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Studies on the Formation of a Permeable Cell Membrane Junction

I. Coupling under Various Conditions of Membrane Contact. Effects of Colchicine, Cytochalasin B, Dinitrophenol

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Summary. Individual cells (macroblastomeres) of newt embryo were brought into contact, and electrical coupling was monitored during the formation of permeable membrane junction. In one set of experiments, the cells were allowed to establish contact at random membrane spots by spontaneously moving cell processes. Coupling became detectable 8–14 min after contact. In another set, contact was imposed, by micromanipulation, at membrane spots of known junctional history. The basic experiment was (i) to make a junction (conditioning junction) at randomly chosen membrane spots, (ii) to pull the cells apart interrupting their electrical coupling (uncoupling), and (iii) to make a new junction (test junction) either at the same spots that contained the conditioning junction or at different ones. The times required for coupling onset at test junctions fell into two classes, depending on whether in the uncoupling step the membrane continuity between the two cells had been broken or preserved. When all membrane continuity had been broken, coupling through the test junctions became detectable within 4–20 min after membrane contact. This was so when the spots of membrane contact contained conditioning junction as well as when they did not. When membrane continuity (but not coupling) had been preserved in the form of submicroscopic strands, coupling through the test junction set in within 1 sec of joining the cells at spots containing conditioning junction. This capacity for rapid coupling persisted for roughly 10 min following the uncoupling step; thereafter the time of coupling onset was of the class with broken membrane continuity. During development of junction, the coupling coefficients rose gradually over 10–30 min from the detectable level (0.03 or 0.05) to a plateau (0.3–0.9). The cells were capable of developing and of maintaining coupling throughout their entire 100-min division cycle. Treatments with colchicine (0.2–1.1 mM) and with cytochalasin B (0.5–1 μM), blocking cytokinesis and division, did not prevent the development or maintenance of coupling. Treatment with dinitrophenol (1 mM) prevented the development of coupling, but not that of cell adhesion, and (3 mM) blocked reversibly the coupling in established junction.

The surface membranes of a wide variety of cells have the capacity of making the kind of junction that is permeable in the cell-to-cell direction (Loewenstein, 1966, 1968; Furshpan & Potter, 1968). In several cell types, this capacity seems to be a diffuse, rather than a local, property of the cell membrane: When pairs of individual cells of the sponges *Microciona* or *Halyclona* or of the embryo of the newt *Triturus* are micromanipulated into contact at randomly chosen spots on their membranes, permeable junctions form wherever the contact happens to be (Loewenstein, 1967*a*; Ito & Loewenstein, 1969). Moreover, when the newly formed junctions between the *Triturus* cells are broken by micromanipulation and the cells are again manipulated into contact, at spots different from the previous ones, a new permeable junctions forms there (Ito & Loewenstein, 1969). Thus, a large part, perhaps all, of the surface membrane of these cells seems capable of junction formation.

In the present paper, we examine the formation of permeable junction more closely. We study the development of electrical coupling (a) across membrane contacts established spontaneously by motile cell processes and (b) across membrane contacts imposed by manipulation. Specifically, in the latter, we compare the time course of the development in membrane regions that had been in previous coupling contact and the time course in regions that had not.

Furthermore, with the thought in mind that microtubules or microfilaments might play a role in the formation of permeable membrane junction, we study the effects of colchicine and cytochalasin B, known inhibitors of microtubule and microfilament assembly.

Materials and Methods

Cell Material

Macroblastomere cells were isolated from *Triturus pyrrhogaster* embryos of the late morula stage (stage 8 of Okada & Ichikawa, 1947) and placed in medium containing (in mM) 81.2 NaCl, 0.9 KCl, 1.2 $CaCl_2$, 0.36 $NaHCO_3$. The isolated cells continued dividing and, in successful experiments, they did so even while impaled on the microelectrodes. The division cycle was 100 min under the experimental conditions (Fig. 1). The incipient cleavage furrow, easily seen, served as a convenient reference to stage the cells. Single cells were generally obtained 80–90 min after furrow formation. At this stage, the daughter cells could be readily separated. The diameter of the cells freshly isolated from the embryo ranged from 0.22 to 0.6 mm; that of the daughter cells, 0.3–0.48 mm. For experimental assemblage of cell junctions, we selected cell pairs of about equal size and stage. We used isolated macroblastomeres or their daughter cells in all experiments, except in those dealing with the effects of cytochalasin B in which we used, in addition, small blastomeres (0.15–0.18 mm diameter) from the outer (black pigmented) cell layer of the embryo.

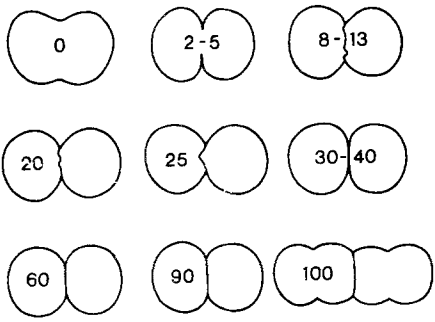


Fig. 1. Division cycle of a macroblastomere. The cells are diagrammed as seen in the dissecting microscope, their animal pole-vegetal pole axis coinciding with the optical axis, except for stage 0 in which the axes are at 90°. The numbers of each diagram are the times (min) in the division cycle; time 0 is start of furrow formation. The wiggly lines at stage 8–25 min represent cell surface wrinkling during the early phases of cell constriction. The cells "round-up" at stage 80–90 min (karyokinesis?) and "relax" at 90–100 min. They could be separated without damage after stage 40 min

Coupling Measurements

Electrical measurements of coupling were taken continuously during junction formation and breakage. The cells were on the bottom of the dish which was covered with yolk and contained the medium. The techniques for measurement of coupling (and of cell isolation) are described elsewhere (Ito & Loewenstein, 1969). The general arrangement of intracellular microelectrodes is diagrammed in Fig. 2. Except for the experiments shown in Figs. 12 and 16, the electrodes were located in the dividing cell halves that were in experimental contact so that coupling measurements could be taken directly across the contact plane. Experiments in which more than one contact occurred between the cells were discarded. Membrane current (i) and voltages (V_1, V_2) were displayed at

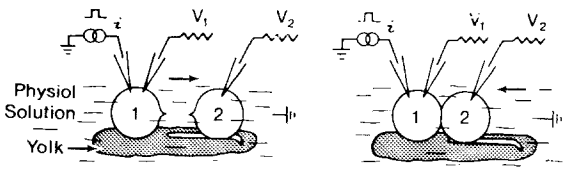


Fig. 2. Diagram of set-up and electrode arrangement. The cells (*1* and *2*) were placed on the yolk-covered bottom of a glass dish filled with physiological solution, and impaled on microelectrodes. One electrode (i) served to inject pulses of current into cell *1* and two other electrodes, to measure the resulting changes in membrane potential (V). For experimental cell contact or separation, the cells were micromanipulated with the aid of the electrodes. The drawings illustrate this for an experiment Type 2 of cell separation *(left)* and reunion *(right)*, where the micromanipulator and a groove in the yolk made by the sliding cell *2* during separation, provided the constraints for rejoining the cells at the membrane regions of former contact. In experiments Type 1, the separated cells were first rotated to the desired angle and then joined

slow speed (3 or 12.5 mm/min) on a multichannel pen recorder system and, simultaneously, for precise determination of coupling onset, at fast speed (10 cm/sec) on a four-trace oscilloscope. Coupling was detectable on the oscilloscope records down to a V_2/V_1 ratio of 0.05; in a few cases, to 0.03. The oscilloscope records were necessary, because in some experiments, the current pulses produced a small resistance coupled artifact in the V_2-channel which masked the low-level membrane-V_2 during the early phase of development of coupling on the pen records. These artifacts were readily distinguished from true membrane-V_2 on the faster and more amplified oscilloscope records. (Samples of oscilloscope records are in Fig. 6 of the second paper of this series.) The marks *c*, denoting coupling onset on the figures of the pen records, were positioned according to the oscilloscope records.

Junction Formation and Breakage

Experimental junction formation was produced by (*a*) micromanipulating a pair of single cells together or (*b*) by placing the cells about 10–20 μ apart and allowing them to make contact by their own movement. In the first method, the two cells were pushed into contact with the aid of the microelectrodes, sometimes while being impaled on them. In the second method, the contact was made by motile processes protruding from the cell surfaces. Such processes formed spontaneously and moved in circles over the cells

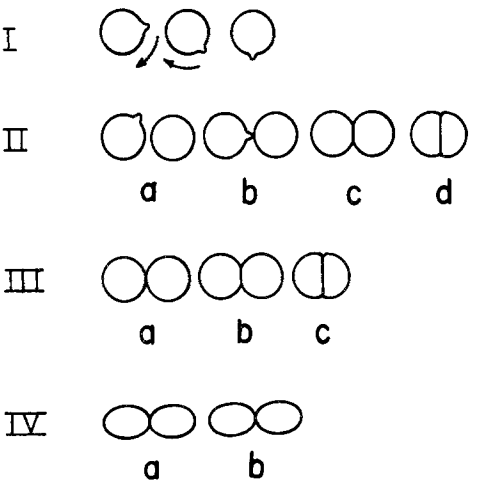


Fig. 3. Diagrammatic representations of: (*I*) Spontaneous circus movement of a cell protuberance. (*II*) The sequence in an experiment on junction formation by spontaneous contact: *a*, initial experimental cell positions: cells are positioned 10 to 20 μ apart by micromanipulation; *b*, spontaneous adhesive contact by motile protuberance. The contact area increases thereafter progressively by spontaneous cell movement; *c*, contact situations, as seen in dissecting microscope, 1–4 min after initial contact; and *d*, after 30–60 min. The electrical measurements in Fig. 5 are from situations *b* to *c*. (*III*) Junction by imposed contact: *a*, initial micromanipulated cell contact; *b* and *c*, the apparent contact area broadens spontaneously. (*IV*) Junction by imposed contact in cells treated with colchicine or cytochalasin B: *a*, initial micromanipulated contact; *b*, the contact area does not change noticeably with time in these "relaxed" cells. All three kinds of contact are adhesive

(Sirakami, 1963), occasionally bridging the gap between the cells. (The movements were more active during the first 10–15 min after isolation of the cells from the embryo.) The cells first adhered at the motile processes and then pulled themselves together increasing their apparent contact area (Fig. 3). In either method of junction formation, coupling measurements were taken only on cells that were in adhesive contact. Adhesion was ascertained after cell impalement by moving the electrodes. *Time zero* for determination of coupling onset was the time of contact as seen in the microscope.

For experimental breaking of cell junctions, the cells were pulled apart by micromanipulation. The cells were then still impaled on the microelectrodes from the preceding coupling measurements and the electrodes were driven apart by micromanipulators. Cell 1, which contained two electrodes, was generally fixed and cell 2 was moved (Fig. 2). To break the junctions, the cells were moved several millimeters apart, except in experiments Type 2. In experiments Type 2, cell separation was limited to 100–300 μ so as to allow rejoining of the cells in a subsequent step, at the same regions in former junctional contact (*see* Results). Wobble was minimized in these experiments by the use of a sliding track: in sliding over the yolk during their separation, the cells indented it slightly; the indentation served as track in the subsequent cell reunion.

Fig. 4 shows photographic examples of cell junctions made by methods *a* and *b* and of the breaking of a junction.

Colchicine, Cytochalasin B and Dinitrophenol

In the experiments on the effect of colchicine and cytochalasin B, the cells were generally carried into contact with the aid of a loop of human hair fixed on a micromanipulated glass capillary. This method of bringing the cells into contact was preferred here to the general method described above, because, after the treatment with colchicine and particularly with cytochalasin B, the cells became very sticky and could not be easily moved without damage over the bottom of the dish. Once the cells were brought together, they were impaled with the microelectrodes for continuous electrical measurement. In a few experiments with colchicine and cytochalasin B, cell contact was made as in the general method. The colchicine (Merck, mol wt 459.14) and the 2,4-dinitrophenol were added to the medium, and the cytochalasin B (Imperial Chemical Industries, mol wt 4800) was dissolved in 1 mg/ml dimethyl sulfoxide, before adding it to the medium. In the case of dinitrophenol, the pH of the medium was adjusted to 7.3–7.6 with Tris-HCl.

All experiments were done at room temperature ranging from 22 to 24 °C.

Results

We performed two classes of experiments on junction formation. In one class, we let the cells make adhesive contact by spontaneous movement of their surface. In another, the adhesive contact was imposed by micromanipulation.

Junction Formation by Spontaneous Contact

Fig. 5*A* illustrates a typical experiment of the first class. Two cells were placed 10–20 μ from each other. They established contact spontaneously by means of a motile protuberance, such as photographed in Fig. 4*a*. As soon

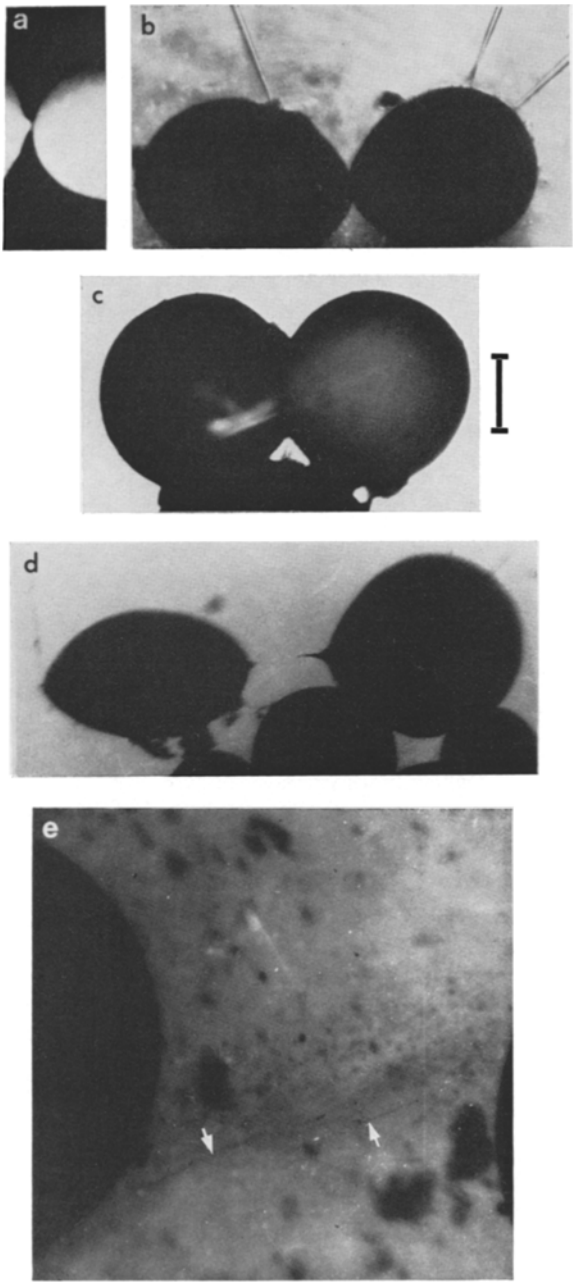


Fig. 4. Photomicrographs: (*a*) Two isolated macroblastomere cells make spontaneous contact. The surface of one of the cells is seen puckered up at the region of contact. The puckering region is a spontaneously moving protuberance of the kind diagrammed in Fig. 3. The cells had originally been placed so as to leave a gap between them, which was closed by the protuberance. (*b*) Two such cells, after firm adhesion. Three intracellular microelectrodes, used for continuous measurement of coupling, are seen. (*c*) Cells in a situation of imposed contact. The two cells were pushed together by micro-

as the adhesiveness of the cell contact was ascertained, the voltage-recording electrodes were inserted into the two cells (the current-delivering electrode was already inside cell *1* and served to test adhesiveness; *see* Materials and Methods), and electrical coupling was monitored continuously. Coupling became detectable 17 min after cell contact and developed over about 40 min to a plateau: the current (i) injected into cell 1, which initially produced no detectable voltage (V) in cell *2*, produced a voltage in this cell, which rose progressively, as an increasing fraction of the test current entered cell *2* via the junction (*see* paper II of this series); the coupling ratio V_2/V_1 rose progressively over the next 20 min from near 0 to 0.8 where it remained constant over the following 10 min of observation ("plateau").

Experiments of this kind were performed on 10 cell pairs in which the contact occurred at different times in the cell division cycle (e.g., Fig. 5*B*). Coupling developed in seven of the cell pairs. The average time of onset of detectable coupling was 12 ± 2.9 (SD) min. "Plateau" coupling was reached in 10–30 min[1].

In three pairs, no detectable coupling developed over 20–30 min of observation. These pairs apparently also did not adhere stably. They came easily apart upon manipulation, without showing the fine ("*A*") strands which were seen during the initial phase of separation of all coupling cells (Fig. 4*d*).

The cells were dividing during the measurements in the experiments of this and the following sections, except for the experiments dealing with the effects of colchicine and cytochalasin B. As is typical for dividing cells, the cell input resistances (V_1/i) and the coupling ratios V_2/V_1 fluctuated in the course of the measurements. The fluctuations are probably due to changes in cell membrane resistivity and in cell membrane area during cytokinesis, and changes in the geometry of current flow (Ito & Loewenstein, 1969);

manipulation. (*d*) The breaking of a junction. Two formerly coupled cells are shown here during the final stage of separation. The cells were slowly driven apart by micromanipulation. The last connection between them, a fine "*A*"-strand, is seen breaking. (*e*) The breaking of a junction in preparation for an experiment *Type 2a*. Here all "*A*"-strands were broken; the remaining "*B*"-elements are diffusely visible *(arrows)* (*see* diagram, Fig. 8). Further cell separation (not photographed) breaks the "*B*"-elements. Calibration line ~ 160 μ for *a*; ~ 100 μ for *b*, *c* and *d*; and ~ 22 μ for *e*

1 We rarely continued our measurements in the "plateau" for longer than 20 min; coupling may conceivably have improved further at a slower rate. The term "plateau" used here and in the following sections merely refers to the phase of coupling development in which the V_2/V_1 coefficient became sensibly constant over 10–20 min of observation.

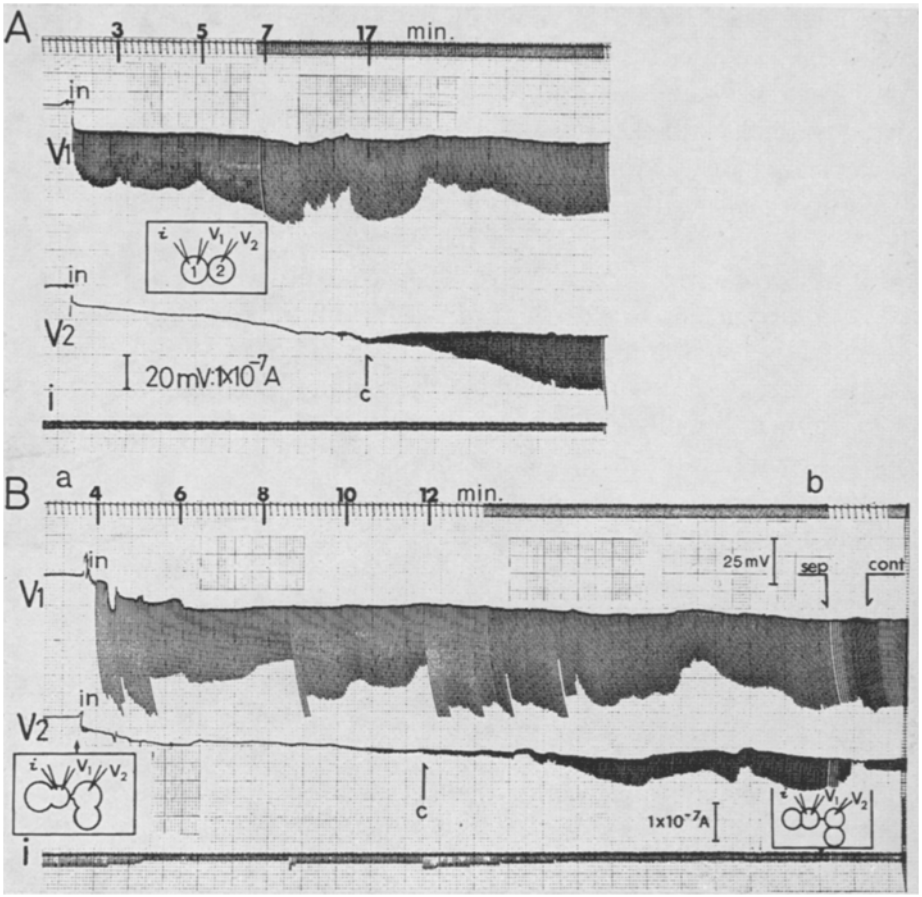


Fig. 5. Coupling by spontaneous cell contact. (*A*) Two cells, isolated from the embryo at stage 40 min in their division cycle, were positioned near each other. They established visible contact spontaneously by means of a small motile protuberance at stage 44 min in their division cycle (*see* diagram Fig. 3*b*, *c*). The voltage (*V*)-recording electrodes were inserted into the cells 2 min after contact [the current (*i*)-delivering electrode was inside cell 1 earlier] and the development of coupling was monitored continuously. The large fluctuations in V_1 occurred in the course of microelectrode manipulation. *Lower trace:* test current, 2×10^{-8} A; rectangular pulse duration 300 msec; frequency, 0.4/sec in the fast records and 0.08/sec in the slow records. *In this and the following figures: time* 0 *is the time of cell contact as seen in the microscope; the frequency of the time marker in the upper trace is 0.1/sec (note change in recording speed); the numbers above the thick time marks give the time after contact in min; downward deflection in the i trace means inward current and in the V traces, cell inside negative; "c" signals onset of coupling as determined on high-speed oscilloscope records (see Materials and Methods), and "in" and "ex" signal entry and exit of voltage-recording electrodes.* (*B*) *a*, Two cells were isolated from the embryo at stage 90 min in their division cycle, and spontaneous contact was established at stage 95 min. At *b* the cells were separated by micromanipulation and then put into contact again after 1 min. The cell diagrams *(insets)* show the electrode location and the cell contact situation at the times marked by the insets' arrows. Frequency of test pulses, 0.33/sec in fast records; 0.1/sec in slow records

when cytokinesis was blocked by colchicine, the cell input resistance was relatively stable (*see* Fig. 17). The fluctuations in input resistance, although cumbersome, did not obscure the results on coupling development; the development of V_2 (or of V_2/V_1) was clear and was the dominant event in the records.

Junction Formation By Imposed Contact

Junction Formation Between Cells not in Previous (Experimental) Contact

Imposed Random Contact. In one series of experiments of this class, pairs of macroblastomeres were pushed together at regions of their surfaces chosen at random. The cells had not been in previous contact for at least 15 min. They were isolated from the embryo at stages 40–80 min before the experiments and were kept separate from each other. (The cell regions of previous contact in the embryo were unknown.) The experiments were, in this and other respects, similar to those of the preceding section, except that here the cell contact was forced by micromanipulation.

The results were similar to those of the experiments on spontaneous cell contact: In one series, the cells developed detectable coupling 11 ± 5.8 (SD) min after establishment of contact (18 cell pairs); and the degree of coupling increased progressively over the subsequent 10–30 min to a "plateau" (Fig. 6). In another series, with material from a different breeding season, coupling set in 21 ± 11 min after contact (41 pairs) and plateau was reached in 12–50 min (Table 1).

One out of the 60 adhesive cell pairs tested did not develop coupling over 106 min of observation. Such a coupling failure may have occurred for several reasons. One possible cause, for instance, may have been excessive Ca influx through a poor membrane seal around the microelectrode in cell *2* (*see* Rose & Loewenstein, 1974); cell *2* had only a 23-mV membrane potential (cell *1*, 41 mV).

Junction Formation Between Cells in Previous Contact

Membrane Contact Conditions. In another series of experiments, cells with a known history of coupling contact were again brought into contact. (The daughter cells of isolated macroblastomeres were used in these experiments.) The general procedure was (a) to establish coupling between a pair of cells by spontaneous or imposed contact *(conditioning junction)*, (b) to pull the cells apart interrupting their electrical coupling *(uncoupling)*, and (c) to force the cells into contact again *(test junction)*. *Step c followed b within 3 min* (except in certain experiments in which the time is specifically stated). Most cells showed no major current leaks during or after the uncoupling. Evidently, after cell separation, the formerly permeable junctional

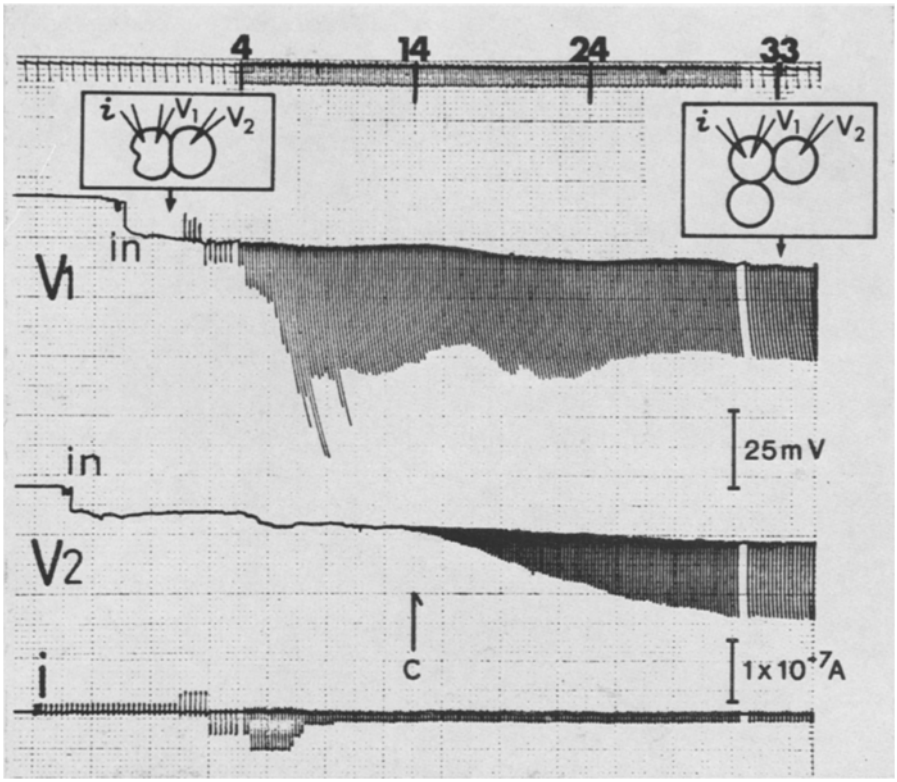


Fig. 6. Coupling by imposed cell contact; contact regions chosen at random. The two cells had been isolated from the embryo 60–70 min before the experiment and kept separate. Contact was established by micromanipulation at stage 80–90 min in the division cycle. Current pulses 1.3×10^{-8} A; 0.23/sec. Current-passing electrode entered cell 1 about 50 sec after entry of voltage-recording electrode

regions of the cells became relatively impermeable and whatever break in cell membrane had occurred sealed in the medium (which contained 10^{-3} M Ca). This resembles the behavior of several other cell systems (Loewenstein, 1967*b*).

Two types of experiments were done that differed in the histories of the regions of test cell contact (Fig. 7):

Type 1) The contacting regions[2] in the test junction were both different from those in the conditioning junction.

Type 2) The contacting regions were both the same as in the conditioning junction.

2 The region of cell contact as seen at 80× magnification in the stereomicroscope. Such regions had apparent lengths of 50 to 70 μ at the time of initial contact.

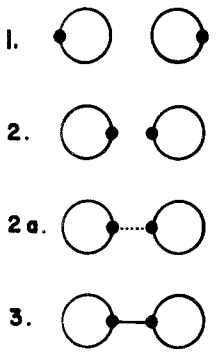


Fig. 7. Scheme of the experiments on imposed contact between cells with a known contact history. The diagram represents the cells after the uncoupling step, in position to be moved into test contact. The black dots represent the membrane regions in contact in the conditioning junction. The numbers denote the experiment type described in the text

All cell membrane continuity was broken in the uncoupling step in the two types of experiments. In experiment Type 1, this was accomplished simply by moving the cells apart over distances of several millimeters. In experiments Type 2, the breaking of membrane continuity was more complex. Here we had to limit the cell separation in the uncoupling step to 100–300 μ, so as to be able to bring together, in the subsequent test junction, the same general membrane regions that were in contact in the conditioning junction (*see* Materials and Methods and Fig. 2). When the cells are moved apart over distances of the latter order, they tend to remain connected by fine strands (*A*) of about 1 μ in diameter that are capable of mediating coupling (Ito & Loewenstein, 1969). The cell separations were always carried beyond the breaking point of such coupling strands. Even so, membrane continuity was often not entirely broken; the cells remained linked by still finer elements (*B*) not mediating detectable electrical coupling (Fig. 8). These elements were not visible in the stereomicroscope. They were discernible in the compound microscope at 600 × magnification, although they were still too fine to give a sharp image (Fig. 4*e*). To sever the *B* elements, a fine micromanipulated needle was passed across the space between the cells. Membrane continuity between the cells was thus as completely broken in the uncoupling step of experiments Type 2 as it was in experiments Type 1.

In a variant of experiment Type 2, membrane continuity was not completely broken:

Type 2a) The contacting regions in the test junction were both the same as in the conditioning junction, but some membrane continuity was preserved

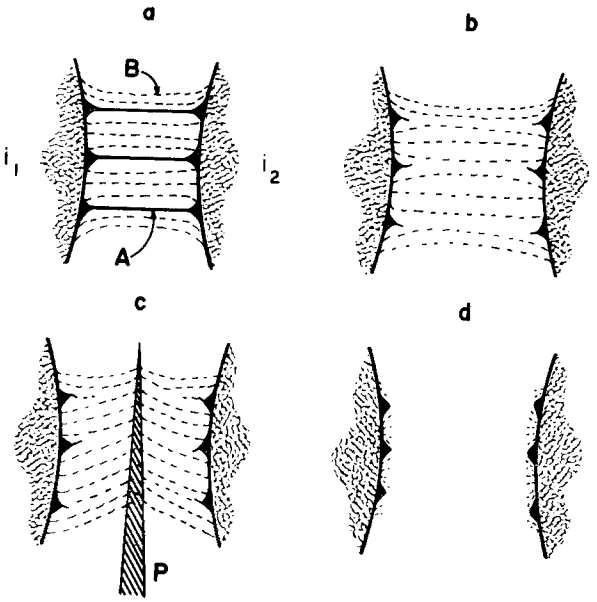


Fig. 8. Diagram of membrane interconnections and their severance during cell separation: (*a*) upon mechanical separation to 50–100 μ, the cells remain connected by *A*- and *B*-elements. The *A*-elements mediate detectable coupling; *B*-elements do not; (*b*) upon cell separation to 100–300 μ, *A*-strands rupture; (*c*) *B*-elements severed with a glass needle (*P*). The diagrams represent the situations before cell reunion in experiment Type 2 (*d*), Type 2a (*b*), and Type 3 (*a*)

after uncoupling. Here cell separation was carried to the point of rupture of all *A*-strands and of complete interruption of electrically detectable coupling during the uncoupling step, but *B*-continuity remained (Fig. 7).

Finally in another type of experiment, the test junction followed partial uncoupling:

Type 3) Test junction was made as in experiment Type 2, but the preceding uncoupling was incomplete. Here the *A*-strands and a degree of coupling (coupling ratio 0.1–0.2) remained after cell separation (50–100 μ).

Results. Experiments Type 1 and 2 gave results similar to those obtained in the experiments on random cell contact described above: coupling became detectable 4–20 min after contact, and increased progressively to a "plateau" (Figs. 9 and 10). The coupling ratios in the "plateau" were on the average 50% below the coupling ratios in the conditioning junction[3]. The highest coupling ratio observed was 0.74, 5% below that of the conditioning junction.

3 Excluded from this tally are the cases in which the conditioning junction was <60 min old.

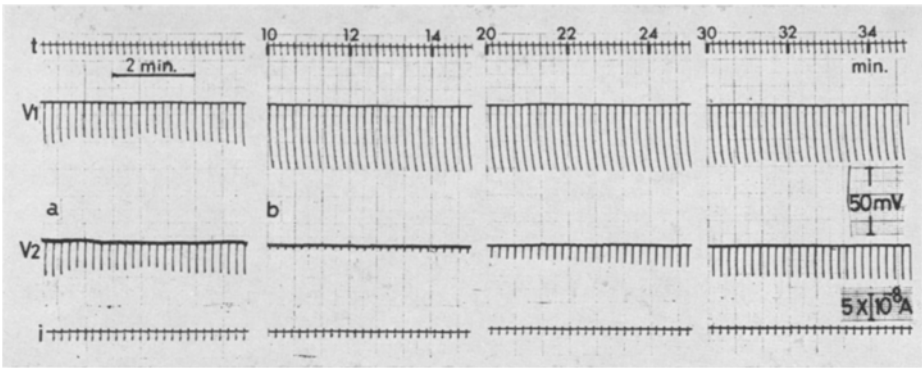


Fig. 9. Coupling by imposed cell contact; *experiment Type 1.* The two cells are daughters of a cell originally isolated from the embryo. At stage 60–70 min, the two daughter cells were pulled apart and kept separate for about 2 min; they were then micromanipulated into contact again (test junction) at regions different, on both cells, from the previous regions of contact. (*a*) The original coupling between the two cells before their separation. (*b*) Development of coupling at the test junction. The record was started when the first V_2 became detectable; breaks in *b* correspond to 5-min interruption of the records. Current pulses 0.09/sec; 1.5×10^{-8} A in *a* and 1.25×10^{-8} A in *b*

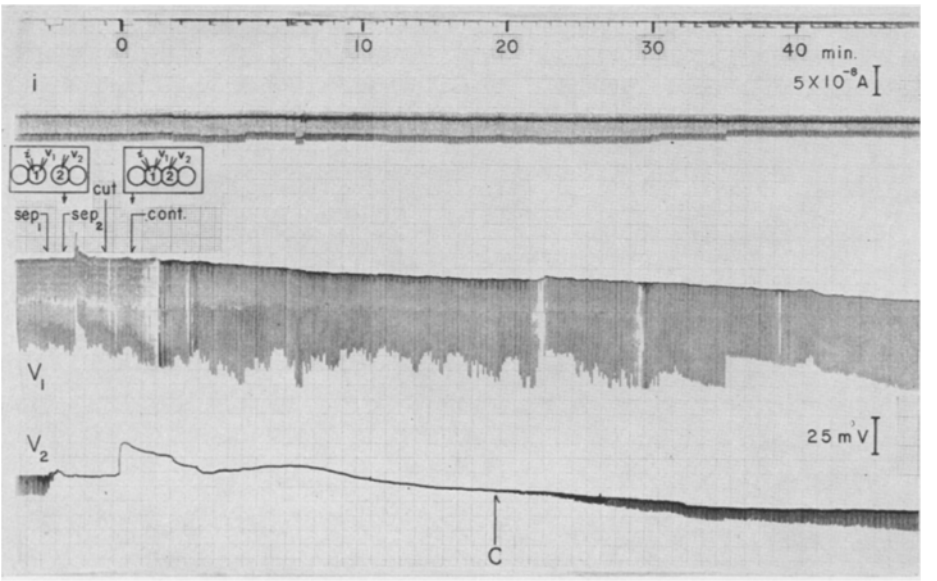


Fig. 10. Coupling by imposed cell contact; *experiment Type 2.* Conditioning junction had previously been established between the two isolated cells. 43 min after the conditioning cell contact the cells were pulled apart in two steps (*sep_1; sep_2*) breaking all "*A*"-connectors. Then the "*B*"-connectors were cut (*cut*) and, 0.33 min thereafter (*time 0*), contact for test junction was made (*cont*) at the former (conditioning) contact regions. Coupling (*C*) in the test junction developed about 19 min after the contact. Current pulse intensity $1.3–3\times10^{-8}$ A; frequency, 0.13/sec after *time 10 min.* (At *time 0–10 min* current pulse frequency and pen recorder speed were higher.) The membrane potentials (at zero current) were about 54 mV at *time 55 min.* The transient depolarization of cell 2 at *time 0* was caused by the movement of the voltage-recording microelectrode during the micromanipulation for cell contact

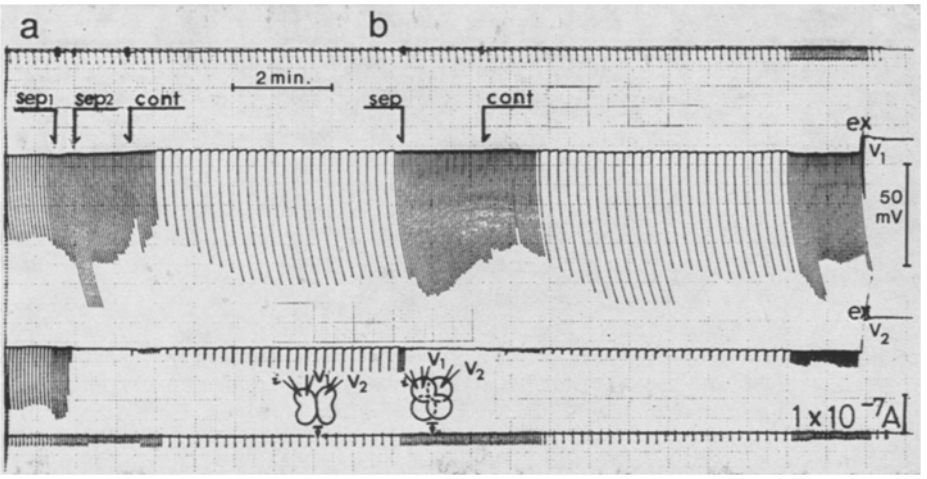


Fig. 11. Coupling by imposed contact; *experiment Type 2a*. The two cells (daughters of a cell isolated from the embryo) had previously been manipulated into coupling contact. (*a*) They were manipulated apart in two steps (sep_1; sep_2) until coupling became undetectable. 70 sec after their uncoupling (at stage 10 min in their division cycle), they were manipulated into contact (*cont*) at the former contact regions. (*b*) Repetition of this experimental sequence at a later stage (17 min) in the division cycle. (After the first separation step in *a*, the cells were still connected by a single "*A*"-strand of $\sim 1\ \mu$ in diameter mediating electrical coupling. This strand and the coupling were broken in the next step, sep_2; "*B*"-continuity remained)

Experiments Type 2a yielded quite different results. Here coupling was often detectable within the order of 1 sec of full cell contact, that is, within the time interval of the test pulses. In one series of experiments, in which the test pulse interval was 3 sec, coupling set in within this time in 13 out of 18 trials. In another series, in which the test pulse interval was 0.75 sec, coupling set in within the latter time in all of five trials. The rise to "plateau" coupling was progressive as in all other types of experiments (Figs. 11; 5*B*, *b*; and 12*b*, *c*).

The capacity for rapid coupling lasted for about 10 min. When test junction was imposed more than 10 min after the uncoupling of the conditioning junction, the times of coupling onset were of the same order as in experiments Type 1 and 2. Fig. 12 shows an example: after a prolonged cell separation (12 min), coupling ensued (*d*), but the coupling onset took 12–16 min, as against < 3 sec in the two preceding couplings in which the separation had been short (*b*, 12-sec separation; *c*, 30-sec separation).

In Fig. 13, the times of coupling onset of the various Type 2a trials (that yielded coupling) are plotted against the times of cell separation (time

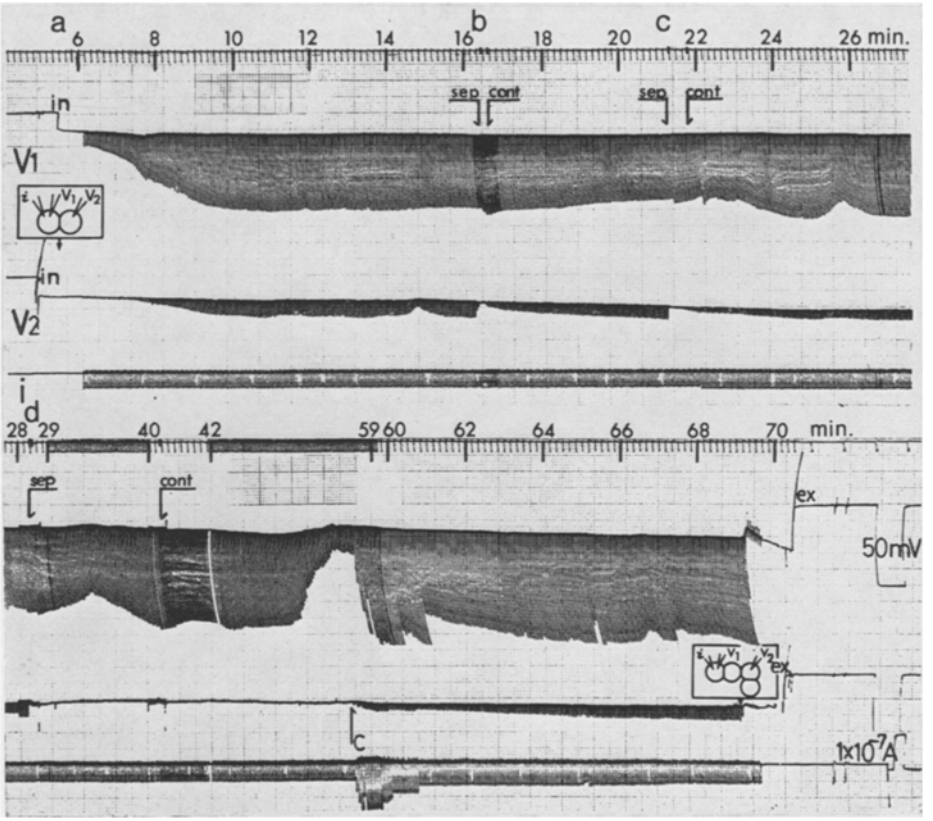


Fig. 12. The capacity for rapid coupling in experiments Type 2a depends on the time the cells were separate after the preceding junction. Two cells were first manipulated into coupling contact at membrane regions of unknown contact history at stage 50 min in the division cycle (*a*); they were then separated *(sep)* and manipulated into contact again *(cont)* in three consecutive Type 2a experiments at the same contact region after separation periods of 12 sec (*b*), 0.5 min (*c*) and 12 min (*d*). Note the marked delay in coupling onset in *d*. Top and bottom records are continuous. Test current pulses 0.33/sec; $5–5.5 \times 10^{-8}$ A, except in *d* where current was adjusted to 1.3×10^{-7} A to compensate for fall in input resistance during formation of cleavage furrow

between uncoupling and test contact). From the scattergram it can be discerned that for >10-min separations, the times of coupling onset tend to be much greater than for <3-min separation; whereas for the former, the times of coupling onset are >7 min in all trials, for the latter these times are <3 sec in 73% of the trials. There are not enough data around and above the 10 min separation time to define the limit of time the rapid coupling capacity is retained; the available data suggest that this limit may lie somewhere in the neighborhood of 10 min.

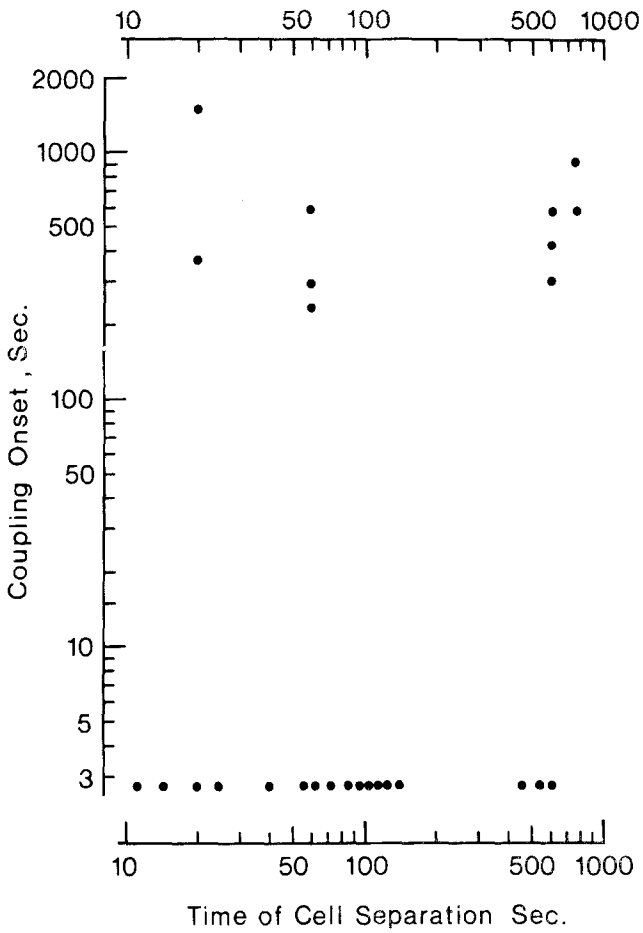


Fig. 13. Time of coupling onset *vs.* the time of cell separation preceding test contact in experiments Type 2a. The resolution of the method for determination of *time of coupling onset* in the various trials was 0.75 or 3 sec. The times of coupling onset <0.75 sec are plotted together with those of <3 sec below the 3-sec-ordinate level

In 27% of the Type 2a trials with <3-min separations, the times of coupling onset were 4–25 min, falling in the range of experiments Type 1 and 2. As discussed further on, we believe that in these cases we exceeded the limits for reversible distension of the *B*-elements; that is, the actual conditions at the time of test contact may have been like those of experiment Type 2 rather than of experiment Type 2a.

In a simple formation of communicating junction, one would expect that the rise of V_2 be accompanied by a fall in V_1, as a progressively increasing fraction of the current injected into cell 1 enters cell 2; and, conversely, upon decoupling, one would expect V_1 to rise. Both changes, in fact, are found in developing sponge cell junctions (Loewenstein,

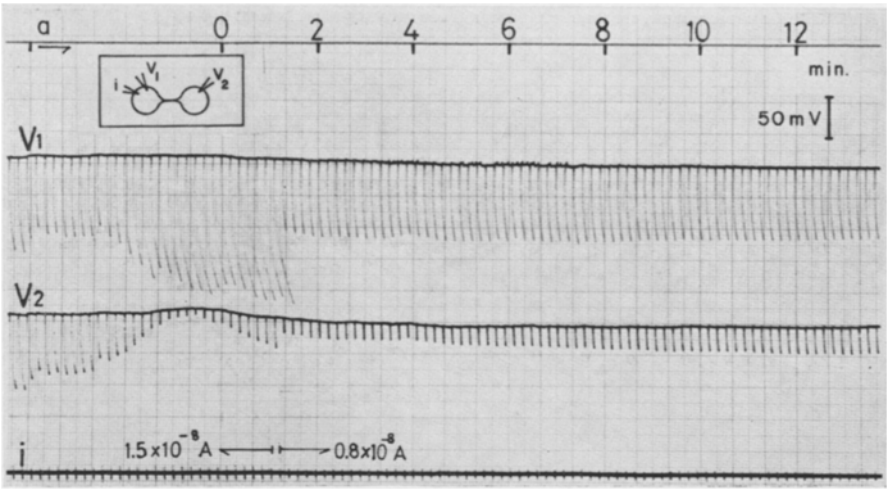


Fig. 14. *Experiment Type 3.* A conditioning junction had previously been established between two isolated cells. At *a* the cells were pulled apart; they remained mechanically and electrically connected by an "*A*"-strand. At the final intercell distance of about 100 μ, the residual coupling ratio was 1/15 of that of the conditioning junction. In the subsequent test step, the cells were pushed together re-establishing full contact *(time 0)*. Current pulse frequency, 0.09/sec; note change in intensity at arrow marks. The membrane potentials were at all times equal in the two cells and ranged from 16 to 20 mV

1967*a*) and in many of the present cases (*see also* Ito & Loewenstein, 1969). However, in some instances in which the interval between cell separation and test contact was short, as in the case shown in Fig. 11, the rise in V_2 was found to be accompanied by a rise in V_1. This may simply have been a manifestation of fluctuation of nonjunctional membrane resistance during cytokinesis (Ito & Loewenstein, 1969). A further possibility is that, during the phase of rising V_1, the cell membrane was recovering from a leak around the electrodes, produced by the manipulation during the preceding cell separation, and that the recovery in input resistance overshadowed the fall in this resistance associated with the coupling process.

Experiments Type 3. Here, by design, a degree of coupling was allowed to persist after (partial) cell separation. The coupling ratios at the time of test contact were 0.08–0.16, 72–80% below the corresponding ratios before "uncoupling". Following complete cell reunion, the coupling ratios at the test junction increased progressively to a "plateau" of 0.54–0.73 (Fig. 14). In one instance, the "plateau" value was equal to the stable coupling ratio of the conditioning junction.

Fig. 15 gives the time courses of the development of coupling for representative cases of the various types of experiment Types 2a, 3, and imposed random contact; and Table 1 summarizes the results of all types of experiments.

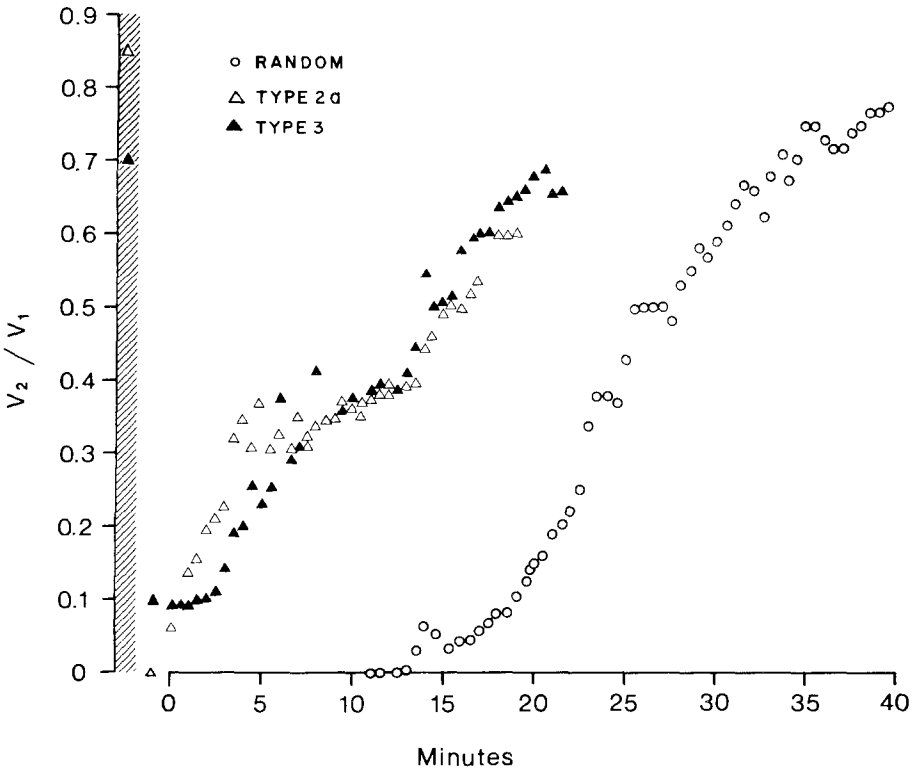


Fig. 15. The time course of coupling development under various experimental conditions. Data from an experiment of imposed random contact (○) (which is representative of all experiment types in which test junction is preceded by total membrane discontinuity), Type 2a (△) and Type 3 (▲). Time zero, test cell contact established. The points in hatched region are V_2/V_1 values during conditioning junction

Junction Formation and Cell Cycle

The cells seem capable of developing coupling and of maintaining coupling throughout the division cycle. For instance, in the experiment shown in Fig. 16, coupling produced by imposed contact, developed at the beginning of the division cycle, near stage 0 min [*random* (*a*)] and again *(Type 2a)* at stage 50 min (*b*), and, except for a 70-sec period of experimental uncoupling, the coupling is seen to have been maintained continuously from stage 0 to stage 66 min. Fig. 5 shows examples of coupling produced by spontaneous contact, which arose at stages 44 min (5*A*) and 16 min (5*B*, *a*). We performed a total of 41 experiments of imposed random contact, with successful development of coupling. In these experiments, the stages at which cell contact occurred and at which coupling set in, span the entire division cycle (Table 2).

Table 1. Time of coupling development

Experiment type	Time of coupling onset [a] (min)			Time to "plateau" coupling [d] (min)		"Plateau" coupling ratio		
	range	mean [b]	No. of cases	range	mean [b]	range	mean [b]	No. of cases
Spontaneous contact	8–14	12 ± 2.9	7	10–30	20 ±6.0	0.20–0.75	0.39±0.25	7
Imposed contact								
Random	4–26[f]	11 ± 5.8	18	10–30	19 ±5.8	0.15–0.72	0.37±0.29	20
	7–47[f']	21.4±11	41	12–50	26.7±8.8	0.16–0.82	0.49±0.21	22
Type 1	4–12	8 ± 3.5	15	10–30	20 ±7.0	0.22–0.74	0.31±0.20	10
Type 2	7–20	12 ± 4.3	7	[e]				
Type 2a	<0.012 or <0.05 [c]	<0.012 or <0.05 [c]	18	[e]				
	4–25	9 ± 6.3	9	8–18	17 ±7.2	0.3 –0.9	0.52±0.22	9
Type 3			3	14–24	19 ±5.0	0.5 –0.7	0.59±0.12	3

[a] Time from cell contact to onset of detectable V_2.
[b] With standard deviation.
[c] Resolution of the method, 1 sec or 3 sec.
[d] Time from detectable coupling onset to time when V_2/V_1 reaches a value sensibly constant over 10–20 min of observation (*see also* footnote 1).
[e] V_2/V_1 rose progressively, as in all other types of experiments (*see* Fig. 10), but the time course was followed fully to "plateau" in few cases only.
[f, f'] The two groups belong to experiment series done in different breeding seasons: f, 1972/1973; f', 1974. Group f' is detailed in Table 2.

The shortest times of coupling onset were found among the cases in which test contact was imposed in the 97–16 min interval of the cell cycle. It is noteworthy that the corresponding coupling developments occurred between stages 0–20 min, the period in the cell cycle in which the most active cytoplasmic surface movements occur (cleavage is completed by stage 20 min). The tendency for the shorter times of coupling onset was clearly unrelated to the time the cells had been isolated from the embryo; the shorter times were seen among the cells that, at the time of test contact, had been isolated for 7 min (the shortest time in isolation) as well as among those which had been isolated for 120 min (the longest time).

Once coupling had been established at the region of membrane contact, it was maintained throughout the cycle, including the cleavage phase. Besides, coupling was also maintained continuously at all times across the

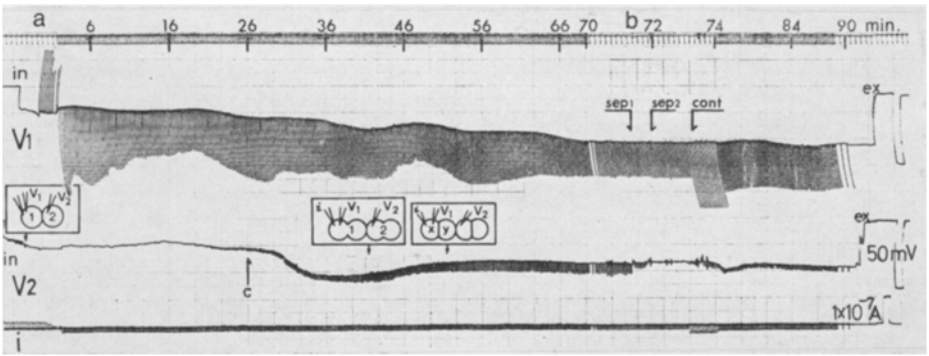


Fig. 16. Development and maintenance of coupling during the cell division cycle. (*a*) Contact (random) was imposed on two isolated cells (*1* and *2*) (separated daughters of an isolated blastomere) at a stage corresponding to 80 min in the division cycle of the parent blastomere. The V_1- and V_2-electrodes were in nonadjacent cell halves in the next cycle, so that coupling was measurable across the planes of imposed contact and of cleavage (the junction between the new daughter cells x and y) in series. (Both cell 1 and 2 cleaved 20–40 min after imposed contact.) In (*b*) the 1–2 cell junction was broken by pulling the cells apart in two steps *(sep*$_1$, *sep*$_2$*)* and then re-made *(cont)* in an experiment Type 2a. The first coupling at the imposed contact (*a*) developed to detectable level at stage 6 min, and the second coupling (*b*) at stage 50 min. Note also the continuous coupling across the $x-y$ plane. (Coarse protoplasmic continuity between cells x and y is lost at stage 40 min; after this stage the coupling between x and y is truly junctional; *see* Ito & Loewenstein, 1969)

cleavage plane. These two points are clearly shown by continuous coupling measurements taken across the junction of daughter cells in experiments on imposed contact in which the electrodes, the plane of cell contact, and the daughter cell junction (the former plane of cleavage) happened to be suitably located. An example is shown in Fig. 16. A further known instance of continuous maintenance of coupling during cell division is that of certain fibroblasts in tissue culture (O'Lague, Dalen, Rubin & Tobias, 1970).

Effects of Colchicine and Cytochalasin B on Junction Formation

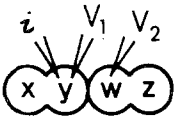
Colchicine. The action of colchicine was investigated in a set of experiments of *imposed random contact.* The cells were in medium containing 0.22–1.09 mM cholchicine since their isolation from the embryo and were kept in this medium throughout the experiments. Movement of cellular processes and cytokinesis were blocked in all cases by the colchicine treatment by the time the experiments on junction formation were begun; the cells were then in the relaxed state, lying rather flat on the bottom of the dish. Unlike in control medium where the apparent area of cell contact increased with time by autochthonous cell movement following the imposed

Table 2. Coupling and cell cycle

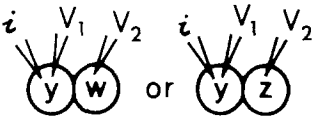
Cell cycle stage (min) at which test contact was made	Cell cycle stage (min) at which coupling set in	Time of coupling onset (min)		No. of cases	Time in isolation[f] (min)
		range	mean[d]		
0–19[a]	14–46	8–30	18± 7.6	9	7–140
20–39	42–67	16–41	25± 9.1	11	33–168
40–59	57– 4	11–43	27±15.2	8	43–248
60–83[b]	86–15[e]	13–47	24±12.1	6	7– 218
84–99[c]	98–12[e]	7–22	13± 5.1	7	29– 225

Junctions by *imposed random contact*. All experiments in breeding season of 1974.

[a] Cleavage furrow completed by stage 20 min. Test junction (y – w) in this group was made between cleaving cells:



In all other groups, test junction is made between mechanically separated daughter cells:



[b] "Rounded-up" stage.
[c] "Relaxed" stage.
[d] With SD.
[e] 2nd cleavage in isolation; all other stages are from 1st cleavage.
[f] The time cells had been in isolation from the embryo, at the moment of test contact.

contact (Fig. 3, *III*, *IV*), the apparent area of cell contact in the colchicine-treated cells stayed limited to about the original apparent area of the imposed contact. The cells, nonetheless, adhered well. This was ascertained before development of coupling and after it. Cell input resistance and membrane potential (at zero current) were similar to those in control medium.

Fig. 17 illustrates a typical experiment and Table 3 summarizes the results of the experiments with colchicine treatment at the concentration of 1.09 mM. The times of coupling onset were similar to those in control medium. The times of development to "plateau" coupling were longer (compare with Table 1, *imposed random contact*, group f'); but we are not confident that the difference is as significant as it appears from the means and their statistics since, unlike the time of coupling onset, the determination of the time of development to "plateau" coupling is imprecise.

In another set of experiments, cell pairs were exposed to colchicine, 0.44 mM, for 25 min after they had established full coupling in control medium. This produced no significant change in the coupling.

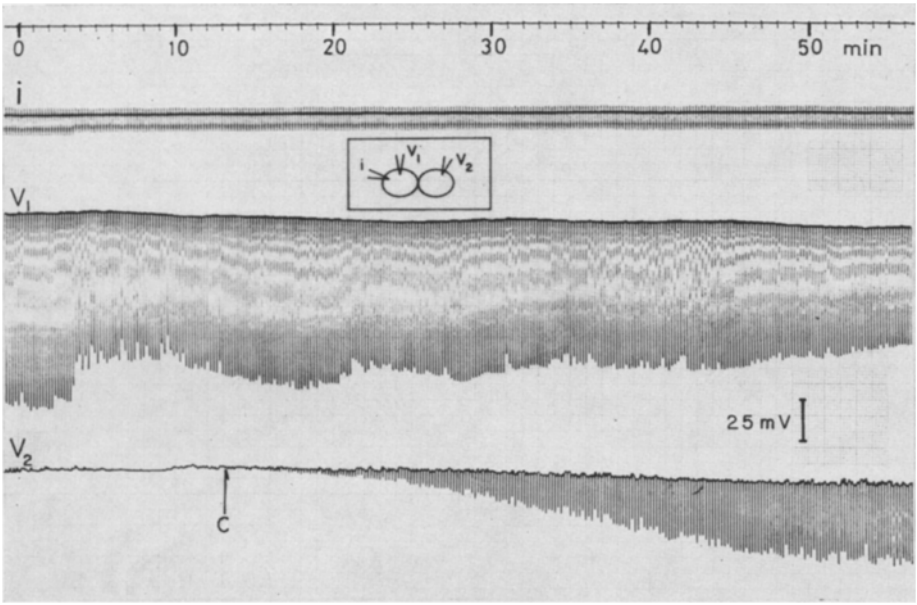


Fig. 17. Coupling in colchicine-treated cells. Contact was made *(time 0)* between two cells *(imposed random contact)*. The cells were in colchicine-containing medium (1.09 mM) throughout the experiment since 53 min before cell contact. Cell division and cytokinesis were blocked; cells were "relaxed" at time of contact. Current pulse intensity, 2×10^{-8} A; frequency, 0.09/sec. Membrane potentials in the two cells (at zero current) were 22–28 mV

Table 3. Coupling in colchicine-treated cells

Exposure to colchicine [a] (min)	Time of coupling onset (min)			Time to "plateau" coupling (min)		"Plateau" coupling ratio		
	range	mean [b]	No. of cases	range	mean [b]	range	mean [b]	No. of cases
16–301 [c]	7–29	16±6.7	18	24–71	38±13.5	0.14–0.92	0.55±0.28	11

Junction by *imposed random contact*. Experiments in breeding season 1974.

[a] Length of time cells were in colchicine medium before test cell contact; cells continued in colchicine medium until time of "plateau" coupling. Concentration of colchicine in the medium, 1.09 mM.

[b] With SD.

[c] Range includes: 2 cases of 16–20 min; 10 cases, 50–92 min; and 6 cases, 114–301 min.

Cytochalasin B. For the study of the effects of cytochalasin B, we used small blastomeres from the outer (pigmented) cell layer of the embryo in addition to macroblastomeres. The isolated cells were placed in medium

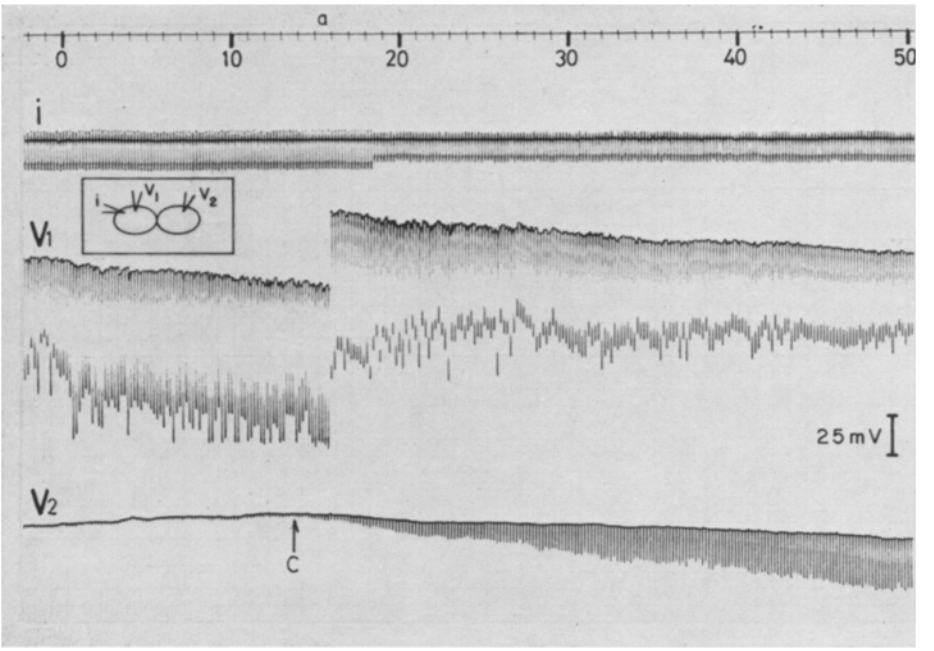


Fig. 18. Coupling in cytochalasin-treated cells. Contact was made *(time 0)* between two cells which had not been in previous contact *(imposed random contact)*. The cells were in cytochalasin medium (1 μM) throughout the experiment since 180 min before cell contact. Cell division and cytokinesis were blocked; cells were extremely "relaxed" at the time of contact. Current pulse intensity, 3.5×10^{-8} and 2.5×10^{-8} A; frequency, 0.09/sec. Membrane potentials (at zero current) were about 30 mV. At *a*, the pen recorder base line of the V_1 trace was shifted. The small downward deflections of the V_2 trace *before* the *c*-mark, are resistive artifacts (*see* Materials and Methods), which here were unusually high; when the V_2-electrode was extracellular touching the outside of the cell, the deflections had the same magnitude as those before the *c*-mark

containing cytochalasin-B concentrations ranging 0.521–5.21 μM; movement of cellular processes and cytokinesis were blocked in both kinds of cells (nevertheless, in concentrations of 0.5–1 μM, the nuclei of the small blastomeres often continued to divide once or twice) and relaxation, which took place within 10 min of the treatment, was even more pronounced than in colchicine-treated cells. The coupling measurements on macroblastomeres treated with cytochalasin B were beset with difficulties. The cell membranes often seemed to seal poorly around the electrodes; in fact, in several instances, the membrane was coarsely ruptured by the electrodes, with visible leakage of protoplasm (a rare event in untreated cells). The number of successful measurements were relatively scant.

Table 4. Coupling in cytochalasin-B-treated cells

Exposure[a] (min)	Time of coupling onset (min)		Time to "plateau" coupling (min)		"Plateau" coupling ratio		Input resistance in cell 1[e] (MΩ)		N
	range	mean[b]	range	mean[b]	range	mean[b]	range	mean[b]	
Cytochalasin B[c]									
30–186	13–25	17±5.3	25–70	50±16.9	0.27–0.76	0.46±0.20	1.77–4.0	3.25±0.91	5
Control[d]									
55–232	10–15	12±2.2	56–92	73±15.2	0.33–0.79	0.61±0.20	1.48–5.9	3.15±1.91	4

[a] Length of time cells were in cytochalasin-B or control medium before test cell contact; cells continued in the respective medium until "plateau" coupling was reached.
[b] With SD.
[c] Medium contained the normal constituents plus 5 μg/ml cytochalasin B and 0.5% dimethyl sulfoxide.
[d] Medium contained the normal constituents plus 0.5% dimethyl sulfoxide.
[e] Before test contact.

In five successful experiments of *imposed random contact* in which the input resistances in cell 1 ranged 1.77–4.0 MΩ, the same order as in control medium (1.48–5.9 MΩ), the cells were found to establish coupling at cytochalasin-B concentrations of 1 μM (Fig. 18). The times required for coupling onset were somewhat longer than in the control medium, but the difference was not significant. (The control medium here is the medium plus 0.5% dimethyl sulfoxide, the solvent in the cytochalasin medium.) There was also no significant difference in the time to "plateau" coupling nor in the "plateau" coupling ratios (Table 4).

The blastomeres from the outer cell layer of the embryo seemed somewhat less susceptible to rupture in cytochalasin medium. It was for this reason that we also used these smaller cells in the experiments. But here too, few measurements with satisfactory input resistance were made. In one, a pair of coupled daughter cells retained their coupling (coupling ratio 0.6) after exposure to 0.5 μM cytochalasin B for 160 min of observation; and in another, a measurement taken on the outer cell layer *in situ*, coupling was retained for 25 min after exposure to 1 μM cytochalasin B.

The cells from the outer layer of the embyro seemed to be less attached to each other and to the deeper cells when the embryo was dissected in cytochalasin B medium than when it was dissected in control medium; the outer cells seemed to stay attached to each other only or mainly at their edges confronting the exterior. This was evidently sufficient for maintaining,

at least, some degree of electrical coupling, a coupling that was measurable on the outer cell layer *in situ* as well as on portions of this layer isolated from the embryo. The presence of coupling in the isolated layer showed clearly that the coupling measured *in situ* on the outer layer was via permeable junction and not simply due to the surface resistance of the embryo (*see* Ito & Loewenstein, 1969).

To sum up, macroblastomeres and small outer blastomeres are capable of establishing and maintaining coupling in cytochalasin medium.

Effects of Dinitrophenol on Junction Formation

Treatment with 2,4-dinitrophenol (DNP) is known to depress the permeability of established junction between *Chironomus* salivary gland (Politoff, Socolar & Loewenstein, 1969). This uncoupling effect is shared by several other metabolic inhibitors which, by depressing Ca^{++} efflux across (nonjunctional) cell membrane and Ca^{++} uptake by mitochondria, cause elevation of the level of free ionized Ca in the cytoplasm. In fact, the junctional uncoupling by DNP has just been shown – by direct, simultaneous measurement of intracellular [Ca^{++}] and coupling in *Chironomus* salivary gland cells – to be associated with a rise in cytoplasmic [Ca^{++}] (Rose & Loewenstein, 1974).

Here we have made an exploration of the effect of DNP on the formation of macroblastomere cell junction. The isolated cells were kept in medium containing 1 mM DNP throughout the experiments. Thirty to 60 min after their exposure to DNP-medium, the cells were micromanipulated into contact, left undisturbed for 60–150 min, and then impaled with the microelectrodes for coupling measurement. Cleavage in the DNP medium was slowed in the smaller cells (300–400 μ) and arrested in the larger ones (500–600 μ). Relaxation was comparable to that in colchicine-medium.

The cells adhered well and stably in the DNP medium, but no detectable coupling was found to develop across the test contacts (five cases). Presumably, as in the case of the *Chironomus* cell junction, the cytoplasmic [Ca^{++}] was elevated by the action of DNP and coupling was thereby prevented.

In another set of experiments, cell pairs with coupling junction established in normal medium (by *imposed random contact*) were exposed to 3 mM DNP. In 10 experiments the coupling ratio was found to decrease 1.6–5 min after DNP exposure, becoming undetectable in 8–14 min (Fig. 19). Here again cell adhesion was retained. In two trials of reversal, coupling across the junction was restored to the original level within 28–33 min of exposure to control medium.

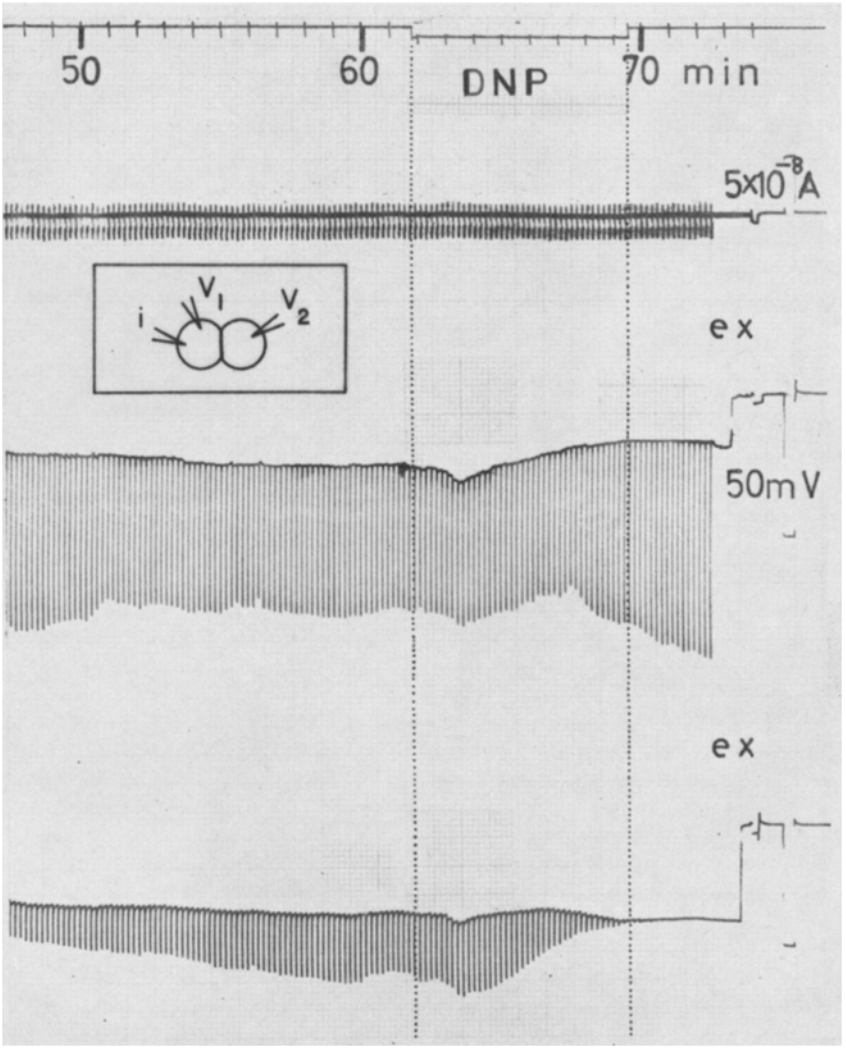


Fig. 19. Uncoupling in a forming junction by dinitrophenol. A junction between two cells had been made at *time 0* by *imposed random contact* in control medium; the record shows the end phase of the establishment of coupling. During the time signalled on the time base, the control medium was replaced by medium containing 3 mM DNP; the cells were in this medium throughout the remainder of the experiment. Note that, aside from the V_2/V_1 ratio, the junctional uncoupling is manifested by the divergence of the membrane potentials at zero current of the two cells. The uncoupled junction remained cohesive. (Cell cleavage and cytokinesis were blocked.) Current pulse intensity, 1.3×10^{-8} A; frequency, 0.09/sec

Discussion

Junction Formation. The present results show that when adhesive contact is established between the embryo cells, the membrane regions in contact form a permeable junction, that is, a coupled, insulated membrane system where molecules of a certain size range can diffuse from one cell interior to another with little loss (*see* Loewenstein, 1966). The basic functional elements of such a system as defined by measurements of electrical conductance and studies with diffusible tracer molecules, are (a) the permeable *junctional membrane* portions containing the membrane diffusion channels and (b) the *junctional insulation* that seals off the interior of the coupled system from the exterior. In a simple model of membrane junction proposed by one of us (Loewenstein, 1966), these elements are pictured forming multiple, parallel *junctional units*, each complete with its individual insulation, providing effective cell-to-cell passageways (Fig. 20, Top). The assembly of such a coupled membrane system seems to take on the order of 10 min: The electrical measurement detects the first signs of coupling 4–47 min after establishment of membrane contact under the various experimental conditions of contact; and a "plateau" in coupling is reached in 10–50 min. The coupling tended to start somewhat earlier when membrane contact was imposed by manipulation than when it occurred between spontaneously moving cell processes (Table 1). But this may merely have been due to our misjudging the moment of adhesive contact (time zero) in the latter case, or due to the fact that the initial contact was broader in the former case, rather than to a more fundamental difference in the process of junction formation. Only in experiments Type 2a, in which full contact was re-established between not entirely discontinuous membrane regions of electrically uncoupled cells, did coupling set in much faster (< 3 sec).

A possible explanation of this fast coupling is as follows: The *B*-elements (the remnants of membrane continuity of conditioning junction in experiments Type 2a; *see* Fig. 8*b*) are elastic cell extensions that contain intact portions of conditioning permeable membrane junction. In the final position in the uncoupling step, the *B*-elements are so stretched that their longitudinal internal (cytoplasmic) resistance becomes too high or the junctional membranes they enclose become too distorted to mediate detectable coupling. (Their diameter is < 0.3 μ and their length, 100–300 μ.) When the *B*-elements are relaxed in the step of test contact, the cell-to-cell path resumes its original dimensions and coupling is restored. In other words, the first detectable coupling (within < 1 sec of test contact) in an experiment Type 2a would be across residual conditioning junction, just as the coupling at the

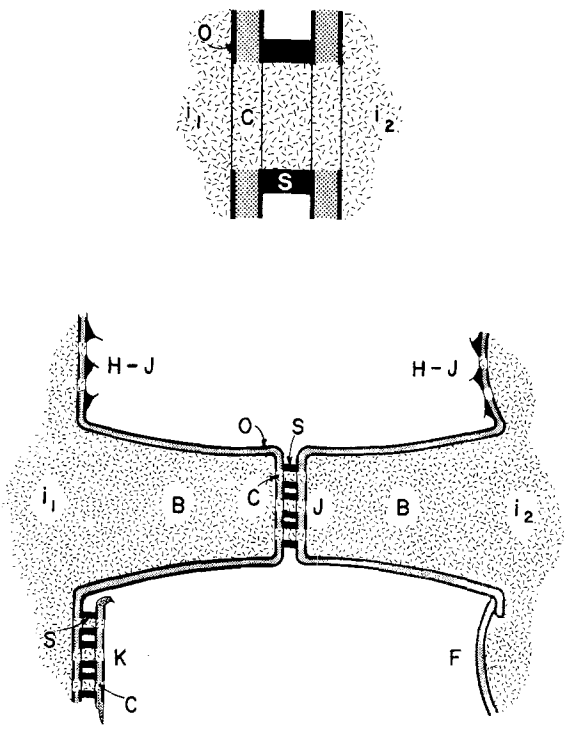


Fig. 20. *Top:* A *junctional unit: C*, permeable *junctional membrane; S, junctional insulation; O*, nonjunctional membrane; *i*, cell interior. In the model (Loewenstein, 1966), many such parallel units, each an effective cell-to-cell passageway, constitute a permeable membrane junction *(J, bottom)* (from Loewenstein, 1966). *Bottom:* Hypothetical representation of the situation of membrane contact in an experiment Type 2a. *B*, a distended *B*-element at the end of the uncoupling step (*see* Fig. 8*b*), containing residual conditioning junction (*J*) with *junctional units* intact. The rest of the conditioning membrane junction shown here has ruptured. Two possible rupture modes are diagrammed: the junction has split in halves (*H*−*J*) or has broken out of the membrane (*F, K*) of one of the cells. The halves and breaks seal in the presence of Ca^{++} in the medium. i_1, i_2 are the interiors of the two cells

time of test contact in experiment Type 3 is clearly across residual conditioning junction. In this light, experiments Type 3 and 2a differ only in that, before test contact, the cell-to-cell conduction path via the *A*-elements is shorter (50–100 μ) and wider than via the *B*-elements, and in that the area of residual conditioning junction in experiment Type 3 is perhaps greater than in Type 2a (the diameter of an *A*-element is of the order of 1 μ). The ca. 10-min (uncoupling step-test contact) time limit for rapid coupling in experiments Type 2a (Fig. 13) may then possibly reflect the time limit for elastic reversibility of the *B*-element.

Another possibility is that the rapid coupling in experiment Type 2a is the result of a facilitation of the process of junction formation by the previous presence of permeable (conditioning) junction. Although this would be a more exciting possibility, it seems fruitless to speculate along these lines further until information is available on the structure of the *B*-elements and of the membrane junction, and on the fate of the junction during the uncoupling.

In all types of experiments on new junction formation, the degree of coupling rose progressively from the moment of detectable coupling onset to the "plateau". As will be shown in the following paper (Ito, Sato & Loewenstein, 1974), this reflects a progressive rise in junctional conductance. Even in experiment Type 3, the degree of coupling rose progressively from the coupling level of residual conditioning junction (Fig. 14), and the times to "plateau" coupling were of the same order in all experiments. It appears, therefore, that the rising phases in the curves of coupling development in experiments Types 1 and 2 reflect the development of new junction, and that in Types 3 and 2a they at least include development of new junction.

The times of coupling onset in the experiments of spontaneous contact (8–14 min), of imposed random contact (4–47 min) and of imposed contact Types 1 and 2 (4–20 min) are of the same order as those found under conditions of random contact in four other cell systems. In cells from the sponges *Microciona* and *Halyclona*, on which the first experimental study of junction formation was made, the times of coupling onset in experiments of imposed random contact ranged from 1 to 40 min (most cases, from 4 to 15 min) (Loewenstein, 1967*a*). In bovine lymphocyte aggregates stimulated with phytohemagglutinin, the time of coupling onset (after stimulation) ranged 1–7 min (Hülser & Peters, 1972; *see also* Sellin, Wallach & Fischer, 1971; Oliveira-Castro, Barcinski & Cukierman, 1973). In certain fibroblasts derived from Novikoff hepatoma, the time of coupling onset under conditions of spontaneous (random) contact in tissue culture ranged 5–10 min (Hammer, Epstein & Sheridan, 1973) and in embryonic chick heart cells, this time, inferred from observation of synchronous beating, ranged 4–38 min (DeHaan & Hirakow, 1972; *see also* DeHaan & Sachs, 1973). The time reported for *Fundulus* embryo cells is longer, 1 hr (Bennett & Trinkaus, 1970). But this value may have been an overestimate; it is based on spot checks of electrical coupling of about 10 cases only, not on continuous measurements.

In our earlier work on newt embryo cells (Ito & Loewenstein, 1969), the experimental conditions were in many cases similar to those depicted in Fig. 2, with one of the cells sliding back and forth on a yolk track during

cell separation (uncoupling) and rejoinder, and the cell separations during the uncoupling were often on the order of 200–300 μ. Although no conscious effort was made at that time to make junctions with a former membrane region in contact, the chance for this to happen under these conditions was high. Some of these experiments were therefore comparable to the present systematic experiments Type 2a and the times of coupling onset were indeed of the short < 3-sec order. We also had cases with times of coupling onset of the long order, but we neglected them. We thought that they represented cases in which we had misjudged the time zero of test contact or cases in which the coupling was retarded because of poor initial membrane contact. For these reasons, we assumed that the shorter values were the more significant ones. In view of the present results, it is clear that this assumption was wrong. The short values must have belonged to the special Type 2a.

Junction Breakage. The experiments in which electrical coupling and input resistance were monitored during cell separation show that the cell membrane resistance stays generally high after the breaking of the cell junction. The input resistance in cell 1 generally increased upon cell separation and in many cases, such as in the experiments of Figs. 11*a* sep_1, 11*b* and 12*b*, the increased value is not far from the expected one, if, after occlusion of the junctional path to cell 2, the current injected into cell 1 is distributed uniformly over the nonjunctional membrane of cell 1 with unaltered resistivity (*see* the second paper of this series). *This means that the former junctional membrane diffusion channels as well as any possible membrane breaks are rapidly sealed upon cell separation.*

Processes of junctional sealing and of membrane sealing, and some of their requirements are known. *Chironomus* salivary gland cell junctions seal when the Ca^{++} activity on the cytoplasmic face of junctional membrane is raised experimentally above $5–8 \times 10^{-5}$ M (Oliveira-Castro & Loewenstein, 1971; Rose & Loewenstein, 1971, 1974; *see also* Politoff *et al.*, 1969), and this presumably is so too when the Ca^{++} activity is raised on the external face of junctional membrane, the face that is normally protected by *junctional insulation* from the high Ca^{++} activity in the external medium (Loewenstein, 1966; Loewenstein, Nakas & Socolar, 1967; *see also* Délèze, 1970). Furthermore, broken (nonjunctional) membranes of various kinds of cells seal in medium containing Ca^{++} (Heilbrunn, 1956) and this seal is tight enough even to block the small inorganic ions (Kuffler & Potter, 1964; Oliveira-Castro & Loewenstein, 1971). For instance, holes of about 2 μ in diameter drilled into *Chironomus* salivary gland cells seal in the

presence of Ca^{++} or Mg^{++}; the restoration of the membrane diffusion barrier is nearly complete in medium with Ca^{++} or Mg^{++} activities of $1–2 \times 10^{-3}$ M (Oliveira-Castro & Loewenstein, 1971). The divalent cation activities in the present medium were more than sufficient for such self-sealing of junctional diffusion channels or membrane breaks.

In some cases, the input resistance fell transiently during cell separation or increased less than expected on the basis of the above considerations (Fig. 5*B*, *b*). This may have been due to slow or poor junctional sealing. However, we think it more likely that this was caused by leakage around the electrodes, as the cells were pulled apart by them.

We don't know the fate of the membrane junction during the cell separation. Two possibilities will be considered: (i) the membrane junction splits in two halves so that each membrane retains the corresponding junctional membrane portions (Fig. 20); and (ii) one of the cell membranes breaks and the other carries along all or parts of the junction with the junctional units complete (although occluded). The two possibilities are represented schematically in Fig. 20. There is electron-microscopic evidence suggesting that both separation modes may occur in cell systems in which the "gap junction" (Revel & Karnovsky, 1967) is the probable site containing the permeable junctional units. In cells of frog heart (Barr, Dewey & Berger, 1965; but *see also* Dreifuss, Girardier & Forssmann, 1966), guinea pig smooth muscle (Barr, Berger & Dewey, 1968), and mouse liver (Goodenough & Gilula, 1972), the gap junction appears to be split in halves when the cells are exposed to hypertonic sucrose medium. In fact, the freeze-fracture electron-micrographs of Goodenough and Gilula (1972) seem to have caught the gap junction in the process of splitting. On the other hand, in rat liver cells, dissociation produced by Ca,Mg-free medium containing collagenase and hyaluronidase or ethylenediamine-tetra-acetic acid, the gap junction is carried seemingly complete by one of the membranes (Berry & Friend, 1969), and when liver cells are ruptured in a Dounce homogenizer, rather complete junctions break off from the rest of the cell membrane (Bennedetti & Emmelot, 1967; Goodenough & Revel, 1970). We have no electron-microscopical information to guide us in the question of which mode of disengagement was operating in the present experiments. Be that as it may, it is clear that the cells promptly selfsealed upon their disengagement in all experiment types.

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