the KCl electrodes. One electrode was positioned in the oocyte; the second was inserted in a nurse cell.

Once all needles and electrodes were in place, the standard volume of FSG was injected into the oocyte. The injection needle was left in place during the incubation period to prevent leakage. A current of 5×10^{-8} A was then passed in such direction as to reverse the normal polarity for 10–30 min.

After both 10 and 30 min of reversed polarity, the fluorescent material was seen in a 20–30 μ m wide band crossing the bridge complex to a single

nurse cell. (Fig. 18) with the longer incubation the band usually spread out somewhat at the periphery of the nurse cell where the electrode had been located. In no case did fluorescence become detectable in more than one nurse cell. The phenomenon was demonstrated in 12 follicles exposed to the reversed current for 10–30 min.

NATURALLY OCCURRING ALTERED POLARITY: In addition to the experimental modification of intercellular bridge polarity, both the potential difference and FSG movement were found in a small number of cases to be naturally reversed. The cases observed all occurred in over-aged animals. The pupae used in these experiments were formed by caterpillars raised in the field during the summer months and stored at 6°C for the rest of the year. The adult molt and associated ovarian development ensue within a few days after the animal has been returned to 25°C. If the pupae are stored for longer than 12 mo at 6°C, ovarian development tends to be abnormal. Productivity declines, and many follicles are resorbed after the initiation of yolk formation.

The cell potentials of many of the unresorbed

follicles in such animals were erratic. Some follicles had a reversed polarity, the oocyte being negative to the nurse cells. Other follicles had no intercellular potential difference, though the cytoplasm was

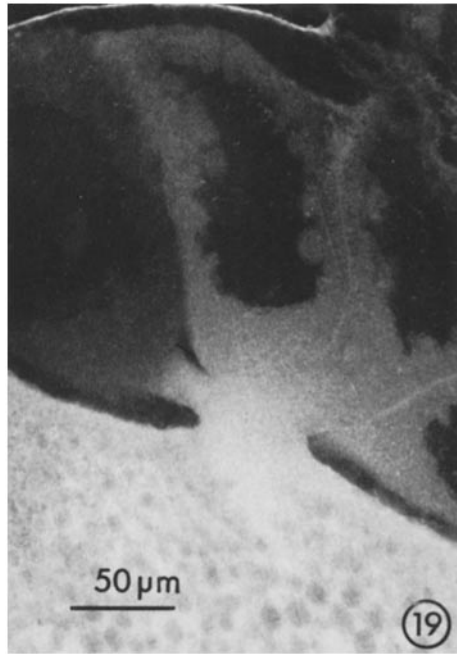


FIGURE 19 Naturally occurring reduction in the barrier to FSG migration in a follicle from an overaged animal. This follicle was injected through its oocyte and incubated in dissecting solution for 1 h. Fluorescence can be seen throughout the oocyte and in all but one of the nurse cells. $\times 260$.

10–20 mV negative to the medium. One animal had follicles with nurse cell to oocyte potential differences as high as 45 mV in the normal direction.

Overaged animals also showed an increase in the ability of FSG to move from the oocyte to the nurse cells. In Fig. 19 a follicle is shown which was frozen 1 h after FSG injection into the oocyte. Only one of the nurse cells failed to show fluorescence. Other follicles from the same animal also showed some fluorescence in their nurse cells. The morphology of these follicles, including the texture and distribution of the yolk, was normal, so that reduced polarity of the bridges, if related to resorption, is an early step in the process. The fact that variation in the electrical and permeability characteristics of the bridges were both most extreme in overaged animals is an additional correlation suggesting that FSG mobility in the bridges is related to the normal 10 mV potential gradient.

DISCUSSION

Polarized bridges are distinguished in morphological terms by the fact that one member of a cell cluster grows to a substantially greater volume than the others. In molecular terms, polarity is characterized by the accumulation in the oocyte of materials known to be synthesized in the nurse cells. Until the observations reported here on FSG movement, however, it was not necessary to postulate any mechanism of movement within the bridge complex other than free diffusion. The movement of RNA toward the oocyte, for instance, could be

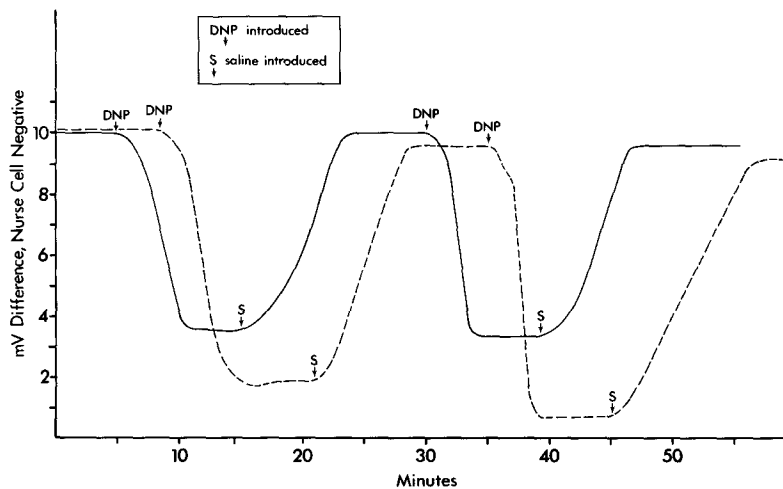


FIGURE 20 The effect of 2,4-DNP on the nurse cell-oocyte potential difference in two follicles. Cecropia saline and saline containing 2,4-DNP alternately bathed the follicles while electrodes in a nurse cell and in the oocyte monitored electric potential changes.

explained by the fact that nurse cells synthesize this material in vastly greater quantities than the oocyte, and a concentration gradient favoring diffusion toward the oocyte necessarily results. Glycogen, by contrast, is known to be synthesized primarily in the oocyte during vitellogenesis (Engels, 1971), and it does not accumulate in appreciable amounts in the nurse cells. This would be explained if it turned out, for instance, that some materials were fixed in place in the oocyte in such a way that they never reach the intercellular bridge complex.

These two factors, origin in the nurse cells and adsorption in the oocyte, are undoubtedly components of the overall tendency toward one-way movement of endogenous materials across the bridge complex. They do not explain, however, the inability of microinjected FSG to cross the bridges from the oocyte to the nurse cells or from nurse cell to nurse cell. For this reason it is necessary to postulate a polarizing force that operates within the bridge complex itself.

In several orders of insects the nurse cells are retained in a syncytial chamber at the apex of the ovary. A "trophic cord," which connects each oocyte to the syncytium, becomes greatly elongated (e.g., a millimeter or more) as the developing follicle is gradually displaced toward the base of the ovary. That the trophic cord is a modified intercellular bridge seems highly probable from recent analyses of its development (Büning, 1972; Huebner and Anderson, 1972). By studying the effects of vinblastine sulfate, Huebner and Anderson (1970) showed that microtubules are crucial for the morphological organization of the cord. They further proposed that microtubules may play a direct role in the transport of materials toward the oocyte.

In insects such as *cecropia*, which combine the oocyte and nurse cells within the same follicle, microtubules are not conspicuous in the intercellular bridges during vitellogenesis. A cluster of microtubules, presumably a remnant of the mitotic spindle, can be seen in newly formed bridges in *Drosophila* (Koch and King, 1969; Mahowald, 1971), but it disappears during subsequent development. The lack of microtubules has also been shown in *Dytiscus* (Steinert and Urbani, 1969) and *Nasonia* (King and Richards, 1969), and it appears, from Fig. 8, to be true of *cecropia* during the stages analyzed here. We therefore suggest that polarized FSG movement in the vitellogenic

cecropia follicle occurs by mechanisms other than that suggested for the trophic cord, and that an electrical potential gradient merits consideration as a component of the phenomenon.

Though the difference in electrical potential between the nurse cells and the oocyte of vitellogenic *cecropia* follicles (Table I) is routinely observed in this laboratory, its physiological basis remains unclear. A charged gel, and hence a fixed charge in either cell would result in such a difference. It could also be generated or enhanced by an ion pump, provided that the cytoplasm in the bridges between the two cells had a high enough resistance to reduce the current flow to a physiologically supportable level. Whatever the cause of the potential difference, it would certainly affect the distribution of any charged molecules or organelles newly introduced into the cytoplasm and sufficiently mobile to reach the intercellular bridge complex.

It must be emphasized that the electrophoretic explanation of polarized movement in the bridge complex is an interpretation and not a conclusion. Thus, an electrophoretic theory requires that the ionic charge carried by the mobile FSG component be negative at the pH of the cytoplasm in the bridge complex, but this in fact is not known. Efforts to confirm the theory by injecting positively charged dyes have thus far been thwarted by the adsorptive qualities of the cytoplasm. As in the case of FSG at low temperatures, the several dyes tried did not spread through the oocyte beyond the site of injection and thus failed to reach the bridge complex.

A further reservation is the possibility that the conditions leading to antipodal migration of FSG, in addition to reducing or reversing the potential gradient as shown, could also have impaired other mechanisms, more directly responsible for polarized FSG movement. The effects of reversed current of FSG distribution represent the most powerful argument in favor of the electrophoretic theory, especially since ooplasmic FSG invaded only one nurse cell, in most cases identifiable as the one that had contained the ground electrode. We can argue from this result that the potential gradient between the cells, if not actually the driving force, is necessary for whatever cytoplasmic organization is in fact responsible for bridge polarity. This is, so far as we know, the first physiological analysis of an intercellular bridge in animal germ cells, and the deficit of information needed to satisfy these reservations is substantial.

With regard to other systems, electrical potential gradients between different regions of cytoplasm within single cells have been reported in melanophores (Kinosita, 1963) and in amoebas (Bruce and Christiansen, 1965), and they have been inferred from the external currents generated by fertilized *Fucus* eggs (Jaffe, 1969).

Kinosita (1963) found that both centripetal and centrifugal migration of melanin granules is accompanied by changes in the potential difference between the arms and the central body of the melanophore. The sign and magnitude of the changes were appropriate for an electrophoretically motivated migration of the granules. Jaffe (1969) proposed that electrical gradients, generated by an asymmetric membrane depolarization, are crucial for the differentiation of the rhizoid tip in the *Fucus* eggs. According to his analysis, a potential gradient would result which was capable of influencing the distribution of ionic materials in a manner affecting polarized growth of the system. The intercellular bridges of vitellogenic insect ovaries, by virtue of their size and polarity, should provide new opportunities to explore the possibility that electrical potential gradients are among the mechanisms used by developing cells to generate regional differences in their cytoplasm.

This work was supported by National Science Foundation grant GB-8568. We also acknowledge with pleasure the assistance of the electronics shop of the Institute of Neurological Sciences, supported by the National Institutes of Health grant T01-GM-01994.

Received for publication 29 December 1972, and in revised form 27 March 1973.

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