The Permeability of *Drosophila melanogaster* Embryos

by

Catherine E. Watson
B. Sc. The University of British Columbia, 1987

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES
Department of Biochemistry

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

March 1990

Department of _BIOCHEMISTRY_

The University of British Columbia
Vancouver, Canada

Date _MARCH 29th, 1990_.

DE-6 (2/88)

ABSTRACT

Drosophila are used extensively for genetic, developmental and now molecular biology research. At present, germline transformation of these organisms can only be achieved by microinjection of P-element vectors into the pole cells of young embryos. The technique of microinjection however, requires a delicate touch and is quite laborious. Therefore, the development of a rapid and simple technique was investigated.

Electroporation, like microinjection, is a physical means of introducing DNA into a cell and is therefore potentially applicable to all cell types. Electroporation involves the use of an electrical current to create pores in the membrane of a cell. Macromolecules, such as DNA may enter a cell via these pores. Electroporation is a quick, reproducible, and efficient method for transforming cells. Through studies of the survival and permeability of *Drosophila melanogaster* embryos exposed to electrical currents, it was discovered that although the survival of the embryos decreased steadily as field strength increased, the embryos did not become permeable to a water soluble dye unless a pulse of 10 kV/cm was applied. Few embryos survived this extreme voltage required for dye uptake. Attempts to introduce DNA into dechorionated *Drosophila* embryos utilizing this technique however, produced no transformants. These results suggested that the remaining protective coatings of the dechorionated embryo were obstructing efficient pore formation, thus preventing DNA penetration.

In view of these results, methods to eliminate the wax layer, present between the chorion and vitelline membrane of laid eggs,

were examined. Wax removal by detergent solubilization, solvent extraction and melting by heating were investigated, yet did not produce a satisfactory procedure.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENT

I wish to extend my sincere gratitude to Dr. G.M. Tener for giving me the opportunity and support to carry out this work. I would also like to thank Dr. Ian Gillam for his helpful suggestions. To the newly knighted Dr. Nina Seto, thanks for all the discussions and encouragement. And a special thanks to those who dared to read this thesis in its early forms. To Hiron Poon, I hereby bequeath my bench space, and "sorry for not letting you sit at your desk half the time."

To those who have come into my life,
and
especially for those who left.

LIST OF ABBREVIATIONS

A	adenosine
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
Amp	ampicillin
BIM	basic incubation media
bp	base pair(s)
BSA	bovine serum albumin
C	cytidine
cm	centimetres
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dH_2O	distilled water
DNA	deoxyribonucleic acid
DNA Pol I	DNA polymerase I
DNase	deoxyribonuclease
DR	*Drosophila* Ringer's solution
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
g	grams
G	guanosine
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
hr(s)	hour(s)

IAA	isoamyl alcohol
K	1000 revolutions per minute
kb	kilobase(s)
kD	kilodalton(s)
kV	kilovolt(s)
L	litre(s)
M	molar
mA	milliampere(s)
μCi	micro curie(s)
μFd	microfaraday(s) (96,494 coulombs)
μg	microgram(s)
mg	milligram(s)
min	minute(s)
μl	microlitre(s)
ml	millilitre(s)
mM	millimolar
μm	micrometre(s)
msec	millisecond(s)
MW	molecular weight
ng	nanogram(s)
nm	nanometre(s)
PBSal	phosphate buffered saline
PBSuc	phosphate buffered sucrose
PBSuc TN	PBSuc with 0.05% TX-100 and 0.01% Nile Blue
PBSuc TX	PBSuc with 0.05% TX-100
r^{506}/r^{506}	*Drosophila melanogaster* strain homozygous for the *rosy* mutation number 506

RNase	ribonuclease
SDS	sodium dodecyl sulphate
SSC	standard saline citrate
τ	time constant
TB	terrific broth
TBE	tris-borate-EDTA
Tris	tris(hydroxymethyl)amino methane
tRNA	transfer RNA
TX-100	triton X-100
V	volts
v / v	volume per volume
w t	homozygous wild-type
w / v	weight per volume
XDH	xanthine dehydrogenase enzyme
Xdh	xanthine dehydrogenase gene
℃	degrees celsius

I. Introduction

A. *Drosophila* as an Organism for Scientific Research

One of the most widely studied organisms in science is *Drosophila* - the fruit fly. Its usefulness stems from the ease in which *Drosophila* stocks can be obtained and maintained. In addition, their relatively short life cycle and ease in mating, as well as their small genome and relatively few chromosomes make them an ideal organism for genetic studies. As a result, a vast amount of information on these insects has been accumulated. They have been used extensively for genetic and developmental studies and now in the growing field of molecular biology.

Studying the expression and regulation of a gene is of great interest to many scientists. Examination of the effects of altering the nucleotide sequence of a gene or its flanking region on the *in vivo* and *in vitro* expression is routinely performed in many laboratories. One problem commonly encountered however, is reintroducing the mutated gene back into the genome of some organisms. Unfortunately, *Drosophila* is a case in point.

B. *Drosophila* Transformation

1. The germline cells

Cloned DNA must be integrated into the germline to obtain stable transformation of a multicellular organism such as *Drosophila melanogaster.*

The *Drosophila* embryo undergoes a number of synchronous nuclear divisions without subsequent cell division during the early stages of embryonic development (figure 1). Shortly after
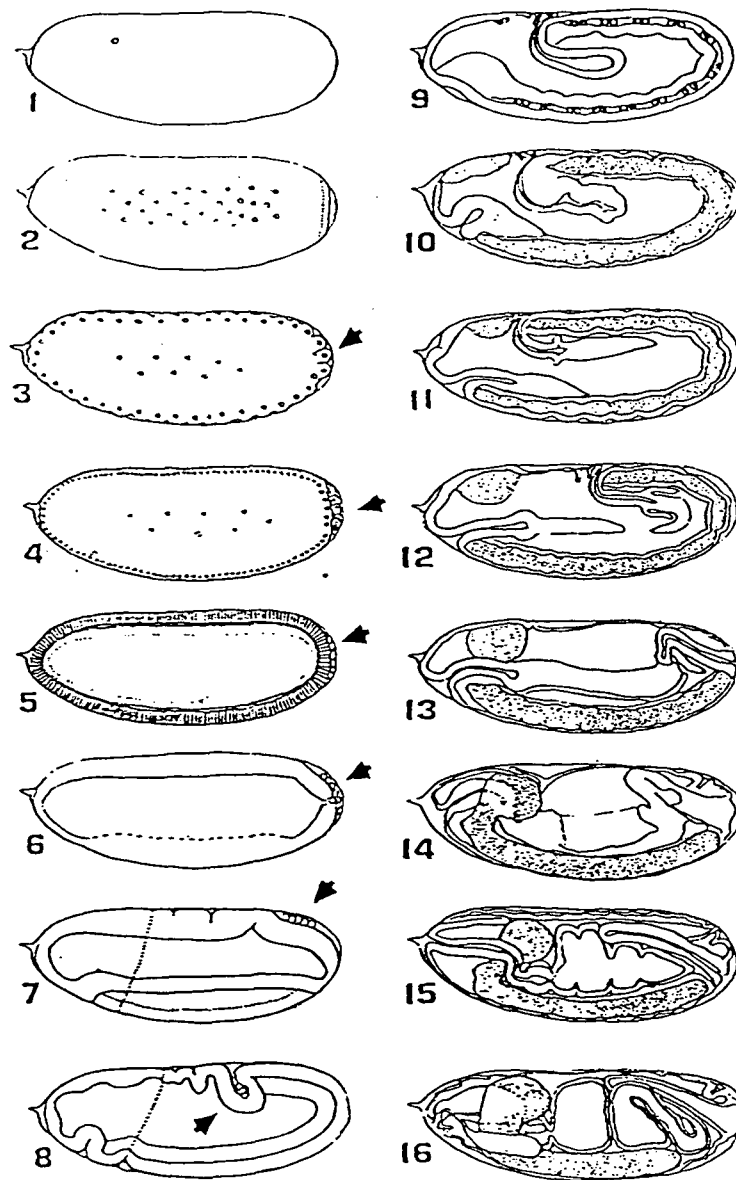
Figure 1.   Embryonic Development of *Drosophila*

Schematic diagrams of 16 stages of *Drosophila* embryogenesis.   The arrows trace the appearance and fate of the pole cells.   (Wieschaus and Nusslein-Volhard, 1986). Transformation must be achieved at or prior to stage 4.

fertilization,several nuclei migrate to the posterior end of the embryo, where they pinch off from the others, and form the pole cells. Subsequently, these pole cells are directed towards the interior of the embryo where they form the gonads of the fly. Consequently, to produce a line of stable transformants, one must introduce the exogenous gene into the pole cells prior to their internalization. This results in a restricted window of time during which transformation may occur. At room temperature, this 'transformation window' is approximately 90 minutes post fertilization.

2. Microinjection

At present, the only method available to introduce DNA into the pole cells of *Drosophila* is by microinjection. In this procedure, the preparation of the embryos is very important. The embryos are collected and dechorionated prior to pole cell formation. The embryos are mounted parallel to one another on double-sided tape with their posterior ends over the edge of the tape, and desiccated slightly. The extent of desiccation is critical since inadequate drying will cause cytoplasmic leakage upon injection, while excessive desiccation causes the embryo to shrivel up (Rubin and Spradling, 1982). Both states cause embryonic development to cease and are therefore unacceptable.

After optimal desiccation has been achieved, the embryos are covered with halocarbon oil (to prevent further water loss), and injected using a small needle (<1 mm in diameter). The sharp tip of the needle is used to pierce the embryo and the DNA solution

expelled (1-5% of the volume of the embryo) into the posterior pole of the embryo. The embryos are allowed to develop in a humid chamber and the hatched larvae are recovered and placed on standard food (Spradling and Rubin, 1982).

The microinjection procedure is most often done at reduced temperature (such as 18 °C) to slow down embryonic development, thereby extending the period of time in which transformation can occur. Also, the microinjections are performed in high humidity to reduce excess desiccation of the embryo. Microinjection however, requires great patience, considerable manual dexterity and is quite labour intensive. Transformation results achieved can vary greatly depending primarily on the expertise of the injector.

3. P-elements

The vectors used for transformation of *Drosophila* are derived from P-elements. P-elements belong to a family of *Drosophila* transposable elements, that are heterogeneous in length (0.5-2.9 kb), but homologous in sequence (O'Hare and Rubin, 1983). The intact (2.9 kb) P-element possesses perfect 31 bp terminal repeats, and three internal open reading frames (encoding a transposase, and presumably a transposase repressor). The smaller P-elements appear to have arisen from internal deletions of the larger element. These non-autonomous P-elements still retain their terminal repeats, and are therefore able to transpose, however they are shorter in length and are unable to produce their own transposase.transposition defective (O'Hare and Rubin, 1983).

The introduction of P-elements into a genetic background

lacking them (termed a M-cytotype), by microinjection (Rubin and Spradling, 1982) or by genetic means (Kidwell *et al* , 1977), produces a condition known as hybrid dysgenesis. This state is defined by such traits as, sterility, male recombination, visible and lethal mutations, reversion of mutations, and chromosomal re-arrangements and non-disjunction (Bregliano and Kidwell, 1983). This phenomenon is due to transposon jumping in the genome. When stable germline transformation is desired, a non-transposable P-element is used.

In germline transformation of *Drosophila*, the gene of interest is cloned into a defective P-element (containing a selectable marker if required). Since the element cannot induce its own transposition, a helper P-element is required to produce the transposase which is required for transformation. Alternatively, strains containing a non-transposable P-element can be used as recipients, thereby eliminating the requirement of the co-injection of the helper element. This increases the frequency of transformation since only the P-element construct must enter the pole cell.

Analysis of P-element transformants revealed transposon integration into random sites throughout the genome (Rubin and Spradling,1982). Although a 8 bp target sequence (GGCCAGAC) has been proposed as the target for P-element insertion (O'Hare and Rubin, 1983), it is not a stringent requirement. The transposed DNA shows little sign of deletions or rearrangement (Spradling and Rubin, 1982 and 1983).

Although microinjection is a relatively efficient method of

transformation of *Drosophila*, giving results ranging from < 1 to 3% of injected embryos (Scholnich *et al* , 1983; Wakimoto *et al* , 1986), the method is extremely tedious. Hence, a new method which is quick and simple, would be preferred. Electroporation has that potential.

C. Electroporation

In 1982, Neumann *et al.* demonstrated that DNA could be introduced into cells by applying an electrical current across a cell suspension containing DNA. This process has since been termed electroporation (or electroinjection). The theory behind this technique is quite simple and stems from observations made by Zimmermann *et al.* (for review see Zimmermann, 1982). When an electrical current is applied to a suspension of membranes, an electrical potential (V) is set up across the membrane. If this potential exceeds the inherent electrical potential of the membrane (V_{max}), reversible membrane breakdown will occur (Zimmermann, 1982).

$$V_{max} = 1.5 \; E_o r \cos \theta \qquad \text{(Equation 1)}$$

$$\text{where} \quad V_{max} = \text{electric potential}$$

$$E_o = \text{field strength (V/cm)}$$

$$r = \text{cell radius}$$

$$\cos \theta = \text{angle between the membrane and}$$

$$\text{the field direction}$$

The lipid molecules become momentarily disorganized and form holes or pores in the bilayer upon reorganization (figure 2). The phospholipids continue to move, closing the pore and re-establishing the integrity of the membrane. If the electrical potential set up
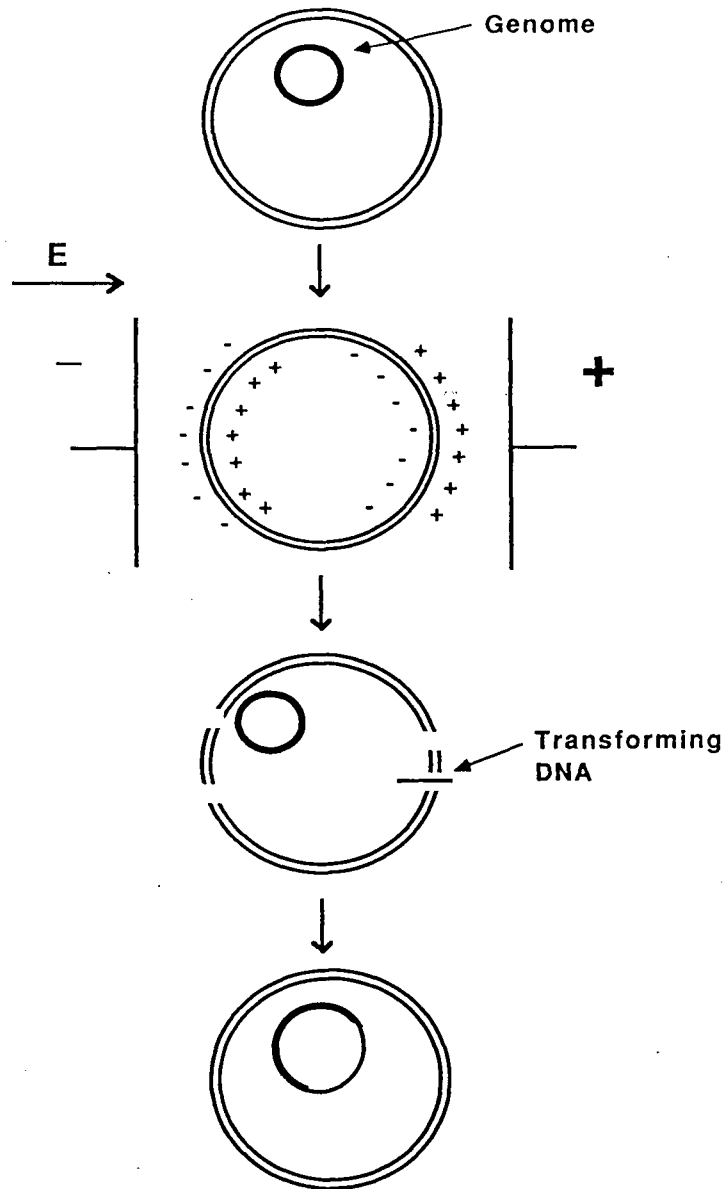
**Figure 2.   Pore Formation and DNA Integration
During   Electroporation**

When a cell is subjected to an electrical field (E), an electrical potential is established across its membrane.   If the potential exceeds the critical potential of the membrane, the lipids become disorganized and upon reorganization form pores.   It is through these pores that the DNA can possibly enter and become integrated into the genome of the cell.   The pores in the membrane gradually re-seal, re-establishing the integrity of the membrane.

across the membrane exceeds a critical value, the membrane undergoes irreversible breakdown resulting in cell death.

DNA can enter the cells through these transient pores and be incorporated into the genome (Toneguzzo *et al.*, 1988). A positive aspect of this mass microinjection, is that the DNA does not enter via pinocytosis or endocytosis and therefore is not subjected to the many degradative enzymes in the lysosomal vesicles (Toneguzzo *et al*, 1986). As a result, the transferred DNA is rarely damaged, as can be the case for chemical transformation procedures (Potter, 1988). In addition, results seem to indicate that the DNA integration is random. If multiple inserts occur they do so at distinct loci, not as tandem arrays. When co-integration of two markers is desired (as may be the case with P-element transformation), electroporation of mammalian cells typically gives between 23-77% of transformed cells as cointegrates (Toneguzzo *et al.*, 1988).

Electroporation has been used successfully for transformation of a wide variety of cell types including cultured cell lines, mammalian primary cell cultures, mammalian embryonic stem cells, isolated intercellular vesicles, dicot and monocot plant cells, trypanosomes, and a variety of bacteria (for review see Andreason and Evans, 1988). The universal applicability of electroporation stems from the fact that it is a physical means of DNA introduction - a mass microinjection, and therefore does not rely on the unique properties of the cell like other procedures do.

Unfortunately, to optimize transfer efficiency, many physical and biological parameters must be investigated for each cell type.

The two most important parameters are field strength and pulse duration. Their optimization will determine the number, size and length of pore opening and hence the permeability of the cell to macromolecules. The importance of field strength in the reversible membrane breakdown required for pore formation was mentioned above (see Equation 1). Once V_{max} has been exceeded, further increases in field strength cause additional pore formation over a wider surface area of the cell (related to $\cos \theta$). The number and size of the pores increase until the applied field strength creates such a great potential across the membrane that irreversible membrane breakdown occurs.

The voltage stored in a capacitor decreases exponentially when the capacitor is discharged. The time required for the peak voltage to decrease to 1/e is called the time constant (τ) and is used to compare pulse lengths. τ is dependent on two variables: the size of the capacitor that is discharged (larger capacitors require more time to release their charge) and the resistance of the media through which the electricity is discharged. The resistance in turn depends on the ionic strength of the solution (higher ionic strength results in lower resistance and hence a shorter τ) and cuvette geometry. An increase in τ results in the pores remaining open longer.

The disadvantage of electroporation is the many parameters that must be examined initially to optimize transfection. Other parameters which must be optimized include:

DNA concentration, topology and method of preparation

cell concentration and growth conditions

pulse wave shape

buffer composition

temperature

incubation time (before and after the pulse)

(Potter, 1988).

The preliminary work can be extensive and determining the ideal conditions labour intensive; however once perfected, the procedure is rapid and simple.

D. Xanthine Dehydrogenase

1. The *rosy* locus

The *rosy* locus of *Drosophila melanogaster* is located on the right arm of chromosome 3, at position 87 DE. This locus encodes the enzyme Xanthine Dehydrogenase (XDH). Flies lacking this protein (*rosy* ⁻ mutants) possess dull, dark red-brown eyes as opposed to the bright red eyes of wild-type (wt) flies. In addition to converting hypoxanthine into uric acid, XDH also oxidizes 2-amino-4-hydroxypterin to isoxanthopterin, as well as catalyzing many other reactions involving pteridines. As a result, *rosy* ⁻ mutants accumulate the substrates hypoxanthine and 2-amino-4-hydroxypterin (Hadron and Schwink, 1956; Mitchell *et al.*, 1958) and the pteridines, biopterin, and sepiapteridine (Hadron and Schwink, 1956; Graf *et al.*, 1959). The absence of this gene does not greatly impair fly survival under normal conditions. However, if the growth temperature is increased to 29 °C, significant increase in pupal

mortality is observed (Glassman, 1965).

The *rosy* locus has been studied extensively. A host of spontaneous, chemical and radiation induced mutants at this site have been isolated, and intensive fine structure analysis of the region has been performed (Cote *et al.*, 1986). In 1987, the *Xdh* gene was sequenced and the intron/exon boundaries defined (Lee *et al.*, 1987; Keith *et al.*, 1987). The *Xdh* gene contains 4 exons and encodes a 1335 amino acid protein of 147 kD. The r^{506} mutation is a 3.4 kb deletion in the coding region of the gene. It encompasses the last one-third of the second exon, the entire third and fourth exon as well as about a kb of 3' flanking DNA (see figure 12). XDH is a soluble protein that functions as a homodimer.

2. Purine selection

The presence or absence of XDH activity in *Drosophila* can be determined by growth on food supplemented with purine (Finnerty *et al*, 1970). The *rosy* ⁻ individuals succumb prior to eclosion, while wt flies develop normally. The mode of purine toxicity is unclear. Purine does not appear to be a substrate for XDH, yet it inhibits the conversion of hypoxanthine to xanthine. As a result, the hypoxanthine accumulates in the malpighian tubules and causes the death of the fly (Glassman, 1965). The concentration of purine required for selection is proportional to XDH activity. Hence, by titration of the purine concentration, *rosy* ⁻ mutants with decreased enzymes activity can also be distinguished from null mutants.

E.   The *Drosophila*   Eggshell

1.   The composition of the eggshell

Drosophila   embryos are unlike the simple,   membrane bound cells typically transformed by electroporation.   They possess many outer protective layers, which together allow the penetration of sperm and the exchange of respiratory gases, yet still shield the embryo from mechanical injury and desiccation.   Protective layers present in plant cells, yeast and gram positive bacteria are removed prior to transformation by electroporation.

The outer protective coating of *Drosophila*   embryos, termed the eggshell,   consists of five distinct layers   (figure 3;   Margaritis *et al.*, 1980).   From the outer most, they are:

exochorion   (300-500 nm)

endochorion   (500-700 nm)

innermost   chorionic   layer (40-50 nm)

'waxy' layer (0.50 nm)

vitelline   membrane   (300 nm)

The exo and endochorions are composed primarily of protein. The exochorion is a dense protective coat, while the endochorion is composed of a rigid network of cavities.   These cavities are filled with air after ovulation and together with the respiratory appendages are responsible for gas exchange (Margaritis *et al*, 1980). The innermost chorionic layer is polycrystalline in nature, though its function is yet undefined.   These three outermost layers are easily removed with a short hypochlorite treatment (Hill, 1945), leaving what is termed a dechorionated embryo.

VIEW B (from outside)

exochorion

endochorion

VIEW A (cross-sectional)

inner chorionic layer

wax layer

VIEW C
(from inside)

vitelline membrane
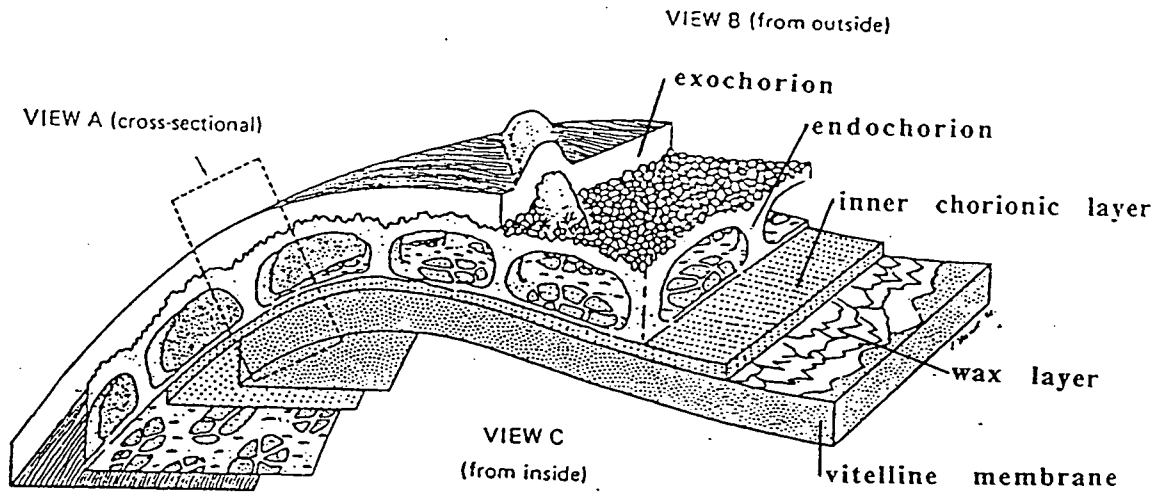
**Figure 3.   The Main Layers of the *Drosophila*
Eggshell**

A three dimensional representation of a fragment of the
Drosophila eggshell main body indicating the relative orientation of
various 2-dimensional views. (Margaritis *et al.* , 1980)

Probably the best physical evidence for the existence of the wax layer comes from Margaritis *et al.* (1980), who used ultrastructural techniques (transmission, scanning and freeze-fracture electron microscopy) to examine the eggshell of *Drosophila*. Through their studies, they revealed the presence of a thin layer of hydrophobic plates which were devoid of proteins or other large molecules, and which produced smooth fracture faces during freeze-fracture studies.

The vitelline membrane is the innermost barrier of the embryo. It is highly protienaeous (74%), consisting primarily of Alanine (29%), Proline (18%), and Serine (17%). The proteins are cross-linked via bonds between the meta carbons of tyrosine. Therefore, the vitelline membrane cannot be dissolved easily (Petri *et al.*, 1976; Fargnoli and Warning, 1982).

2. Embryo permeability

Drosophila embryos are essentially a closed system, impermeable to everything except respiratory gases and water vapour. Even dechorionated, the embryos remain impervious to water soluble molecules. The primary reason for this is believed to be a the thin wax layer which is laid down between the chorion and the vitelline membrane just prior to ovulation (Davies, 1947). This layer has been compared to an insects cuticle because of their functional similarity in waterproofing, but the two layers originate from different cell types (Cummings *et al.*, 1971). The existence of this layer prohibited embryo fixation as well as *in vivo* studies of metabolism and protein synthesis which required the

uptake of water soluble molecules. Therefore, methods to remove this barrier were investigated. Three methods that were found to remove the wax layer (thus making the embryo permeable to water soluble molecules) were detergent solubilization, solvent extraction and heat removal.

Detergents such as Triton-X 100 (TX-100) and sodium dodecyl sulphate (SDS) have been shown to solubilize the wax layer, thus making the embryo permeable to water soluble molecules (Eudy *et al.*, 1969; Sayles *et al.*, 1973). The detergents are able to displace the wax molecules and form micelles, thus removing the wax from the surface of the embryo.

Solvents such as heptane and octane have also been used to increase the permeability of *Drosophila* embryos (Limbourg and Zalokar, 1973). Since the hydrophobic wax molecules are more soluble in the organic solvent than in an aqueous environment, the solvent extracts the wax from the embryo surface, leaving an embryo which is freely permeable to small water soluble molecules.

Evidence for the presence of a wax layer was first derived from observations that heating the *Drosophila* embryo makes it very sensitive to the osmotic state of the incubation buffer (King and Koch, 1963). For example, an embryo placed in a saturated saline solution developed normally, unless the solution was heated to 45 °C. The increased temperature caused the embryo to lose water rapidly in the hypertonic solution, shrivels up and cease development. This observation implies that the wax barrier melted, thus resulting in an embryo that is extremely permeable to water.

F. Scope of this Thesis

The original goal of my thesis was to develop electroporation as an alternative method for the germline transformation of *Drosophila*. However, the protective layers of the dechorionated embryo (especially, the wax layer present between the chorion and the vitelline membrane) proved a greater obstacle to the efficient formation of electrically induced pores than anticipated. Therefore, methods to remove this layer without harming the embryo were investigated. The approaches examined included, detergent solubilization, solvent extraction and temperature effects. The results of both the electroporation studies and the wax removal experiments are presented here.

II. MATERIALS and METHODS:

A. Materials

All reagents and chemicals were purchased from MCB (Matheson Coleman and Bell) Reagents, Aldrich Chemical Company, BDH Inc., Fisher Scientific Company, or Nichols Chemical Company Ltd. The antibiotics, lysozyme, spermine, spermidine, BSA, and purine were purchased from Sigma Chemical Company. The Bacto-tryptone, Bacto-agar, and yeast extract were obtained from Difco. The soy flour for the fly food was made by Stone-Buhr, the agar from USBC, the glucose and sucrose from BDH and BC Sugar respectively, and the methyl-p-hydroxybenzoate from BDH. The double-sided tape was made by 3M and the Miracloth by Calbiochem. The electroporators used were either a Bio-Rad gene pulser equipped with a capacitance extender or a BRL Cell-Porator. The *E. coli* DNA Pol I, Bam H1 and the electrophoresis grade agarose were acquired from Pharmacia. The herring sperm DNA was a gift from Dr. D.A. Sinclair. The nylon membranes (Hybond N) were by Amersham. Nile Blue, Methyl Red and Blue Dextran were purchased from Allied Chemical, MCB, and Pharmacia respectively. The pentane and heptane were from BDH, while the decane was from Sigma. The detergent TX-100 was purchased from J.T. Baker Chemicals Co.

B. Buffers and Solutions

The composition of the buffers and solutions used were as follows:

Basic Incubation Media (BIM): 9 mM $MgCl_2$, 10 mM $MgSO_4$, 3 mM NaH_2PO_4, 68 mM glutamic acid, 67 mM glycine, 4.1 mM malic

acid, 0.1 mM Sodium acetate, 10 mM glucose, 5.5 mM $CaCl_2$. (Limbourg and Zalokar, 1973)

50 X Denhardt's: 10 g/L Ficoll, 10 g/L polyvinylpyrolidone, 10 g/L BSA.

Drosophila lysis buffer: 100 mM Tris-HCl (pH 8.0), 50 mM NaCl, 50 mM EDTA, 0.15 mM spermine, and 0.5 mM spermidine.

Drosophila Ringer's (DR): 110 mM NaCl, 1.9 mM KCl, 2.4 mM $NaHCO_3$, 0.8 mM $CaCl_2$, 0.07 mM NaH_2PO_4.

6 X Gel Loading Buffer: 0.25% bromophenol blue, 0.25% xylene cyanol, and 15% Ficoll 400.

Hepes Buffered Saline (HBSal): 21 mM HEPES (pH 7.05), 137 mM NaCl, 5 mM KCl, 0.7 mM NaH_2PO_4, 6 mM glucose.

10 X Nick Translation Buffer: 0.5 M Tris-HCl (pH 7.2), 0.1 M $MgCl_2$, 1.0 mM DTT, 500 µg/ml BSA.

Nick Translation Elution Buffer: 10 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.25 mM EDTA.

Phosphate Buffered Saline (PBSal): 2.6 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 137 mM NaCl.

Phosphate Buffered Sucrose (PBSuc): 0.272 M sucrose, 7 mM potassium phosphate [1.47 mM KH_2PO_4, 5.53 mM K_2HPO_4, (pH 7.4)], 1 mM $MgCl_2$.

PBSuc TN: PBSuc, 0.05% TX-100, and 0.01% Nile Blue.

PBSuc TX: PBSuc and 0.05% TX-100.

20 X SSC: 3 M NaCl, 0.3 M sodium citrate pH 7.0.

TBE: 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA (pH 8.0)

TE8: 10 mM Tris-HCl, 1 mM EDTA, (pH to 8.0)

C.   DNA   Amplification   and   Purification

1.   Bacterial   strains

The *E.coli* strain DH5α was obtained from Dr. D.A. Sinclair and kept as frozen stocks (1 ml aliquots in 15% glycerol at -70 °C). The bacteria were grown on SOB (2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$), SOC (SOB supplemented with 20 mM glucose) or TB (12 g/L Bacto-tryptone, 24 g/L yeast extract, 4% glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4) broth or plates (15 g of Bacto-agar/litre of broth). Plasmid selection was achieved by addition of Ampicillin [25 μg/ml (broth) or 100 μg/ml (plates)]

2.   Vectors

The vectors pCarnegie 20 (Rubin and Spradling, 1983) and pπ25.1 (Spradling and Rubin, 1982) were obtained from Dr. C.H. Newton. pCarnegie 20 contains a full length P-element inserted into pUC8. The P-element possesses a polylinker into which the 7.2 kb Hind III fragment of the *rosy* gene was cloned. pπ25.1 is a vector containing a 2.9 kb P-element and approximately 1.8 kb of flanking *Drosophila* DNA cloned into the Bam HI site of pBR322.

3.   Bacterial   transformation

The plasmids were transformed into *E. coli.* DH5α competent cells essentially as outlined by D. Hanahan (1985), with the following modifications. A 200 μl aliquot of frozen competent cells was thawed, 2-4 ng of plasmid DNA was added and the solution set on ice for 30 min. The cells were given a heat shock (90 seconds at 42 °C) and immediately placed back on ice. SOC (800 μl) was added and the suspension incubated at 37 °C for 45 min. The bacteria were plated

on SOB-Amp plates and grown overnight at 37 °C. A liquid culture was then prepared by inoculating 5 ml of TB-Amp with a single isolated colony from the plate and grown at 37 °C for 12-16 hrs. 1 ml stocks were prepared in 15% glycerol and stored at -70 °C.

4. Plasmid isolation

    a. Mini preps

Plasmid DNA was isolated from overnight cultures (5 ml of TB-Amp inoculated with a single isolated colony and grown at 37 °C for 12 hrs) by the alkaline lysis method (Birnboim and Doly, 1979) as follows: 100 μl of ice cold Glucose-Tris buffer [50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), and 4 mg/ml lysozyme] was added to the pellet of 1.5 ml of culture. After a 5 min. incubation at room temperature, 200 μl of fresh, ice cold NaOH solution (0.2 N NaOH, and 1% SDS) was added, the tube inverted to mix, then placed on ice for 5 min. Subsequently, 150 μl of ice cold KOAc solution (3 M potassium, and 5 M acetate) was added, the tube vortexed gently in an inverted position and incubated on ice for 5 min. The solution was spun for 5 min. in a microfuge.

The supernatant was transferred to a fresh tube and extracted with one-half volume of Tris-equilibrated phenol [phenol extracted repeatedly with 0.1 M Tris-HCl (pH 8.0) until the pH of the phenol was above 7.6] and one-half volume of chloroform. The aqueous phase was transferred to a fresh tube and the DNA precipitated with 2 volumes of cold 95% ethanol for 10 min. The DNA was pelleted, washed twice (95% then 70% ethanol), dried and finally resuspended in 20-50 μl of TE8 [10 mM Tris-HCl, 1 mM EDTA, (pH 8.0)] containing 20 μg/ml RNase A.

b.   Large scale plasmid isolation

A 500 ml culture was prepared by inoculating TB-Amp with 0.5 ml of a 5 ml culture, and incubating at 37 °C overnight. The cells were pelleted in 250 ml tubes (6 K for 5 min. in a GSA rotor), the supernatant poured off, and 5.0 ml of cold Glucose-Tris buffer (all solutions are the same as for the mini preps) added. The suspension was transferred to a fresh centrifuge tube to which 20 mg of lysozyme was added and the tube incubated at room temperature for 5 min.   10 ml of cold NaOH solution was then added, the solution mixed by inversion and placed on ice for 10 min.   7.5 ml of cold KOAc solution was then mixed in by vortexing gently, and the solution was centrifuged (10 K for 20 min. at 4 °C, in a SS34 rotor).

The supernatant was transferred to a fresh tube and extracted with 8.0 ml of Tris-equilibrated phenol and 2.0 ml of chloroform for 10 min. on a rotating wheel. The extraction was followed by centrifugation (10 K for 10 min. at 15 °C, in a SS34 rotor). The aqueous phase was transferred to a fresh tube, the nucleic acids precipitated (0.6 volume of propanol-2 at room temperature for 10 min.), and pelleted by centrifugation (8 K for 10 minutes at 20 °C, in a SS34 rotor). The pellet was washed with 95% ethanol, dried, then resuspended in 400 µl TE8 supplemented with 200 µl of 250 mM EDTA.

CsCl (4.2 g) and 300 µl of EtBr (10 mg/ml) were added, the solution vortexed and incubated on ice (in darkness) for 15 min. The RNA was pelleted by centrifugation (10 K for 10 min. at 4 °C, in a SS34 rotor).   An additional 200 µl of EtBr was added to the supernatant, and the solution transferred to a Beckman quick-seal

centrifuge tube. After centrifugation (50-55 K for 12-17 hrs at 20 °C, in a VTi65 rotor), the lower of the 2 resulting bands was withdraw with a 1 ml syringe equipped with a 21 G 1/2 needle. The solution was placed in a tube to which 2 volumes of dH_2O were added. Extraction of the EtBr was then performed with water-saturated butanol (8-9 extractions), the final aqueous phase being transferred to a 30 ml Corex tube and the DNA precipitated with 0.1 volume of 2.5 M Sodium acetate (pH 5.2), and 2 volumes of cold 95% ethanol overnight at -20 °C. The tube was centrifuged (5 K for 20 min. at 4 °C, in a SS34 rotor), the pellet washed once with 95% ethanol and twice with 70% ethanol, dried, then resuspended in 200-400 µl TE8 and transferred to a microcentrifuge tube. The DNA was reprecipitated with 0.1 volume of 2.5 M Sodium acetate (pH 5.2) and 2.5 volumes of cold 95% ethanol, pelleted by centrifugation (10 min. in a microfuge). The pellet was washed once with 95% ethanol and once with 70% ethanol, dried, and finally resuspended in 50-100 µl TE8. Purity of the DNA was determined by A 260/280 nm and mini gel electrophoresis.

D. Southern Analysis of *Drosophila* Genomic DNA

1. Rapid phenol extraction of genomic DNA

Genomic DNA was extracted from flies as outlined in Jowett (1986), with the following modifications: 200-400 flies (equal numbers of males and females) were ground to a powder in a small mortar containing approximately 2 ml of liquid nitrogen. The resulting powder was scraped into a 30 ml Corex tube containing 1

ml/100 flies of lysis buffer [100 mM Tris-HCl (pH 8.0), 50 mM NaCl, 50 mM EDTA, 0.15 mM spermine, and 0.5 mM spermidine], and 10 µl/100 flies of Proteinase K (10 mg/ml in 50% glycerol) was added. The solution was then incubated at 37 °C for 2 hrs (with occasional mixing), extracted once with 1 volume of Tris-equilibrated phenol, then twice with one-half volume Tris-equilibrated phenol and one-half volume chloroform/isoamyl alcohol (IAA) (10:1), and finally with one volume of the chloroform mixture.

The resulting aqueous phase was transferred to a tube containing 0.1 volumes of 2.5 M Sodium acetate (pH 5.2) and 2 volumes of cold 95% ethanol. The DNA was precipitated at -20 °C for 1-2 hrs. The DNA was pelleted (8 K for 10 min. at 4 °C, in a SS34 rotor), the pellet briefly dried in a vacuum desiccator and redissolved in 400 µl TE8. After transfer to a microcentrifuge tube, 100 µg/ml of RNase A was added and the tube incubated at 37 °C for 30 min. Extractions with phenol/chloroform and chloroform alone were performed. The DNA was precipitated, pelleted, washed twice with ethanol (95% then 70%), dried as before, and finally resuspended in 50-100 µl TE8. The concentration of the DNA was estimated by assay on a mini agarose gel using bacteriophage λ DNA as a standard.

2.   Enzymatic digestion of genomic DNA

Restriction enzyme digests of *Drosophila melanogaster* genomic DNA were usually carried out in a total volume of 200 µl. Approximately 2-5 µg of DNA were digested with 2-3 units/µg of restriction enzyme using the buffer supplied by the manufacturer

supplemented with 0.5 mM spermine) at 37 °C for 2-5 hrs (complete digestion was monitored by loading 10 μl and 2 μl loading buffer on a 0.7% mini agarose gel). The restricted DNA was then ethanol precipitated, washed, dried and resuspended in 15 μl TE8. Loading buffer (5 μl) was added, and the DNA loaded on a 0.7% agarose gel (1 X TBE, 0.7 mg/ml EtBr). The gel (25 x 20 cm) was run for 10 min. at 100 V, then at 30-50 V for 18-24 hrs.

3.   Southern Analysis

   a.   Southern transfer

The DNA in the agarose gel was transferred to a nylon membrane by the method of Southern (1975). The gel was covered with denaturing solution (1.5 M NaCl, 0.5 M NaOH) and incubated at room temperature for 1 hr (with gentle shaking). The gel was rinsed with dH$_2$O, then submerged in neutralizing solution (1 M Tris-HCl (pH 8.0), and 1.5 M NaCl) for 1 hr(room temperature and gentle shaking), and rinsed again. The gel was placed on a piece of filter paper supported by a glass plate or a sponge in a dish of 10 X SSC . A nylon (Hybond-N) filter cut to size, 2-3 pieces of filter paper soaked in 10 X SSC, a 2-3 inch stack of paper towels, a glass plate and finally lead weights were place on top of the gel. The transfer was allowed to proceed at room temperature for 8-12 hrs. The filter was removed, rinsed in 6 X SSC, wrapped in Saran Wrap®, exposed to UV light for 3 min. and stored at -20 °C till required.

   b.   Preparation of nick-translated probe

A 50 μl solution containing the following: 5 μl 10 X Nick Translation buffer, 2.5 mg/ml BSA, 1.0 μl 0.5 M β—mercaptoethanol,

2.0 µl 0.5 mM dGTP, dTTP, and dCTP, 2.0 µl 35 µM dATP, 3-5 µl [α–^{32}P]dATP, 0-1 µl DNase I (10 pg/µl), 1.0 µl 10 mM CaCl$_2$, 1 µg plasmid DNA, and 10 units *E. coli.* Pol I was incubated at 15-16 °C for 1.5 hrs. The reaction was stopped by the addition of 150 µl of 1% SDS/10 mM ETDA. The solution was heated at 65 °C for 10 min., then 5 µl *E. coli* tRNA (10 ng/ml) were added. The probe was loaded onto an LKB Ultragel AcA 54 column, and eluted with Nick Translation Elution Buffer [10 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.25 mM EDTA]. Six-drop fractions were collected while monitoring the separation with a Geiger counter. The tubes containing the most incorporated radioactive were pooled, their combined volume measured and the radioactivity assessed by the Cerenkov method. Incorporation of 10^7 cpm/µg DNA was typical.

c.   Hybridization of filters

The genomic DNA laden filter was placed in a heat sealable bag with 10 ml of Prehybridization solution (6 X SSC, 0.5% SDS, 5 X Denhardt's, and 100 µg/ml denatured herring sperm DNA) prewarmed to 68 °C. The bag was sealed and the filter incubated at 68 °C for 4 hrs. The nick-translated probe was then added to the bag, the bag resealed and incubated at 68 °C for a further 12-18 hrs.

d.   Washing of filters

The filter was removed from the bag, placed in a solution of 1 X SSC and 0.5% SDS prewarmed to 68 °C and incubated at 68 °C for 45-60 min. The solution was changed and a further 1 hr incubation was performed. Two 1-hour washes in 0.1 X SSC and 0.5% SDS at 68 °C were then done, and the filter was wrapped in Saran Wrap$^®$ and

exposed to X-ray film for 1-3 days depending on radioactivity retained on the filter.

E. *Drosophila* Embryos

1. *Drosophila melanogaster* strains

Isogenic wild-type Oregon R flies were obtained from Dr. G.M. Tener. The r^{506}/r^{506} mutants were obtained from V.K. Lloyd (UBC). Both strains were maintained in sponge stoppered 160 ml Corning® glass dilution bottles containing 30 ml of enriched fly food (1 litre of tap water, 100 g soy flour, 20 g yeast extract, 17 g agar, 1 g citric acid, 9 g trisodium citrate, 40 g glucose, 40 g sucrose, 17 ml 10% methyl-p-hydroxybenzoate in 95% ethanol, and two of the following antibiotics: 20 mg streptomycin, 10 mg tetracycline, or 30 mg ampicillin).

2. Embryo collection and dechorionation

Flies 5-8 days old were placed in small milk bottles. The opening was covered by a small Petri dish containing 1% agar lightly spread with yeast paste (yeast powder with 2% acetic acid and 5% ethanol) placed inside an adapter. The collection plates were changed every 25-35 minutes (after an initial 2-3 hour prelay where they were changed about every hour). The embryos were loosened from the agar with dH_2O and a paint brush, and poured into a 1.5 cm diameter buchner funnel lined with a disk of Miracloth. The embryos were dechorionated by washing them with 3% sodium hypochlorite (Javex or Sunbrite), then were rinsed with dH_2O.

3.   Assessment of embryo survival

The ratio of the number of first instar larvae which hatched within 48 hrs post treatment to the total number of embryos recovered after treatment was used to assess embryo survival. Survival to subsequent life cycle stages was evaluated by transferring of the larvae to vials of enriched food and the number of adults which pupated and eclosed counted. Fertility was determined by mating individual virgins to 5 virgins of the opposite sex.   Matings which produced progeny were deemed to be from fertile individuals.

4.   Assessment of embryo permeability

Embryo permeability was assessed by their ability to take up a water soluble dye such as Nile Blue or Methyl Red.   Embryos that became visibly coloured, were deemed permeable.   The percentage of permeable embryos was calculated by dividing the number of coloured embryos by the total number of embryos recovered after treatment.

5.   Tape-mounted embryos

Two layers of double-sided tape cut into thin (1 mm) strips were placed on a glass slide.   The Miracloth containing the dechorionated embryos was placed on the slide and the slide placed in the bottom half of a petri dish lined with a disk of filter paper moistened with dH_2O or PBSuc. (This dish when covered with a lid is referred to as a humid chamber.)   The embryos were then mounted side by side on the tape in groups of 25-50, using a paintbrush.   The lid was then placed on the. dish.   Individual tapes were treated as

desired, placed on a new slide, covered with halocarbon oil and the slide then placed back in a humid chamber.

6.   Non-mounted embryos

Dechorionated embryos were washed off the Miracloth with the appropriate buffer (containing 0.05% TX-100) into a cuvette. After the desired treatment, the solution containing the embryos was poured out of the cuvette into the buchner funnel lined with a fresh disk of Miracloth, the cuvette rinsed out thrice with buffer and the embryos rinsed briefly with buffer. The cloth was then placed on a slide, covered with halocarbon oil and placed in a humid chamber, or put directly onto the moistened filter paper in the chamber.

a.   Detergent-treated embryos

Dechorionated embryos were washed off the Miracloth with PBSuc (containing the desired amount of TX-100), into a 0.2 cm cuvette. The embryos were incubated for 20 minutes, then collected on Miracloth, rinsed with buffer and placed in a humid chamber (PBSuc). The embryos were allowed to develop at room temperature.

b.   Solvent-treated embryos

Dechorionated embryos were washed off the Miracloth into a 1 cm diameter cup made of 100-mesh stainless steel and the cup submerged in about 2 ml of solvent for the desired time. The solvent was drawn out of the cup with paper towels, and the embryos rinsed with buffer. The embryos were then rinsed out of the cup and into the lined funnel with buffer, and the embryos

treated as above (non-mounted embryos).

c.   Heat-treated embryos

Dechorionated embryos were washed into a 0.2 cm cuvette with 500 µl of PBSuc TN. The cuvette was placed in a water bath at a specified temperature, and the solution incubated for 20 minutes. The embryos were then collected on Miracloth, rinsed with PBSuc, and placed in a humid chamber (PBSuc). The embryos were allowed to develop at room temperature.

7.   Electroporation of embryos

Dechorionated embryos were either rinsed off the Miracloth into a 0.2 or 0.4 cm cuvette with the appropriate buffer, or tape-mounted and the tape placed down the side of the cuvette. The embryos were preincubated at room temperature for 1-5 minutes, pulsed at the desired field strength and capacitance, then incubated a further 1-15 minutes at room temperature (any embryos not in solution were removed). The suspended embryos were collected on Miracloth, and rinsed with buffer. The tape-mounted embryos were removed from the cuvette, the tape placed on a slide, and the embryos covered with halocarbon oil. Both sets of embryos were then placed in a humid chamber (PBSuc) and allowed to develop at room temperature.

F.   Purine   Selection

1.   Purine titre

Vials containing five male and ten female flies of the following strains were prepared:

1. +/+ X +/+
2. r^{506}/r^{506} X r^{506}/r^{506}
3. r^{506}/r^{506} X +/+

The first two crosses produce homozygous wt and r^{506} F_1 flies respectively, while the third cross produces F_1 $+/r^{506}$ heterozygotes. The vials each contained 5 ml of enriched food. The flies were left to lay embryos for 2-3 days then transferred to a fresh vial. 100 µl of purine solution (the desired quantity of purine (w/v) dissolved in dH_2O) was added to the vacated vial. The flies were transferred every 2-3 days for 8 days. The vials were left at room temperature, and the number of adults which eclosed from each vial was tallied.

2. Purine selection of putative transformants

Ten males and 20 females from each putatively transformed line were mated in glass vials containing 5 ml of enriched food. The flies were allowed to lay embryos for 2 days then were transferred to a fresh vial. 100 µl of purine (0.2% (w/v)) was added to the vacated vial. The flies were transferred every 2 days for 8 days. The embryos were allowed to develop at room temperature. Lines surviving the purine selection process were scored as containing a wild-type *Xdh* gene.

III. RESULTS and DISCUSSION

A. Effect of the Exposure of *Drosophila* Embryos to an Electrical Current

1. Survival of embryos exposed to increasing field strengths

Dechorionated embryos were exposed to a range of electrical field strengths. Increases in the field strength lead to corresponding decreases in the ability of the embryos to survive subsequent to the pulse (figure 4). The survival decreased fairly steadily over the range investigated. At 10 kV/cm only about 10% of the treated embryos hatched. The survival of embryos mounted on tape (data not shown) was often considerably lower (almost half) than for embryos suspended in 0.05% TX-100.

2. Permeability of embryos exposed to increasing field strengths

Dechorionated embryos became receptive to the uptake of the dye Methyl Red, only at high field strength (figure 5). To achieve dye uptake above background, over 10 kV/cm at a capacitance of 25 μFd was required. Above this value, greater than 75% of the treated embryos became permeable to the water soluble dye. However, less than 10% survived the treatment. In contrast, embryos pulsed over a range of voltages in the presence of Blue Dextran or [3H]-Dextran, were unable to take up detectable amounts of dye or radioactivity (data not shown).

3. Survival of embryos exposed to increasing capacitance

Dechorionated embryos were exposed to a field strength of 1 kV/cm over a range of capacitances (0-1980 μFd) in a BRL Cell-Porator. As expected, survival decreased steadily as capacitance and therefore τ increased (figure 6). When capacitance was varied over

100

80

60

% Survival

40

20

0

0   2000   4000   6000   8000   10000
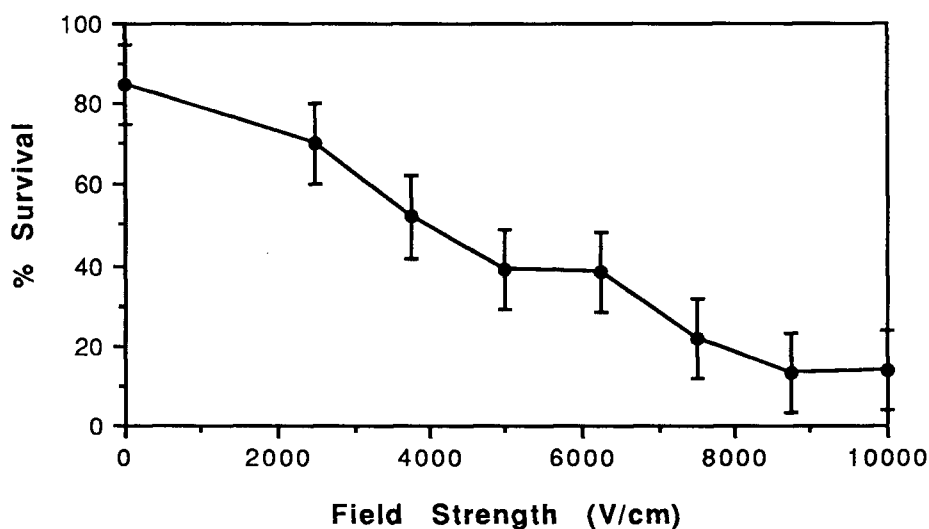
Field Strength (V/cm)

**Figure 4.   The Effect of Increasing Field Strength on
Embryo   Survival.**

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 2 hrs. Subsequently, the egg laying plates were changed every 30 min.. The embryos from each collection were dechorionated, rinsed with dH_2O, then rinsed off the Miracloth into a 0.2 cm cuvette with 500 μl of PBSuc TX.   The cuvette was left at room temperature for 5 min., the embryos resuspended by tapping and the suspension pulsed at the indicated field strength (25 μFd). τ's ranged from 1.7 (10 kV/cm) to 2.6 msec. (2.5 kV/cm).   The embryos were incubated a further 15 min. at room temperature (any eggs not in solution were removed).   The embryos were collected on Miracloth, rinsed with PBSuc and placed in a humid chamber (PBSuc).   The percentage of embryos that survived to first instar larvae to hatch within 48 hrs of the treatment is given.
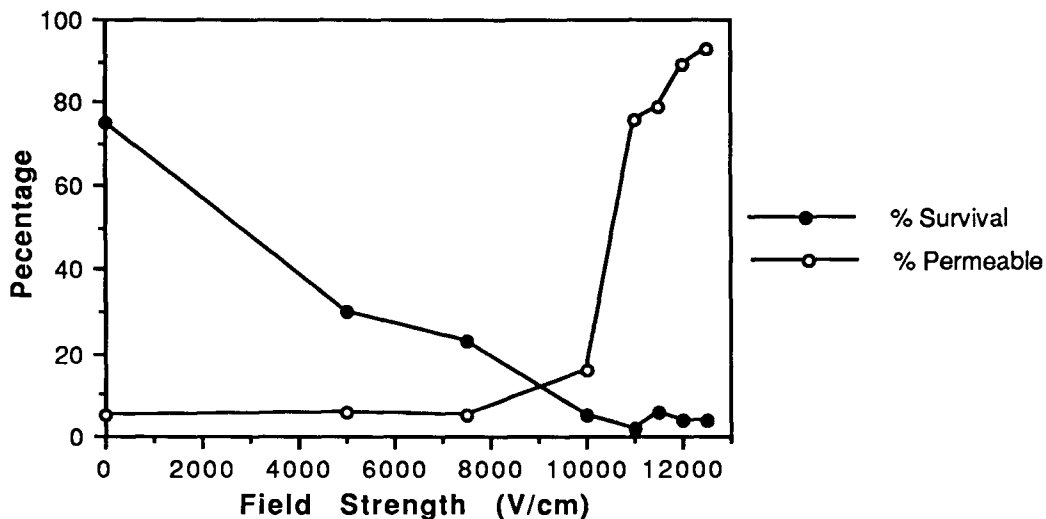
Figure 5. **The Effect of Increasing Field Strength on Embryo Survival and Permeability to the Dye Methyl Red.**

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 2 hrs. Subsequently, the egg laying plates were changed every 30 min.. The eggs from each collection were dechorionated, rinsed with dH_2O, then rinsed off the Miracloth into a 0.2 cm cuvette with 500 µl of PBSuc TX containing Methyl Red (0.01% w/v). The cuvette was left at room temperature for 5 min., the embryos resuspended by tapping and the suspension pulsed at the indicated field strength (25 µFd). τ's ranged from 1.3 (12.5 kV/cm) to 2.1 msec. (5 kV/cm). The embryos were incubated a further 15 min. at room temperature (any eggs not in solution were removed). The embryos were collected on Miracloth, rinsed with PBSuc and placed in a humid chamber (PBSuc). The number of embryos collected after treatment (coloured and not) was recorded, as was the number of larvae that hatched within 48 hrs of the treatment.
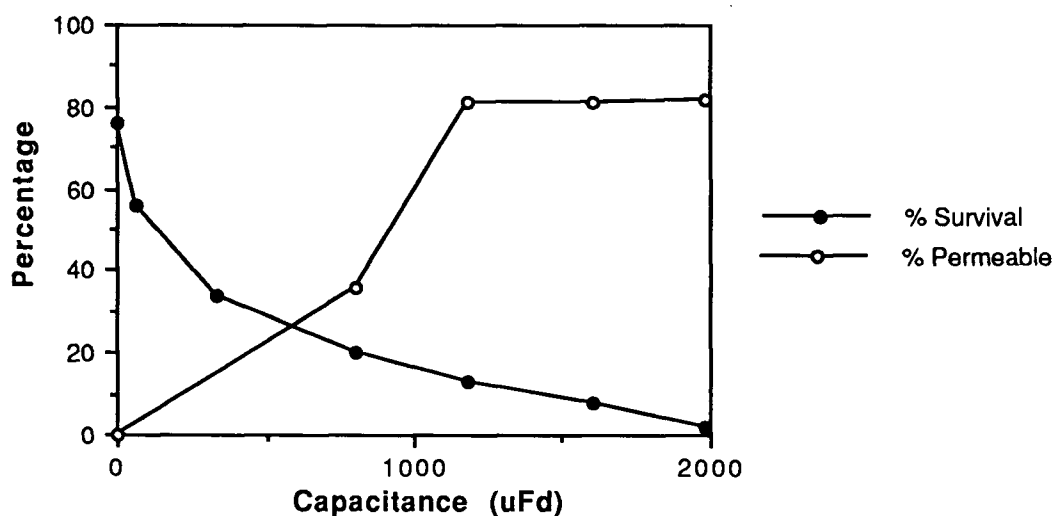
Figure 6.   The Effect of Increasing Capacitance on Embryo Survival and Permeability to the Dye Nile Blue.

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 3 hrs. Embryos were then collected at 30 min. intervals. The embryos were dechorionated, rinsed with dH_2O, washed into a 0.4 cm cuvette using 900 μl PBSuc TN. The eggs were incubated for 5 min., and pulsed at 400 volts (1 kV/cm) on the low resistance setting (BRL Cell-Porator) at the indicated capacitance. After a 15 min. post-pulse incubation, the eggs were collected on Miracloth, rinsed with PBSuc, placed in a humid chamber (PBSuc) and allowed to develop at room temperature. The number of embryos collected after treatment (coloured and not) was recorded, as was the number of larvae that hatched within 48 hrs of the treatment.

a range of field strengths, the typical survival curve which showed a decreased survival corresponding to an increased voltage (see above), was observed for each capacitance (figure 7). However, a corresponding downward shift of the survival curve accompanied each increase in capacitance.

4. Permeability of embryos exposed to increasing capacitance

Dechorionated embryos were pulsed (1 kV/cm) in PBSuc TN at various capacitances (figure 6). Permeability to the dye became extensive at 1180 μFd and above. At this point, about 80% of the treated embryos were able to take up the Nile Blue. Interestingly, increasing the capacitance from 1180 to 1980 μFd did not show any further significant increase in permeability.

5. Survival of embryos exposed to an electrical current in the presence of DNA

Pulsing of embryos in the presence of DNA did not reveal any significant change in survivability of the treated individuals (figure 8). A slight decrease in survival was observed when either the voltage or the capacitance (data not shown) was varied. However, this is more likely a variation among trials (see below), than due to the presence of the DNA.

6. Discussion

An electrical pulse has been used to introduce water soluble macromolecules into a number of cell types (reviewed by Potter, 1988; Shigekawa and Dower, 1988). The above results indicate that *Drosophila melanogaster* embryos also become permeable to water soluble dyes after electroporation. At high field strength (10 kV/cm
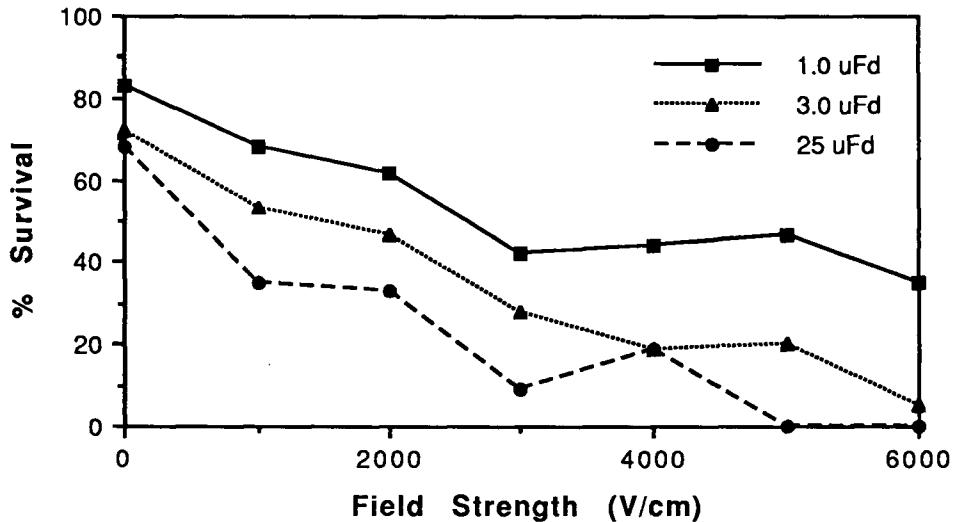
Figure 7.   **The Effect of Varying Capacitance on the Field Strength Survival Curve.**

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 3 hrs. Embryos were then collected at 30 min. intervals, dechorionated, rinsed with dH$_2$O, and mounted on tape in groups of 25 (5-6 tapes/collection). Embryos older than approximately 1 hr were not used. The slide containing the mounted embryos was placed in a developing chamber (PBSuc) until the tape was required (0-5 min.). The tape was placed down the side of a 0.4 cm cuvette containing 800 µl PBSuc, and preincubated for 1 min.. The embryos were then pulsed at the indicated field strength, and incubated one more minute. T's were typically 0.2 (1.0µFd), 0.5 (3.0 µFd) and ranged from 3.1 to 4.6 msec. (25.0 µFd). The tape was then removed from the cuvette, placed on a new slide and covered with halocarbon oil. The slide was placed back in a humid chamber (dH$_2$O) and the embryos allowed to develop. The % survival was calculated from the number of embryos mounted in relation to the number of larvae that hatched within 48 hrs.
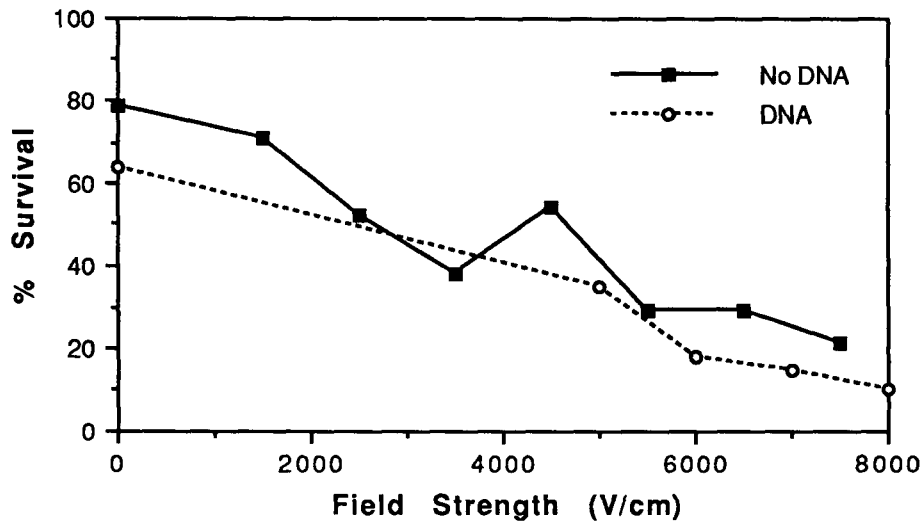
**Figure 8.   The Effect of the Presence of DNA During
Pulsing on Survival of Embryos
Exposed to an Electrical Current.**

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 3 hrs. Embryos were then collected at 30 min. intervals. The embryos were dechorionated, rinsed with dH_2O and mounted on tape (20 eggs/tape, 3-4 tapes/collection).   The tapes were placed individually down the side of a 0.2 cm cuvette containing 400 μl of PBSuc (no DNA) or Electroporation Buffer (10.3 μg/ml pCarnegie 20, 0.545 μg/ml pπ25.1 in PBSuc), incubated for 30 seconds in the

buffer and pulsed at the desired field strength (25 μFd). Τ's were usually approximately 1.9 msec.   Forty-five seconds post pulse, the tape was removed from the cuvette, placed on a glass slide, and the embryos covered with halocarbon oil.   The slide was then placed back in a humid chamber (dH_2O).   Embryos older than 1 hr were discarded.   The percentages survival was calculated from the number of embryos mounted (after aging) in relation to the number of larvae that hatched within 48 hrs of the treatment.

or greater) or high capacitances (800 μFd or greater), significant numbers of the treated embryos become permeable to dyes such as Methyl Red or Nile Blue. However, under these conditions fewer than 20% of the embryos survived the treatment. These observations brings up two questions:

1. Why is such a high voltage required to make the embryos permeable to the water soluble dye?

2. What is causing embryo death at low field strength?

The criterion for the extreme field strength to produce dye uptake is interesting. According to the equation $\Delta V_{max} = 1.5\ E_o r$ (Neumann $et\ al.$, 1982), the critical membrane breakdown potential is proportional to the radius of the cell (r). Smaller cells therefore require larger field strengths (E) for membranes breakdown to occur. Electroporation of the large mammalian cells typically occurs at < 1 kV/cm (Andreason and Evans, 1988), while for the small bacteria protoplasts, about 6 kV/cm is required (Skigekawa $et\ al.$, 1988). However, the size of the $Drosophila$ embryo is significantly larger than most cells commonly transformed by electroporation, yet they required an extremely high field strength for dye uptake to be observed. This inconsistency presumably results from the remaining layers of the $Drosophila$ eggshell, in particular the wax layer. A higher field strength would presumably be required to disorganize the compact wax molecules, than the more fluid phospholipids in a typical bilayer structure.

Therefore the extraction of this protective layer prior to electroporation should aid in the electroporation process. Its

removal would make the embryo permeable to small water soluble molecules, and as a result, should decrease the critical voltage required to create pores large enough for high molecular weight molecules (such as DNA) to move through the vitelline membrane. Transformation of the de-waxed embryo by electroporation could then be re-examined. This, of course, assumes that the vitelline membranes do not pose another barrier to the electroporation process.

The death of the embryos pulsed at lower voltages was puzzling. It was expected that the electric field would have little effect on cell survival until the critical breakdown potential of the membrane was exceeded. However, in the above experiments, survival consistently declined with increasing voltage (even at very low parameters). Yet, the embryos were not permeable to water soluble molecules suggesting that pores had not formed. One possible explanation for this phenomenon is that regardless of the integrity of the membrane, the electric field was affecting the development of the embryo.

The movement of charged molecules in an electric field is well documented and characterized. Hence, the idea of it taking place within an intact cell is not revolutionary. Similarly, the charged nature of DNA is important in the electroporation process. It has been shown that although the pores formed by the electrical current are important to this technique, the electrophoretic movement of the charged DNA through the pores, is equally important (Winterbourne et al, 1988). Electrical currents have also been used to extract plasmid DNA from cells (Calvin and Hanawalt, 1988).

With this in mind, it is possible that a high electric field could result in electrophoresis of developmentally important molecules, such as morphogens (Manseau and Schupbach, 1989), or even the nuclei themselves. Such movement could result in these molecules being absent from their site of action at the required time. This type of mass electrophoresis would therefore have dire consequences on embryonic development.

B.   Electroporation of *Drosophila*   Embryos

1.   Survival of electroporated embryos from embryo to adult

The effect of the presence of DNA in the electroporation buffer on the development of embryos exposed to an electrical current was examined. Exposure of embryos to an electrical pulse in the absence of DNA did not greatly effect their ability to develop to adults if they survived to the first instar larval stage (figure 9). On average, in the absence of DNA, 81% of the individuals able to hatch after being exposed to a field strength from 0 to 7.5 kV/cm, subsequently went on to eclose. When DNA was present in the electroporation buffer during pulsing, the ability to develop from larvae to adult appearred to depend on the magnitude of the pulse. At low field strength, 6 kV/cm and below, survival to eclosion (75%) was similar to embryos pulsed in the absence of DNA (81%). However, above 6 kV/cm, a significant number of the larvae did not develop into adults when DNA was present in the electroporation buffer. The average % eclosed from the surviving larvae was only 43%.
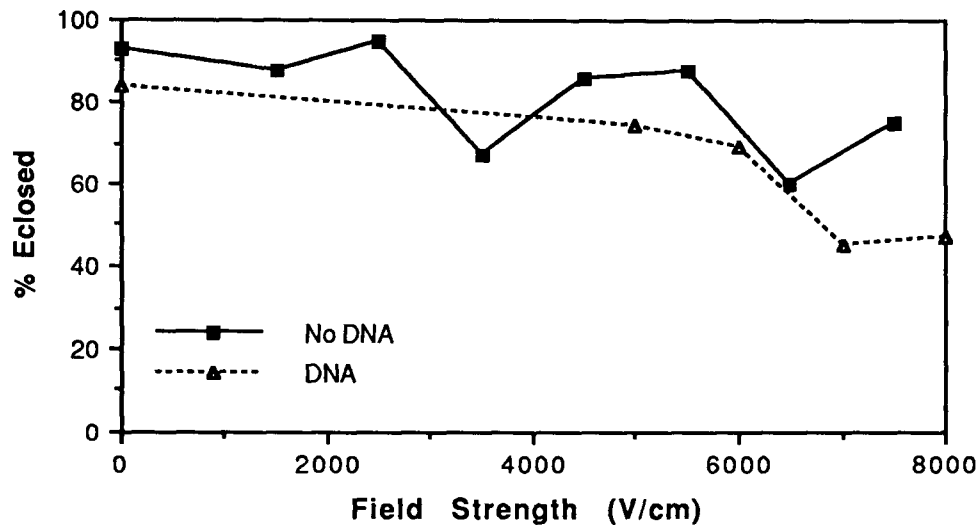
Figure 9. **The Effect of the Presence of DNA During Pulsing on the Development of Embryo to Adult.**

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 3 hrs. Embryos were then collected at 30 min. intervals. The embryos were dechorionated, rinsed with dH$_2$O and mounted on tape (20 eggs/tape, 3-4 tapes/collection). The tapes were placed individually down the side of a 0.2 cm cuvette containing 400 µl of PBSuc (no DNA) or Electroporation Buffer (10.3 µg/ml pCarnegie 20, 0.545 µg/ml pπ25.1 in PBSuc), incubated for 30 seconds in the buffer and pulsed at the desired field strength. τ's were typically approximately 1.9 msec.Forty-five seconds post pulse, the tape was removed from the cuvette, placed on a glass slide, and the embryos covered with halocarbon oil. The slide was then placed back in a humid chamber (dH$_2$O). Embryos older than 1 hr were removed. Embryos that hatched within 48 hrs of treatment were rescued from the humid chamber and transferred to enriched food. The larvae were allowed to develop at room temperature. The number of flies to eclose in relation to the number of larvae placed in the food vial is given.

2. Phenotypic selection of putative transformants

To screen for transformants, individuals that eclosed after treatment, were mated individually to r^{506}/r^{506} flies. The progeny from these crosses were scored for eye colour phenotype. The results of the screen are recorded in Table I. From these data, it appeared that increases in field strength had given rise to increased transformation. However, the presence of F_1 wild-type progeny in control lines suggests other possibilities (see discussion).

3. Chemical analysis of putative transformants

a. Purine selection titre

The presence of an active *Xdh* gene can also be determined by growth of the larvae on food supplemented with purine.

The r^{506} strain and the wt strain were allowed to lay embryos on normal food for 3 days. They were then removed and 100 µl of purine was added to the vials containing the embryos. Homozygous wild-type (+/+) and heterozygous (+/r^{506}) flies (the F_1 generation of a cross between r^{506}/r^{506} and wt) were able to survive on the purine supplemented food at all concentrations examined (figure 10). In contrast, although the homozygous mutants (r^{506}/r^{506}) produced good larval growth, few flies eclosed even at the lowest concentration of purine used (0.1%). By 0.2% purine none eclosed.

b. Purine selection of putative transformants

From the above titre, 0.2% purine was adequate to eliminate all non-transformed progeny. The selection was performed on the phenotypically determined putative transformants and the results are given in Table I. Seven putative transformants, originating from

Table I.   The Analysis of the Electroporation of r^{506}/r^{506} Embryos with pCarnegie 20

Field Strength (V/cm)	No. Treated	% Hatched	% Eclosed	% Fertile	No. F_0wt	No. F_1wt	No. Purine Selected	No. Southern Analysis
0	74	64	86	100	2	2	ND	ND
5000	92	35	75	75	0	2	0	0
6000	106	18	50	67	1	3	2	0
7000	277	15	46	73	1	4	1	0
8000	238	10	47	62	2	5	4	0

r^{506}/r^{506} flies were prelaid in collection bottles for 3 hrs. The eggs were then collected at 25-30 min. intervals.   The eggs were dechorionated, rinsed with dH_2O, mounted on tape and kept in a developing chamber (dH_2O) until needed. Individual tapes were placed down the side of a 0.2 cm cuvette containing PBSuc, incubated for 15 seconds and pulsed at the indicated field strength (25 μFd). Thirty seconds post pulse, the tape was removed, placed on a glass slide, the embryos covered with halocarbon oil, embryos older than 2 hrs removed, and the slide placed in a developing chamber (dH_2O). Embryos that hatched within 48 hrs of treatment were transferred to standard food and allowed to develop at room temperature.   Eclosing adults were scored for eye colour and mated individually to r^{506}/r^{506} virgins. F_1 adults were also scored for phenotypic expression of XDH then brother/sister mated, and their progeny subjected to purine selection (see Materials and Methods).   Fly lines which gave positive results to the chemical selection were examined by Southern Analysis. ND = no data available.
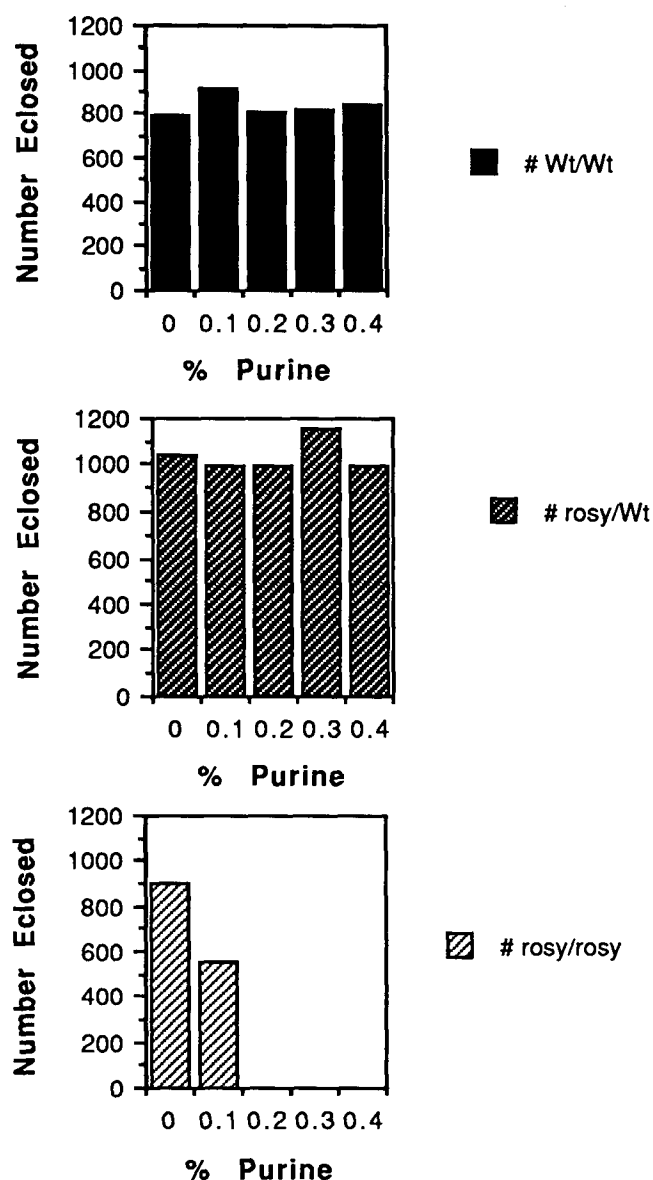
Figure 10.   Purine Selection Titre.

Five male and ten female flies were placed in a glass vial containing 5 ml of enriched food. The flies were left to lay eggs for 2-3 days then transferred to a fresh vial. 100 µl of dH$_2$O containing the indicated percentage of purine (w/v) was added to the vial containing the developing larvae. The vials were left at room temperature, and the number of adults to eclose were counted. The numbers reported are a summation of duplicate experiments run over 10 days (8 vials in total). +/+ = wild type Oregon R male and female matings, r/r = r^{506}/r^{506} male and female matings, and r/+ = r^{506}/r^{506} males mated to wild type Oregon R females.

5 F_0 individuals were found: 2M2, 2M3, 4M1, 6M1a, 6M1b, 6M1c, and 6F2. Lines of these flies were maintained by brother/sister matings.

4. Molecular analysis of putative transformants

Southern analysis was performed on genomic DNA extracted from the seven 'transformed' lines, as well as wt and r^{506}/r^{506} homozygotes. A representative of the results is given in figure 11. The mating strategy used (brother/sister) created heterogeneous populations of r^{506}/r^{506}, $r^{506}/*$, and $*/*$. (Where $*$ represents the *Xdh* gene at a undefined locus in the putative transformants) The figure suggests that the Eco RI bands of all putative transformants can be explained as originating from either a wt or r^{506} locus. The 9.2 kb band is common for both r^{506} and wild-type genes and comes from the 5' end of the *Xdh* gene (see figure 12). In the wild-type gene, there is also a 4.6 kb Eco RI fragment (3' end) that hybridizes to pCarnegie 20. However, in r^{506}, the 3.4 kb deletion includes the 3' Eco RI site. As a result, the next Eco RI site (0.6 kb 3') becomes the 3' end of the second band, creating a 1.8 kb fragment.

5. Discussion

The presence of DNA in the electroporation buffer had little effect on the development of the flies from larvae to adult except at high field strength. This suggests that the DNA may have entered the embryo during the high voltage pulse, and interfered with development due to integration into developmentally important genes. This however seems unlikely since no transformants were isolated, and as shown above, the embryos were not permeable to
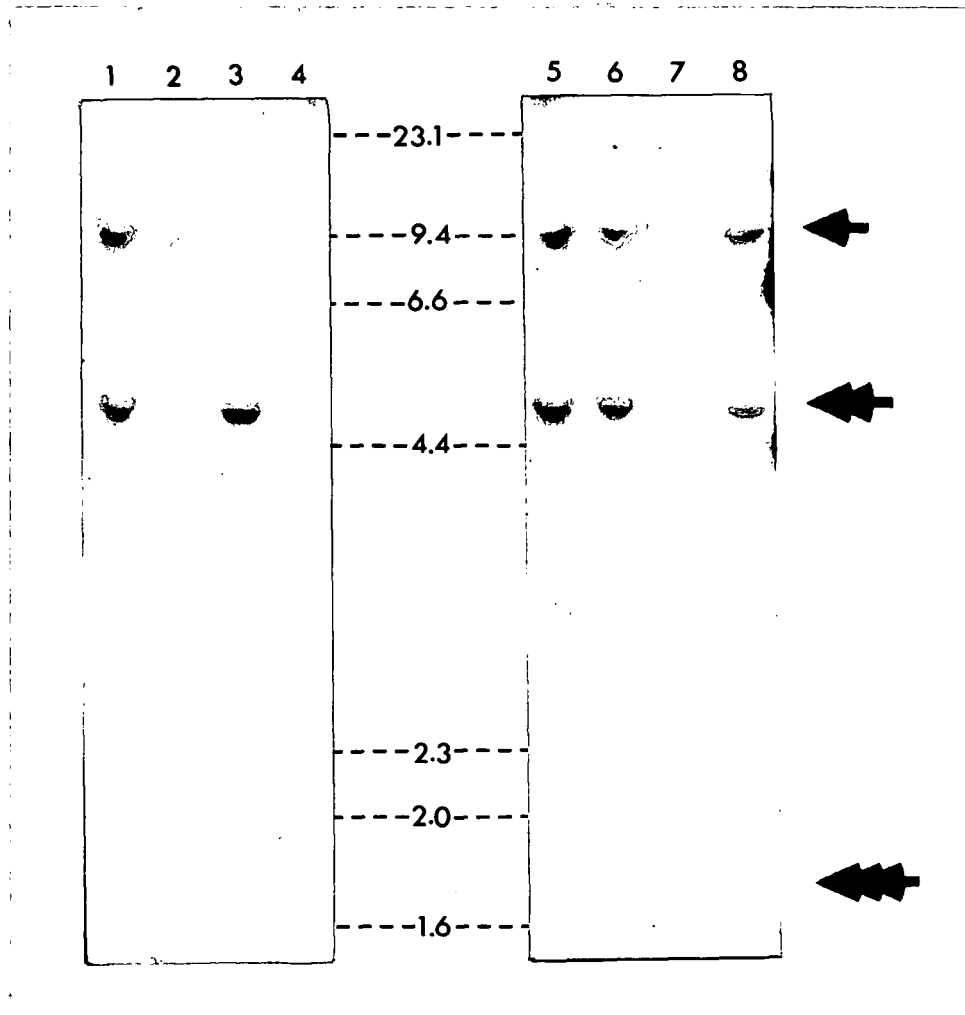
Figure 11. **Southern Analysis of Putative Transformants.**

Genomic DNA from lines created by brother/sister mating of suspected transformants were digested with Eco RI and run out on a 0.7% agarose gel (25 x 20 cm). The gel was cut in two length wise, and the DNA was transferred to nylon membranes (Hybond-N). The membranes were hybridized individually with pCarnegie 20 (nick-translated with $[\alpha^{32}P]dATP$) at 68 °C for 16 hrs. The membranes were then washed at 68 °C with 1 X SSC, 0.5% SDS, then 0.1 X SSC, 0.5% SDS. The membranes were exposed to X-ray film for 2 days. Fly line are (1) 2M3, (2) r^{506}/r^{506}, (3) +/+, (4) 2F2, (5) 6M1a, (6) 6M1b, (7) 6F2, (8) 4M1. The bands indicated are ( ) 9.2, ( ) 4.6, and ( ) 1.8 kb.

Figure 12. *rosy* Locus of *Drosophila   melanogaster*

The *rosy* locus located on chromosome 3 at position 87 DE is represented here. The *Xdh* gene is shown as a rectangle with the 4 exons in black and the 3 introns in white. The position of the 3.4 kb r^{506} deletion is given, as is the Hind III fragment that was cloned into the P-element vector pCarnegie 20. Relevent restriction enzyme sites are shown. O kb is designated as the Eco RI site in exon 2.

water soluble molecules below 10 kV/cm. Therefore, the reason for the increased mortality of the larvae of embryos treated at high field strength is unknown.

The presence of wild-type flies in the F_0 generation could be the result of 3 different genetic events.

1. Somatic transformation of the embryos (Scavarda and Hartl, 1984; Maxwell and Maxwell 1988; Toneguzzo *et al*. 1986). As integration did not occur in the germline, this would cause expression of the *Xdh* gene in the treated individual, but not in its progeny.

2. Transformation of the embryo very early in its development such that some somatic and germline cells possess the transformed *Xdh* gene. This would result in an F_0 possessing wild-type eyes, and its progeny also being wild-type with respect to eye colour.

3. The r^{506}/r^{506} stock used for electroporation could have been contaminated with a wild-type fly at some point. Therefore a few of the treated embryos would have actually been $r^{506}/+$ heterozygotes and would give F_0 flies with wild-type eyes even if untreated. These flies would of course pass the wild-type gene onto half on their progeny (F_1).

As wild-type F_0 flies from control trials were also isolated, the third possibility seems most likely. One remote possibility is that transformation occurred without pulsing. Yoon and Fox (1965) showed that transformants could be isolated by simply incubating premature embryos collected using an 'ovitron', in solution containing DNA (Fox and Yoon, 1966). A small percentage of such

embryos are permeable to water soluble molecules since the wax layer secretion does not occur in embryos collected in this way. However, as no transformants were detected by Southern analysis, this seems very unlikely.

The visual difference between *rosy* $^-$ and wild-type eye colour is subtle, but distinct. The presence of even 1% of the normal amount of XDH has been reported to give wild-type eye colour (Chovnick *et al*, 1977). A problem with this selection method is that the eye colour does not fully developed until 1-2 days after eclosion. In addition, the desire to see wild-type eye colour when the difference is so slight, makes miss classification a problem. For these reasons, a chemical method for distinguishing between *rosy* $^-$ and wild-type flies was utilized as a secondary screen.

Purine selection has been proven extremely useful for selection of wild-type flies over *rosy* $^-$ mutants (Finnerty et al, 1970). The 0.2% titre eliminated any leakage of the r^{506}/r^{506} that occurred at 0.1%, yet allowed heterozygotes to develop uneffected. Utilization of this selection on the putative transformants eliminated half of the phenotypically selected fly lines. However, as the amount of purine required to kill the flies depends on the quantity of functional XDH expressed, it is possible that poorly expressing transformants were eliminated.

Southern analysis of the purine selected lines showed no evidence for germline transformation. All the bands derived from the putative transformants can be explained as coming from either a r^{506} or wild-type *Xdh* gene. As a result, one must conclude that

none of the wild-type F_1 flies were in fact transformed by the pCarnegie 20 vector. The most obvious explanation for the presence of wild-type individuals in the F_0 and F_1 generations is contamination of the r^{506}/r^{506} stock with a wt fly.

C. The Survival and Permeability of Dechorionated Embryos
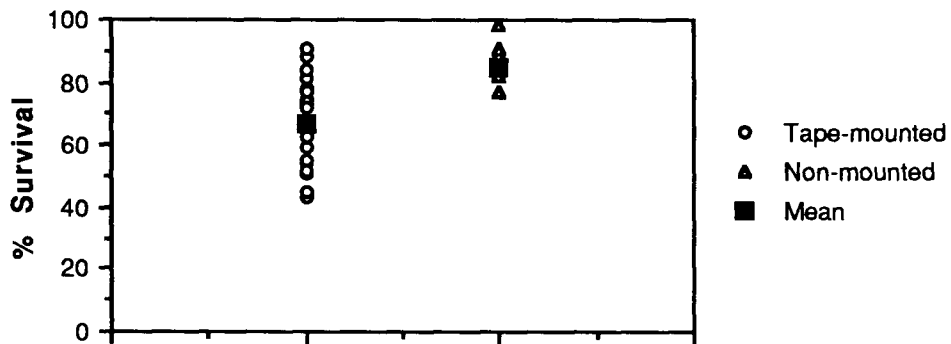
1. Variation in the survival of dechorionated embryos

Variation in survival of untreated embryos was observed. The variability followed an approximately normal distribution (figure 13). However, the parameters of the distribution differed with the mode of preparation of the embryos. r^{506}/r^{506} embryos dechorionated, mounted on tape, covered with halocarbon oil, and placed in a humid chamber, have great variability and a low mean survival ($x=67\%$; $s=12.5$). In contrast, with r^{506}/r^{506} embryos simply dechorionated and placed in a humid chamber much less variation was observed. The standard deviation was half that above ($s=5.9$), while the mean survival was 25% higher ($x=85\%$). The data were only spread over a 20% margin (77-98%) compared to the 50% distribution (43-91%) for tape-mounted embryos. Consequently, the procedure of mounting embryos on tape was abandoned when it was found that addition of 0.05% TX-100 to the buffers would prevent the embryos from sticking to the sides of the cuvette and each other.

2. Permeability of dechorionated embryos

Incubation of dechorionated embryos in buffers containing 0.05% TX-100 and 0.01% Nile Blue revealed that these embryos were basically impermeable to water soluble macromolecules such as

A.

B.
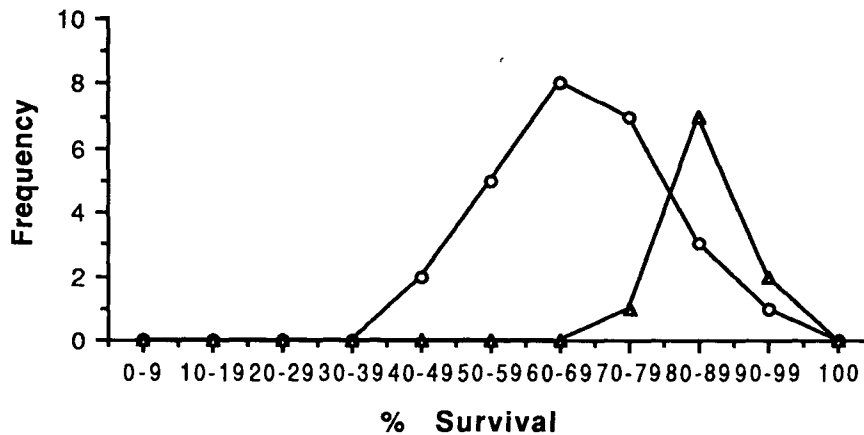
Figure 13. **Variability in the Survival of Dechorionated Embryos**

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 2-3 hrs. Embryos were then collected at 25-35 minute intervals. The embryos were dechorionated, then treated in one of two ways. Tape-mounted embryos were arranged side by side on double-sided tape in groups of about 25-50, covered with halocarbon oil and then placed in a humid chamber (dH_2O). Non-mounted embryos were placed directly in a humid chamber (dH_2O) after having their chorion removed. The percentage of embryos to hatch within 48 hrs of collection is given. A. The points represent different collections on the same day, as well as collections from different days, and for the tape mounted embryos, different tapes from the same collection are also included. The mean for all trials is also given. B. The number of trials in which the percentage survival was within the range indicated is graphed.

dyes. Less than 6% of the treated embryos were able to take up visible quantities of dye (data not shown).

3. Discussion

Dechorionated embryos remain essentially a closed system. Water vapour and respiratory gases can permeate the remaining portions of the eggshell, but even small molecules such as nucleotides, amino acids, sugars or water soluble dyes cannot. However, the permeability of the dechorionated embryos to water vapour makes desiccation their primary cause of death.

Removal of the chorion from *Drosophila* embryos takes only about 2-3 minutes. However, the time required to properly mount the embryos on tape can be considerable (up to 15 minutes) and is proportional to the number of embryos mounted and the dexterity of the experimenter. This difference in the time that the vulnerable embryos are exposed to the environment is significant. Although the mounting on tape takes place in a humid chamber (to increase the local humidity), tape-mounted embryos still showed visible signs of desiccation after only a few minutes. It was not common practice to completely control the environment surrounding the specimens. Several trials were performed in a chamber of controlled humidity (75-85%) and temperature (17-18 °C); nevertheless, little difference in percentage survival was observed. Therefore, the poor survival and variation observed between tape-mounted trials in comparison to simply dechorionated embryos were likely due to differences in preparation time and the resulting extents of desiccation. These results also suggest that 80% humidity was insufficient to prevent

significant desiccation.

Due to the variability in survival between preparations it was hard to draw conclusions from numerical values obtained, or see small differences in survival. However, survival trends were reproducible and were somewhat informative.

A small percentage of dechorionated embryos (< 6%) were able to incorporate the blue dye. One possible explanation for this is that the wax layer is not impermeable in these embryos. Davies (1947) observed that when blowflies were induced to lay embryos continuously, 5-10% of the embryos were osmotically sensitive. This suggested that the wax layer which is secreted just prior to laying had not solidified in the embryos laid last. These embryos therefore would not possess an intact water barrier and would remain permeable to water soluble molecules until the wax dried.

D. Permeability of Embryos Treated with TX-100
1. Survival of embryos treated with increased concentrations of TX-100

Dechorionated embryos were incubated in PBSuc containing increasing concentrations of TX-100 (figure 14). The results indicate that survival was not remarkably affected at low concentrations (below 0.5%) of TX-100. After a 20 minute incubation in buffer, survival remained high (about 75%). A zero point control (no TX-100) could not be performed due to the adhesive nature of the embryos in the absence of detergent. However, the average survival of untreated dechorionated embryos was 85% (see above).
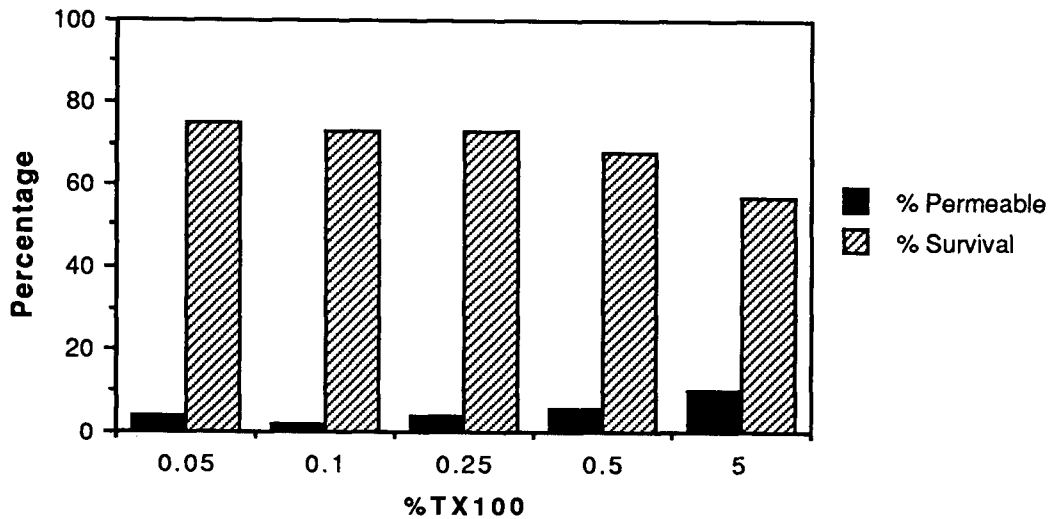
Figure 14. **The Effect of Triton-X 100 Concentration on Embryo Survival and Permeability to the Dye Nile Blue**

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 3 hrs. Subsequently, dechorionated embryos from 30 min. collections were washed into a 0.2 cm cuvette with 500 µl of PBSuc containing Nile Blue (0.01% w/v) and varying amounts of TX100 (v/v) . The cuvette was incubated at room temperature for 20 min. The embryos were then collected on Miracloth, rinsed with PBSuc, and placed in a humid chamber (PBSuc). The number of embryos collected after treatment (coloured and not) was recorded, as was the number of larvae which hatched within 48 hrs post treatment.

Increasing the TX-100 concentration to 5.0% showed a marked decrease in percentage survival (to below 60%).

2. Permeability of embryos treated with increased concentrations of TX-100

Dechorionated embryos were incubated in PBSuc containing Nile Blue and various concentrations of TX-100. Increasing the concentration of TX-100 had limited effect on embryo permeability during a 20 minute incubation (figure 14). At 5.0% TX-100, the percentage of embryos able to take up the Nile Blue dye was still less than 10%.

3. Discussion

Due to the low survival, great variation in survival, not to mention laborious preparation of tape mounted embryos, a system for using freely suspended embryos was sought. Prior investigators have demonstrated that detergents such as SDS and TX-100 at low concentrations, can be used to eliminate the 'stickiness' of the dechorionated embryos, yet did not interfere with the continued development of the embryos. In addition, detergents have also been used to make the *Drosophila* embryo permeable to radiolabelled nucleotides (Eudy *et al*, 1969; Sayles *et al*,1973).

A low concentration of TX-100 (0.05% (v/v)) was found to be adequate for embryo suspension. The embryos could easily be suspended in buffer, and although they settled to the bottom of the cuvette due to gravity, they were readily dispersed and resuspended by simply by taping the cuvette. As previously stated, these non-mounted embryos were much easier to prepare and use, and also

gave higher and less variable percentage survival values (85 $+/-$ 6%).

Incubation of the dechorionated embryos in buffer containing a small amount of TX-100 (up to 0.5%) did not effect embryos survival. However, at higher concentrations (eg 5%) a noticeable increase in mortality was apparent. This is in agreement with Sayles *et al.* (1973), who reported that *Drosophila* embryos incubated in *Drosophila* Ringer's containing TX-100 developed normally providing the detergent concentration did not exceed 0.5%, and the exposure was limited to 2 hours.

A 20 minute incubation in the detergent containing buffer was not sufficient to make the embryo permeable to the dye. Sayles *et al.* used at least a two hour incubation to achieve incorporation of [^{3}H]-amino acids or uridine into the embryo. However, with the constraint of transforming the pole cells (which form 1.5 hours after fertilization at room temperature), a lengthy incubation was not feasible. Decreasing the temperature at which the experiment was performed to 17 °C would extend the transformation window and therefore permit a longer incubation. However, a quicker method would be preferred.

E. Permeability of Embryos treated with Solvents
1. Survival of heptane-treated embryos incubated in different buffers

Survival of dechorionated embryos incubated in heptane for one minute was extremely low. When such embryos were incubated for 30 minutes in any of the typical buffers, mortality ranged from 70-

100% (figure 15). Although the percent survival varied between trials, PBSuc gave the highest survival (18%). In fact, over a number of trials, 28% survival (PBSuc) was the highest recorded for heptane-treated embryos.

2. Permeability of heptane-treated embryos incubated in different buffers

Heptane-treated embryos are extremely permeable to dyes such as Nile Blue (figure 15). 80-95% of treated embryos became coloured, regardless of the buffer used for the incubation. This is a 18-19 fold increase in permeability as compared to controls (5%).

3. Survival of embryos treated with different solvents

Dechorionated embryos incubated for one minute in a variety of n-alkyl solvents showed a very poor ability to survive (figure 16). Following a thirty minute incubation in PBSuc containing 0.05% TX-100 and 0.1% Nile Blue, survival was typically around 10% regardless of the solvent used.

4. Permeability of embryos treated with different solvents

The three solvents were all able to increase the permeability of the embryo to the Nile Blue dye (figure 16). 90% or more of the solvent-treated embryos were able to take up the dye. Although solvent treatment of *Drosophila* embryos made them very permeable to the dye Nile Blue, as well as other dyes such as Toluidine Blue, Acridine Orange, and Methyl Red (data not shown), larger molecules were still excluded. Figure 17 shows the results of an incubation of solvent-treated embryos for 30 minutes in PBSuc containing 0.05% TX-100 and 0.4% Blue Dextran. No visible coloration
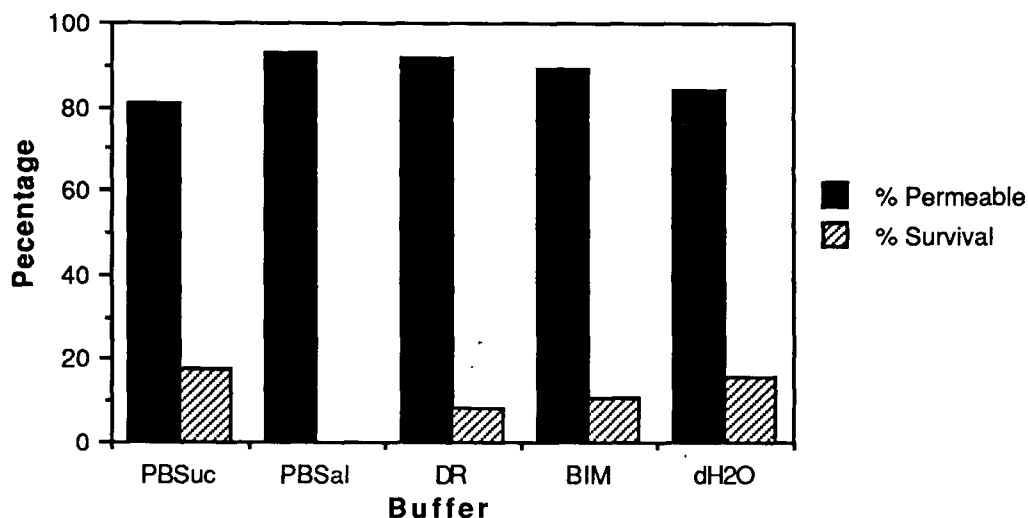
Figure 15. **Survival and Permeability of Heptane-Treated Embryos**

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 2 hrs. Subsequently, the egg laying plates were changed every 30 min. The embryos from each collection were dechorionated, rinsed with dH_2O, then rinsed off the Miracloth into a 100 mesh stainless steel cup using dH_2O. The embryos were then submerged in dH_2O saturated heptane for 30 seconds and the excess solvent drawn off with paper towels. The embryos were rinsed with the buffer indicated , collected on Miracloth, then quickly washed off the Miracloth into a 0.2 cm cuvette with 500 µl of the indicated buffer. The buffers each also contained TX-100 (0.05% v/v) and Nile Blue (0.01% w/v). After a 30 min. incubation in the solution, the embryos were collected on Miracloth and rinsed with the appropriate buffer (without detergent and dye). The cloth disk was laid on a glass slide, the embryos covered with halocarbon oil, and the slide placed in a humid chamber (indicated buffer). The number of embryos collected after treatment (coloured and not) was recorded, as was the number of larvae that hatched within 48 hrs of the treatment.
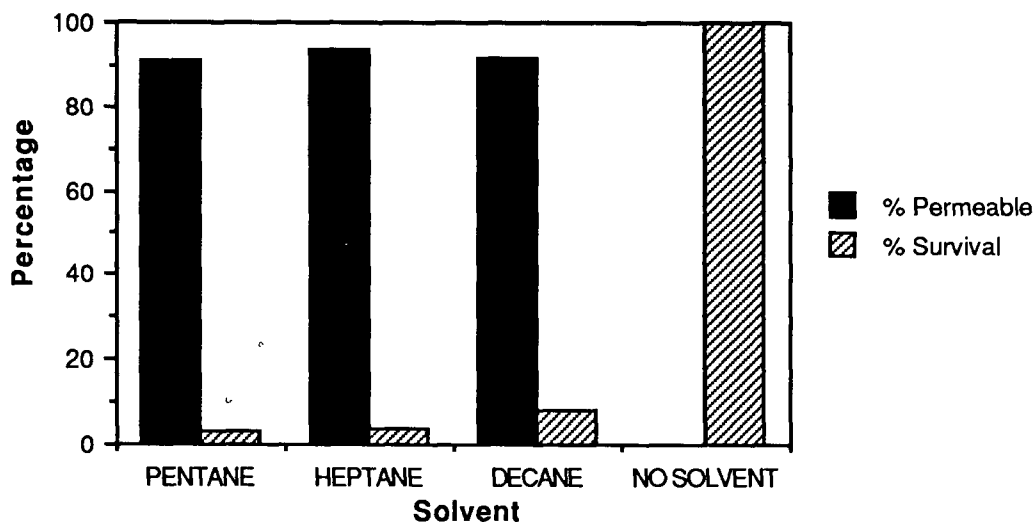
Figure 16. The Effect of Different Solvents on Embryo Survival and Permeability to the Dye Nile Blue.

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 2 hrs. Embryos were then collected at 30 min. intervals. The embryos were dechorionated rinsed using dH_2O and washed into a 100 mesh stainless steel cup with dH_2O. The cup was then submerged into the indicated solvent for 30 seconds. The residual solvent was blotted away with paper towels. The embryos were then rinsed with PBSuc, collected on Miracloth, and washed into a 0.2 cm cuvette with PBSuc TN. The cuvette was incubated at room temperature for 20 min., at which time the embryos were collected on the cloth disk, rinsed with PBSuc and placed in a humid chamber (PBSuc). The number of embryos collected after treatment (coloured and not) was recorded, as was the number of larvae that hatched within 48 hrs of the treatment. No = embryos were incubated in PBSuc instead of solvent.
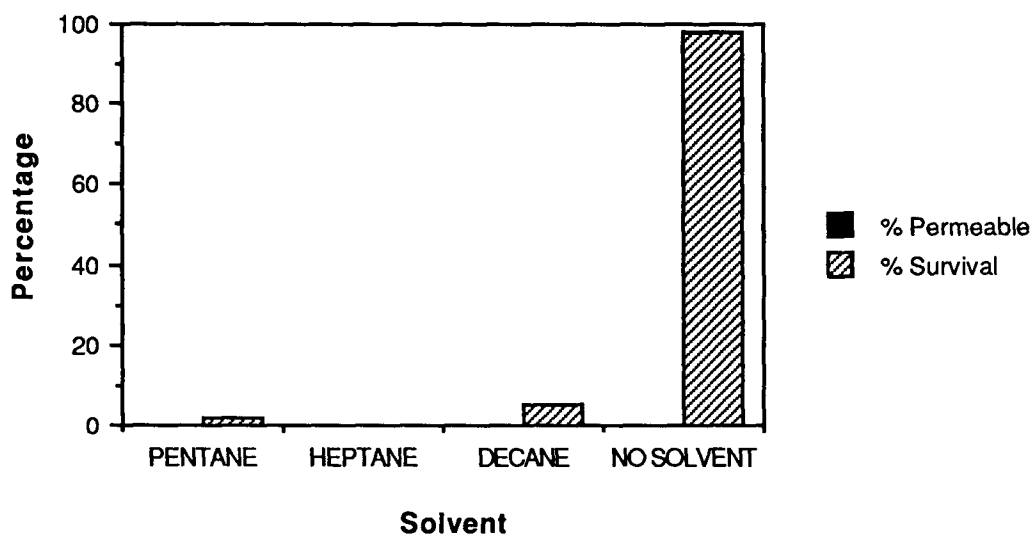
Figure 17. **The Effect of Different Solvents on Embryo Survival and Permeability to Blue Dextran.**

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 2 hrs. Embryos were then collected at 30 min. intervals. The embryos were dechorionated rinsed using dH_2O and washed into a 100 mesh stainless steel cup with dH_2O. The cup was then submerged into the indicated solvent for 30 seconds. The residual solvent was blotted away with paper towels. The embryos were then rinsed with PBSuc, collected on Miracloth, and washed into a 0.2 cm cuvette with PBSuc TX containing Blue Dextran (0.4% w/v). The cuvette was incubated at room temperature for 20 min., at which time the embryos were collected on the cloth disk, rinsed with PBSuc and placed in a humid chamber (PBSuc). The number of embryos collected after treatment (coloured and not) was recorded, as was the number of larvae that hatched within 48 hrs of the treatment. No = embryos were incubated in PBSuc instead of solvent.

of any of the treated embryos was observed.

5. Discussion

The embryos of *Drosophila melanogaster* are generally impermeable to water soluble metabolites, even upon removal of the chorion. In 1973, Limbourg and Zalokar reported that dechorionated *Drosophila* embryos briefly submerged in heptane or octane developed normally, yet the procedure made them permeable to radiolabeled amino acids. Embryo survival was reported to be equivalent to controls (95%) when solvent treatment did not exceed eight minutes.

Unfortunately, in attempts to duplicate such studies, solvent-treated embryos did not survive to levels even closely resembling that of controls. The high mortality rate could be due to one of two things: residual solvent or excessive desiccation. Different solvents ranging from pentane to decane were examined, thus investigating a range of volatilities and water solubilities of the solvents. Pentane is extremely volatile and therefore should have evaporated quickly during subsequent procedures. Also, in trials where the embryos were washed with buffer, although solvents are not extremely soluble in aqueous solutions, the extensive washing would have removed traces of the solvents. However, the use of all three solvents resulted in extensive death of treated embryos. Therefore, the presence of residual solvent after treatment was not a major factor in the death of solvent-treated embryos.

Experiments were typically not performed under humid conditions. However, several trials were performed in a room in

which the humidity was held constant at 75-85%. The results of these experiments were comparable to those performed in an uncontrolled environment. Therefore, either desiccation was not the reason for the increased death or more likely, the treated embryos are so vulnerable to desiccation that they require higher than 85% humidity to avoid extensive dehydration.

Although typically 80% of the treated embryos failed to develop further after the solvent treatment, the procedure did make the embryos extremely permeable to water soluble dyes such as Nile Blue. It could be suggested that it was the dead embryos that took up the dye, however in controls (no solvent treatment) up to 25% of the embryos failed to develop to the larval stage, yet only 0-6% of these treated embryos were able to take up the dye. Therefore, the solvent treatment was removing some barrier (the waxy layer), thus making the embryos permeable to water soluble substances. While the small dyes are readily absorbed by the solvent-treated embryos, Blue dextran (MW 2 million) uptake was not detected in any of the treated embryos. Since embryos remain impermeable to the high molecular weight dextran, the vitelline membrane must still be intact, thus preventing the uptake of large molecules.

As the solvent treatment removes the waterproofing barrier of the embryo, the nature of the buffer in which they are incubated may become important in terms of tolerance to the metabolites present and the osmotic difference between the buffer and the cytoplasm of the embryos. Previous investigators have used *Drosophila* Ringer's solution or a cell culturing solution (BIM

(Limbourg and Zalokar, 1973)) for their incubations, yet these buffers did not result in an improvement in the ability of the embryos to survive. In fact, as figure 15 illustrates, the different medias had little effect on embryo survival. However since mortality was so extensive (even for solvent-treated embryos not subsequently incubated in buffer), the effect of the different buffers on the solvent-treated embryos cannot be assessed accurately.
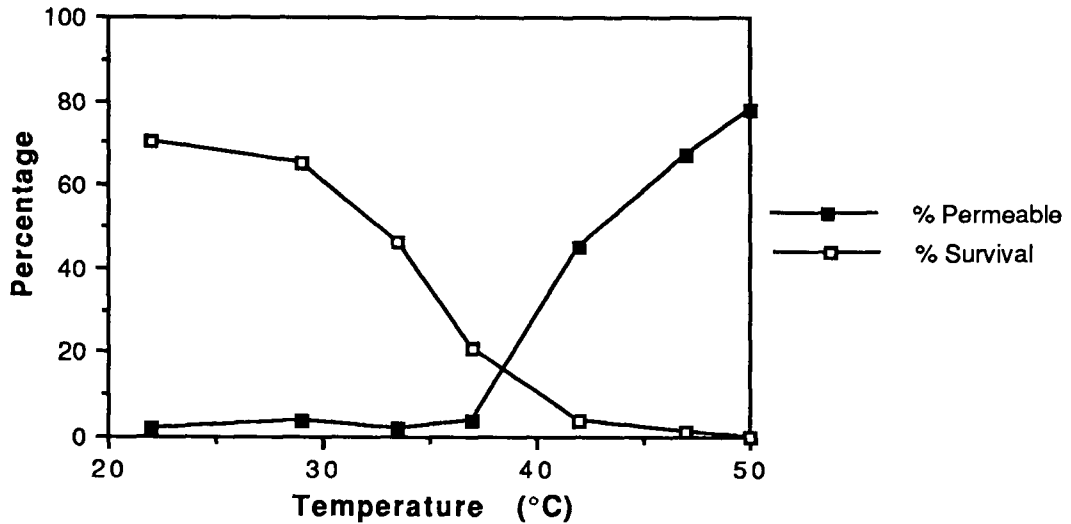
F. Effect of Temperature on Embryo Permeability

1. Survival of embryos at increasing temperatures

Survival of dechorionated embryos incubated for 20 minutes in PBSuc containing TX-100 decreased with an increase in incubation temperature (figure 18). Survival decreased slightly between room temperature (22 °C) and 29 °C, then rapidly above this. By about 42 °C, few if any embryos survived the incubation.

2. Permeability of embryos at increasing temperatures

The effect of temperature on the permeability of dechorionated embryos was investigated. At low temperatures, the embryos were impermeable to the Nile Blue dye, however, as the incubation temperature was raised, their permeability increased (figure 18). Incubation in PBSuc containing 0.5% TX-100 and 0.01% Nile Blue for 20 minutes had little effect on embryo permeability at low temperatures. An increase in permeability was not observed until 42 °C, where the percentage of embryos able to take up the dye jumped from about 4% (37 °C) to 45%. A dramatic increase. Increasing the temperature further, resulted in even greater

Figure 18. **The Effect of Temperature on Embryo Survival and Permeability to the Dye Nile Blue**

r^{506}/r^{506} flies (five to six days old) were placed in a laying bottle in the dark, and allowed to lay eggs for 2 hrs. Dechorionated embryos from 30 min. collections were washed into a 0.2 cm cuvette with 500 μl of PBSuc TN. The cuvette was placed in a water bath at the indicated temperature for 20 min.. The embryos were then collected on Miracloth, rinsed with PBSuc, and placed in a humid chamber (PBSuc). The number of embryos collected after treatment (coloured and not) was recorded, as was the number of larvae that hatched within 48 hrs of the treatment.

permeability. By 50 °C, almost 80% of the treated embryos were capable of dye uptake.

3. Discussion

In 1945, Wigglesworth showed that transpiration through the cuticle of many insects increased drastically above a critical temperature. In 1963, King and Koch showed that for *Drosophila* embryos which were already laid, incubation in saturated saline solutions had no effect on survival unless the temperature of the solution was raised to 45 °C. At this point, the embryos shrunk and as a result, failed to develop further. Davies (1947) also demonstrated this in blowfly embryos (critical temperature around 38 °C). These observations contributed to the hypothesis that the major waterproofing substance of such embryos was a wax layer.

Figure 18 confirms that the above is true for *Drosophila melanogaster*. The permeability of the embryos increased dramatically between 37-42 °C, therefore suggesting that the critical temperature for melting the waxy layer was above 37 °C. The apparent discrepancy between these results and the published literature may be the result of the detergent present in the buffer aided in the extraction of the wax molecules, or simply that King and Koch did not examine temperatures between room temperature and 45 °C. (They did not present any data over this range.)

Unfortunately, at a temperature at which the embryos were quite permeable (42 °C), few survived the incubation. Perhaps by increasing the TX-100 concentration, or using another detergent with a lower critical micelle concentration, the temperature at which

permeability is observed could be lowered to a point where greater embryo survival makes the procedure viable.

G. Conclusions

Exposure to an extreme field strength (10 kV/cm) was required to produce a *Drosophila melanogaster* embryo which was permeable to water soluble dyes. However, such conditions were detrimental to the normal development of the embryos. It therefore appears that the removal of the thin wax layer, without harming the embryos, must first be achieved before electroporation can be utilized efficiently on *Drosophila* embryos. The removal of this layer can be achieved by three methods: detergent solubilization, solvent extraction and heating.

Detergent solubilization of the wax did not occur quickly enough at room temperature to make the procedure feasible for use in transformation experiments. It is possible that by decreasing the incubation temperature, the time available for germline transformation would be extended. However, this would cause the wax to become more rigid, thus requiring either more detergent (above 5.0% TX-100, which would be toxic to the embryo), or an even longer incubation to remove the barrier. The temperature could also be increased, thus combining heating and detergent solubilization to remove the wax. However, this increase in temperature would reduce the transformation window. Also the use of 0.5% TX-100 had little effect on increasing the permeability of the embryo at survivable temperatures, hence higher concentrations (perhaps 5.0%)

or alternate detergents would have to be investigated.

Finding a viable combination of detergent concentration, incubation temperature and transformation time seems improbable at this time. Solvent extraction, on the other hand, seems promising. The technique is rapid, and may even eliminate the need to remove the embryos chorion. A procedure which keeps the embryo in a very humid, or aqueous environment throughout the procedure still remains to be found.

Once the wax is removed, the embryo should become more receptive to the electrical current. (Assuming the vitelline membrane does not also interfere with the process.) The field strength required to achieve pore formation would presumably decrease, thereby decreasing the effects of electrophoresis of developmentally important factors and increasing the probability of recovering transformants.

IV. REFERENCES

Andreason, G.L. and Evans, G.A. (1988) Introduction and expression of DNA molecules in eukaryotic cells by electroporation. BioTechniques **6**, 650-660.

Birnbiom, H.C. and Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res. **7**, 1513-1523.

Bregliano, J.C. and Kidwell, M.G. (1983) Hybrid dysgenesis determinents. In Sapiro, J.A. (Ed.) Mobile Genetic Elements. Academic Press, New York, pp 363-410.

Brown, M. (1975) A photographic study of development in the living embryo of *Drosophila melanogaster*. J. Embryol and Morph. **33**, 789-801.

Calvin, N.M. and Hanawalt, P.C. (1988) High-efficiency transformation of bacterial cells by electroporation. J. Bacteriol. **170**, 2796-2801.

Chovnick, A. (1973) Gene conversion and transfer of genetic information within the inverted region of inversion heterozygotes. Genetics **75**, 123-131.

Chovnick A., Gelbart, W. and McCarron, M. (1977) Organization of the *rosy* locus in *Drosophila melanogaster*. Cell **11**, 1-10.

Cote, B., Bender, W., Curtis, D. and Chovnick, A. (1986) Molecular mapping of the *rosy* locus in *Drosophila melanogaster*. Genetics **112**, 769-783.

Cummings, M.R., Brown, N.M., and King, R.C. (1971) The cytology of the vitellogenic stages of oogenesis in *Drosophila melanogaster*. III. Formation of the vitelline membrane. Z. Zellforsch. **118**, 482-492.

Davies, L. (1947) Laboratory studies on the egg of the blowfly *Lucilia sericata* (M.G.). J. Exp. Biol **25**, 71-85.

Eudy, W.W., Bernheim, N.J., and Dobrogosz, W.J. (1969) Procedure for incorporation of radioisotopically labeled metabolites into embryos of dipterous insects. Anal. Biochem. **29**,136-142.

Fargnoli, J. and Waring, G.L. (1982) Identification of vitelline membrane proteins in *Drosophila melanogaster*. Dev. Biol. **92**, 306-314.

Finnerty,V.G., Duck, P. and Chovnick, A. (1970) Studies on genetic organization in higher organisms. II. Complementation and fine structure of the maroon-like locus of *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA **65**, 939-946.

Fox, A.S. and Yoon, S.B. (1966) Specific genetic effects of DNA in *Drosophila melanogaster*. Genetics **53**, 897-911.

Glassman E. (1965) Genetic regulation of Xanthine Dehydrogenase in *Drosophila melanogaster*. Federation Proceedings. **24**, 1243-1251.

Graf, G.E., Hadron,E. and Ursprung, H. (1959) Experiments on the isoxanthopterin metabolism in *Drosophila melanogaster*. J. Ins. Physiol. **3**, 120-124.

Hadron, E. and Schwinck, I. (1956) A mutant of *Drosophila* without isoxanthopterine which is non-autonomous for the red eye pigments. Nature **177**, 940-941.

Hanahan, D. (1985) Techniques for Transformation of *E. coli*. In Glover, D.M. (Ed.) DNA Cloning. Vol I: A practicle approach. IRL Press, Oxford, pp109-135.

Hill, L. (1945) Chemical removal of the chorion from *Drosophila* eggs. *Drosophila* Information Service **19**, 62.

Jowett, T. (1986) Preparation of nucleic acids. In Roberts, D.B. (Ed.) *Drosophila, a practicle approach*. IRL Press, Oxford, pp 275-286.

Keith, T.P., Riley, M.A., Kreitman, M., Lewontin, R.C., Curtis, D., and Chambers, G. (1987) Sequence of the structural gene for Xanthine Dehydrogenase (*rosy* locus) in *Drosophila melanogaster*. Genetics **116**, 67-73.

Kidwell, M.G., Kidwell, J.F., and Sved, J.A. (1977) Hybrid dysgenesis in *Drosophila melanogaster*: A syndrome of aberrant traits including mutation, sterility and male recombination. Genetics **86**, 813-833.

King, R.C. and Koch, E.A. (1963) Studies on the ovarian follicle cells of *Drosophila*. Quart. J. Micr. Sci. **104**, 297-320.

Lee, C.S., Curtis, D., McCarron, M., Love, C., Gray, M., Bender,W., and Chovnick, A. (1987) Mutations affecting expression of the *rosy* locus in *Drosophila melanogaster*. Genetics **116**, 55-66.

Limbourg, B., and Zalokar, M. (1973) Permeabilization of *Drosophila* eggs. Dev. Biol. **35**, 382-387.

Mahowald, A.P. (1962) Fixation problems for electron microscopy of *Drosophila* embryos. *Drosophila* Information Service **36**, 130-131.

Maniatis, T., Fitsch, E.F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Manseau, L.J., and Schupbach, T. (1989) Anterior-posterior pattern formation in *Drosophila* embryogenesis and oogenesis. Trends in Genetics **5**, 400-405.

Margaritis, L.H., Kafatos, F.C. and Petri, W.H. (1980) The eggshell of *Drosophila*. I. Fine structure of the layers and regions of the wild-type eggshell. J. Cell Sci. **43**, 1-35.

Maxwell,.I.H. and Maxwell, F. (1988) Electroporation of mammalian cells with a firefly luciferase expression plasmid: kinetics of transient expression differ markedly among cell types. DNA **7**, 557-562.

Mitchell, H.K., Glassman, E. and Hadron, E. (1958) Hypoxanthine in *rosy* and *maroon-like* mutants of *Drosophila melanogaster*. Science **129**, 268-269.

Neumann E., Schaefer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982) Gene transfer into mouse lyoma cells by electroporation in high electric fields. EMBO J. **1**, 841-845.

O'Hare, K., and Rubin, G.M. (1983) Structures of P transposable elements and their sites of insertion and excision in the *Drosophila melanogaster* genome. Cell **34**, 25-35.

Petri, W.H., Wyman, A.R., Kafatos, F.C. (1976) Specific protein synthesis in cellular differentiation. III. The eggshell proteins of *Drosophila melanogaster* and their program of synthesis. Dev. Biol. **49**, 185-199.

Potter, H., Weir, L., and Leder, P. (1984) Enhancer-dependent expression of human κ immunoglobulin genes introduced into mouse pre-B lymphocytes by electroporation. Proc. Natl. Acad. Sci. USA **81**, 7161-7165.

Potter, H. (1988) Review: Electroporation in biology: Methods, applications and instrumentation. Anal. Biochem. **174**, 361-373.

Rubin, G.M. and Spradling, A.C. (1983) Vectors for P element-mediated gene transfer in *Drosophila*. Nucleic Acid Res. **11**, 6341-6351.

Rubin, G.M. and Spradling, A.C. (1982) Genetic transformation of *Drosophila* with transposable element vectors. Science **218**, 348-353.

Sayles, C.D., Procunier, J.D., and Browder, L.W. (1973) Radiolabelling of *Drosophila* embryos, Nature, New Biol. **241**, 215-216.

Scavarda, N.J. and Hartl, D.L. (1984) Interspecific DNA transformation in *Drosophila*. Proc. Natl. Acad. Sci. USA. **81**, 7515-7519.

Scholnick, S.B., Morgan, B.A. and Hirsh, J. (1983) The cloned Dopa Decarboxylase gene is developmentally regulated when reintegrated into the *Drosophila* genome. Cell **34**, 37-45.

Shigekawa, K. and Dower, W.J. (1988) Electroporation of eukaryotes and prokaryotes: A general approach to the introduction of macromolecules into cells. BioTechniques **6**, 742-751.

Southern, E.M. (1975)   Detection of specific sequences among DNA fragments separated by gel electrophoresis.   J. Mol. Biol. **98,** 503-517.

Spradling, A.C. and Rubin, G.M. (1982)   Transposition of cloned P elements into *Drosophila* germ line chromosomes.   Science **218,** 341-347.

Spradling, A.C. and Rubin, G.M. (1983)   The effect of chromosomal position on expression of the *Drosophila* Xanthine Dehydrogenase gene.   Cell **34,** 47-57.

Toneguzzo, F., Hayday, A.C. and Keating, A. (1986)   Electric field-mediated DNA transfer: Transient and stable gene expression in human lymphoid cells.   Mol. Cell. Biol. **6,** 703-706.

Toneguzzo, F., Keating, A., Glynn, S. and McDonald, K. (1988)   Electric field-mediated transfer: Characterization of DNA transfer and patterns of integration in lymphoid cells.   Nucleic Acid Res. **16,** 5515-5532.

Wakimoto, B.T., Kalfayan, L.J. and Spradling, A.C. (1986) Developmentally regulated expression of *Drosophila* chorion genes introduced at diverse chromosomal positions.   J. Mol. Biol. **187,** 33-45.

Wieschaus, E. and Nusslein-Volhard, C. (1986)   Looking at embryos. In Roberts, D.B. (Ed.) *Drosophila, a practicle approach*.   IRL Press, Oxford, pp 199-227.

Wigglesworth, V.B. (1945)   Transpiration through the cuticle of insects. J. Exp. Biol. **21,** 97-114.

Winterbourne D.J., Thomas, S., Hermon-Taylor, J., Hussain, I.and Johnstone, A.P., (1988)   Electric shock-mediated transfection of cells. Biochem. J. **251,** 427-434.

Yoon, S.B. and Fox, A.S. (1965)   Permeability of premature eggs from *Drosophila* collected with the 'ovitron'.   Nature **206,** 910-913.

Zalokar, M. (1971)   Fixation of *Drosophila* eggs without pricking. *Drosophila* Information Service **47,** 128.

Zimmermann, U. (1982) Electric field-mediated fusion and related electrical phenomena. Biochem. Biophys. Acta **694**, 227-277.

o