on coupling 21 minutes after return to normal solution, when the surface membrane resistance has fully recovered to normal. What is significant here is that, while the resistive voltage in cell II is smaller than in the control, the resistive voltage in cell I is actually larger (Fig. 1). This may be expected upon uncoupling. As the resistance (r_c) across the cell junction increases, a decreasing fraction of the current reaches cell II, whereas an increasing fraction flows through the outer surface of cell I alone (Fig. 2).

The junctional resistance in this experiment increased from a normal value of approximately 24×10^6 ohms to 70×10^6 ohms. This is nearly the highest degree of uncoupling we were able to obtain by repeated washing of the prep-

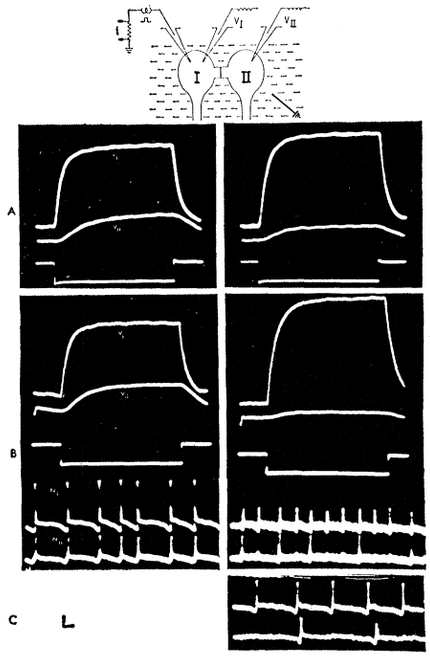

Fig. 1. Uncoupling of a nerve cell junction. Rectangular current pulses (i) of 1×10^{-8} amperes are passed between cell interior and exterior, and the resulting resistive membrane voltages V_I and V_{II} are recorded simultaneously in the two cells. (Left column) V_I, V_{II}, and samples of spontaneous nerve impulses N_I and N_{II} in the normally coupled cells; (right column) after uncoupling of the cells by bathing the preparation A in Ca^{++}-free saline; (B) in saline containing 2.5 mM EDTA; and (C) 3 mM EDTA. All records of the right column were taken after the preparation was bathed in normal saline for a time sufficient for full reversal of the Ca^{++} effects on the nonjunctional surface membranes; thus the records give purely the irreversible effects of Ca^{++}, as they relate to junctional coupling. Calibration: 10 mv for all records; 20 msec for V and i records; 200 msec for N records.

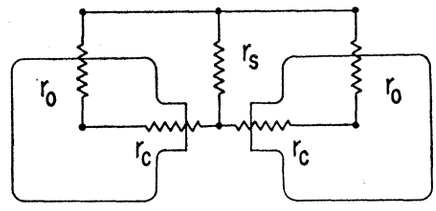

Fig. 2. Electrical analog of cell junction. r_o, Resistance across the nonjunctional cell surface membrane; r_c, across the cell junction; r_s, along the intercellular surface exterior. In the normally coupled junction r_s is high in relation to r_c.

aration in Ca^{++}-free solutions. For more uncoupling, chelating agents had to be used.

Figure 1B illustrates a case in which disodium ethylenediamine tetraacetate (EDTA) was used. Junctional resistance increased from approximately 30×10^6 ohms to 190×10^6 ohms. This is approximately the level of junctional resistance at which interaction of impulse activity is cut off between the cells. Normally the cells produce impulses in synchrony. This is one of the functional adaptations of this type of junction (*4*). When the junctional resistance reaches the level above, synchronization first fails partially (Fig. 1B); and then completely (Fig. 1C).

Magnesium ion seems to substitute for Ca^{++} in maintaining intercellular communication. In a series of experiments in which all the $CaCl_2$ of the bathing solution was replaced by equimolar $MgCl_2$, no uncoupling was found. Subsequent removal of $MgCl_2$ resulted in uncoupling.

It appears from the present results that Ca^{++} is normally required for maintaining electrical connection between these cells. At present we have nothing definite to offer as to the mode of Ca^{++} action. But some light is thrown on this question by the fact that, when uncoupled, the cells seal themselves off and actually increase their total surface resistance.

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6. The solution contained in mmole per liter: NaCl, 113.5; KCl, 4.3; $CaCl_2$, 1.8; maleic acid, 10; glucose, 10; tris buffer, 10; NaOH ~ 10 adjusted to pH 7.4 (from Nicholls and Kuffler, *5*). The Ca^{++} concentration of the test solution referred to in the text as Ca^{++}-free was below 10^{-5} mM, the level of Ca^{++} contamination in the present experiments. In the test solutions containing EDTA, the chelator was added to the Ca^{++}-free solution in the concentrations stated for each case. Molarities and osmolarities were constant within 2 percent, and pH within 3 percent in all solutions.
7. Equilibrium between the actual exterior of the nerve cells and the bathing solution is reached here within 30 seconds as shown for a variety of ions by Nicholls and Kuffler (*5*).

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Uncoupling of an Epithelial Cell Membrane Junction by Calcium-Ion Removal

Abstract. *Calcium takes part in maintaining ion communication between salivary gland cells (*Chironomus thummi*). Its withdrawal from the cell systems results in virtual disconnection of ion communication, at Ca^{++} concentrations which do not noticeably affect cell adhesion. The junctional membrane surfaces, which are normally quite freely permeable to ions, become as impermeable as the nonjunctional membrane surfaces; each cell seals itself off irreversibly as a unit. In maintaining ion communication Mg^{++} substitutes for Ca^{++}.*

This report deals with the action of Ca^{++} on an epithelial cell junction. Among the various epithelial cells which are now known to present intercellular ion communication (*1*), we chose the salivary gland cells of the midge *Chironomus thummi* for the present experiments. These have junctional membrane complexes of the septate type (*2*). The choice was guided by the size of the cells (100 to 200 μ), by their simple arrangement in single chains, and, particularly, by the stability of their cell surface membrane resistance (*3*).

The glands were isolated in saline (*4*), and a series of cells were impaled with microelectrodes arranged for measurements of resistive membrane voltage (Fig. 1).

Removal of Ca^{++} results in functional uncoupling of the cells. Figure 1 illustrates an example in which the concentration of free Ca^{++} is lowered to $5 \times 10^{-4} M$ by addition of disodium ethyleneglycol-bis(β-amino ethyl ether)-*N'*,*N'*-tetraacetate (EGTA) to the bathing fluid. The first signs of uncoupling occur within 1 minute of EGTA application; and maximal uncoupling occurs after about 5 min. The resistive membrane voltage in cell II, produced by a current of 5×10^{-8} ampere, diminishes after EGTA application from its normal value of about 20 mv to nearly zero, whereas that in cell I increases steeply from about 30 mv to a peak of 120 mv, as the cell uncouples from the other fourteen of the chain, which were formerly all interconnected. The junctional coupling resistance, as computed by transmission-line analysis of voltage attenuation along various cells of the chain (*1*) increases by several orders of magnitude during uncoupling.

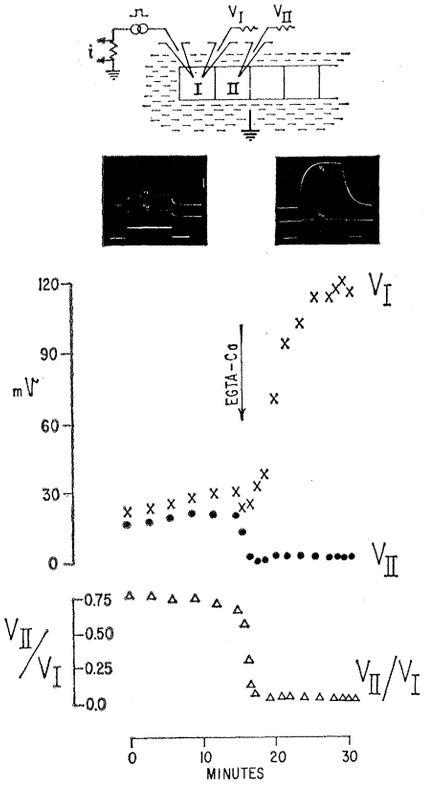

Fig. 1. Uncoupling of a gland-cell junction by Ca removal. Rectangular pulses of current (5×10^{-8} ampere) were passed between cell interior and the grounded cell exterior, and the resulting resistive membrane voltages (V) were recorded in adjacent cells I and II. The upper ordinates show membrane voltages V_I and V_{II}. The lower ordinates show the coupling ratio V_{II}/V_I. At the arrow, the control solution surrounding the preparation was replaced by one containing a complex of 1 m*M* EGTA and 1.49 m*M* Ca, to give a free Ca^{++} concentration of 0.5 m*M*. The insets show oscilloscope records of membrane voltage (V_I, V_{II}) and membrane current ($i = 5 \times 10^{-8}$ ampere) before (left) and after (right) uncoupling. Calibration: 20 mv; current pulse duration, 120 msec.

In addition, Ca^{++} removal causes a decrease in general surface membrane resistance, as it does in other cells. But this effect differs markedly in rate from the effect on junctional coupling. During the first 8 to 40 minutes after application of the chelator (*5*), the effect on junctional coupling predominates by far, as is reflected in the steep initial rise of the curve of V_I (Fig. 1). Thereafter, the surface resistance diminishes markedly and, as a result, V_I falls toward zero (not shown in Fig. 1). This effect of Ca^{++} removal is, at least, partly reversible, while that on junctional coupling is not reversible at all.

Unlike the uncoupling in the nerve-cell junction (*6*), uncoupling in the epithelial cell junction could not be demonstrated by treating the preparation with Ca^{++}-free solutions alone; a chelating agent had to be present. It was therefore necessary to ascertain (i) that uncoupling was not due to the chelator molecules themselves; and (ii) that among the possible metal ions, it was the Ca ion whose removal from the tissues causes uncoupling.

To clear up the first point, we applied different combinations of EGTA and Ca. The concentrations of free Ca^{++} in these combinations were constant and slightly too high for uncoupling, while those of free EGTA were varied over a wide range. No uncoupling ensued under these conditions, even when the free EGTA concentration was one order of magnitude greater than that required to produce uncoupling at lower levels of free Ca^{++}.

The second point was tested for the two likely ions, Ca^{++} and Mg^{++}, with chelators of different affinities with these ions. Disodium ethylenediaminetetraacetate (EDTA) and EGTA provided a convenient set of chelators for this purpose: the stability constants, at the *p*H used, of the Ca complexes of EGTA and EDTA are rather similar, within one order of magnitude; on the other hand, the Mg complexes of EGTA and EDTA differ by five orders (*7*). The minimum concentrations required to produce detectable uncoupling by EGTA and EDTA were tested and were found to be of the same order ($10^{-3} M$). Thus, removal of Ca, not of Mg, leads to uncoupling.

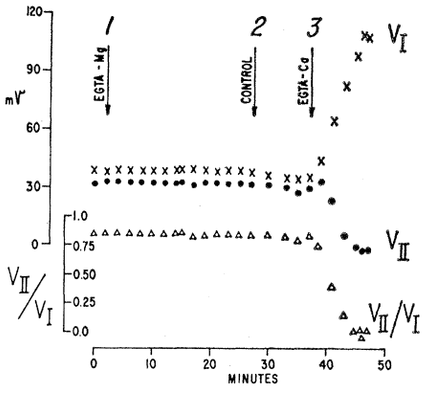

Fig. 2. Uncoupling prevented in the presence of Mg^{++}. Electrical arrangement and notations as in Fig. 1. At arrow No. 1 the control solution was replaced by a "Ca^{++}-free" solution containing 2.5 m*M* EGTA–2.5 m*M* $MgCl_2$. At arrow No. 2, return to control solution; arrow No. 3, solution containing 2.5 m*M* EGTA–2.5 m*M* $CaCl_2$, with free Ca^{++} being 0.1 m*M*.

Uncoupling does not occur when there is a sufficiently high concentration of Mg^{++} in the extracellular fluid. Figure 2 shows an example in which the preparation is bathed in a solution, of low Ca^{++} concentration, containing an EGTA-Mg complex. The free EGTA is here far in excess of that required for uncoupling (at this level of free Ca^{++}) in absence of Mg^{++}; yet there is no detectable uncoupling.

From our results emerges Ca^{++} as an important factor in intercellular communication. In both the epithelial cell junction here and the nerve cell junction (*6*) electrical coupling relates to the cellular content of Ca^{++}, in all likelihood to that in the membrane junctions themselves. Sufficient removal of Ca^{++} leads to complete and irreversible interruption of intercellular communication. Moreover, a phenomenon of interest in itself is that this interruption is associated with transformation of the normally low-resistive membrane material of the junction into one of relatively high resistivity.

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3. The experiments required that the resistance of the surface membrane remain stable for long periods when the cells were impaled with microelectrodes. This condition is rare in epithelial cells; in this regard, experiments with nerve cells have a clear advantage. Of the various epithelia tested, *Chironomus* salivary gland cells provided the best material, and experiments lasting 1 to 2 hours could be run under stable conditions. But even in these relatively sturdy cells, satisfactory results could be obtained only at the expense of considerable number of trials and material.
4. The normal saline solution had the following composition: 87 m*M* NaCl; 2.7 m*M* KCl; 1.3 m*M* $CaCl_2$; 10 m*M* tris buffer. Changes in Ca^{++} concentration and additions of chelators in the test solutions were done by equimolar substitution for NaCl of the normal solution. The molarity of all solutions was thus constant, and the osmolarity constant within 1 percent. The *p*H was 6.3 in all solutions. The Ca^{++} contamination in "Ca^{++}-free" solutions was below 10^{-6}*M*.
5. The length of this span depends on the free Ca^{++} concentration.
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Properties of Bursicon: An Insect Protein Hormone That Controls Cuticular Tanning

Abstract. *On gel filtration the retention volume of bursicon indicates a molecular weight of about 40,000. On disc electrophoresis bursicon migrates toward the anode and appears at an* R_F *(relative to the marker dye) of 0.3 to 0.4. The properties of fractions with bursicon activity in blood, brain, and ganglion of a fly* (Sarcophaga bullata), *and in blood, ganglion, and corpora cardiaca of a roach* (Periplaneta americana) *are similar, but not identical. Bursicon in the blood is more heat labile, and the activity in brain and corpora cardiaca shows two peaks in electrophoresis, instead of one as in the other fractions.*

The action of a hormone which mediates tanning in the cuticle of adult flies has been reported (*1, 2*). At the time of emergence from the puparium the blood is devoid of this hormone. If the head is separated from the body by a ligation on the neck at this time, the rest of the body never tans. Injection of blood from a tanning fly into a ligatured fly causes the latter to tan. The hormone controlling this process is derived from the median neurosecretory cells in the brain and from the abdominal region of the combined thoracico-abdominal ganglion (*3, 4*). This hormone seemed to be entirely different from the conventional insect hormones and was given the name *bursicon* (from the Greek bursikós, pertaining to tanning) (*4*).

Early in the investigation it became clear that bursicon is a protein. It is nondialyzable and is destroyed by alcohol, acetone, several proteases, and trichloroacetic acid (*1, 3, 4*). After precipitation with ammonium sulfate, the activity can be largely recovered.

The following experiments were designed to provide evidence for the protein nature of bursicon. All experiments were carried out with the blowfly *Sarcophaga bullata*, except where stated otherwise.

The methods for detecting and testing for bursicon activity have been described previously (*4*). For a quantitative evaluation of hormone activity, a classification was devised whereby different degrees of tanning were expressed by numbers of plus (+) signs, with three-plus (+++) standing for maximal effect. In instances of suboptimal activity, the abdomen is more intensely tanned than the thorax. Thoraces and abdomina were evaluated separately and the total number of plus signs in all flies in a test was added. (Thus, the maximum value for any fly is six plus signs, three each for thorax and abdomen; and when five flies are used in an experiment, full tanning is expressed by the value 30.)

Blood containing hormone activity was processed through Bio-gel P-200 columns as described (*5*). Samples (100 μl) were placed on columns (14 cm by 0.8 cm), and 20 fractions (0.5 ml each) were collected by elution with 0.005*M* phosphate buffer, *p*H 7.8. The protein content of the fractions was measured with the Folin-Ciocalteu reagent (*6*). The bulk of the blood proteins was excluded from the gel and was contained in fractions 4 and 5, with the amount of protein in subsequent fractions rapidly tapering off. The slight rise in optical density of fractions 11 and 12 was probably due to the presence of polypeptides and amino acids that reacted with the Folin-Ciocalteu reagent (Fig. 1).

In order to get a measure of the molecular weight of bursicon, carbonic anhydrase (mol. wt., 31,000) (Worthington Biochemical Corp.) and hexokinase (mol. wt., 51,000) (Sigma Chemical Co.), were added to blood already containing the hormone activity and processed through the column. The retention value for bursicon and each enzyme was determined. Carbonic anhydrase was measured potentiometrically according to March and Anderson (*7*), and hexokinase according to Andrews (*8*). Maximum bursicon activity was found in fractions 6–8, with the peak almost certainly in fraction 7 (Fig. 1). Tests at higher dilutions of the active fractions, which would have permitted us to determine differences of activity in these three fractions, were omitted. A single peak was obtained in other experiments. Hexokinase showed a peak in fraction 6, and carbonic anhydrase in fractions 8 and 9. The experiment was repeated several times, with a very slight shifting of peaks of activity on different occasions, but the retention volume of bursicon was always roughly intermediate between that of the two enzymes. From this it is concluded that the molecular weight of bursicon is of the order of 40,000.

Active and inactive blood (5-μl sample) was submitted to polyacrylamide disc electrophoresis for acidic proteins,

Table 1. Electrophoretic separation of bursicon activity from various tissues of *Sarcophaga bullata* and *Periplaneta americana*, at *p*H 8.5. Fraction 1 is the fastest moving fraction right behind the marker dye.

Preparation	Bursicon activity of fractions 1 to 11										
	1	2	3	4	5	6	7	8	9	10	11
	Sarcophaga										
Active blood *	3	3	4	1	3	28	25	8	2	3	3
Inactive blood †				1	0	4	4	1	1		
MNC	22	5	5	6	4	5	6	12	10	1	
'Abdominal' ganglion	7	5	6	3	2	19	18	4	6	5	
	Periplaneta										
Corpora cardiaca	18	6	4	1	5	18	20	19	6	2	
Last abdominal ganglion	2	5	3	7	12	18	13	8	5	2	

* From 30-minute-old fly, containing bursicon. † From fly less than 1 minute old, with no bursicon activity.