caused numerous fluorescent spots to develop on papillae (Fig. 2b) which were similar to, but considerably smaller than, the fluorescent "callose rejection reaction" noted by others (*8*). Cycloheximide has also been shown to block migration of pollen nuclei into the germ tubes of *Trigonella* (*3*), *Impatiens* (*4*), and *Tradescantia* (*5*); and to prevent or retard entry of nuclei into metaphase with pollen from *Ornithogalum* and *Tradescantia* (*5*).

Sarfatti *et al.* (*10*) observed that treating pollen from *Lycopersicon* with actinomycin D overcame self-incompatibility. The concentric array of endoplasmic reticulum found in incompatible pollen tubes of *Lycopersicon* suggested that incompatibility might lead to a general cessation of protein synthesis (*11*).

That cycloheximide failed to block germination in situ of *Brassica* pollen on cross-stigmas (compatible), and facilitated germination of self-stigmas (incompatible), supports the previous results in vitro indicating that pollen contains all the enzymes required for tube elongation (*7*). That the cycloheximide-treated self-pollen germinated and adhered to papillae of tissues of self-stigmas, whereas untreated self-pollen did not, indicates that pollen protein synthesis must be required for expression of incompatibility in vivo. These data for *Brassica* pollen in situ are in agreement with the hypothesis that the control of pollen germination in vivo and in vitro by cycloheximide is indirect and results from the blockage, in pollen, of the synthesis of proteins that regulate the expression of self-incompatibility.

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In vitro Growth of Imaginal Disks from *Drosophila melanogaster*

Abstract. *Imaginal disks were cultured in a new medium in vitro. Optimal growth occurred when the medium was supplemented with insulin and a juvenile hormone analog and conditioned by larval fat body. The disks grow in vitro by normal cell division and maintain their capacity to differentiate into normal patterns of adult cuticular structures.*

During the embryogenesis of *Drosophila melanogaster* and related insects, some of the cells are programmed (that is, determined) to become imaginal disk cells (*1*). These cells do not immediately differentiate or become polytene as do larval cells. Instead, they proliferate as determined cells throughout larval life.

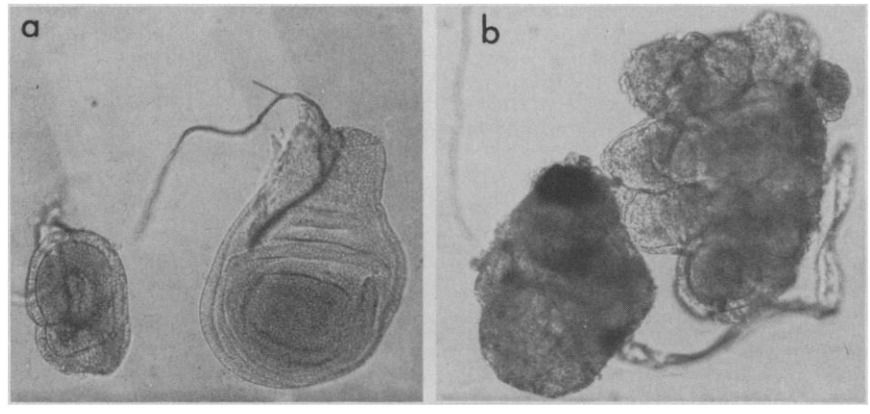

Fig. 1. Wing and leg imaginal disks. (a) Disks dissected from mature third instar larvae. (b) Explanted disks cultured for 2 weeks in medium XCS in vitro.

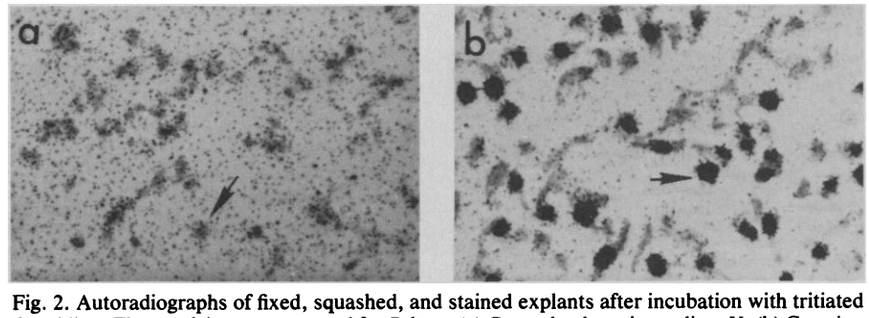

Fig. 2. Autoradiographs of fixed, squashed, and stained explants after incubation with tritiated thymidine. The emulsion was exposed for 7 days. (a) Control culture in medium X. (b) Growing culture in medium XCS. Arrow indicates intense grain density over a nucleus.

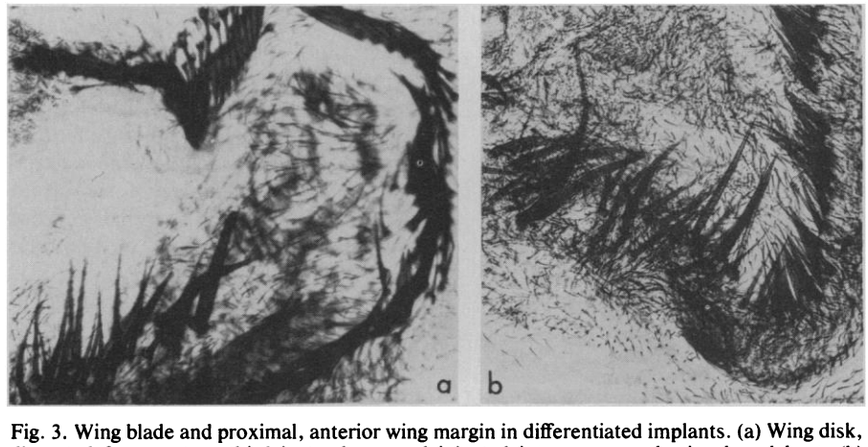

Fig. 3. Wing blade and proximal, anterior wing margin in differentiated implants. (a) Wing disk, dissected from mature third instar larva and injected into metamorphosing larval host. (b) Fragment of an explant cultured in medium XCS for 2 weeks and injected into metamorphosing larval host.

During metamorphosis, the disks give rise to the external structures of the imago or adult (*2*). The proliferative phase of disk development can be prolonged by injecting disks from mature larvae into the abdominal cavity of adult females (*3*). Such in vivo culturing interrupts normal development after determination has occurred but before terminal differentiation begins. Hadorn (*4*) observed that imaginal cells growing in cultures of this type can accurately transmit their determined state from one cell generation to another, while retaining their capacity for terminal differentiation. Because of this separation in time between their determination and differentiation and because of the availability of mutants, the imaginal disks of *Drosophila* offer exceptional advantages as a system for studying the process of determination.

Biochemical studies are notably absent among the different approaches that have been applied in recent years to the study of imaginal disk determination. Believing that biochemical approaches require a method for growing imaginal disks in vitro, we have developed a system for in vitro culture designed to be analogous to the in vivo culture method. This report is a description of imaginal disk growth in vitro.

Each culture was initiated with approximately 50 imaginal disks that were dissected from late, third-instar, wild-type (Urbana-S) larvae that had been grown axenically. The disks were slightly wounded with a tungsten needle and transferred to 0.07 ml of medium in an organ culture dish (*5*). The cultures were incubated at 25°C in a 5 percent carbon dioxide atmosphere. Maximal growth occurred in medium X (Table 1), which was supplemented with Altosid (*6*), a juvenile hormone analog (31.0 ng per 100 ml = $10^{-9}M$) and bovine insulin (0.04 unit per 100 ml) and conditioned by all of the fat body from five larvae. The use of insulin was suggested by the finding that it is present normally in the hemolymph of *Drosophila* larvae (*7*). The medium was conditioned either by preincubation with fat body, in which case the medium was replaced twice weekly in the disk cultures, or by coculturing of the disks with fat body, in which case the medium was replaced every 2 weeks. The composition of medium X reflects partial analyses of larval hemolymph that have been published (*8*), as do the compositions of media used to grow cells in vitro derived from *Drosophila* embryos (*9*, *10*). One major difference between those complete media and our conditioned and supplemented medium (XCS) is that there is no mammalian serum in XCS. In fact, the main reason for designing a new medium was our observation that every kind of serum we tested caused disks to degenerate.

The disks grow in vitro as organized compact masses just as they do when cultured in vivo in adult females. Figure 1 shows a wing and a leg disk before and after 2 weeks in culture. Whereas disks dissected from larvae are basically flat, as they continue to grow in vitro they become convoluted. After 4 weeks in culture the explants appear to be an order of magnitude larger than their original size. For comparison, disks in unsupplemented, nonconditioned medium X do not even double in size after 4 weeks in culture although the cells are still alive by the criterion of trypan-blue exclusion. After only 2 weeks, disks cultured in medium X supplemented with 5 to 15 percent heat-inactivated fetal calf serum disintegrate and the cells incorporate trypan blue.

Table 1. Composition of medium X. The *p*H is adjusted to 6.8; the osmolarity is 333 milliosmoles per liter. Abbreviation: MW, molecular weight (average).

Component	Amount (mg per 100 ml)
$MgSO_4 \cdot 7H_2O$	123.0
$MgCl_2 \cdot 6H_2O$	319.2
KH_2PO_4	20.0
K_2HPO_4	25.6
KCl	44.6
$KHCO_3$	30.0
Potassium citrate	31.2
Potassium fumarate	80.0
Potassium α-ketoglutarate	87.0
Potassium acetate	75.0
Sodium citrate	312.2
Sodium 1,4-piperazinediethanesulfonate, Pipes	360.4
Calcium succinate monohydrate	51.8
Lactalbumin hydrolysate	900.0
L-Proline	100.0
L-Tryptophan	10.0
L-β-Alanine	5.0
L-Cysteine	10.0
L-Serine	25.0
γ-Aminobutyric acid	7.0
Bactopeptone	500.0
Trehalose	400.0
Glucose	440.0
L-α-Phosphatidylcholine, dimyristoyl	0.1
L-α-Phosphatidylcholine, distearoyl	0.1
Cholesterol	0.26
Yeastolate	200.0
Vitamin A, *trans*-retinol	0.01
Vitamin E, D-α-tocopherol	0.01
Sodium α-glycerophosphate	162.0
Calcium phosphorylcholine	129.0
Phosphorylethanolamine	7.0
Bovine serum albumin, fraction V	1000.0
Polyvinylpyrrolidone, MW = 10,000	25.0
Polyvinylpyrrolidone, MW = 40,000	100.0
Polyvinylpyrrolidone, MW = 360,000	900.0

We have examined whether the growth of disks in medium XCS occurs by an increase in the number of cells or an increase in the size of cells. This issue is especially relevant to *Drosophila* cells because they have the genetic potential to become polytene. Tritiated thymidine (0.05 μg/ml, 83 μc/μg) was added to disks cultured for 4 weeks in medium XCS. As a control, we used disks cultured in medium X. After 3 days, the explants were fixed in 45 percent acetic acid, squashed, postfixed in ethanol, dehydrated, and coated with nuclear track emulsion (NTB-2). The cells were stained with Ehrlich hematoxylin after autoradiography. The nuclei of the control culture had only background levels of grain density. By contrast, more than half of the cells in the growing culture had a high density of grains over their nuclei (Fig. 2). The sizes of the cells in both cultures were comparable. This result indicates that the disks grow in vitro by normal cell division.

To test the differentiation capacity of disks grown by this in vitro method, three explants were each cut into disk-size fragments and injected into the abdominal cavity of metamorphosing larvae (*11*). Of 15 implants recovered, 13 contained normal patterns of differentiated adult structures. The other two had necrotic material. We identified structures derived from wing, haltere, and leg disks among the 13 differentiated implants. Figure 3 shows a part of one of these implants that contained wing structures.

Imaginal disk cells also grow in primary in vitro cultures derived from embryos (*10*), although they require a period of growth in vivo before they can differentiate normally (*12*). Our method differs significantly by starting with mature imaginal disks. This not only limits the range of cell types in each culture but will also permit the analysis in vitro of disks from pupal lethal mutants, which can first be distinguished from their nonlethal siblings during larval life.

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5. Falcon, catalog number 3010. To maintain adequate humidity, sponges, cut to fit the outer ring, were placed in the dishes and saturated with sterile *Drosophila* Ringer solution (*11*).
6. Altosid, formerly ZR-515, is isopropyl 11-methoxy-3,7,11-trimethyldodeca-2,4-dienoate. It is a product of Zoecon Corporation.
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Tris Buffer Attenuates Acetylcholine Responses in *Aplysia* Neurons

Abstract. *The commonly used buffering agent tris(hydroxymethyl)methylamine (tris) antagonizes the action of iontophoretically applied acetylcholine on neurons of* Aplysia californica. *Concentrations of 5 to 10 millimolar tris markedly reduced both excitatory and inhibitory responses.*

The buffering agent tris(hydroxymethyl)methylamine (tris) is widely used in biological solutions. In studying synaptic systems in *Aplysia*, we have found that tris at concentrations ranging from 5 to 10 m*M* is a potent inhibitor of both excitatory and inhibitory responses to iontophoretically applied acetylcholine.

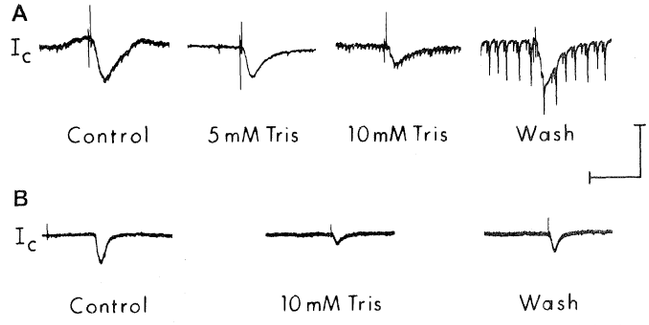
Experiments were performed on neurons found in the pleural and abdominal ganglia of *Aplysia californica*. Ganglia were dissected and pinned, according to standard procedures (*1*), in a bath containing Instant Ocean at *p*H 7.9. Cells were impaled with a single microelectrode for voltage clamping in a manner previously reported (*2*). A second electrode was placed near the cell to iontophoretically apply acetylcholine or dopamine to the neuron under study. To ensure that perfusion or drug action did not alter the position of the iontophoretic electrode relative to the cell, we made frequent visual observations and carefully measured the time to peak of the response. The tris used in these experiments was obtained from Sigma Chemical Company and from Fisher Scientific Co. Several different lots were used.

The data from the first experiment, shown in Fig. 1A, reveal the effect of tris on an excitatory response to iontophoresed acetylcholine. Cell R_{15} was voltage-clamped to −60 mv. Pulses of acetylcholine were applied as the cell was bathed in Instant Ocean at *p*H 7.9. Excitatory current responses are shown. The replacement of the control solution with Instant Ocean containing 5 m*M* tris at *p*H 7.9 attenuated the response. At 10 m*M* tris further reduced the response, and washing for 30 minutes allowed recovery of the current to control levels.

Figure 1B shows data from a similar experiment on an inhibitory response. In this case one of the medial cells from the left pleural ganglion was voltage-clamped to −70 mv (below the reversal potential for the inhibitory postsynaptic response in this cell) and a "reversed" inhibitory (inward) current was recorded as acetylcholine was pulsed. This response was strongly attenuated by 10 m*M* tris, but washing for 15 minutes produced a partial recovery. For both excitatory and inhibitory responses, the control response amplitude could be restored by increasing the strength of the acetylcholine pulse. Hence, the blocking action of tris on acetylcholine responses could easily have been missed in previous studies if the control levels were established in the presence of tris.

We considered the possibility that contamination of the tris was responsible for these effects. However, the experimental results shown above were repeatedly obtained with tris from different lots and different sources. Moreover, these results have been independently confirmed by Parmentier and Zbicz (*3*). Using the land snail *Helix aspersa*, they also observed that 10 m*M* tris produced marked attenuation of an excitatory response to iontophoresed acetylcholine.

The time required for tris to act varied somewhat from experiment to experiment. Measurable effects were often seen after 1 minute, but on some occasions they took up to 10 minutes. The abdominal ganglion neurons are invaginated by glial processes and are encased in a connective tissue sheath (*4*), both of which may reduce the availability of charged molecules to the neuronal surface. Since the pK_a of tris is 8.3 (K_a is the association constant), solutions near *p*H 8.3 contain both charged and uncharged forms of tris. Solutions with a higher *p*H contain more of the uncharged form, which might facilitate penetration.



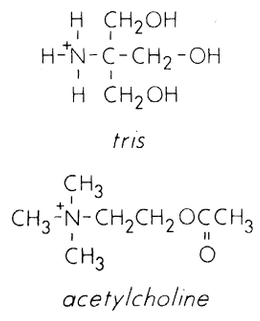


Fig. 1 (left). Voltage clamp data showing currents following iontophoresis of acetylcholine. Inward (depolarizing) currents are downward deflections. (A) Current underlying acetylcholine excitation of abdominal ganglion cell R_{15} clamped to −60 mv. Attenuated responses are obtained with 5 and 10 m*M* tris. Washing for 30 minutes restores the control response and elicits increased spontaneous synaptic activity. (B) Current underlying acetylcholine-induced inhibition of a neuron in the left pleural ganglion. This neuron was clamped to − 70 mv (below the reversal potential for the inhibitory response in this cell) and thus inward, rather than outward, currents are seen. Tris at 10 m*M* strongly attenuates the response, and washing for 15 minutes partially reverses the effect. Calibration: 15 seconds and 15 na in (A) and 36 seconds and 6 na in (B). Fig. 2 (right). Chemical structures of tris and acetylcholine. Tris is shown in its charged form.