ELECTRICAL PROPERTIES, pH, AND ION ACTIVITIES IN LOCUST EGGS

CHANGES IN ELECTRICAL PROPERTIES AND OOPLASMIC ACTIVITIES OF Na^+, K^+, H^+, Ca^{++}, AND Cl^- DURING EGG DEVELOPMENT IN THE LOCUST

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TITLE: Changes in Electrical Properties and Ooplasm Activities of Na^+, K^+, Cl^-, Ca^{++}, and H^+ During Egg Development in the Locust

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ABSTRACT

This thesis addresses two hypotheses: 1) ooplasmic ion activity is regulated during water uptake by locust eggs, and, 2) activities of Ca^{++} and H^{+} are maintained at levels appropriate for their use as signals for developmental processes, including activation, in insect eggs. Hypothesis 1 is based on the perturbing effect of large changes in intracellular Na and/or K activity on enzyme function in eukaryotic cells. Hypothesis 2 is based on the ionic hypothesis of activation developed from studies of eggs of marine invertebrates.

Electrical potential difference across egg membranes (PD_{egg}), and ooplasmic activities of Na^{+}, K^{+}, Cl^{-}, Ca^{++} and H^{+} were measured with double-barrelled ion-selective microelectrodes. Locust eggs maintained a measurable potential difference across egg membranes throughout development. Input resistance (R_{in}) decreased by approximately 5-fold in eggs after fertilization suggesting that the chorion is not the major barrier to ion movements into and out from the egg. Chilling and anoxia decreased the contribution of a metabolic component to PD_{egg}. The effects of hypercapnia on PD_{egg} and ooplasmic pH suggest that the metabolic component may be a H^{+}-pump.

Chloride diffusion contributes to PD_{egg} in eggs after fertilization; contributions of other ions were relatively small. Potential differences measured in internally perfused locust eggs after osmotic lysis of the serosal epithelium indicated that the chloride-dependent component of PD_{egg}

is developed across the chorion and/or serosal cuticle. In vivo values of PD_{egg} are probably less negative than values measured in control saline because chloride content of ground water is low.

Ooplasmic sodium and potassium activities remained at typically intracellular levels during water uptake, possibly due to release from internal stores. Chloride activities were typical of extracellular fluids and were not regulated during water uptake.

Measurements of pH and pCa in locust eggs are consistent with the increases in these parameters predicted by the ionic hypothesis of activation. Calcium activity in the ooplasm of unfertilized locust eggs (pCa 6.4 - 4.9) appeared to be at a level appropriate for the use of calcium as a signal or second messenger. Calcium activity increased 100-fold within 1 day of fertilization, and 1000-fold by day 3. Calcium entry from external sources at fertilization and release from internal stores later in development may contribute to the progressive increase in ooplasmic Ca^{++} activity. The ooplasm likely alkalinizes after oviposition as ambient pCO_2 declines. Available data suggest a metabolically-dependent proton pump may control ooplasmic pH in locust eggs, in contrast to the Na^+/H^+ exchanger implicated in alkalinization of marine invertebrate eggs.

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General Introduction

Evolution of the cleidoic egg has played a pivotal role in the successful exploitation of the terrestrial environment by insects. In contrast to the eggs of marine invertebrates, insect eggs are characterized by large quantities of yolk and by their protective coverings, which permit the passage of respiratory gases and water, but protect the egg and limit the loss of ions and non-electrolytes. Grasshopper and locust eggs, for example, are partially collapsed while stored within the body of the female, but increase their water content by up to 233 % by taking up water from the substratum after oviposition (Roonwall, 1936b). If ion activities are correspondingly reduced during this increase in water content, enzyme function may be perturbed. Large changes in sodium and potassium activities in eukaryotic cells are known to alter protein structure, thereby altering enzyme function (Somero, 1986).

It is not known if ion activities are regulated in the ooplasm of locust eggs during water uptake. Ionoregulation has been studied in frog eggs through the measurement of isotope fluxes (Morrill *et al.*, 1977). Observation of ionoregulation is difficult in insect eggs, however, because the protective coverings of the egg bind or limit the fluxes of radioisotopes, and also complicate the use of compounds which inhibit ion transport or metabolism. Although ion selective microelectrodes (ISME's) have been used in toad, avian and echinoderm eggs (Robinson, 1985; Davis *et al.*, 1988; Shen and Steinhardt, 1978), they have not been used to

measure ion activities in chorionated insect eggs. Moreover, although changes in ion activities (particularly H^+ and Ca^{++}) play important roles in the activation of eggs in many phyla (Whitaker and Steinhardt, 1986; Jaffe, 1985), little is known about the roles of changes in the activities of these ions as signals controlling the activation or development of insect eggs.

This thesis describes measurements of membrane properties and ion activities (H^+, Na^+, K^+, Ca^{++}, and Cl^-) in the developing eggs of the locust before and after oviposition and fertilization. The data permit evaluation of the extent and mechanism of ionoregulation. Possible developmental roles for changes in ion activities are considered. Relevant background information is provided in sections 1.1 - 1.3, below, and specific goals of the thesis are outline in section 1.4.

1.1 Reproduction in Locusts

1.1.1 Female reproductive system

The reproductive system in a mature female locust consists of paired ovaries which occupy most of the abdomen. Each ovary is comprised of a number of ovarioles (egg tubes) attached to the corresponding lateral oviduct at their bases. Their apices converge at the midline to form a single ligament anchored in the mesothorax. Each ovariole contains a series of oocytes at different stages of development, with the most mature nearest the opening to the oviduct. The two lateral oviducts merge to form the common oviduct which broadens to form the vagina. The blind anterior end of the lateral oviducts house the accessory glands which

produce the proteinaceous foam secreted during oviposition (Uvarov, 1966). The spermathecal duct extends from the spermatheca to the vagina, entering just anterior to the gonopore. The spermatheca stores the spermatozoa transferred at copulation within the spermatophore produced by male. The vagina ends at the gonopore which forms the opening of the genital chamber between the valves of the ovipositor. Eggs are coated with spermatozoa as they pass the opening of the spermathecal duct and exit the gonopore during oviposition. Penetration of the sperm into the egg occurs approximately 90 minutes later (Lanot *et al.*, 1987).

1.1.2 Egg growth and membrane formation

Vitellogenin, the major nutritive yolk protein in the egg, is produced in the fat body of the female and is sequestered from the maternal bloodstream by the developing oocyte (Kunkel and Nordin, 1985). Vitellogenin is a complex of lipid, carbohydrate and protein components with a molecular weight of about 550 kDa (Chen *et al.*, 1978). Within the egg, yolk proteins are stored in large membrane-bound yolk spheres (Gupta, 1967). Clusters of vitellogenin-specific receptors are found in the oocyte plasma membrane. A detailed study of vitellogenin uptake has shown that these receptors are pinched off from the plasma membrane to form endocytotic spheres containing vitellogenin. These small spheres (0.05 - 0.08 um in diameter) subsequently merge to form yolk spheres as large as 20 - 30 um (Petavy, 1986; Rohrkasten and Ferenz, 1985, 1986). Vitellogenesis begins when eggs are 1.8 - 2.0 mm long and continues until the eggs have reached their full length of 6.5 mm (Lanot *et al.*, 1986). Yolk spheres are distributed throughout the egg with larger spheres

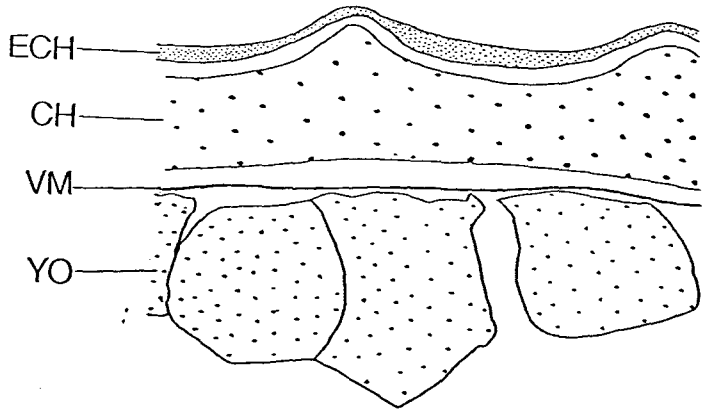
predominating near the centre of the egg and smaller spheres at the periphery (Petavy, 1986).

The lipid component of vitellogenin is modified slightly around the time of uptake into the oocyte although the total lipid content remains constant (Kunkel and Nordin, 1985; Rohrkasten and Ferenz, 1985). The resulting 570 kDa compound is cleaved 2 - 3 days after fertilization to form vitellin, a 445 kDa compound (McGregor and Loughton, 1974; Rohrkasten and Ferenz, 1985).

Formation of the egg coverings is initiated in the ovary and continues after oviposition (Uvarov, 1966). The arrangement of the egg coverings at selected stages of development is schematicized in Figure 1. The vitelline membrane is secreted by the ovum and is the first layer external to the oolemma. In grasshoppers it is a delicate structure which disappears shortly after development begins (Slifer, 1937). The next layer out is the chorion, which is secreted by the follicle cells when the egg is about 5.8 mm long (Lanot *et al.*, 1986). The chorion is divided into two layers, the endochorion, consisting of a mesh work of fine fibres with spaces between, and a thinner exochorion which shows a faint hexagonal pattern, reflecting the shape and arrangement of the follicle cells. When the egg passes into the oviduct it acquires an extrachorion, which is probably a mucous secretion.

As the embryo begins to develop, a cellular layer known as the serosa or serosal epithelium is formed from the blastoderm. The serosa lines the inner surface of the chorion, surrounding both the yolk system and the embryo. Because of the extent of the serosa, the ventral portion

Figure 1 Changes in egg coverings of locusts in unfertilized eggs (unf.), and in eggs 3 days and 7 days after fertilization at 37° C (after Slifer 1937, modified). Hatching occurs on day 10 at this temperature. Abbreviations are as follows: ech, extrachorion; ch, chorion; vm, vitelline membrane; yo, yolk sphere; yc, yellow cuticle; wc, white cuticle; ss, serosal space; se, serosal epithelium.



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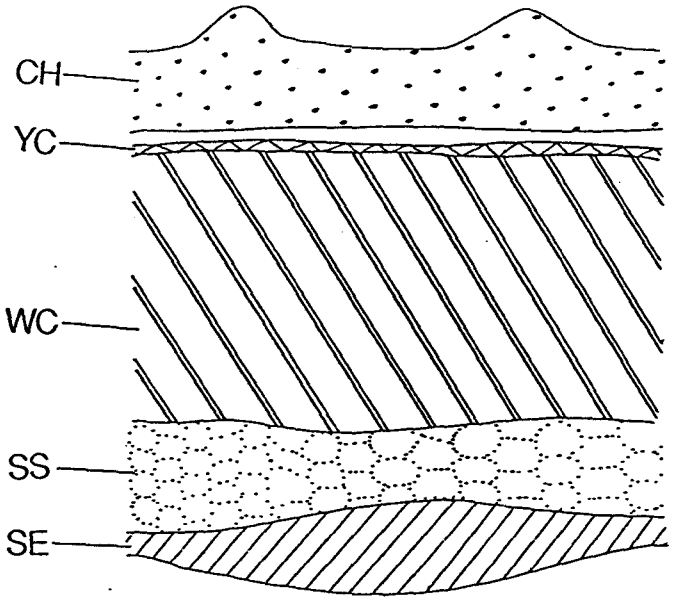

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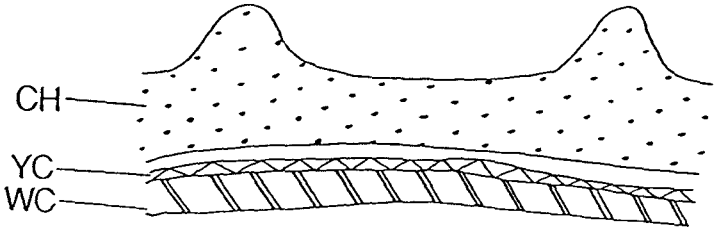


DAY 7

of the embryo is separated, in fact, from its source of nutrients. The serosal epithelium secretes two further acellular membranes. The inner white cuticle, which is laminated and consists primarily of chitin, becomes thicker than the chorion but is resorbed subsequently and almost disappears before hatching. The outer yellow cuticle is a thinner layer which resembles the cuticulin of the insect integument. The fluid which fills the space between the serosal epithelium and the serosal cuticle forms a tanned lipid membrane if exposed to air (Jones, 1958). When the eggs swell during water absorption, any damage to the serosal cuticle is repaired in this way.

At the time of oviposition locust eggs are pale-yellow, banana-shaped cylinders 5.5-6.0 mm long (Roonwall, 1936a). The anterior end is rounded, whereas the posterior end is blunter and contains the micropyle, a ring of 35 - 43 easily visible pores through which the sperm enter the egg. Each pore forms a funnel-shaped canal, running obliquely through the chorion and tapering from 8.5 - 20 um in diameter at the exterior to 2 um at the interior end (Roonwall 1936a; O'Donnell and Solweij, 1990).

1.1.3 Oviposition

During oviposition, the female first forms a small hole in the soil by extending her abdomen while opening and closing the ovipositor valves to push soil particles away. When the hole is complete, the female secretes a frothy secretion which is produced by the accessory glands and subsequently hardens to form the egg pod. Pulsating movements of the abdomen propel the egg through the lateral and common oviducts to, and then out of, the vagina. The egg is extruded at the bottom of the hole,

micropylar end down. The abdomen gradually contracts as the hole fills with eggs and frothy secretion. The upper portion of the hole is filled with froth which hardens to form a 'plug'.

1.1.4 Embryo growth

Meiosis is arrested during prophase I until chorionation, when high titres of ecdysone appear to reinitiate meiosis up to metaphase I (Lanot *et al.*, 1987). This second meiotic arrest is removed at the time of egg laying (Lanot *et al.*, 1987). Fertilization occurs by sperm entry through the micropyle (Wigglesworth, 1982). The egg pronucleus and the sperm nucleus move together and enter mitosis 3 - 4 hours after oviposition (Lanot *et al.*, 1987). Although a yolk-free area of cytoplasm forms around the daughter nuclei (Sanders *et al.*, 1985), the daughter cells are not enclosed within plasma membranes. These syncytial cells divide synchronously for the first 2 divisions (Roonwall, 1936a). Subsequently, the cells remain syncytial but for the next 6 cleavages mitosis is metachronous, so that a progressive wave of cleavage is initiated at the posterior end of the egg and travels forward. Cells continue to divide and some begin to migrate anteriorly along the periphery of the egg about 7 hours after fertilization (Roonwall, 1936a). Plasma membranes completely encapsulate the nuclei and yolk-free areas of ooplasm of the cells at the egg periphery by 18 - 23 hours after fertilization at 33° C (Roonwall, 1936a), or about 12 - 16 hours at 37° C (McGregor and Loughton, 1974).

The serosal epithelium is complete and the germ disk, which will form the embryo, is a double layer of cells by 24 hours post fertilization

(McGregor and Loughton, 1974). The layer of the germ disk in contact with the yolk forms the mesoderm and the outer layer forms the ectoderm (Roonwall, 1936a). The embryo develops and differentiates so that the head, thorax, and abdomen are clearly visible by day 2 - 3 (McGregor and Loughton, 1974).

A process known as blastokinesis begins on day 4. The embryo moves along the concave side of the egg, around the posterior end, and moves along the convex side so that the head of the embryo points upward, toward the anterior of the egg. The embryo also rotates 180^{o} on its long axis during this time, so that its ventral surface faces the concave side of the egg (Uvarov, 1966). Blastokinesis is completed and rudimentary organ systems are visible in the embryo on day 5 (McGregor and Loughton, 1974).

Ectoderm and mesoderm from the ventral side of the embryo migrate around the periphery of the yolk and ooplasm to join at the dorsal midline during days 3 - 6 (Saunders *et al.*, 1986). This process is known as dorsal closure. In the fully developed hopper, the mesoderm encapsulating the yolk and ooplasm forms the foregut (Uvarov, 1972; Roonwall, 1936a). Development is completed on days 7 - 9, and hatching occurs on day 10 (McGregor and Loughton, 1974).

1.1.5 Water uptake

Although the egg is a closed system with respect to nutrients, eggs of orthopterans such as locusts and crickets are laid in moist environments and absorb water during embryogenesis. The mass of the locust egg increases more than two-fold by water uptake, and its water

content increases from 52 to 82 percent during development (Roonwall, 1936b). The mechanism of water uptake is not well understood, but appears to be an active process (Edney, 1977). The serosal epithelium, but not the embryo, is required (Grellet, 1971).

The egg membranes are permeable to water and to non-polar molecules with a diameter as large as that of glucose at all times during development (Browning, 1969). Vitellogenin can also pass through the chorion and vitelline membrane, since vitellogenesis continues until ovulation, when the egg passes into the oviduct (Lanot *et al.*, 1986; Rohrkanten and Ferenz, 1985). Small molecules pass through the coverings of living eggs much less rapidly than through the dead egg coverings, however, suggesting that the cells of the serosal epithelium, or at least their outer cell membranes, control the movement of solutes into and out from the egg (Grellet, 1971; Browning, 1972).

1.1.6 Electrophysiological properties of locust eggs

Immature, unchorionated locust oocytes at varying stages of development exhibit an inside negative membrane potential which becomes more negative as the oocyte develops, reaching a maximum of -40 mV in eggs longer than 0.6 mm (Woolberg *et al.*, 1976). The membrane potential of vitellogenic locust oocytes is dependent primarily on potassium diffusion across the vitelline membrane, but a ouabain-sensitive Na+/K+ pump also contributes approximately 8 % to the total potential difference (Woolberg and Cocos, 1981).

Mature, chorionated eggs maintain a potential difference between the ooplasm and bathing saline of more than -40 mV, inside negative (O'Donnell

and Solweij, 1990). These eggs produce calcium-dependent action potentials of long duration (> 100 sec) when depolarized (O'Donnell and Solweij, 1990). Intracellular recordings indicate that excitability is localized to the area around the micropyle (O'Donnell and Solweij, 1990). Extracellular current measurements with the vibrating probe technique also indicate that experimentally injected current traverses the egg membranes primarily near the micropyle (O'Donnell and Shipley, 1991).

After activation these eggs maintain a potential difference less negative than unfertilized eggs (-34 mV), and their input resistance (0.27 megohms) is lower than unfertilized chorionated eggs (1.3 megohms) and comparable to that of pre-chorionated eggs (0.24 megohms; O'Donnell and Solweij, 1990; Woolberg *et al.*, 1976). Input resistance (R_{in}) provides a measure of the relative difficulty with which ions traverse the egg membranes. Fertilized eggs are also inexcitable in calcium salines, but will produce action potentials in salines containing barium (O'Donnell and Solweij, 1990), which passes through many types of calcium channel more readily than calcium (Hille, 1985). The membrane is therefore still excitable but the more conductive membranes act as a shunt which reduces the overshoot produced by calcium influx through voltage-dependent channels (O'Donnell and Solweij, 1990).

1.2 Activation

Activation is the process in which a mature but metabolically rather dormant unfertilized egg (Busa and Nuccitelli, 1984) is transformed into an active zygote. Activation is characterized by changes in membrane

properties, increases in the rates of oxygen consumption, DNA and protein synthesis, formation and fusion of pronuclei, and cell division. Activation is usually initiated by fertilization, although eggs of many species can be activated parthenogenically.

1.2.1 Ionic hypothesis of activation

The ionic hypothesis of activation states that many of the developmental processes initiated during activation are dependent, directly or indirectly, upon changes in the intracellular activities of ions, especially Ca^{++} and H^{+} (Whitaker and Steinhardt, 1985). Changes in Ca^{++} activity and pH act as signals, alone or in concert, to initiate the various processes necessary for embryo development. This hypothesis is based primarily upon studies of sea urchin eggs, where interaction of the fertilizing sperm with the egg surface triggers the release of calcium from intracellular stores, such as specializations of the endoplasmic reticulum (Gilkey *et al.*, 1973, Gardiner and Grey, 1983).

The increase in calcium activity initiates a subsequent increase in calcium efflux from the egg; the increase in cytoplasmic calcium activity, therefore, is transient (Steinhardt and Epel, 1974). In addition to increasing intracellular calcium activity, the egg-sperm interaction depolarizes the egg. Before fertilization, the sea urchin egg has a high specific membrane resistance (Hagiwara and Jaffe, 1979). Fertilization initiates a regenerative action potential dependent on voltage-gated calcium entry. The rising phase of the action potential is thought to correlate to the fertilization current (Whitaker and Steinhardt, 1985). The falling phase overlaps with the rising phase of a prolonged

depolarization called the activation potential, which results from sodium-influx through calcium-gated channels (Dale *et al.*, 1978). The depolarization caused by the action potential and activation potential may act as a fast, temporary block to polyspermy (Jaffe, 1980; Hagiwara and Jaffe, 1979), because sperm will not bind to egg membranes with a positive potential (Jaffe, 1986). The fast-block begins about 3 sec after fertilization and reduces the probability of sperm entry to 5 % (Rothchild and Swann, 1952). In *Xenopus* eggs, it is hypothesized that the rise in cytoplasmic calcium opens chloride channels. Chloride efflux in pond water also produces a positive-going fertilization potential which acts as a fast, temporary electrical block to polyspermy (Cross, 1981).

Calcium also controls the permanent block to polyspermy which is established by elevation of the fertilization membrane 30 - 60 sec after fertilization (Rothchild and Swann, 1951; Byrd and Collins, 1975). The increase in calcium activity triggers the progressive, wave-like exocytosis of cortical granules in the egg and the consequent formation of the fertilization membrane (Whitaker and Steinhardt, 1985).

The increase in cytoplasmic calcium activity also activates protein kinase C which initiates cytoplasmic alkalinization through activation of a Na^+/H^+ exchange. Alkalinization of the cytosol is dependent on millimolar activities of sodium in the bathing fluids (Johnson *et al.*, 1976; Shen and Steinhardt, 1978). The timing of this alkalinization correlates with the timing of acid efflux after fertilization (Whitaker and Steinhardt, 1978; Johnson *et al.*, 1976), and it is hypothesized that the intracellular calcium release activates a sodium-hydrogen exchange mechanism (Johnson *et al.*, 1976). Lower intracellular pH may

metabolically repress the eggs (Busa and Nucitelli, 1984), preventing accidental premature activation. Alkalinization of the egg cytoplasm alone activates protein synthesis but the increase in calcium accelerates the rate of this synthesis (Winkler *et al.*, 1980). DNA synthesis has a similar dual ionic control, but it has an absolute requirement for both the cytoplasmic alkalinization and calcium activity increase (Whitaker and Steinhardt, 1981).

Another calcium activated kinase which converts NAD to NADP has been isolated in sea urchin eggs (Epel *et al.*, 1981). This increase in NADP increases "the readily available reducing power [for biosynthesis] in cells" (Stryer, 1981, p. 333).

1.2.2 Activation in deuterostomes and protostomes

The primary role of intracellular calcium increase and the secondary role of egg alkalinization can account for the morphological, physiological and electrical changes in sea urchin eggs after fertilization. Jaffe (1985) has extended the ionic hypothesis to account for activation of protostomes as well as deuterostomes.

The deuterostomes include echinoderms, hemichordates, and vertebrates. These animals are characterized by radial cleavage, indeterminant developmental patterns, and formation of the coelom from outpockets of the gut in a process termed enterocoely. After fertilization deuterostome eggs exhibit a progressive wave of cortical granule exocytosis and membrane elevation which moves from the animal pole to the vegetal pole (Jaffe, 1985).

Alkalinization in deuterostome eggs probably plays a subordinate

role to that of calcium during activation. In frog eggs cytosolic pH has been shown to alkalinize by 0.2 pH units after fertilization (Webb and Nuccetelli, 1985). Ascidian eggs, however, maintain constant cytosolic pH during and after fertilization (Russo *et al.*, 1989). In eggs of *Medaka* (a teleost fish), pH is linked to the calcium threshold for propagation of the exocytotic wave (Gilkey 1983). Calcium injection will activate the eggs at intracellular pH 6.5 and 7.0, but the amount of calcium needed to initiate the exocytotic wave is 3-fold larger at pH 6.5.

Protostomes, including annelid, arthropod and molluscan phyla, are characterized by spiral cleavage patterns, determinate development, and formation of the coelom by a splitting of the mesoderm in a process termed schizocoely. Some protostome eggs, mostly mollusc and annelid phyla, extrude a bulb of cytoplasm called a polar lobe immediately before the first cleavage. The polar lobe remains continuous with the egg cell and is absorbed into only one of the daughter cells after the first cleavage (Gilbert, 1988). In contrast to eggs of deuterostomes, exocytosis is not universal in protostome eggs, and when it does occur, as in *Limulus*, it is a prolonged process, from 3 - 60 minutes, beginning simultaneously at a variety of locations on the egg (Bannon and Brown, 1980). The increase in internal calcium activity also occurs synchronously over the entire egg, and leads to a more prolonged increase in cytoplasmic calcium activity (>10 min) than in deuterostomes (1-10 sec locally; Jaffe, 1985). In an echinoid worm, sodium leakage through or near the inserted sperm membrane depolarizes the membrane potential of the egg initiating this voltage-dependent calcium entry (Jaffe *et al.*, 1979). Entry of external calcium through voltage-dependent channels appears to be a characteristic

of all activating protostomes examined to date (Jaffe, 1985).

Both a rise in intracellular calcium activity as well as intracellular alkalinization appear to be required for successful activation of protostome eggs. Reducing sea water pH from approximately 8.0 to 7.1 within 3 minutes of fertilization inactivates eggs in several protostome species and permits refertilization (Jaffe, 1985).

1.3 Ionoregulation

The need for regulation of the activities of various ions in the ooplasm of the eggs varies with developmental stage. Before fertilization and for the first 16 hours after fertilization, the ooplasm is an intracellular compartment within the egg and bathes the arrested egg nucleus or the dividing syncytial daughter nuclei. After the daughter cells have been encased in plasma membrane, and the serosal epithelium has formed, however, the ooplasm has become extracellular with respect to these cells. More simply, intracellular levels of ions (86-140 mM K^+, 5-61 mM Na^+, 11-48 mM Cl^-, and sub-micromolar Ca^{++}) might be expected up to 16 hours after fertilization, and extracellular levels of ions (10-20 mM K^+, 85-110 mM Na^+, 85-115 mM Cl-, and millimolar Ca^{++}; Stobbart and Shaw, 1974; Djamgoz, 1987) thereafter.

1.3.1 Effects of changes in intracellular ion activity levels

Intracellular activities of potassium, sodium, calcium, and chloride affect protein synthesis and mitosis. Studies have shown that potassium activity is highest during mitosis in mouse cells (Lau *et al.*, 1988;

Boonstra *et al.*, 1981), and that increasing potassium activity (a_K) increases protein production in *Xenopus* eggs (Horowitz and Lau, 1988). Increasing potassium activity while maintaining low intracellular sodium activity, however, inhibits mitosis in frog eggs if a_{Na}/a_K is approximately 0.06 (Ziegler and Morrill, 1977). Intracellular chloride activities ranging from 45 mM in wheat germ to 75 mM in rabbit reticulocytes also inhibit protein synthesis (Weber *et al.*, 1977). In sea urchin eggs, intracellular calcium activity has been shown to increase during specific phases in the mitotic cell cycle: nuclear membrane breakdown, the metaphase/anaphase transition, and cleavage (Poenie *et al.*, 1985; Poenie *et al.*, 1986). Increases in calcium activity within mouse fibroblasts have also been shown to induce DNA synthesis (Dulbecco and Elkington, 1975). Similarly, calcium waves have been suggested as the signal for metachronous cleavage of syncytial nuclei in insect eggs (Wolf, 1985). In the gall midge these waves begin 22-26 minutes after fertilization, progressing from the posterior end of the egg at approximately 7 um/min. The calcium wave traverses the egg in about 60 minutes, and a new wave is initiated approximately every 20 minutes (Wolf, 1985).

1.3.2 Effects of extracellular ions on cell function

In many cells, uptake of glucose and amino acids, and regulation of intracellular pH and calcium is dependent on the sodium gradient, ie. high extracellular and low intracellular sodium activities (Schultz, 1986). Any change in the sodium gradient would therefore change sodium-coupled ion- or nutrient-transport. External potassium activity affects the membrane potential of cells if, as for many insect cells, they are

selectively permeable to potassium (Djamgoz, 1987; Dawson *et al.*, 1989; Verachtert *et al.*, 1989). Increasing external potassium would therefore make the potential difference across the cell membrane less negative and decreasing external potassium would make the potential difference more negative.

Cell-cell adhesion has been shown to be calcium-dependent in a variety of animal cells and low external calcium activities have been used to dissociate clusters of invertebrate cells (Hynes, 1972; Spiegel and Spiegel, 1975; Fink and McClay, 1980). Calcium has also been shown to be integral to cell-cell adhesion and communication in the neurogenic region of developing *Drosophila* embryos (Fehon *et al.*, 1990).

1.4 Goals of the Present Thesis

Chapters 2-4 investigate three sets of questions concerning the roles of ions in activation of locust eggs and ionoregulation during embryonic development.

1. Can the techniques used to measure the potential difference across the egg in chorionated unfertilized eggs be used in older eggs *i.e.* after serosal cuticle formation? What is the basis for the potential difference across the chorionated eggs, and is a significant electrical potential difference maintained across the egg as it develops? The term cleidoic is used to indicate the presence of a egg shell, and therefore, a barrier to movement of materials other than respiratory gases and water into and out of the

egg. Part of this chapter concerns the degree to which a cleidoic egg is, in fact, a closed system with respect to ions. The input resistance in locust eggs has been shown to decrease after fertilization, but it is not known if this change is due to an increase in permeability of a specific ion species (O'Donnell and Solweij, 1990). The basis of the potential difference after chorionation is also unknown.

Do the eggs exhibit a prolonged alkalinization after fertilization? Measurements of pH in eggs, the role of pH in activation, and pH regulation are discussed separately from Ca (Chapter 4) because a significant component of the membrane potential appears to be due to a metabolically-dependent proton pump.

2. What are the activities of the major cations (Na^+, K^+) and anions (Cl^-) within the ooplasm and are these activities regulated or do they change only as a result of the tripling of egg water content? Is the tripling of egg water content associated with corresponding decreases in ion activity? Ion activities in the ooplasm before fertilization and for 16 hours immediately following fertilization reflect the ion activities encountered by the syncytial nuclei during division. It is not known if the sodium, potassium and chloride are regulated to levels appropriate for protein synthesis and cell division. Also, are the ion activities maintained to levels that are compatible with enzyme action? It is not known if the ion activities in the ooplasm are regulated to

values typical for an extracellular or an intracellular milieu in insects after the ooplasm is surrounded by serosal epithelial cells.

3. What is intracellular calcium activity in locust eggs, relative to other types of eggs? Is there evidence for a transient or sustained increase in calcium activity around the time of oviposition? When all of the cells in the egg are still syncytial, is the calcium activity comparable to levels found appropriate for protein synthesis and mitosis in other eggs? Since many cells dissociate when extracellular Ca is reduced to intracellular levels, does the calcium activity change once the syncytial cells have been enclosed in plasma membrane and the serosal epithelium has begun to form?

Chapter 2

Ooplasmic pH and Electrical Properties of Developing Locust Eggs

The membranes surrounding locust eggs change dramatically during development, and only the chorion, once formed, remains relatively unchanged during subsequent development. As described in Chapter 1, the oolemma is transformed after fertilization; the serosal epithelium is formed and secretes the white and yellow cuticles. Changes in the structure of the egg coverings raise the possibility of accompanying changes in electrophysiological properties.

Recent evidence for calcium action potentials in chorionated locust eggs indicates that ions can cross the egg membranes present before fertilization from the surrounding fluids (O'Donnell and Soloweij, 1990), although the basis for the resting potential has not been examined. The potential difference across the egg coverings (PD_{egg}) of chorionated eggs declines from about -50 mV prior to oviposition to about -26 mV within 1 day after oviposition (O'Donnell and Soloweij, 1990), but comparable measurements have not been made later in development. Moreover, although the membrane potential of vitellogenic locust eggs is determined mainly by the passive distribution of potassium ions and by a ouabain-sensitive electrogenic Na^+/K^+ pump (Wollberg and Cocos, 1981), the relative contributions of ion diffusion and metabolic ion pumps to the potential difference across the membranes of chorionated eggs are unknown.

Changes in electrical properties might also result in or be related to changes in the activities of ions in the ooplasm. For example, if inside negative potentials are maintained during development, protons will

tend to accumulate in the ooplasm. Moreover, metabolic activity of the embryo and the surrounding yolk itself will result in the release of acidic equivalents into the ooplasm and a further tendency for egg pH to decline. Ooplasmic pH may also be perturbed by changes in egg water content, which increases from 52 % to 82 % of egg mass during development (Roonwall, 1936b).

Measurements of ooplasmic pH are also of interest in view of the role of alkalinization as a stimulus for activation of the eggs of other protostomes such as annelids and molluscs (Jaffe, 1985). It is not known if insect eggs undergo alkalinization after oviposition and/or fertilization.

This chapter addresses these and related ideas through the use of double-barreled pH-sensitive microelectrodes. Simultaneous measurement of electrical potential and pH in locust eggs have been made at intervals from the time before oviposition until embryogenesis is nearly complete. The results indicate that egg pH is sensitive to changes in pCO_2, but that it is closely regulated despite large scale changes in potential difference across cellular and acellular membranes.

2.1 MATERIALS AND METHODS

2.1.1 Egg Collection

Locusta migratoria were reared at 35° C on a diet of bran and fresh wheat seedlings. Plastic cups filled with moist sand were provided as sites for oviposition. Eggs are buried in the sand encased in a foamy secretion which hardens to form a pod. Cups were removed daily, covered

with plastic to minimize moisture loss and incubated at 37° C.

Chorionated unfertilized eggs are referred to in the figures as 'unf.' eggs. These were collected from the lateral oviducts of gravid females which were decapitated and dissected under control saline. The eggs were rinsed with and maintained in control saline until transferred to the experimental chamber. Eggs collected 1 - 9 days after oviposition are referred to as d1 - d9 eggs, corresponding to the embryonic stages 1 - 9 in McGregor and Loughton, (1974). Eggs were obtained as needed from intact pods which were removed from the sand and gently broken open. Fine forceps were used to remove individual eggs from the pod and to remove foam adhering to them. The remaining portion of the pod was placed between two pieces of moist filter paper to prevent desiccation.

2.1.2 Physiological Salines

Composition of the experimental salines are given in Table 1. The control saline has been used previously for electrophysiological studies of ovarian eggs of *Locusta migratoria* (O'Donnell and Solweij, 1990). Anoxic saline was produced by bubbling control saline with N_2, and hypercapnic salines were produced by bubbling 7% CO_2 saline with a 7% carbon dioxide/air mixture or bubbling control saline with 100% carbon dioxide. Inhibitors of metabolism or ion transport were dissolved in control saline at a concentration of 1 mM. Chilled salines were produced by surrounding the saline inflow tubing and experimental chamber with ice.

Table 1.

The composition of the experimental salines (concentrations in mM).

	Control	10x K^+	0.1x Cl^-	0.1x Na^+	7% CO_2 pH 6.9
NaCl	129	51.6	----	12.9	129
KCl	8.6	86	8.6	8.6	8.6
$MgCl_2$	8.5	8.5	1.6	8.5	8.5
$CaCl_2$	2	2	2	2	2
Glucose	34	34	34	34	34
BIS-TRIS	15	15	15	15	----
Na Isethionate	----	----	129	----	----
NMDG Cl	----	----	----	116.1	----
$MgSO_4$	----	----	6.9	----	----
$NaHCO_3$	----	----	----	----	11.9
NaH_2PO_4	----	----	----	----	0.1

2.1.3 Superfusion

Eggs were superfused in a chamber constructed from a 50 mm length of 6 mm diameter polyvinyl chloride tubing. Chamber volume when filled to a depth of 3 mm was approximately 0.7 ml. Saline flowed through the chamber and parallel to the long axis of the egg at 4 - 8 ml per minute.

2.1.4 Electrophysiology

Double-barreled pH-microelectrodes were made from thick-septum theta-glass capillaries (TST 150, 1.5 mm o.d., WPI, New Haven, Ct.) cut into 6 cm lengths. Capillaries were washed in nitric acid, rinsed in distilled water, and dried at 200° C on a hot plate for at least 20 minutes. A three to four cm length of one barrel was filled with distilled water prior to pulling in a Narishige micropipette puller. After pulling, the water filled the tip of one barrel within 10 s. The stem of the micropipette was then pushed through a plastic septum at the top of a 5 ml vial containing 1 ml of dimethyldichlorosilane (Sigma). The empty barrel was thereby exposed to silane vapour for 20 - 30 s, and the water prevented silanization of the other barrel. The micropipette was removed from the vial and placed on a hot plate for a minimum of 20 minutes to complete silanization. A drop of a neutral carrier based on tridodecyl amine (Hydrogen ionophore I, cocktail B, Fluka) was injected into the shank of the silanized barrel through a disposable plastic syringe pulled out over a low flame to a fine tube (Thomas, 1978). Movement of the cocktail to the tip of the electrode by capillary action was aided by pushing fine glass fibres towards the tip, and air bubbles were removed by application of heat from a soldering pencil, or with glass

fibres. The barrel was then backfilled with a solution composed of 100 mM Na citrate and 100 mM NaCl at pH 6. The reference barrel was filled with 0.5 M KCl.

The pH electrodes were calibrated before and after each impalement with control saline adjusted to pH 6.90 and 7.90 with HCl. The mean slope of the pH electrodes was 57.8 mV (n=48). Tip resistances of the reference barrel were 10 to 20 megohms.

Microelectrode tips were broken back to about 1 um outside diameter if the electrode was noisy, the 90% response time to a step change in pH exceeded 5s, or the slope of the electrode was less than 50 mV per unit change in pH. A modification of the method of Tripathi *et al.* (1985) was used for tip breakage, as described in a separate paper (O'Donnell and Machin, 1991).

Potential differences (PD's) from the pH sensitive (V_{pH}) and reference barrels (V_{ref}) of the double-barreled theta-glass microelectrodes were measured through chlorided silver wires inserted into the stem of each barrel and connected to high input impedance differential electrometers (WPI FD 223) with input impedances > 10^{14} ohms. The potential difference of the reference barrel (V_{ref}) was measured relative to a Ag/AgCl half cell connected to the bathing solution by a 3 M KCl agar bridge. V_{pH} was filtered through a low pass RC filter (time constant, 1 s) to reduce noise resulting from the high impedence (>10^{10} ohms) of the pH-sensitive barrel. V_{ref} and the difference (V_{pH}-V_{ref}) were recorded using an AD converter (Axon Instruments) and computer-based data acquisition system sampling at 10 Hz (AXOTAPE, Axon Instruments), and also on a two-channel chart recorder (Linear Instruments, Reno, Nv., USA).

For experiments in which PD_{egg} but not egg pH was measured, both barrels of a theta-glass microelectrode were filled with 3M KCl and either barrel was used to record potentials.

2.1.5 Impalements

Eggs were placed in the experimental chamber and observed at 80x magnification with a Leitz dissecting microscope. Unfertilized and d1 eggs were impaled by advancing the microelectrode with a hydraulic micromanipulator (Narishige) until the tip touched or dimpled the chorion. The microelectrode tip was then transiently advanced 30 - 50 micrometers and immediately retracted the same distance. This procedure impaled the egg to a depth of about 5 - 10 micrometers beneath the egg surface. Eggs on d2 or later were impaled by advancing the microelectrode tip about 100 um after surface contact to traverse the serosal cuticle and epithelium. Eggs were impaled at sites at least 1 mm away from the embryo.

Criteria for acceptable impalements were as follows: 1) The slope of the pH-selective barrel measured before and after impalement changed less than 2 mV. 2) V_{ref} and $V_{pH}-V_{ref}$ were stable to within ± 1 mV for 1 minute or more after impalement. 3) Outputs from both barrels measured in the bathing saline before and after impalement changed by less than 1 mV. 4) Preliminary experiments showed that potentials less negative than -40 mV for unfertilized, d3, d4, and d5 eggs, -15 mV for d1 eggs, or -20 mV for d2 eggs were unstable and usually declined to near zero mV within a few minutes. Eggs were immediately discarded, therefore, if PD_{egg} did not reach and maintain values exceeding these guidelines within the first minute after impalement.

2.1.6 Input Resistance

Egg input resistances were measured after impalement with two microelectrodes. Current was injected through one microelectrode electrode while the deflection in PD_{egg} was measured through a second theta-glass microelectrode. Current injection electrodes were pulled from thin walled capillaries (1 mm o.d., 0.78 mm i.d., WPI) and had resistances of 1 - 5 megaohms when filled with 3 M KCl. Current intensity was monitored through a current-to-voltage converter connected to the bath through 3 M KCl^- agar and Ag/AgCl half cells.

2.1.7 Dechorionation

For some experiments, d1 eggs were dechorionated with 1 % sodium hypochlorite for 60 seconds. Dechorionation increased the visibility of dyes injected to determine microelectrode tip location. Eggs became flaccid during hypochlorite treatment. They were rinsed subsequently in distilled water, then placed in control saline for 2 minutes, where they regained their original turgidity. Dechorionated eggs were placed on a layer of 3 % agar in control saline and incubated in Petri dishes at 37° C until day 3 when they were transferred to the experimental chamber.

2.1.8 Dye Injections

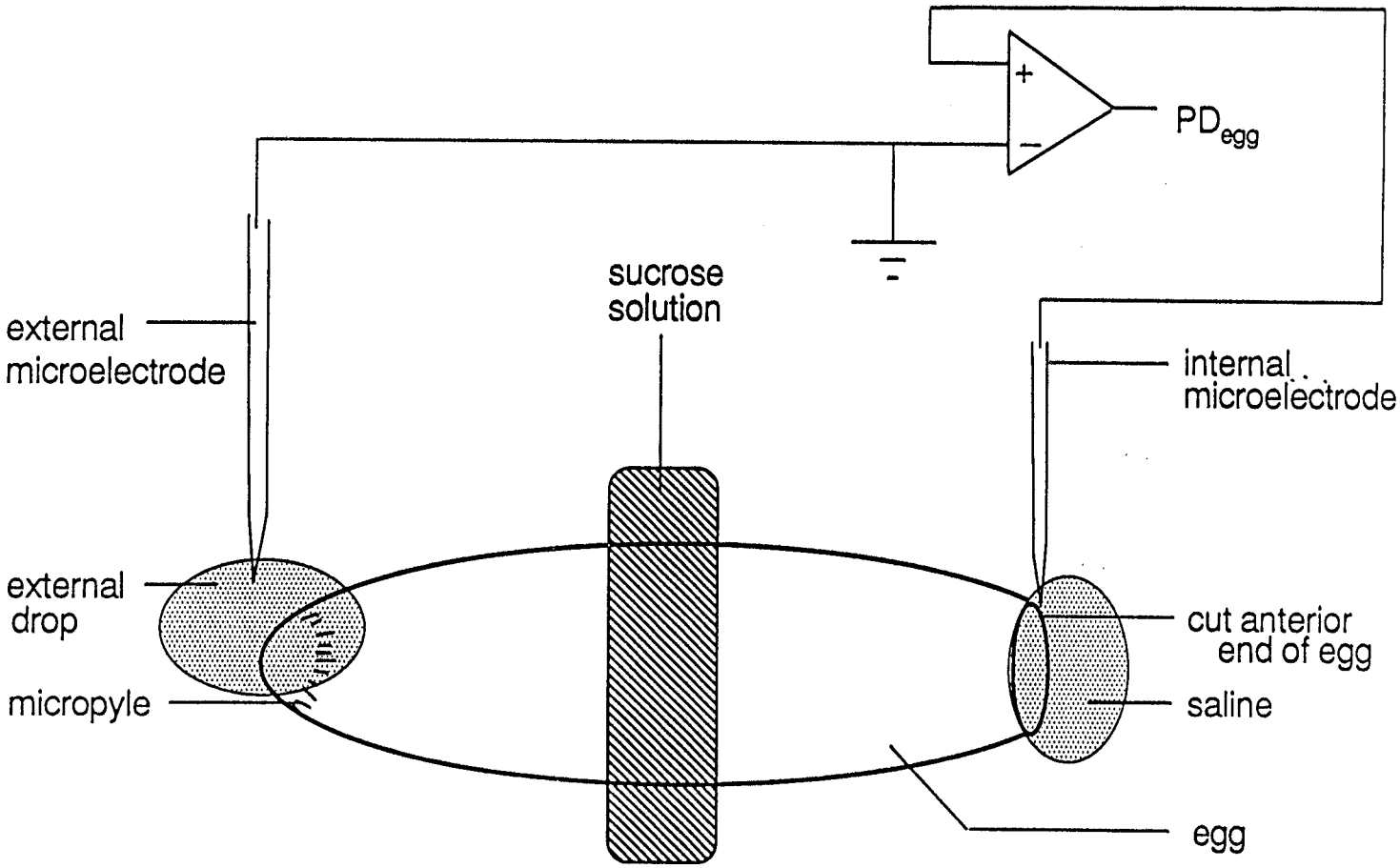
Single-barrelled microelectrodes were pulled from filamented capillary glass (1.0 o.d. 0.58 i.d., WPI) and were filled with 5 mM amaranth in 3 M KCl. Microelectrode tip position was marked by pressure injection of the dye. Pressure was applied through a hand-held syringe connected by polyethylene tubing to the back of the electrode (Gillette

et al., 1981). PD_{egg} measured through the same microelectrode did not change more than 5 mV during injection.

2.1.9 Internal perfusion

Internal perfusion permits control of the fluid bathing the inside of the egg membrane. The arrangements for recording PD_{egg} in internally perfused eggs under mineral oil are shown in Figure 2. A sucrose gap electrically isolated the external fluids from the internal fluids of the perfused egg. A solution of 340 mM sucrose in high resistance water (> 16 x 10^6 ohm cm) ringed the centre of the egg and was kept in place with wetted glass pins and a thin strip of filter paper around the egg at its midpoint. The sucrose solution was replaced every 3 minutes to prevent increases in conductivity of the gap solution arising from leaching of salts from the acellular membranes of the egg. An external theta glass microelectrode filled with 3 M KCl was placed in a drop of saline at the posterior of the egg. The saline was based on the ionic composition of d3 ooplasm found in Chapter 3, and contained 61 mM KCl, 10 mM NaCl, 0.25 mM $CaCl_2$. Osmolality was adjusted to 340 mOsm with glucose, and Bis tris (15 mM) was used as a buffer. pH was adjusted to 7.2. PD_{egg} was first measured by impalement, and the microelectrode was then withdrawn. The anterior end of the egg was then cut off with fine scissors and egg contents were flushed using a plastic tuberculin syringe pulled to a fine tip (100 um) over a low flame. A second theta glass microelectrode was then placed in contact with the internal solution, and the potential difference measured with respect to the external microelectrode. Measured

Figure 2 Experimental arrangement of internal perfusion of d3 eggs. The experiments were performed under mineral oil in petri plates lined with paraffin wax. Saline and sucrose drops were held in place with glass pins. A 1 mm wide strip of filter paper encircled the egg to pull the sucrose bubble under the egg.


+
−
PD_{egg}
sucrose
solution
external
microelectrode
internal
microelectrode
external
drop
micropyle
cut anterior
end of egg
saline
egg

potential differences were corrected for shifts in reference potential resulting from different chloride concentrations in external and internal fluid droplets.

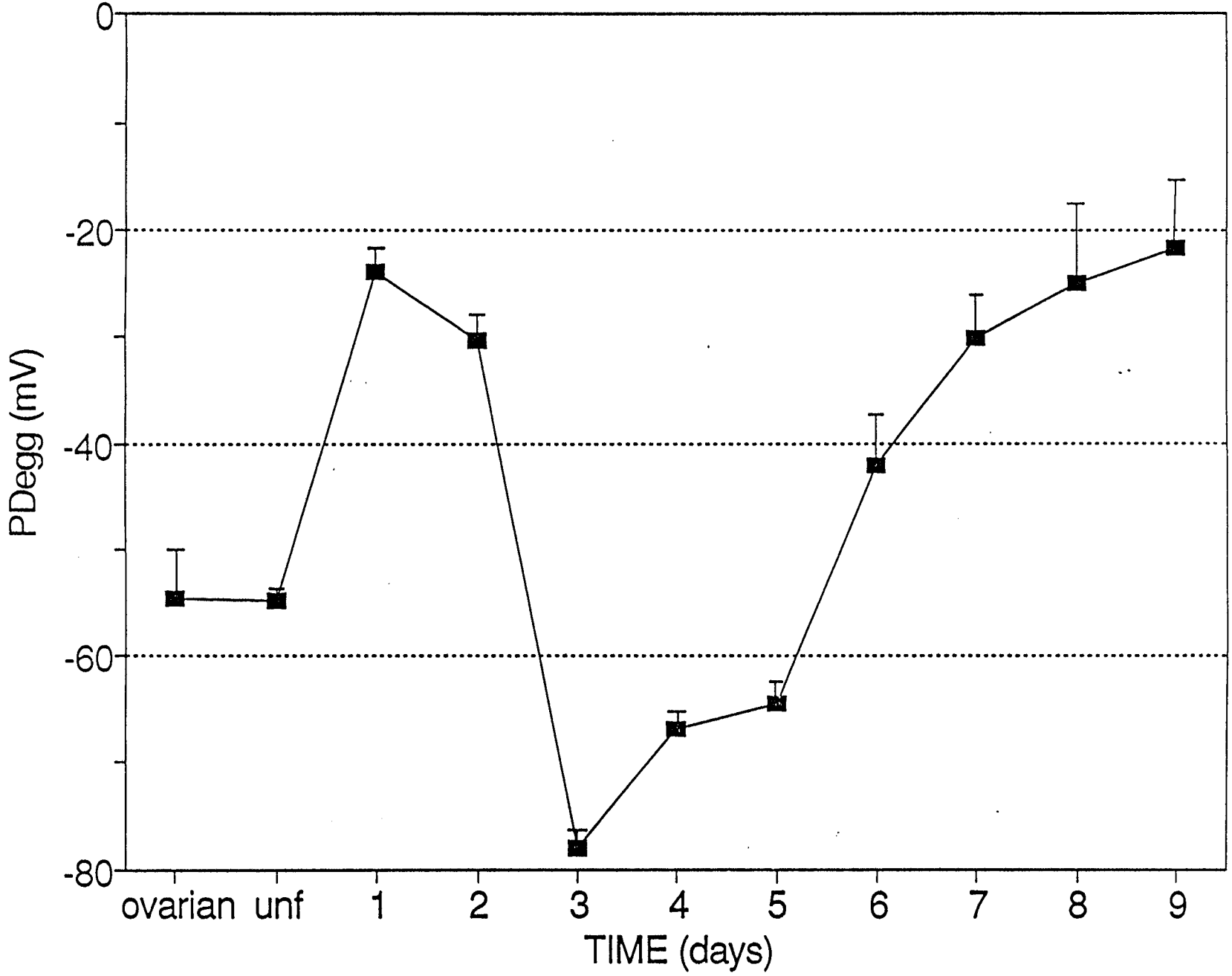
2.2 RESULTS

Microelectrode measurements of PD_{egg} for eggs bathed in control saline at various stages of development are shown in Figure 3a. Attempts to obtain stable recordings of PD_{egg} on d10 eggs, the day of hatching at 37° C, were unsuccessful. For eggs at all earlier stages of development, the egg membranes sealed around the electrode glass sufficiently tightly to permit the entire egg to be lifted off the bottom of the perfusion chamber with no detectable (<0.5 mV) change in potential.

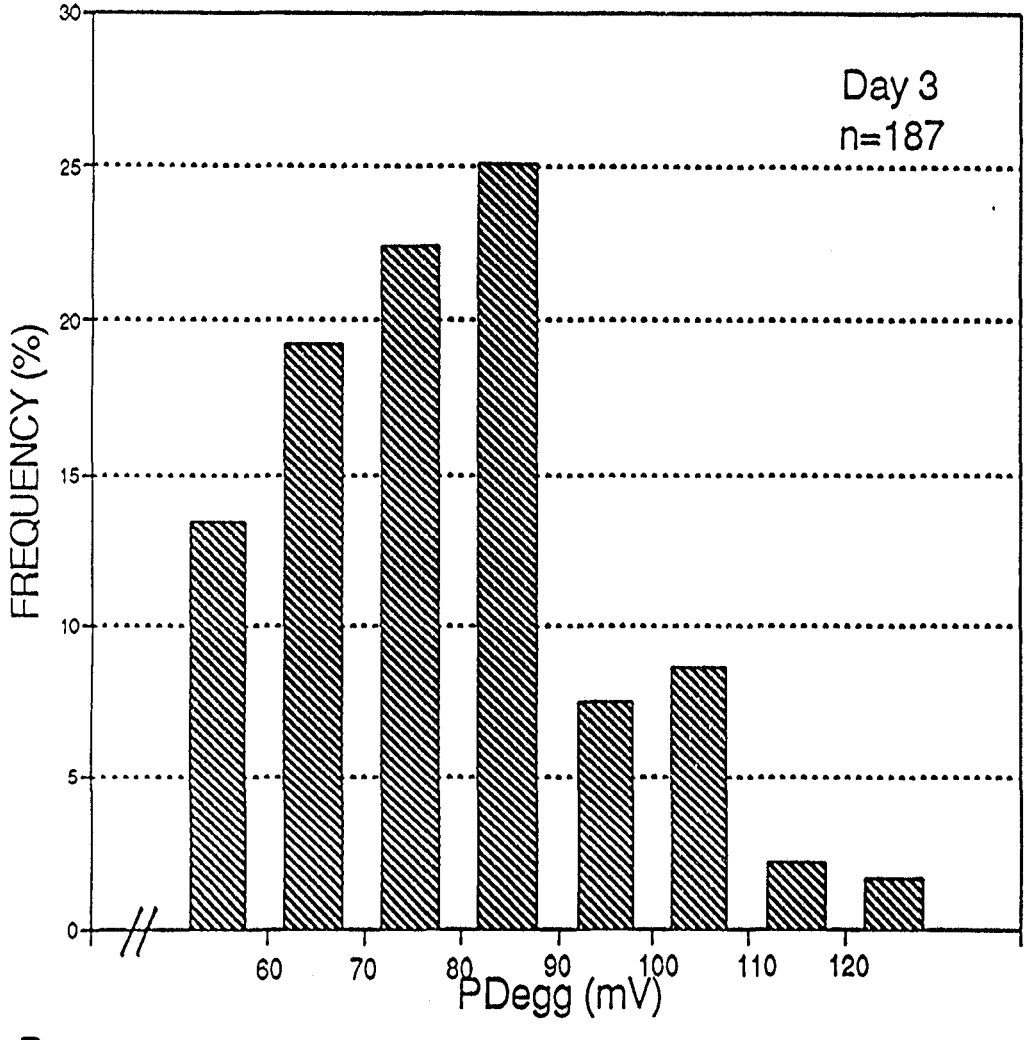
PD_{egg} changed substantially during development, particularly between d1, when the serosal epithelium is formed, and d3, when the rate of water uptake by the embryo is maximal, and both yellow and some white serosal cuticle are present. Values of PD_{egg} more negative than -100 mV were found in 12% of d3 eggs (Figure 3b). Potentials more negative than -90 mV were not seen in d4 eggs, although the average PD_{egg} was only 10 mV less negative for d4 eggs than for d3 eggs (Figure 3c).

It is important to point out that oviposited eggs are not normally in contact with fluids of the same ionic composition and osmolality as control saline. Nevertheless, the large changes in PD_{egg} for eggs at different stages bathed in the control saline suggested that the physiological properties of the cellular and/or acellular membranes surrounding the egg change substantially during development, and that a

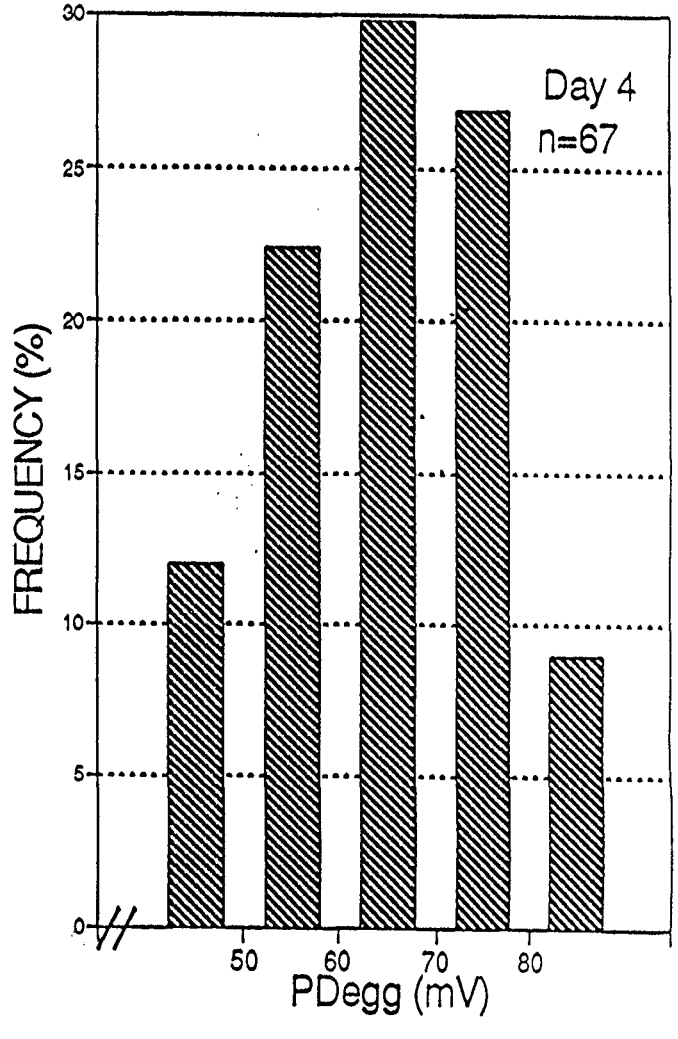
Figure 3 (A) Potential difference across membranes of developing Locusta eggs (PD_{egg}, in mV) bathed in control saline. Data points denote means ± S.E., n=8 for ovarian eggs, 24-187 for days 0-5, and 6-14 for days 6-9. Frequency histograms of PD_{egg} for d3 (B) and d4 (C) eggs.



A.


Day 3
n=187
FREQUENCY (%)
PDegg (mV)
0
5
10
15
20
25
30
60
70
80
90
100
110
120

B.


Day 4
n=67
FREQUENCY (%)
PDegg (mV)
0
5
10
15
20
25
30
50
60
70
80

C.

potential difference of a magnitude comparable to that found in nerves or muscles (Djamgoz, 1987) can be maintained across the egg membranes.

Dye injection experiments indicated that the microelectrode tip was positioned in the ooplasm, and not in the serosal fluid, or within aserosal cell or yolk sphere. For eight dechorionated d3 eggs in which dye injected through the microelectrode clearly outlined yolk spheres, the corresponding mean PD_{egg} was -73 $\pm$ 4 mV, comparable to the value for d3 with the chorion intact (Figure 3a). Less negative (-44 $\pm$ 6, n=4) and more variable potentials (-61 to -24 mV) were obtained when dye injection indicated that the microelectrode tip was positioned external to the serosal cells *i.e.* in the serosal fluid. Dechorionation of the egg increases the size of the serosal space in *Locusta* eggs (Jones 1958), and the serosal space could only be impaled after dechorionation. Attempts to impale the serosal space in chorionated eggs were unsuccessful (n=17). Dechorionation did not affect the viability of the eggs. Hatching rates were comparable for dechorionated eggs (82%, n=108) and control eggs incubated on agar (80%, n=10).

These results indicate that in dechorionated eggs PD_{egg} is composed of two potential differences in series. The serosal space is at a potential of about -44 mV relative to the bath, and this potential difference is established across the acellular membranes which separate the latter two compartments. Subtraction of the latter value from PD_{egg} indicates that the ooplasm is at a potential of about -29 mV relative to the serosal space. This second potential difference is established across the serosal epithelium.

Measurements of egg input resistance also indicated changes in the physiological properties of the egg membranes during development. Input resistance (R_{in}) provides a measure of the effectiveness of the cellular and acellular membranes of the egg as barriers to ion diffusion. A decrease in R_{in} represents an increase in ion conductance. Input resistance declined dramatically after oviposition, from 1.2 $\pm$ 0.2 (n=11) megohms in unfertilized eggs to less than 0.2 megohms in d1 - 5 eggs, when the embryo occupied approximately 60% of the egg volume (Figure 4). Measurements of R_{in} were not made at later stages of development because of the difficulty of avoiding the embryo during impalement, and because of the possibility that the presence of a large embryo would cause non-uniform current flow during the current injections used to determine R_{in}. Changes in PD_{egg} were independent of changes in R_{in}. For example, PD_{egg} increased from -23 mV on d1 to -78 mV on d3 (Figure 3) although there was little change in R_{in} (Figure 4). In contrast to the dramatic changes in PD_{egg} and R_{in} for eggs bathed in control saline, ooplasmic pH remained near 7.25 for eggs collected before or up to 5 days after oviposition (Figure 5). The corresponding Nernst equilibrium pH was calculated from external pH and measured values of PD_{egg} on each day. The difference between measured and equilibrium values of ooplasmic pH suggest that this parameter is closely regulated during development. Possible methods of regulation will be discussed below.

There was no significant increase of ooplasmic pH in fertilized d1 eggs relative to unfertilized eggs. An increase might be expected if fertilization and/or oviposition were associated with a sustained alkalinization. However, it is important to point out that the data for

Figure 4 Input resistance in developing Locusta eggs. Input resistance was calculated from changes in PD_{egg} during current injections. Current intensity was measured through a virtual ground circuit which functioned as a current to voltage converter. Data points denote means $\pm$ S.E., n = 5-11.

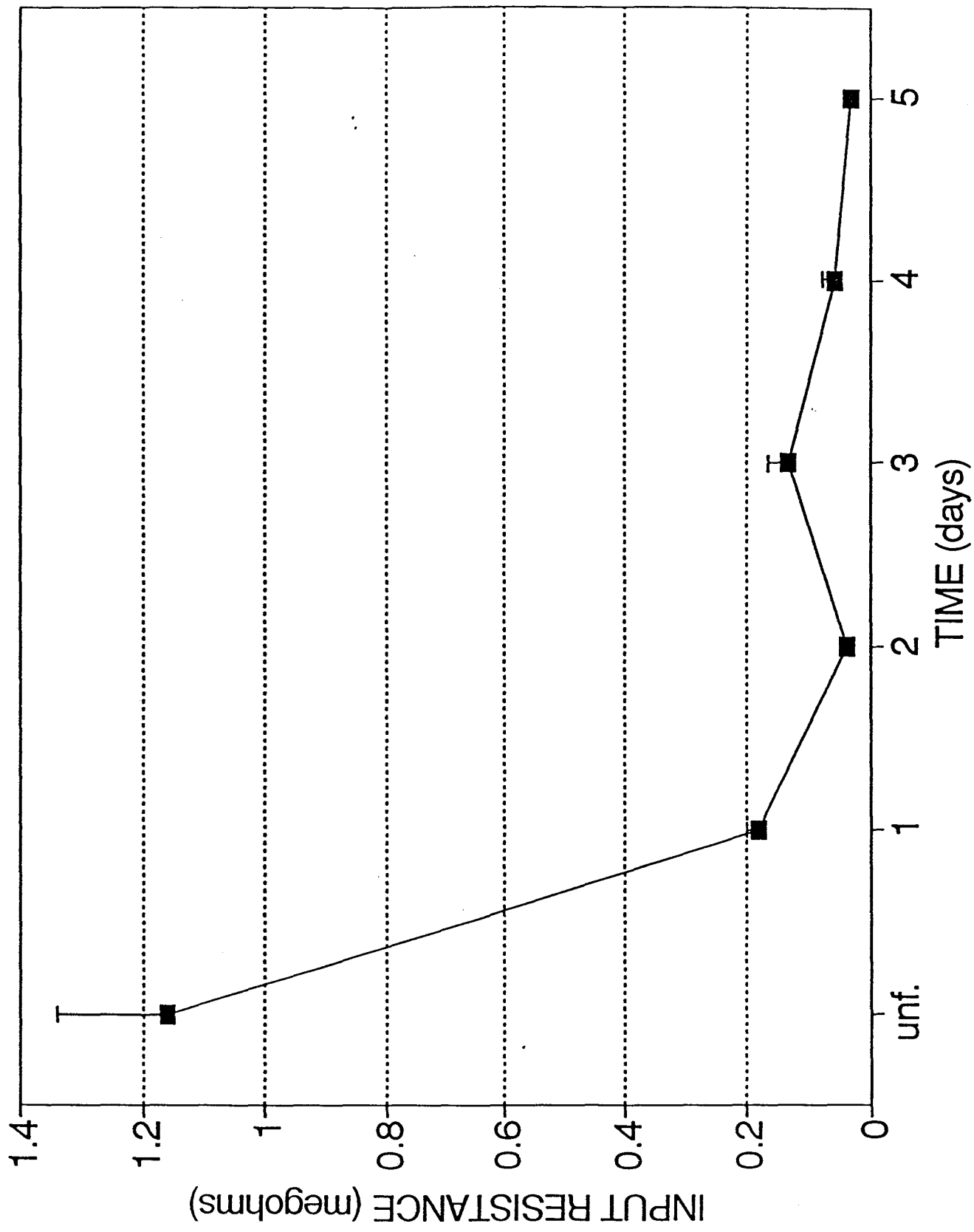

INPUT RESISTANCE (megohms)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
unf.
1
2
3
4
5
TIME (days)

Figure 5 Ooplasmic pH (pH_i) and equilibrium pH (pH_{eq}) during development. Values of pH_{eq} were calculated from external pH and the measured PD_{egg}, using the Nernst equation. Data points denote means $\pm$ S.E., n = 6-13.

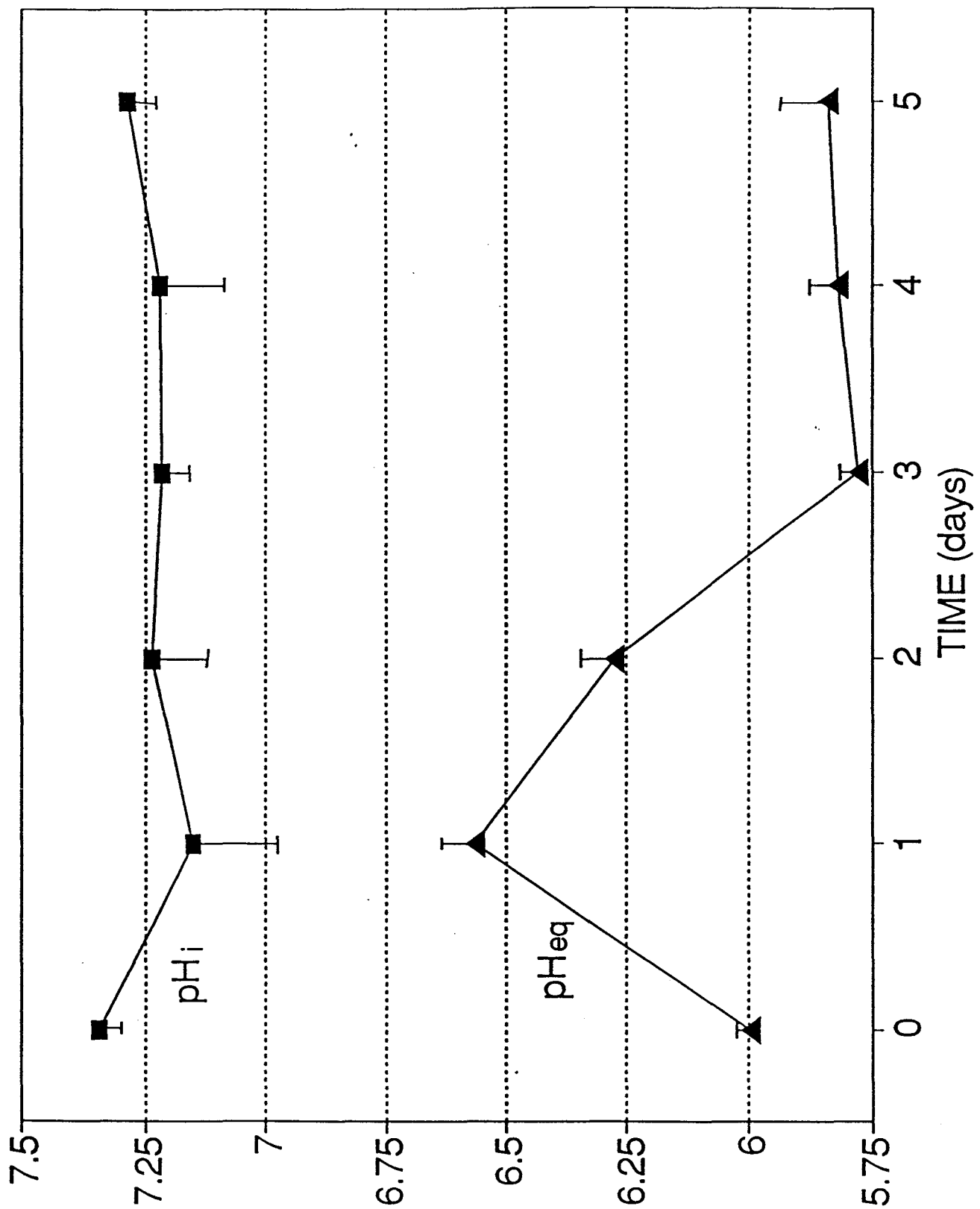

7.5
7.25
7
6.75
6.5
6.25
6
5.75
pH
pHi
pHeq
0
1
2
3
4
5
TIME (days)

Figure 3 were obtained from eggs bathed in saline equilibrated with air. *In vivo*, grasshopper eggs may experience a change in pCO_2 from about 4 - 5 % in haemolymph before oviposition (Harrison, 1988) to < 0.033 % as the foam covering the oviposited eggs equilibrates with air. The effects of changes in ambient pCO_2 on ooplasmic pH and PD_{egg} were examined directly, therefore, in d0 eggs. Ooplasmic pH decreased by about 0.2 units and PD_{egg} hyperpolarized by about 66 % (Figures 6, 7), when the perfusion medium was changed from saline equilibrated with air to saline equilibrated with 7% CO_2 at a pH of 6.9 throughout. PD_{egg} hyperpolarized by 22% (13 mV) in saline at pH of 5.6 bubbled with 100% CO_2 within 6 minutes. The smaller response to more extreme hypercapnia was primarily due to the reduction of PD_{egg} in acidic media. PD_{egg} in unfertilized eggs became 17 $\pm$ 3 mV less negative at pH 5.6 than at pH 6.9 in control saline equilibrated with air.

PD_{egg} was much less sensitive to hypercapnia at later stages of development (Figure 7). We suggest below that the serosal fluid and/ or acellular egg coverings may act as an unstirred layer which reduces the gradient in pCO_2 between the bathing saline and serosal epithelial cells in d1 or older eggs.

Further experiments were designed to assess the contribution of cellular and acellular membranes to the measured value of PD_{egg}. The cellular membranes lining the egg are the oolemma in unfertilized eggs, and the serosal epithelium in d1 or older eggs. Potential differences could arise from diffusion of ions down electrochemical gradients across cellular and/or acellular membranes, and from electrogenic pumping of ions across cellular membranes. The former can be examined by ion substitution

Figure 6 Representative recording of PD_{egg} (upper trace) and pH_i (lower trace), obtained during impalement by a double-barreled pH sensitive microelectrode in an unfertilized egg bathed alternately in saline equilibrated with air or 7% CO_2, at pH 6.9 throughout. Solid arrows indicate impalement of egg and withdrawal of electrode. The electrode was calibrated in saline at pH 6.9 and 7.9 before and after impalement. Deflections in pH indicated by open arrows are artifacts resulting from the differing response speeds of V_{ref} and V_{pH} to a change in potential.

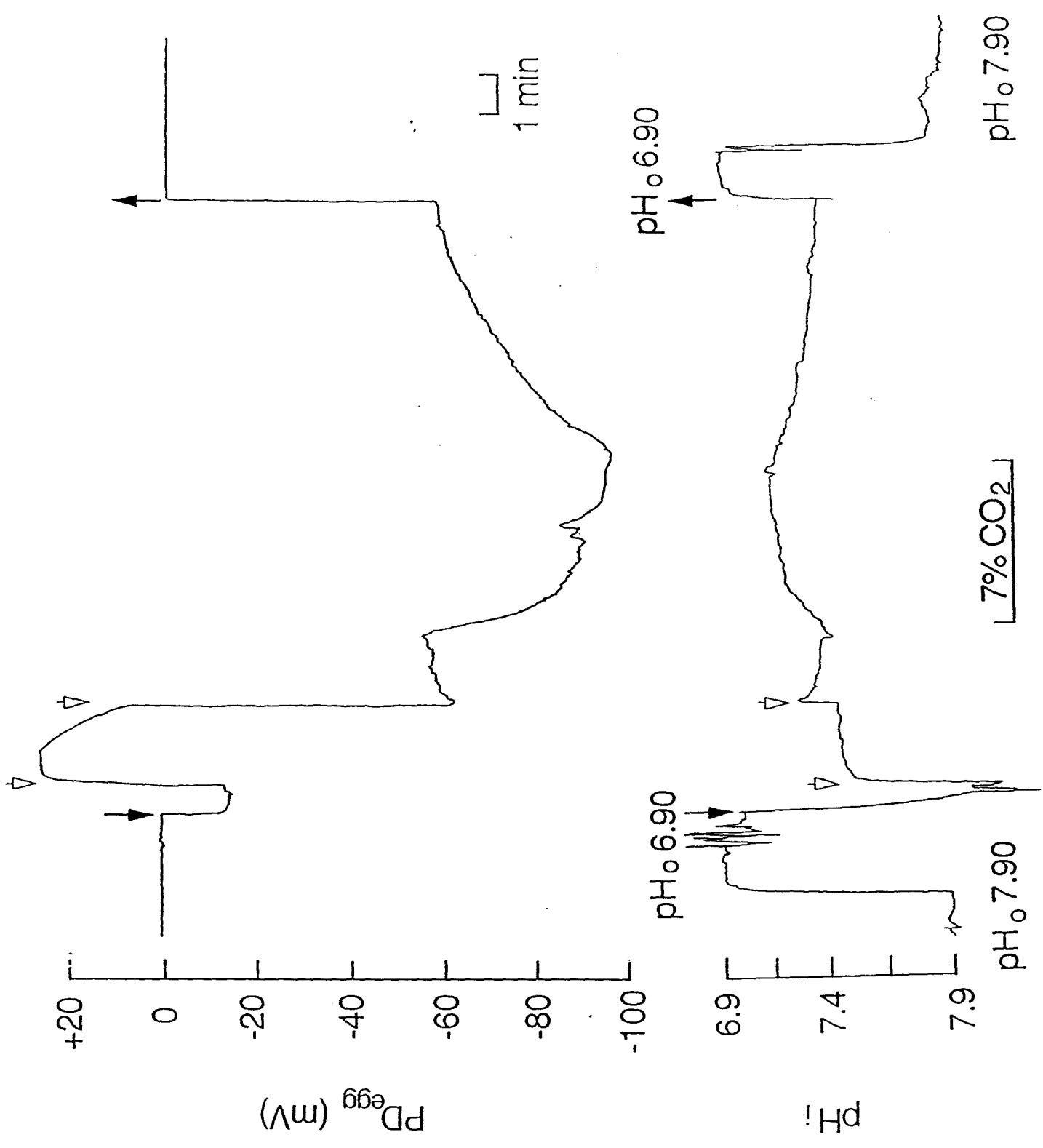
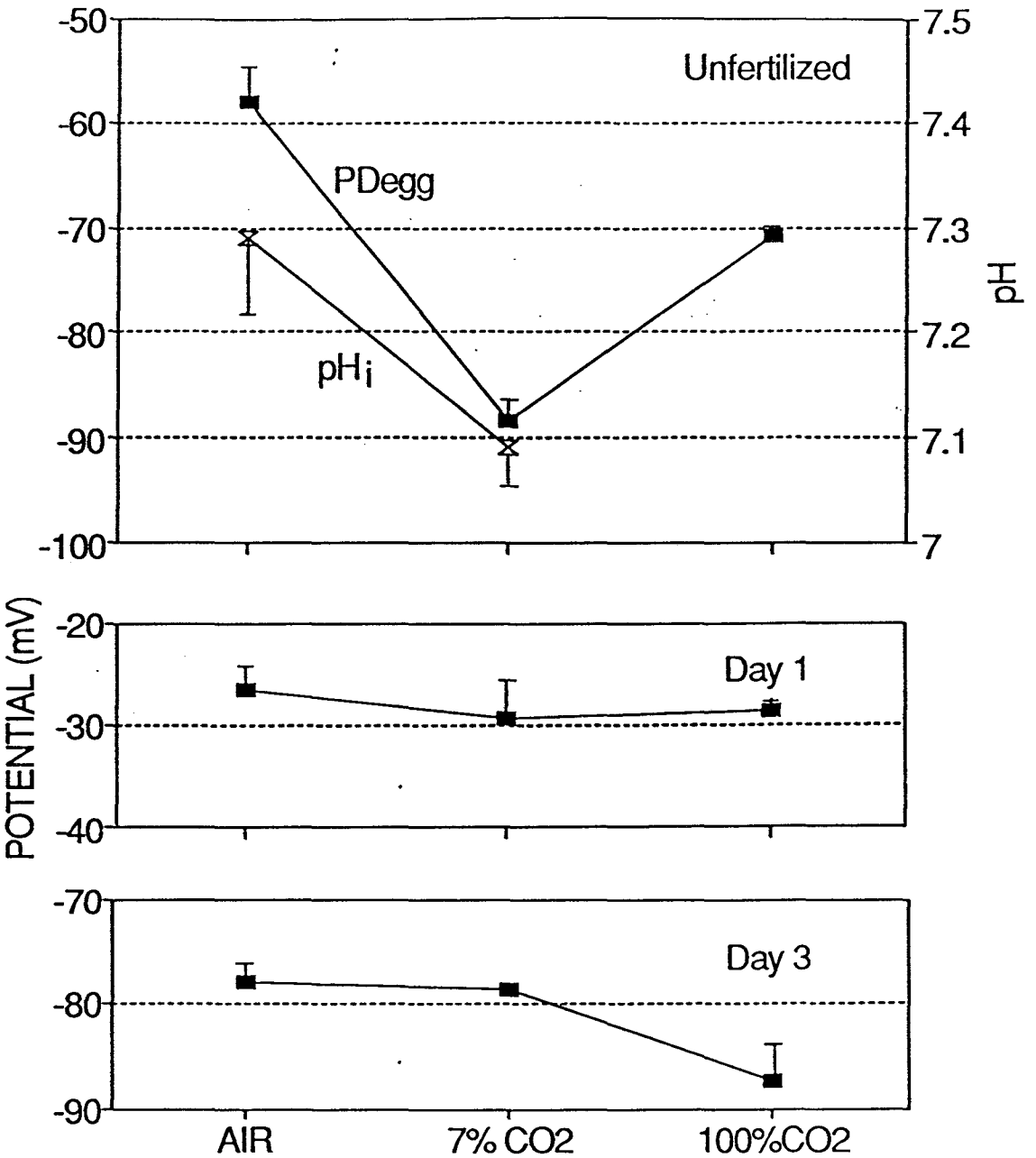

1 min
pH o 6.90
pH o 7.90
7% CO2
pH o 6.90
pH o 7.90
+20
0
-20
-40
-60
-80
-100
PDegg (mV)
6.9
7.4
7.9
pHi

Figure 7 Summary of PD_{egg} and pH_i measurements in developing eggs bathed in saline bubbled with: air (pH 6.9), 7% CO_2 (pH 6.9), and 100% CO_2 (pH 5.6). Open symbols, pH_i, closed symbols, PD_{egg}. Each point denotes mean $\pm$ SE. n = 4-9 for day 0, 5 for day 1, and 3-5 for day 3.


Unfertilized
PDegg
pHi
-50
-60
-70
-80
-90
-100
7.5
7.4
7.3
7.2
7.1
7
pH
POTENTIAL (mV)
Day 1
-20
-30
-40
Day 3
-70
-80
-90
AIR
7% CO2
100%CO2

experiments, and the latter by metabolic inhibition. Both passive potentials created by ion diffusion, and an active potential created by an electrogenic pump have been demonstrated in the vitellogenic eggs of *Drosophila* (Miyazaki and Hagiwara, 1976) and *Locusta* (Wollberg and Cocos, 1981). Diffusional and electrogenic pump contributions to PD_{egg} were examined in unfertilized, d1, and d3 eggs. These stages were chosen because d1 and d3 eggs had the least and most negative PD_{egg} respectively, and unfertilized eggs provided information about chorionated eggs prior to fertilization and oviposition.

Ion substitution experiments indicated that PD_{egg} in unfertilized eggs was relatively unaffected by changes in ionic composition of the bathing saline (Figure 8). The effect of a ten-fold reduction in Cl^- concentration on PD_{egg} in d1 and d3 eggs increased 3-fold to 19 mV and 21 mV, respectively. These changes were about 30 % of the ideal Nernst response (58 mV at 24° C). A ten-fold change in saline K^+ concentration produced less than a 3 mV potential change in chorionated eggs, in contrast to a change of 50 mV in vitellogenic eggs (Wollberg and Cocos, 1981). Small but consistent changes of about 5 mV in response to a ten-fold change in saline Na^+ concentration implied that, although the contribution of Na^+ to PD_{egg} was small, sodium ions were able, none the less, to cross the membranes.

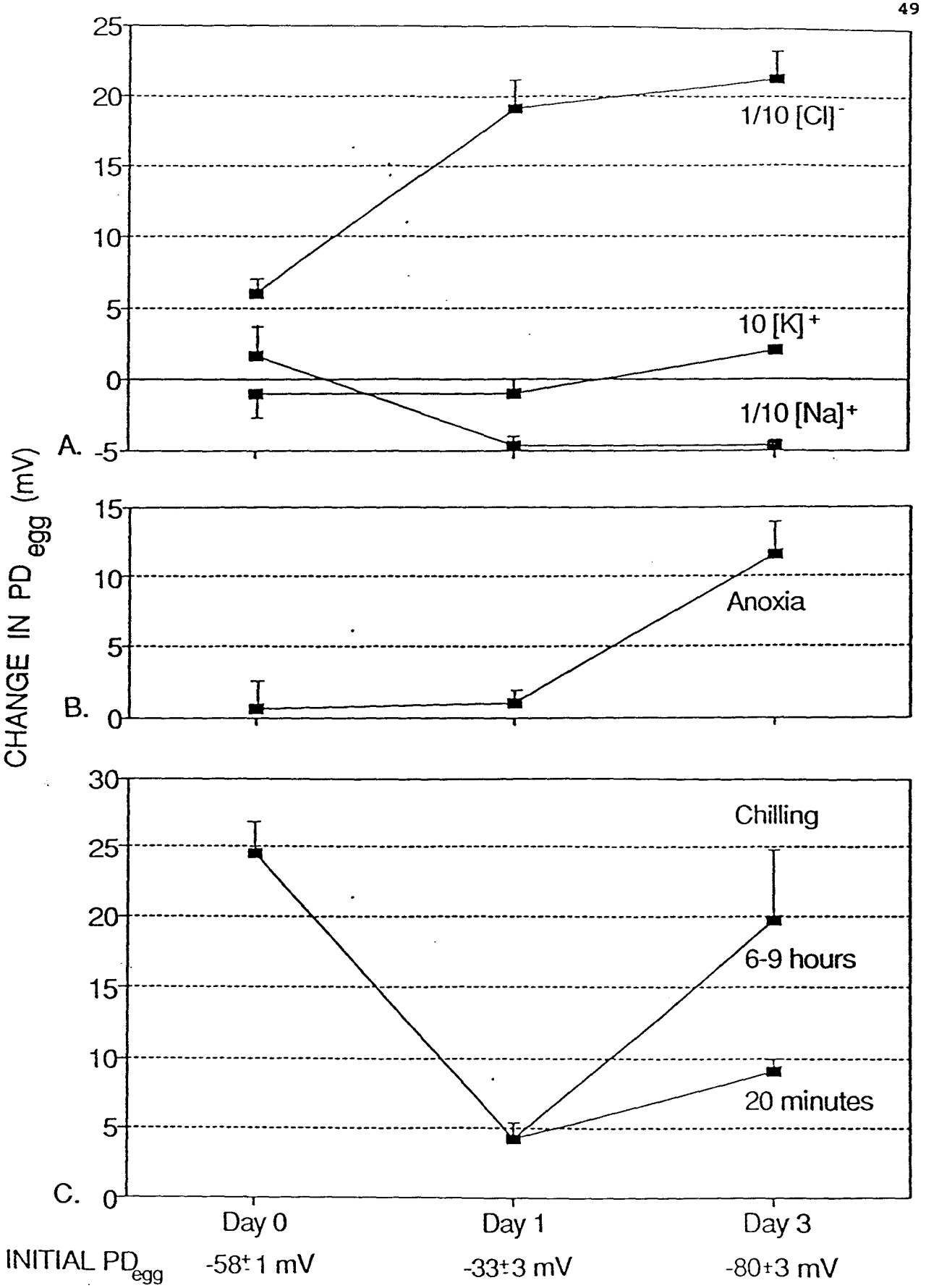
PD_{egg} in d3 eggs which had been dechorionated but retained the serosal cuticle was -73 ± 8 mV ($n = 9$), only 5 mV less negative than the value for d3 eggs with the chorion intact (Figure 3). The change in PD_{egg} in response to a ten-fold reduction in bathing saline sodium concentration was -5 ± 0.4 mV ($n=7$), similar to the change recorded for chorionated

Figure 8 Changes in PD_{egg} in developing locust eggs in response to chilling, anoxia, or alteration of bathing saline composition. All data points indicate means ± S.E.

(A) Changes in PD_{egg} when the bathing saline was changed from control saline to one in which potassium concentration was elevated 10-fold (10 $[K^+]$) , chloride concentration was reduced 10-fold (1/10 $[Cl^-]$, or sodium concentration was reduced 10-fold (1/10 $[Na^+]$). Each point denotes mean ± S.E., n = 6.

(B) Change in PD_{egg} when the bathing saline was changed from air-equilibrated to N_2-equilibrated control saline. N=3 on day 0, 4 on day 1, 17 on day 3.

(C) Changes in PD_{egg} measured when bathing eggs alternately in saline at room temperature (24° C) and at 6° C for 20 minutes each (n=13 on day 0, 4 on day 1, 3 on day 3) The results of prolonged exposure (6 to 9 hours) of day 3 eggs to chilling (n=33) are also shown.


25
20
15
10
5
0
-5
A.
1/10 [Cl]⁻
10 [K]⁺
1/10 [Na]⁺
15
10
5
0
B.
Anoxia
30
25
20
15
10
5
0
C.
Chilling
6-9 hours
20 minutes
CHANGE IN PD egg (mV)
Day 0
Day 1
Day 3
INITIAL PD egg
-58±1 mV
-33±3 mV
-80±3 mV

eggs. PD_{egg} changed by 14 ± 2 mV (n = 7) in response to a 10-fold reduction in the chloride concentration of the saline bathing dechorionated d3 eggs, 5 mV less than the value found in d3 eggs with the chorion intact. These results suggest that the chorion made only a minor contribution to PD_{egg} for eggs bathed in control saline.

A ouabain-sensitive electrogenic pump contributes about 8.5% of the membrane potential observed in vitellogenic locust eggs (Wollberg *et al.*, 1975). The presence of a metabolically dependent pump in the cellular membranes of chorionated eggs was indicated by the effects of chilling or anoxia on PD_{egg} (Figure 8). Chilling unfertilized eggs decreased PD_{egg} by approximately 43 %. Eggs on day 3, when the serosal epithelium lines the egg and the uptake of water is maximal (Chapter 3), were most sensitive to anoxia. Addition of ouabain or the metabolic inhibitors dinitrophenol or KCN at concentrations of 1 mM in control saline for 10 to 20 minutes did not significantly alter PD_{egg} in unfertilized, d1, or d3 eggs (n = 3, each day). As discussed below, these compounds may not have been able to cross the chorion. Long term chilling (6-9 hours, Figure 8C) may deplete reserves of compounds such as ATP, furthur reducing PD_{egg}.

Experiments with internally perfused d3 eggs provided further evidence for the existence of a potential difference across the serosal epithelium. D3 eggs were flushed free of yolk and both external and internal surfaces were bathed in a saline approximating the ooplasm of d3 eggs (10 mM Na^+, 61 mM K^+, 71.5 mM Cl^-, and 0.25 mM Ca^{++}; Chapter 3). Under these conditions, in which there were no differences in internal and external concentrations of the predominant inorganic ions, a potential difference of 18 ± 4 mV was measured, equal to 28% of the PD_{egg} (-62 ± 4

mV) measured by impalement of the eggs with microelectrodes before internal perfusion. This potential difference was abolished (0 ± 2 mV; n = 7) when the serosal cells were first lysed by flushing the eggs with distilled water for 3 min, and the internal solution was replaced with the identical saline based on d3 ooplasm.

Chilling experiments suggested a PD of about 20 mV is attributable to a metabolic pump across the serosal epithelium, slightly larger than the 18 mV suggested by the present experiment. This small discrepancy may result partly from imperfect electrical isolation of internal and external solutions by the sucrose gap. Any leaching of ions out of the egg or lateral diffusion from fluids in the spaces between the fibrous endochorion will tend to increase the conductivity of the sucrose gap solution. A partial short-circuit would result, and the potential difference across the membranes of the perfused egg would be underestimated.

Internal perfusion of the eggs also demonstrated that the potential difference across the acellular membranes was sensitive to differences in transmembrane chloride concentration. After osmotic lysis of the serosal cells, a potential of 13 ± 2 mV, inside negative, was measured when the internal and external solutions were 15 mM KCl/135 mM K acetate and 150 mM KCl respectively. This value equals 23% of the ideal Nernst response for a 10-fold change in chloride concentration, and is approximately 2/3 of the value (37%) measured by microelectrodes in comparable experiments with intact eggs. Potential differences measured in perfused eggs after lysis of the serosal cells may also be underestimates if the electrical isolation by the sucrose gap is imperfect, as discussed above.

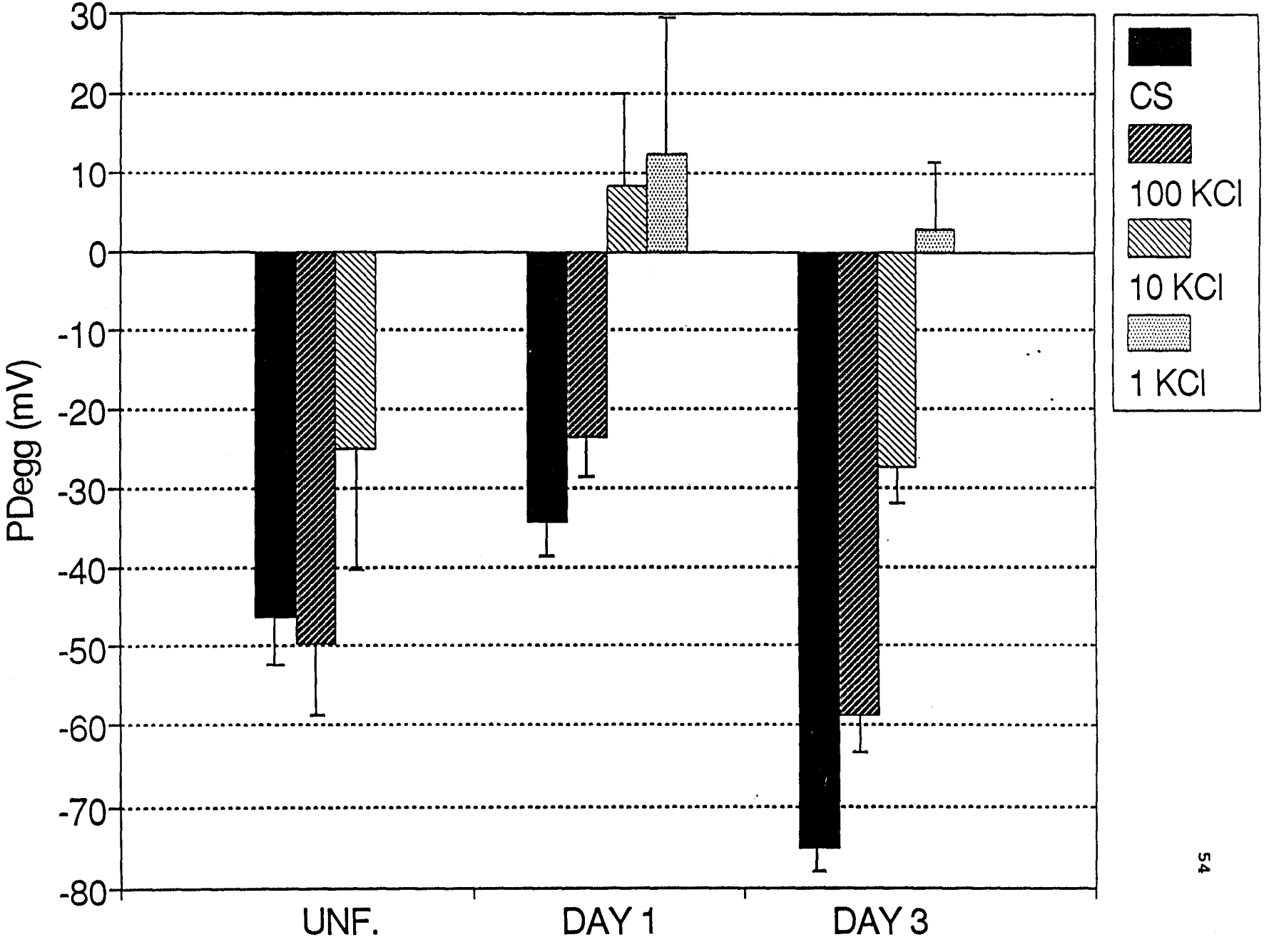
As noted above, oviposited eggs are not normally in contact with ionic strengths and osmolarities comparable to those of control saline. Although groundwater ionic composition is highly variable, a K^+ concentration of about 1 mM is representative (Karanth, 1987). The effects of different concentration of KCl on PD_{egg} are shown in Figure 9. PD_{egg} became less negative in low ionic strengths, as would be expected if the egg membranes were more permeable to chloride than to potassium. Values of PD_{egg} in unfertilized eggs was not measured in 1 mM KCl because depolarization initiated repetitive action potentials and precluded accurate measurement of resting potentials.

2.3 DISCUSSION

This study has demonstrated that ooplasmic pH and the potential difference across cellular and acellular membranes can be readily measured during locust egg development by double-barrelled pH-sensitive microelectrodes. Oviposited locust eggs are capable of maintaining a substantial potential difference across their membranes. The value of PD_{egg} varies with the age of the eggs, and can exceed -100 mV for d3 eggs bathed in a saline similar to locust haemolymph in ionic composition.

Changes in PD_{egg} and R_{in} are consistent with an important role for cellular membranes in determining the electrical properties of the egg. Although the exochorion is added to the egg during oviposition, PD_{egg} of d1 egg measured in standard conditions declines to less than one-half the value recorded in unfertilized eggs, and R_{in} declines five-fold. Dechorionation has little effect on PD_{egg} measured in d3 eggs. In

Figure 9 PD_{egg} in developing locust eggs bathed in control saline (CS), 100 mM KCl, 10 mM KCl, and 1 mM KCl. Means $\pm$ SE, n=3 (day 0), n=6 (day 1), n=8 (day 3).


30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
PDegg (mV)
UNF.
DAY 1
DAY 3
CS
100 KCl
10 KCl
1 KCl

addition, PD_{egg} becomes first more negative (d3) and then less negative (d4-d8) during the period of serosal cuticle secretion, and R_{in} remains low. It is worth noting that the most negative potentials correspond to the period of maximal water uptake on d3-d4. The results suggest that the acellullar membranes of the egg are not the major determinants of input resistance, and that the chorion has only a minor influence on the potential difference between the ooplasm and the fluids bathing the external surfaces of the egg.

The importance of the cellular membranes of the orthopteran egg in retarding solute loss has been emphasized in previous studies using radioisotopic and gravimetric techniques to examine permeabilities (Grellet, 1971; Browning, 1972). The egg coverings are permeable to water at all times (Browning, 1969), and also to non-polar molecules with a diameter as large as that of glucose. It has been calculated that the non-living membranes would permit the loss of all the small molecules from the egg in less than 1 month (ibid), comparable to the total developmental period at temperatures less than about 25° C (McGregor and Loughton, 1974). Small molecules pass through the coverings of living eggs much less rapidly than through dead egg coverings, suggesting that the cells of the serosal epithelium, or at least their outer cell membranes, control the movement of solutions into and out of the egg (Grellet, 1971; Browning, 1972).

PD_{egg} is likely to be near zero or slightly positive for eggs in contact with soil water. The net potential will reflect the summation of two potentials in series across the cellular and acellular membranes of the egg. A metabolically-dependent ion pump contributes to the inside

negative potential across the oolemma of unfertilized eggs or the serosal epithelium later in development. This hyperpolarizing potential is counteracted by a depolarization across the acellular membranes, particularly the serosal cuticle, due to the diffusion of Cl^- from egg to the external solution. Chloride conductance increases after oviposition, and is found in internally perfused eggs after lysis of the serosal cells, and in dechorionated eggs. These results suggest that the chloride conductance is a characteristic of the serosal cuticle. The absence of a significant potassium conductance and the contribution of chloride to the potential difference across the membranes of fertilized locust eggs are in contrast to the electrical properties of vitellogenic eggs. The membrane potential of vitellogenic eggs is determined mainly by the diffusion of K^+ and by a ouabain-sensitive electrogenic pump (Wollberg and Cocos, 1981). The Cl^- dependence of PD_{egg} in fertilized locust eggs also contrasts with the K^+ dependence of PD_{egg} in diapausing eggs of *Melanoplus differentialis* (Jahn, 1935). In addition to differences in species and the stage of the eggs examined, the saturated KCl solution stirred into the yolk of the grasshopper eggs in the earlier study would disrupt cellular membranes and might permeablize the acellular membrane, changing the electrical properties of the eggs. Permeabilization of acellular membranes by exposure to 3M KCl was observed in a study of *Drosophila* eggs by Overall and Jaffe (1986).

Chloride-sensitivity of PD_{egg} after oviposition may offer a physiological advantage to fertilized eggs. Chloride diffusion in low ionic strengths will tend to depolarize the egg, so that protons are closer to electrochemical equilibrium. In contrast, a significant

potassium conductance would lead to highly negative values of PD_{egg} in low ionic strengths, and a tendency for loss of Cl^- and organic ions, and ooplasmic acidification would result.

Contribution of a metabolic pump to PD_{egg} was indicated by the depolarizing effects of chilling and anoxia, and also by the results of internal perfusion experiments, in which a potential difference was observed across the cellular membranes in the absence of any chemical gradients for ion diffusion into or out of the egg. Correlations of the location of injected dyes with electrical potentials also indicated the presence of a substantial potential difference across the serosal epithelium. Anoxia had little effect on PD_{egg} in unfertilized and d1 eggs, possibly because the metabolism of unfertilized eggs is more capable of anaerobic metabolism in the reduced oxygen tensions which may prevail when eggs are crowded together in the lateral oviducts after ovulation.

A proton pump is a likely candidate for the metabolic ion pump of chorionated eggs. This pump could be located in the oolemma of unfertilized eggs, and in the serosal cells of older fertilized eggs. Hyperpolarization of PD_{egg} by ooplasmic acidification during exposure to hypercapnic salines is consistent with the stimulation of proton pumps by low pH (Steinmetz, 1988). Low temperatures and anoxia could impede H^+ pumping through effects on ooplasmic reserves of ATP.

Proton pumps have been implicated in the movement of H^+ across the oolemma of vitellogenic *Cecropia* follicles (Stynen *et al.*, 1988), and a comparable mechanism might well be retained in locust eggs after chorionation. A possible advantage of retention of a proton pump after chorionation is a contribution to ooplasmic pH regulation in the absence

of an external source of Na^+, as would be the case for an Na^+/H^+ antiporter. Although proton pumping was not detected in the study of Woolberg and Cocos (1981), the effects of such a pump on PD_{egg} in vitellogenic eggs prior to chorionation might be masked by greater currents through the ouabain-sensitive Na/K pump at that stage.

The most probable location for the proton pump in fertilized eggs one day or more after oviposition, is on the apical surface of the serosal cells, facing the serosal fluid. Internal perfusion experiments suggested that about 18 mV of PD_{egg} is contributed by the serosal epithelium. In this respect, the serosal epithelium resembles 'tight' vertebrate and invertebrate transporting epithelia of the gut or excretory system.

The diffusion barrier created by the chorion and serosal cuticles makes the analysis of the potential difference more difficult in chorionated eggs than in other preparations, like insect nerve or muscle. Although the metabolic pump of the cellular membranes and the chloride conductance of the acellular membranes contribute substantially to PD_{egg} for eggs bathed in control saline, there must be additional potential sources. In unfertilized eggs, up to 43% of PD_{egg} is metabolically dependent, and about 14% is chloride dependent, leaving about 43% unexplained. One possible contribution is that of ion diffusion across the oolemma between the ooplasm and the space between the oolemma (the vitelline membrane) and the chorion. This latter compartment would be physically isolated from the rapid changes in bathing saline ion composition, and the contribution of ion diffusion across the oolemma to PD_{egg} could not be assessed, therefore, by ion substitution experiments in chorionated eggs. Similarly, for d3 eggs, about 30 % of PD_{egg} can be

explained on the basis of chloride diffusion across the acellular membranes, and about 12 to 25% can be attributed to a metabolic pump, again leaving nearly half of the observed PD_{egg} to be explained. In this case as well, diffusion across the serosal cells, from ooplasm to serosal fluid, could not be detected by ion substitution experiments because of the barrier formed by the overlying serosal cuticle and chorion.

2.3.1 Effects of oviposition on ooplasmic pH

Regulation of egg pH was suggested by the finding that egg pH remained near 7.25 during prolonged immersion of unfertilized or d1 - 5 eggs in control saline, even though the inside negative potential would favour acidification of ooplasmic pH. The mechanism of this regulation is unclear, although the proton pump suggested above might well form an important component. Ooplasmic pH regulation could be accomplished by transport of acidic equivalents not just out of the egg, but into the interior of the yolk spheres, which form a large proportion of total egg volume, particularly during the early stages of development. Yolk spheres are known to be acidified during vitellin processing in *Blatella germanica* (Nordin 1991 cited by Nordin *et al.*, 1990).

Ooplasmic pH of unfertilized eggs bathed in air-equilibrated saline did not differ significantly from that of oviposited d1 eggs. However unfertilized eggs acidified by 0.2 pH units when bathed in a saline equilibrated with a 7% CO_2/air mixture. Although haemolymph pCO_2 is approximately 4-5% in locusts (Harrison, 1988, 1989), we used a 7% solution because more extreme hypercapnia may occur in the localized

environment of the eggs due to close packing of the eggs and the effects of muscular contractions.

It appears likely, therefore, that eggs may undergo a slight alkalinization through the combined effects of reduced pCO_2 and declining (ie. less negative) values of PD_{egg} as the fluids around the egg equilibrate with the lower ionic strengths of soil water. This alkalinization may act as a signal for the initiation of development, perhaps in conjunction with an increase in intracellular calcium content following calcium entry through voltage-dependent channels (O'Donnell and Soloweij, 1990).

Chapter 3

Ooplasmic Activities of Sodium, Potassium, and Chloride

Insect eggs must be supplied prior to oviposition with all of the ions and nutrients required for embryogenesis, in contrast to marine eggs which can take up ions as needed during development. Leakage of ions such as sodium and potassium out of the egg must be minimized, and this need is consistent with the low permeability of the egg membranes to these ions inferred from the ion substitution experiments of chapter 2.

Eggs of many orthopterans, like locusts and crickets, are laid in moist environments and take up water during development. The mass of the locust egg, for example, increases more than 2-fold by water uptake. It is not known if the ooplasmic activities of sodium, potassium, and chloride decrease proportionately during the increase in water content. Results in chapter 2 indicate that pH is regulated within the ooplasm, and raise the possibility of regulation of the activities of sodium, potassium and chloride. Regulation of these ions during water uptake may be evaluated by measuring the ion activities in the ooplasm at this time.

Such measurements would also reveal whether the ooplasmic ion activities are typical of intracellular values ($[K]/[Na] \gg 1$) or of extracellular values ($[K]/[Na] \ll 1$). One consequence of early embryogenesis is that the ooplasm becomes extracellular with respect to the serosal epithelium and the embryo. It is not known if ooplasmic activities of sodium, potassium and chloride change from intracellular to extracellular levels when the serosal epithelium forms.

This chapter reports the first measurements of potassium, sodium and chloride ion activities in the ooplasm of an insect egg at selected stages of development. This chapter also examines measurements of egg water content, and whole egg ion concentrations. The results suggest that sodium and potassium activities are maintained at values substantially different than those expected on the basis of dilution of the ooplasm by water uptake.

3.1 Materials and Methods

3.1.1 Egg Collection and Superfusion

Animals were maintained and eggs were cellected in the same manner as for Chapter 2. Eggs were superfused in the same chamber and under the same conditions as in Chapter 2.

3.1.2 Physiological Saline

The same control saline was used as in Chapter 2. A Ca^{++} free saline was used in measurements of Na^{+} by microelectrodes because of interference by extracellular concentrations of Ca^{++} (Steiner*et al.*, 1979).

3.1.3 Electrophysiology

Ion activities and PD's were measured using the same apparatus and techniques described in Chapter 2 for measurements of pH and PD_{egg}.

K^{+} electrodes contained a cocktail based on the Corning ion exchanger 477317 (IE-190, WPI) and were backfilled with 0.5 M KCl. The

reference barrel was filled with 3 M sodium acetate. Potassium electrodes were calibrated using the 'mixed solution' technique (Armstrong and Garcia-Diaz, 1980; Lee, 1981) with solutions of 150 mM KCl and 15 mM KCl/135 mM NaCl. Corresponding activities were calculated using an activity coefficient of 0.75 (Lee, 1981). Mean slope was 56 $\pm$ 1 mV/decade change in a_K (n=30).

Sodium electrodes contained the neutral carrier ETH 227 (Sodium ionophore I, cocktail A, Fluka, Ronkonkoma, NY) and were backfilled with 0.5 M NaCl. The reference barrel was filled with 0.5 M KCl. Sodium electrodes were calibrated with solutions of 150 mM NaCl and 15 mM NaCl/135 mM KCl. Mean slope was 53 $\pm$ 1 mV/decade change in a_{Na} (n=38). Preliminary experiments indicated that Na^+ electrodes based on ETH 157 (Sodium ionophore II, cocktail B, Fluka) were less sensitive to interference from Ca^{++} but were more prone to interference from intracellular levels of K^+.

Cl^- electrodes contained the Corning exchanger 477913 (IE-173, WPI) and were backfilled with 0.5 M KCl. The reference barrel was filled with 3 M sodium acetate. Chloride ion exchanger microelectrodes were calibrated in solutions of KCl. The mean slope was 59 $\pm$ 5 mV/decade change in a_{Cl} (n=63).

Potential differences from ion sensitive (V_{ion}) and reference barrels (V_{ref}) of double-barreled theta-glass microelectrodes were measured through chlorided silver wires inserted into the stem of each barrel and connected to differential electrometers (WPI FD 223) whose input impedance exceeded 10^{14} ohms. The potential difference of the reference barrel was measured relative to a Ag/AgCl electrode connected to the bathing solution by a

3 M KCl/agar bridge. Noise resulting from the high input impedance (10^9-10^{10} ohms) of the ion-sensitive barrel was reduced using a low pass RC filter with a time constant of 1 s. The difference (V_{ion}-V_{ref}) and V_{ref} were recorded on a two-channel chart recorder (Linear Instruments, Reno, NV) and a computer-based data acquisition system (AXOTAPE, Axon Instruments).

3.1.4 Impalements

Criteria for acceptable impalements were the same as in Chapter 2.

3.1.5 Chloride Analysis

The Corning ion exchanger 477913 is sensitive to a number of organic anions such as HCO_3^- which may be present in eggs. Ooplasmic chloride activities in unfertilized, d2 and d3 eggs were checked, therefore, using a second technique. A Ag/AgCl wire is an almost perfect chloride electrode over a wide range of activities (Dow, 1981; Thomas, 1978), but is not sensitive to organic anions. Silver/silver chloride electrodes are susceptible to interference from other halogens, but the presence of significant quantities of these in biological fluids is unlikely. Eggs were torn open with forceps on a wax-lined petri plate, and 1 ul of egg contents was collected in a calibrated 1-5 ul micropipette (Drummond Scientific Co., Broomall, PA). Samples were diluted 1:1 with nanopure water added to the micropipette to reduce coagulation on the Ag/AgCl wire. Volume measurements were accurate to approximately $\pm$ 5%. The Ag/AgCl electrode and a 3 M KCl microelectrode were placed immediately into the drop and the potential difference was measured with respect to the KCl

electrode. The system was calibrated before and after each measurement with drops of 150 mM NaCl, 15 mM NaCl/135 mM Na acetate, and 1.5 mM NaCl/148.5 mM Na acetate. The slope of Ag/AgCl electrodes was 50 mV/decade change in chloride activity (n=7).

3.1.6 Atomic Absorbtion

Whole egg potassium and sodium concentrations were evaluated using an atomic absorption spectrometer (AAS; Varian, model 1275, Toronto, Ontario). Eggs were weighed in tared 200 ul centrifuge tubes using a Mettler balance (model AE 240) with a resolution of 10 ug. Concentrated nitric acid (20 ul) was added to the tube and the samples were incubated for 3 hours at 45° C. Digests were diluted with 100 ul of nanopure water, and centrifuged at 9000 rpm for 2 minutes. A 115 ul aliquot of the supernatant was diluted in 2.5 ml of nanopure water, then analyzed in the AAS. Blanks and calibrating standards were prepared in the same manner as the experimental samples.

Egg water volume was calculated from the relationship of wet weight to water content determined in a separate group of eggs. Water content was calculated from the difference in wet and dry weights. Eggs were dried at 50° C for 24 h, then cooled over silica gel before weighing.

Preliminary experiments indicated that measurement of potassium and sodium concentrations by AAS in samples of ooplasm collected from ruptured eggs was not feasible. It was difficult to adequately separate yolk spheres from ooplasm after centrifugation of eggs, and the 500-fold dilution for AAS magnified any initial small errors in volume measurement.

3.1.7 Statistics

Data are presented as means ± 1 S.E.M. Significance of differences between means were calculated by Student's t-tests.

3.2 RESULTS

3.2.1 Egg Water Content

Egg water content decreased slightly but significantly ($p<0.05$) from 3.8 ± 0.04 mg (n = 12) in unfertilized eggs to 3.5 ± 0.03 mg in d1 eggs (Figure 10). Water content remained low (3.7 mg) in d2 eggs, then increased to 6.7 mg in d3 eggs and continued to increase to 9.8 mg in d5 eggs (Figure 10). Roonwall (1936) reported a similar increase in water content from 3.3 mg in unfertilized eggs to 10.5 mg in eggs undergoing blastokinesis (d4 to d5 at 37° C; McGregor and Loughton, 1974).

The decline in sodium and potassium concentration (Figure 11) measured by AAS mirrored the increase in egg water content (Figure 10). Mean water content on d5 was 154 % of the value in unfertilized eggs. A range of about 145 % - 163 % of the unfertilized value would include variations resulting from differences in water uptake in eggs oviposited up to 8 h before collection or analysed up to about 8 h after the collection. As a result, potassium and sodium concentrations would both be expected to decrease to between 52 % and 70 % of the values measured in unfertilized eggs. Potassium and sodium concentrations measured in d5 eggs were 51 % and 73 %, respectively, of their values in unfertilized eggs, close to these estimates.

Figure 10 Water content in developing locust eggs. Data points are means ± S.E for 12 eggs.

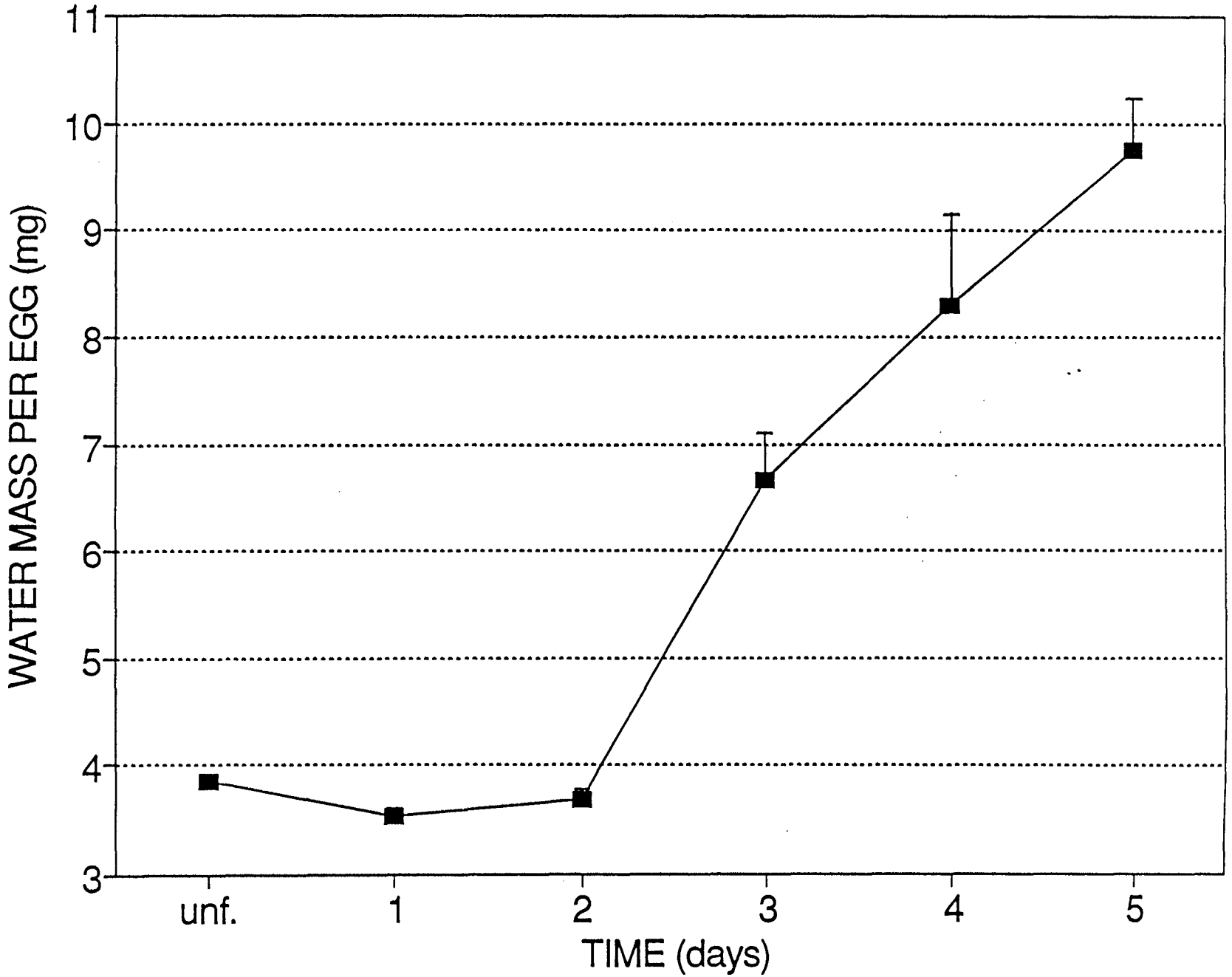
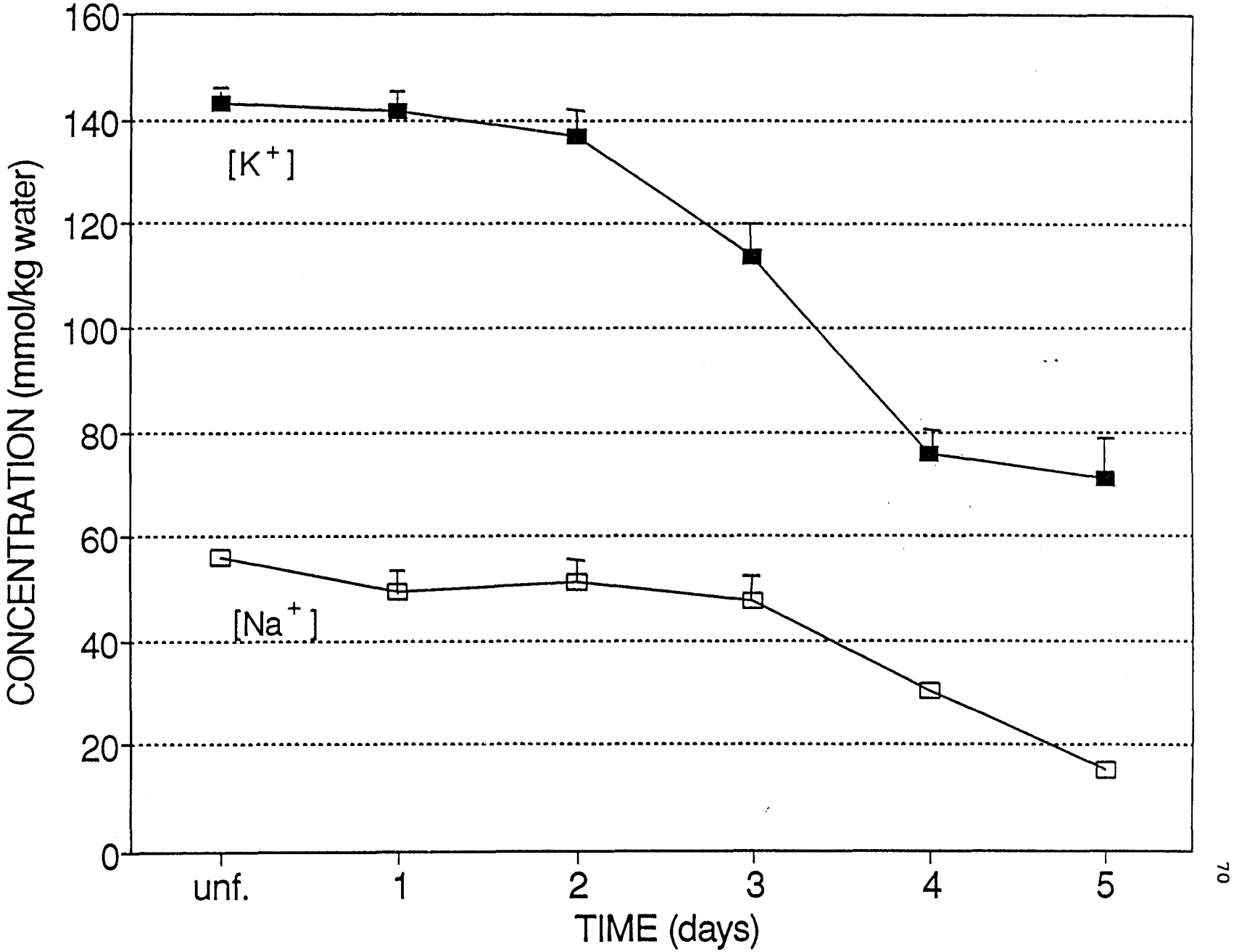

11
10
9
8
7
6
5
4
3
WATER MASS PER EGG (mg)
unf.
1
2
3
4
5
TIME (days)

Figure 11 Concentrations of potassium (closed symbol) and sodium (open symbol) in whole eggs measured by AAS. Data points denote means $\pm$ S.E., n = 12.


160
140
120
100
80
60
40
20
0
CONCENTRATION (mmol/kg water)
[K+]
[Na+]
unf.
1
2
3
4
5
TIME (days)

70

The mean PD_{egg} in unfertilized eggs was -54 mV and became less negative in d1 and d2 eggs (-24 mV and -31 mV respectively), then more negative in d3, d4, and d5 eggs (-72 mV, -66 mV, and -65 mV respectively). PD_{egg} on d3 was within 6 mV of that reported in Chapter 2, and PD_{egg} in unfertilized, d1, d2, d4, and d5 eggs were within 1 mV.

3.2.2 Potassium and Sodium

Comparison of measurements made with potassium selective ISME's at different stages in egg development indicated that ooplasmic potassium activity did not remain constant, but increased after fertilization and before d1, and subsequently decreased before d5 (Figure 12). The ooplasmic activity of sodium and potassium increased from the values in unfertilized eggs on d1 and d2, then decreased sharply on d3 and remained low (Figure 13). The reductions in sodium and potassium activities between d1 and d2 were within 2 mM of those expected from the 4% increase in water content. Similarly, reductions in activities from d2 to d3 were within 2 mM for potassium and 4 mM for sodium of the values expected from the 81% increase in water content over this period.

However, changes in a_K and a_{Na} could not be predicted on the basis of the corresponding changes in water content around the time of oviposition or after d3. Measured activities of potassium and sodium in d1 eggs were 114 mM and 29 mM respectively, much greater than the values of 91 and 9 mM, respectively, estimated from the decline in water content. Similarly, sodium activity remained constant and potassium activity actually increased 10 mM between d3 and d5, despite a 46% increase in

Figure 12 Representative recordings of PD_{egg} (lower traces) and V_K-V_{ref} (upper traces) obtained during impalements with double-barrelled potassium sensitive microelectrodes in an unfertilized (A), d1 (B), and d5(C) eggs. Downward and upward arrows indicate impalement and electrode withdrawal, respectively. Impalement of eggs was performed in control saline. Electrodes were calibrated before impalement (not shown) and after microelectrode withdrawal in the solutions indicated. Calibration solution activities, as shown in figure, were calculated using 0.75 as the activity coefficient.

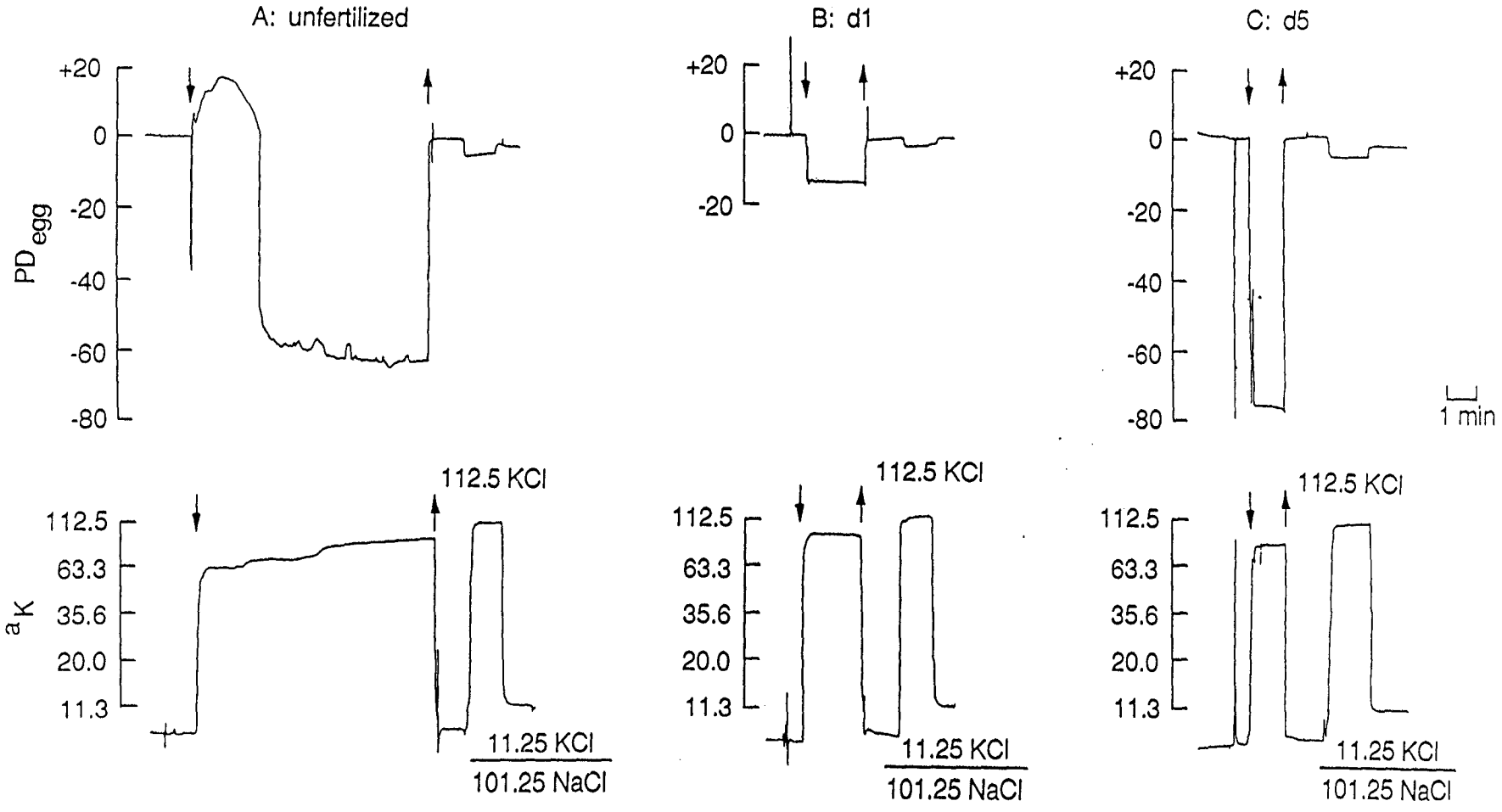
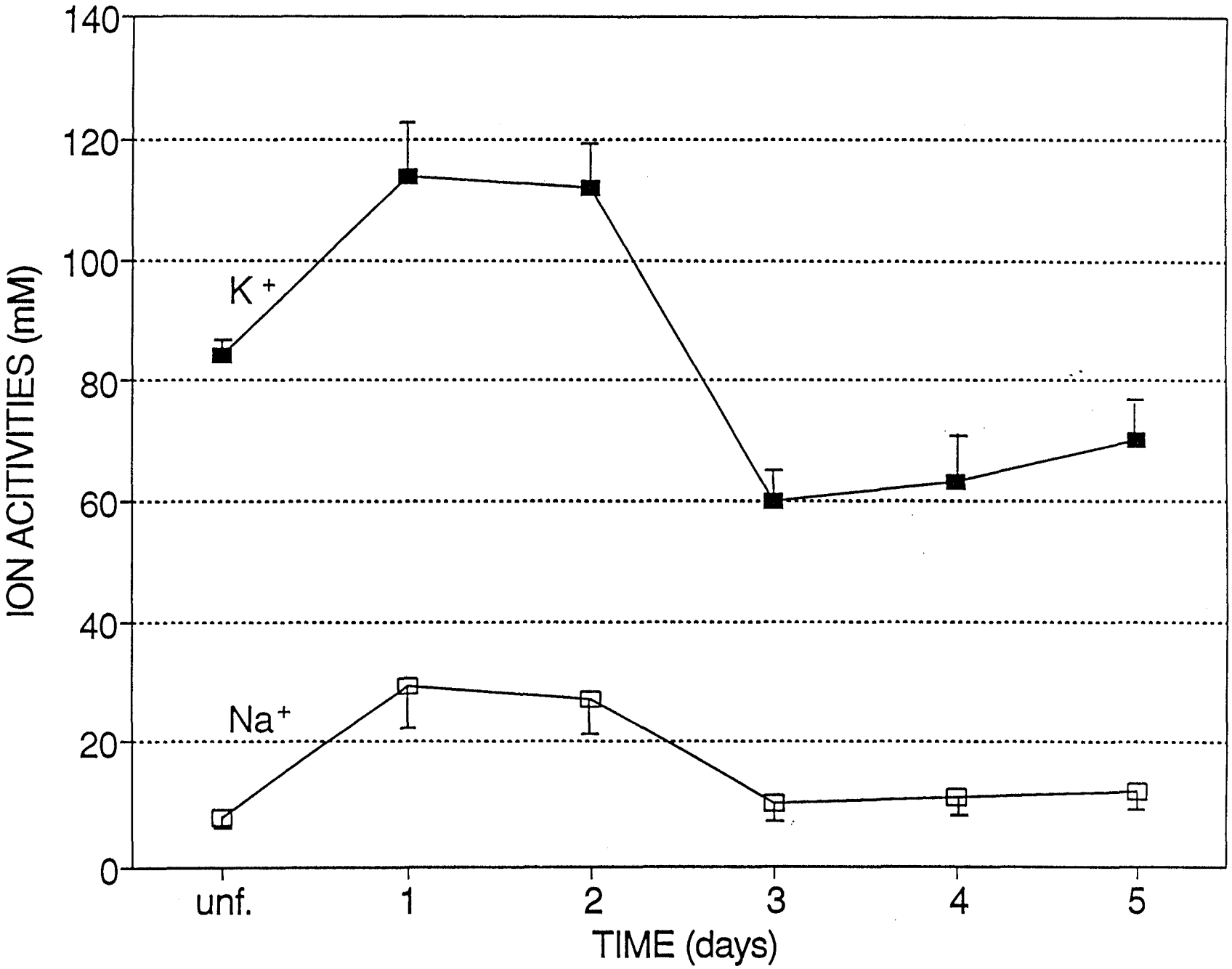

A: unfertilized
B: d1
C: d5
+20
0
-20
-40
-60
-80
PD egg
1 min
112.5 KCl
112.5
63.3
35.6
20.0
11.3
a K
11.25 KCl
101.25 NaCl

Figure 13 Summary of a_K (closed symbols) and a_{Na} (open symbols) measured by ISMEs in the ooplasm of developing eggs. Each data point denotes mean $\pm$ S.E., n= 6 - 14.


140
120
100
80
60
40
20
0
ION ACITIVITIES (mM)
K+
Na+
unf.
1
2
3
4
5
TIME (days)

water content. These results suggest that ooplasmic activities of sodium and potassium are regulated at these times.

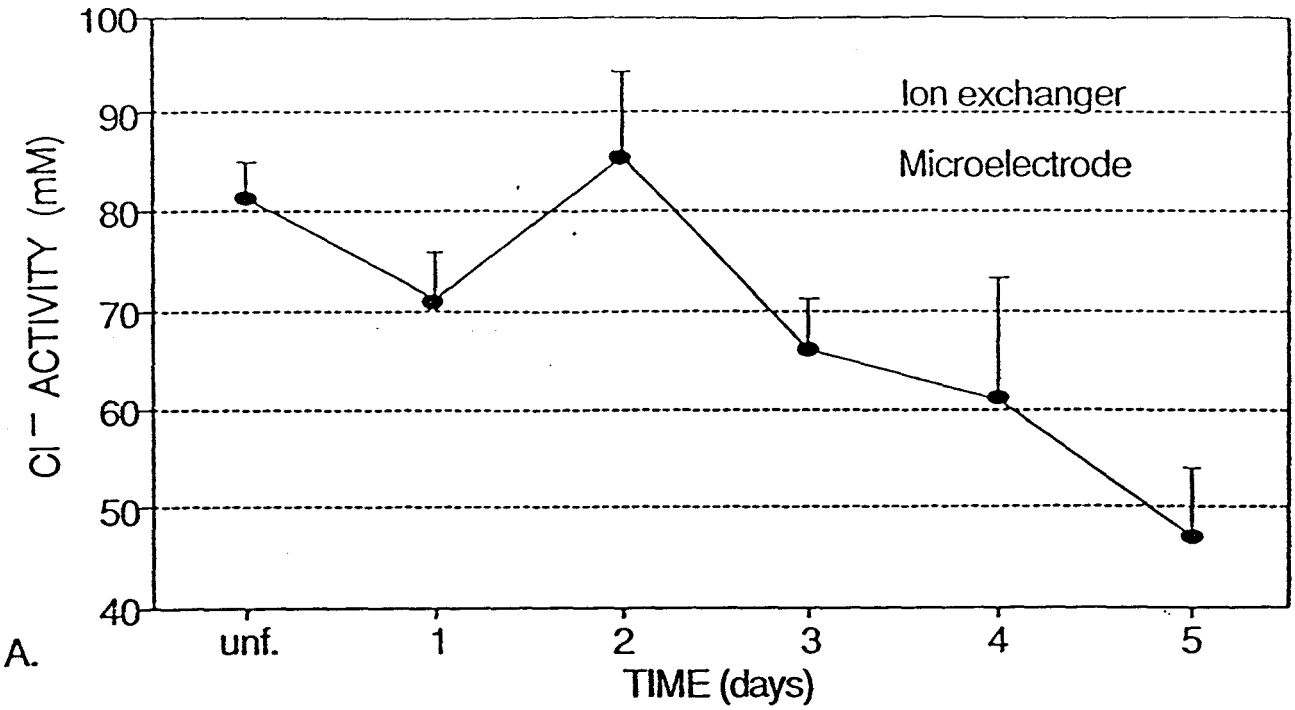
This view is supported by calculations of activity coefficients. These calculations assume that the activity coefficients in whole eggs are the same as those in the ooplasm. Although the ion content of the chorion was low (Hawkins, unpublished results), differences in activity coefficients between ooplasm and yolk spheres may be substantial, and the calculated values should be considered approximations, only. The activity coefficient for potassium increased from 0.59 in unfertilized eggs to 0.82 on d2 and 0.98 on d5. The activity coefficient for sodium increased from 0.15 in unfertilized eggs to 0.53 on d2 and 0.80 on d5. These changes also suggest regulation of ooplasmic ion activity during water uptake, and possible mechanisms are discussed below.

3.2.3 Chloride

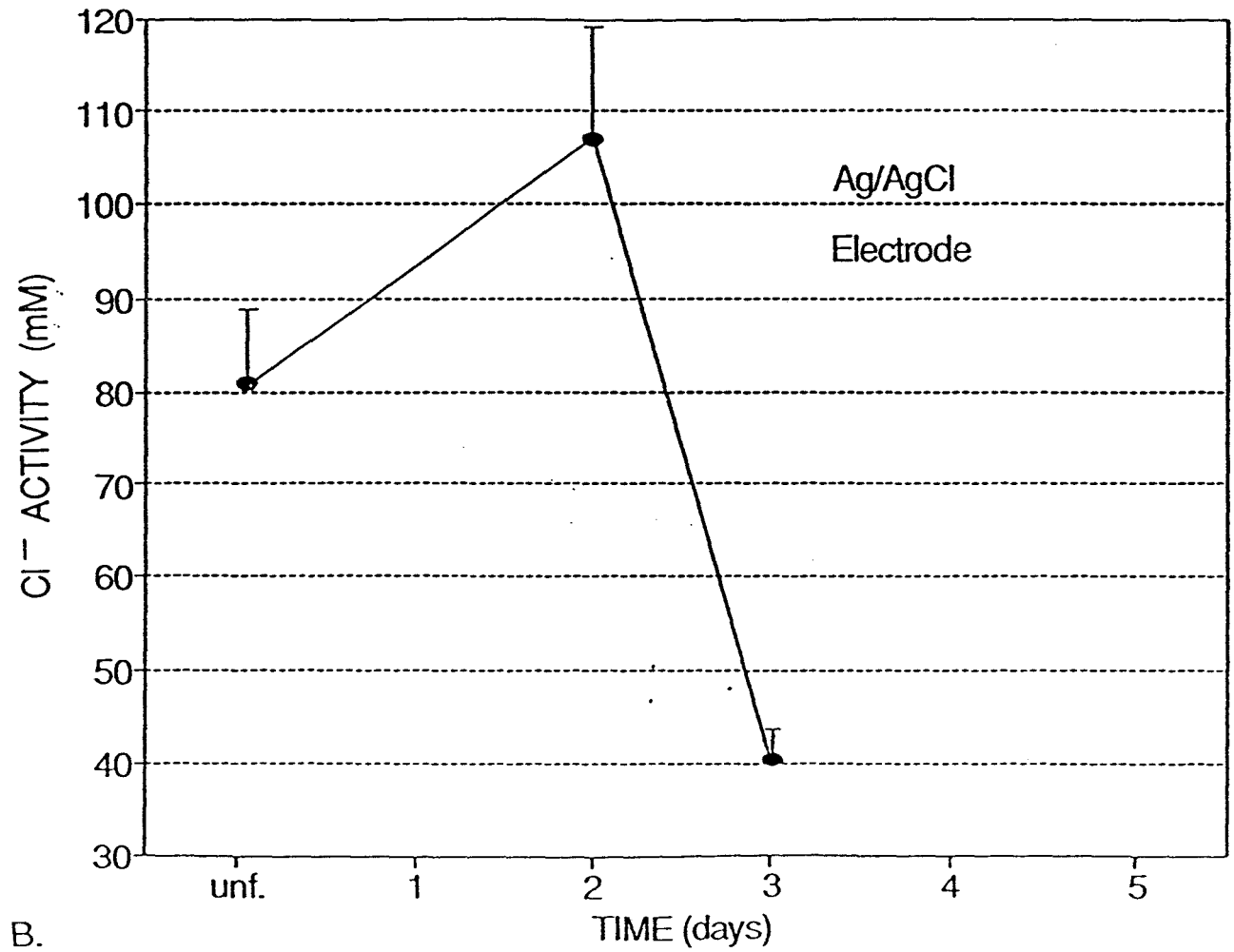
Mean ooplasmic chloride activity in unfertilized eggs measured by ISMEs was 81 mM (figure 14). Comparison of this value with the sum of sodium and potassium activities in the ooplasm of unfertilized eggs (93 mM) indicated that Cl^- is the predominant anion at this stage. Chloride activity remained near this value until d2, then decreased steadily to 47 mM by d5 (Figure 14). The pattern of decline of chloride activity did not resemble the pattern of decline of potassium and sodium activities (Figure 13), nor did it closely follow the pattern of decline expected on the basis of water uptake by the eggs (Figure 10). Chloride activities in d3, d4, and d5 eggs (Figure 14a) exceed the values of 42 mM, 38 mM and 33 mM, respectively, expected from water uptake.

Figure 14 (A) Summary of chloride activities measured by chloride ion exchanger microelectrodes in the ooplasm of developing eggs. Each point denotes mean ± S.E., n = 7 - 14.

(B) Chloride activities in the egg ooplasm measured by Ag/AgCl wire electrodes. Data points denote means ± S.E., n = 6 - 8.


Ion exchanger
Microelectrode
Cl⁻ ACTIVITY (mM)
100
90
80
70
60
50
40
unf.
1
2
3
4
5
TIME (days)

A.


Ag/AgCl
Electrode
Cl⁻ ACTIVITY (mM)
120
110
100
90
80
70
60
50
40
30
unf.
1
2
3
4
5
TIME (days)

B.

These discrepancies might result from interference by other anions, such as bicarbonate (Baumgarten, 1981). Independent estimates of ooplasmic chloride activity were obtained, therefore, with fine Ag/AgCl wire electrodes, which are not affected by organic anions (Thomas, 1978). In unfertilized eggs, chloride activity measured by Ag/AgCl electrodes was 81 mM, equal to the value measured by ion exchanger microelectrodes. This agreement suggested that the concentrations of ions which interfere with chloride ion exchanger electrodes were negligible in eggs at this stage. Similarly, chloride activities in d2 eggs measured by Ag/AgCl electrodes and ion exchanger microelectrodes were 108 mM and 84 mM respectively. The difference was not significant, presumably because of the variability of the data.

In contrast, the chloride activity measured by Ag/AgCl electrodes was 41 mM on d3, well below the 66 mM measured by ion exchanger microelectrodes. The difference may be attributable to the effects of interfering anions on the potentials recorded by the chloride ion exchanger microelectrodes. McGregor and Loughton (1977) have shown a 7-fold increase in amino acids between d2 and d3, but preliminary experiments have shown that chloride ion exchanger microelectrodes are not sensitive to the free amino acid milieu they describe. Other experiments confirmed previous results (Baumgarten, 1981) showing that chloride ion exchanger microelectrodes have selectivity coefficients ($\log_{ClX}$) around -0.2. A value of $\log_{ClX}$ of -1 indicates that the electrode is 10 times more selective for Cl^- than for the interfering anion X, whereas a value of -0.2 indicates that the electrode is only 0.63 fold more selective. Chloride activity values for d4 and d5 were also considered unreliable

because of the possible presence of interfering anions. Chloride activity was not measured in d4 and d5 eggs by Ag/AgCl electrodes because the large size of the embryo and amnion at these stages made collection of just ooplasm difficult.

Chloride activity measured by Ag/AgCl electrodes in d3 eggs was 41 mM (Figure 14), smaller than the activity of 67 mM predicted on the basis of water uptake. Possible explanations for this discrepancy are discussed below.

3.3 Discussion

This paper is the first to use ion-selective microelectrodes to measure ooplasmic activities of sodium, potassium, and chloride in developing insect eggs. Estimates of sodium and potassium concentrations in whole eggs have also been obtained by AAS. In addition, chloride levels in isolated ooplasm have been checked using Ag/AgCl wire electrodes which are less susceptible than ion exchanger microelectrodes to interference from organic anions (Thomas, 1978; Dow, 1981).

We considered the possibility that increases in apparent sodium activity in d1 and d2 eggs relative to unfertilized eggs might result from increases in the activity of an interfering ion. For example, the sodium sensor used in our experiments (ETH 227) is known to be sensitive to calcium (Steiner *et al.*, 1979). Extreme interference might result in apparent sodium activities, as measured by ISMEs, exceeding the concentrations, as measured by AAS (ie. activity coefficient > 1). However, sodium concentration in d1 eggs measured by AAS is 50 mM (figure

2), much larger than the activity of 29 mM measured by ISMEs (figure 4). Moreover, direct measurement of ooplasmic calcium activity by ISMEs (Chapter 4) indicates that interference by calcium would introduce an error of no more than 3 mM in sodium activities measured in the present study in d1 and d2 eggs.

Comparisons of our results with studies of other tissues indicate that sodium activities, but not those of potassium, remain at typically intracellular levels for all egg stages examined. Results obtained using techniques which measure concentrations can be converted into activities using estimated activity coefficients (Lee, 1981; Dow *et al.*, 1984; Gupta *et al.*, 1980). Ooplasmic sodium activities of 8 - 29 mM in locust eggs are similar to intracellular activities of 4 - 49 mM measured in insect muscle and epithelium (Djamgoz, 1987; Gupta *et al.*, 1980) and 5 - 30 mM in other animal cells (Lee, 1981).

Sodium activity in d1 eggs is more than 3.5 times the activity in unfertilized eggs, implying that sodium is released into the ooplasm at this time. In addition, although the decline in ooplasmic sodium between d2 and d3 is consistent with dilution due to water uptake, sodium activities in d3 - d5 eggs did not decrease further, even though water content increased a further 46 %. Both results suggest that sodium may be released into the ooplasm at these times from stores within the egg, perhaps from the yolk spheres.

Ooplasmic potassium activity of 84 - 118 mM in unfertilized, d1 and d2 eggs is similar to intracellular activities of 60 - 120 mM in insect muscle and epithelium (Djamgoz, 1987; Dow *et al.*, 1984) and 80 - 135 mM in various cell types from vertebrates and invertebrates (Burton, 1968).

In contrast, ooplasmic potassium activity from d3 to d5 (60 - 70 mM) is lower than intracellular activities of 75 - 95 mM in locust muscle (Wood, 1963), but similar to the activity of 61 mM measured in locust rectal epithelium (Hanrahan and Phillips, 1984). Nonetheless, potassium levels in egg ooplasm are well above the haemolymph values of 14 mM in locusts (Stobbart and Shaw, 1974).

Although ooplasmic potassium activity declines during egg development, it appears to be regulated above the levels expected from changes in egg water content. Insect eggs can develop in low ion strength solutions, ie. soil water, or even in distilled water (Lees, 1976; Hunter-Jones and Lambert 1961), so the basis for this regulation seems more likely to be the release of sequestered potassium than uptake from the surroundings. Potassium and sodium are sequestered in other egg systems (Palmer *et al.*, 1978; Baltus, 1977; de Laat, 1975) and the yolk has been suggested as a site of storage in frog eggs (Palmer *et al.*, 1978). Similarly, sodium and potassium are transferred from yolk to embryo during the last half of development in chicken eggs (Davis *et al.*, 1988). Earlier studies of butterfly eggs have shown that yolk spheres bind calcium for subsequent release during embryogenesis (Przelecka *et al.*, 1986). In locust eggs small yolk spheres near the periphery of the egg are digested immediately after fertilization, and the remainder are digested from d3 until hatching (Petavey, 1986; Bassemir, 1977). Similarly, amino acid release from vitellin is high on d1, d3 and d4 (McGregor and Loughton, 1974). Although direct evidence is lacking, our results would be consistent with regulation of ooplasmic potassium and

sodium activities by release of these ions from yolk spheres concurrent with release of the products of vitellin processing.

The measured levels of ooplasmic sodium and potassium have several consequences for embryogenesis. The increase in ooplasmic potassium activity from approximately 80 mM before fertilization to 120 mM on d1 may stimulate protein synthesis and mitosis. The membranes surrounding the dividing cells within the egg are not completed until 12 - 16 hours after fertilization (Roonwall, 1936a) and so ooplasm ion activities reflect the activities surrounding the dividing nuclei. Previous studies have shown that potassium activity in mouse cells is highest during mitosis (Lau *et al.*, 1988; Boonstra *et al.*, 1981), and that high potassium activity increases protein production in *Xenopus* eggs (Horowitz and Lau, 1988). The concurrent increase in sodium activity in the first day after fertilization may also enhance mitotic activity. Mitosis in frog eggs is inhibited when intracellular potassium activity increases but that of sodium remains low, *i.e.* when the ratio of a_{Na}/a_K is approximately 0.06 (Ziegler and Morrill, 1977). This ratio remains high during early development of locust eggs, increasing from 0.09 in unfertilized eggs to 0.24 in d1 eggs.

High ooplasmic potassium activity is also relevant to discussions of the membrane potential of cells in contact with the ooplasm, including the serosal epithelium and the inside layer of embryo cells which will form the foregut (Wigglesworth, 1972; Roonwall, 1936a). If the membranes of these cells are selectively permeable to potassium, as for many insect cells (Djamgoz, 1987; Dawson *et al.*, 1985; Verachtert *et al.*, 1989), they

would be continuously depolarized by contact with the potassium-rich ooplasm.

Maintenance of typically intracellular levels of sodium in ooplasm is also pertinent to discussions of intracellular ion regulation by cells in contact with the ooplasm. In many cells uptake of glucose and amino acids and regulation of intracellular pH and calcium is dependent on the sodium gradient, ie. high extracellular and low intracellular sodium activities (Schultz, 1986). Utilization of the sodium gradient may be more difficult for cells in contact with the ooplasm. An important role of the serosal fluid may be to provide an ionic milieu suitable for sodium-coupled ion transport across the outward-facing membranes of the serosal cells. Sodium activity in the serosal fluid was not measured in this study, but the sodium activity of perivitelline fluid in *Drosophila* eggs is 74 mM, similar to haemolymph sodium activity of 80 mM (Van der Meer and Jaffe, 1983).

Chloride activity in the ooplasm is typical of extracellular values in insect tissues, in marked contrast to the levels of sodium and potassium. Ooplasmic chloride activities in eggs before fertilization and until d2 are 70 - 110 mM, well above the 7 - 42 mM range of intracellular values in insect muscle and epithelia (Djamgoz, 1987; Hanrahan and Phillips, 1984; Dow *et al.*, 1981), but close to the 65 - 105 mM found in orthopteran haemolymph (Woodring, 1985; Stobbart and Shaw, 1974).

High chloride activities inhibit protein synthesis in a variety of eukaryotic cell types (Weber *et al.*, 1977). Chloride activities necessary for this inhibition range from 45 mM in wheat germ to 75 mM in rabbit reticulocytes (Weber *et al.*, 1977). The small decline of chloride

activities in ooplasm from 84 mM before fertilization to 70 mM on d1, in conjunction with the increases in a_K and a_{Na}/a_K, may favour increased protein synthesis during mitosis.

Discrepancies in chloride activity in d3 eggs measured by ion exchanger microelectrodes (66 mM) and Ag/AgCl wire electrodes (41 mM) might be accounted for if the eggs contained high levels of bicarbonate (41 mM). However, bicarbonate concentrations in locust haemolymph are nearly 4-fold less (7.5 - 11.5 mM ; Harrison, 1988, 1989). It seems more likely that other interfering anions, in addition to bicarbonate, are present in the ooplasm. The total concentration of amino acids, calculated from data in McGregor and Loughton (1977), is about 0.4 mM, and amino acids at this concentration did not interfere with our ion-exchanger microelectrodes. Selectivity coefficients for acetate and propionate are as high as 0.22 and 0.4, respectively, (Baumgarten, 1981) indicating that as little as 10 - 15 mM of these or similar organic acids could account for up to 25 mM of the apparent chloride activity. The selectivity coefficient for urate is 1.25 (Davis *et al.*, 1988), indicating that urate produces a larger response than an equal concentration of chloride. As little as 1.4 mM urate, therefore, could increase the apparent chloride activity by 25 mM. Locusts use uric acid as their primary form of nitrogenous waste, and deposition of uric acid has been found in muscle tissue (Candy and Kilby, 1975). Grasshopper eggs contain 0.035 mg of uric acid per egg (Bodine, 1946) which would be approximately 0.021 mM in locust eggs, and so uric acid may contribute to the interference in chloride ion-exchanger microelectrodes.

The presence of anions other than chloride is also suggested by the anion deficit in d3 eggs. The sum of the potassium and sodium activities in d3 eggs is 70 mM whereas chloride activity is 42 mM. The anion deficit of about 30 mM, therefore, indicates that significant levels of other anions contribute to maintenance of electroneutrality. Some of these anions may be the result of metabolism, for example oxidation of fatty acids accounts for up to 75% of oxygen consumption in grasshopper eggs (Beenakkers, *et al.*, 1981), and produces organic anions such as acetoacetate and succinate.

Chapter 4

Ooplasmic Calcium Activity

This chapter describes the first use of double-barrelled Ca^{++}-selective electrodes to measure intracellular calcium levels in unfertilized and fertilized insect eggs. The results are compared with measurements in eggs of other phyla and are discussed with respect to the developmental physiology of insect eggs. Calcium is discussed separately from measurements of potassium, sodium, chloride, and pH in developing locust eggs because the time frame of interest is shorter, and because Ca^{++} may play physiological roles that differ greatly in comparison to other ions.

As discussed in Chapter 1, calcium may play a primary role in egg activation, and in the control of cleavage during early development.

On the other hand, calcium is a minor contributor to total osmolality within the ooplasm, in contrast to the roles of potassium, sodium, and chloride as the primary inorganic osmolytes. Typical pCa, the negative log of calcium activity, in egg cells of other species is approximately 6.7 (Poenie *et al.*, 1985; Robinson, 1985; Igusa and Miyazaki, 1986), at least 4 orders of magnitude less than ooplasmic activities of potassium, sodium, or chloride described in Chapter 3.

Chapter 3 also considers the extent to which monovalent ion activities in the ooplasm of developing eggs change from typically intracellular to typically extracellular values. It is also of interest to determine if the calcium activity changes from that typical of an intracellular to that typical of an extracellular milieu. As the

syncytial nuclei and surrounding ooplasm in the egg become encapsulated by the plasma membrane about 16 hours after oviposition (Roonwall, 1936a), the ooplasm becomes extracellular with respect to the cells of the embryo. If ooplasmic calcium activity remains at intracellular levels (pCa 7 - 8), the integrity of junctions between cells of the embryo and the serosa may be compromised since many cell types tend to dissociate when exposed to low levels of extracellular calcium (Hynes, 1972; Spiegel and Spiegel, 1975; Fink and McClay, 1980).

4.1 Materials and Methods

4.1.1 Egg Collection and Superfusion

Animals were maintained and eggs were collected in the same manner as for experiments in chapter 2. Eggs were superfused in the same chamber and under the same conditions as in chapter 2.

4.1.2 Electrophysiology

Calcium activity and PD in eggs were measured using the same apparatus and techniques described in Chapter 2 for measurements of pH and PD_{egg}. Calcium electrodes contained the neutral carrier ETH 129 (Calcium ionophore II, cocktail A, Fluka), and were backfilled with 200 mM KCl, 10 mM ethyleneglycol-bis-(B-aminoethyl ether)N,N,N',N'-tetraacetic acid (EGTA) and 5 mM $CaCl_2$ to yield a pCa of 7. The reference barrel of the electrode was filled with 500 mM KCl.

The neutral carrier ETH 129 is sensitive to calcium levels as low as pCa 9.2 (Schefer *et al.*, 1986), whereas the detection limit of the

neutral carrier used in many earlier studies, ETH 1001 (Calcium ionophore I, cocktail A, Fluka), is above pCa 7.4 (Lanter *et al.*, 1982). The newer sensor is also much less sensitive to interference by other cations. For example, selectivity coefficients of ETH 129 for potassium and magnesium are -7.2 and -6.7, respectively (Schefer *et al.*, 1986). Corresponding values for ETH 1001 are -5.4 and -4.9, respectively (Lanter *et al.*, 1982).

Electrodes were calibrated with solutions used by Peracchi (1990). All solutions contained 5 mM $CaCl_2$, 100 mM KCl, and a calcium buffer. To yield the values of pCa and pH given in brackets, the following buffers were used: 1. (pCa 5, pH 8.42), 10 mM nitriloacetic acid (NTA) and 10 mM N-tris[Hydroxymethyl]methyl-3-amino-propanesolfinic acid (TAPS); 2. (pCa 6, pH 7.70), 10 mM N-hydroxyethylethylenediamine-triacetic acid (HEEDTA), 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES); 3. (pCa 7, pH 7.29), 10 mM EGTA, 10 mM 3-[N-morpholino]propane-sulfonic acid (MOPS); or 4. (pCa 8, pH 7.80), 10 mM EGTA, 10 mM HEPES. Importantly, the sensitivity of ETH 129 is more than two orders of magnitude greater for calcium than for hydrogen, so changes of 2 pH units or less in the calibrating solutions do not introduce significant interference (Schefer *et al.*, 1982). Mean slope was 25 $\pm$ 2 mM per decade change in a_{Ca} (n=11). The success rate for production of double-barrelled calcium selective electrodes, as determined by a response of 20 mV or more to a 1 unit change in pCa of the calibration solutions, was about 50 % (64 of 123 electrodes). However, as explained in the results, only a small percentage of electrodes maintained calcium selectivity after egg impalements.

4.1.3 Impalements

Eggs were superfused with pCa 5 calibrating solution during impalement, and impalements were performed using the techniques described in chapter 2. The criteria for an acceptable impalement were: 1. the slope of the calcium-selective barrel measured before and after impalement changed less than 2 mV, 2. V_{ref} and $V_{ion}-V_{ref}$ were stable to within $\pm$ 1 mV for 1 minute or more after impalement, 3. Output from both barrels measured in pCa 5 calibrating solution before and after impalement changed by less than 2 mV. The fourth criteria used in chapter 2, the minimum PD_{egg} for eggs on each day, was not applied to impalements in this chapter because the superfusion solution for measurement of ooplasmic calcium was not the same as the solution for activity measurement of ooplasmic potassium, sodium, chloride or pH. The lower chloride activity in the pCa 5 saline made PD_{egg} measured in this saline slightly less negative than PD_{egg} measured in the control saline used in chapter 2.

4.2 Results

The number of successful measurements of ooplasmic calcium activity was small (Table 2), because most electrodes lost Ca^{++} selectivity during impalement. Loss of selectivity usually occurred because egg turgor pressure caused ooplasm to flow into the microelectrode tip, thereby displacing the ionophore cocktail. Recalibration was successful after withdrawal of the electrode from the egg in only 17 % (11/64) of the impalements attempted.

Table 2.

Ooplasmic calcium activities, presented as pCa, and PD_{egg} measured in locust eggs at different stages in development.

Time	pCa	PD_{egg} (mV)
unf	4.9	-49
unf	6.4	-77
d1	4.5	-23
d1	4.8	-24
d1	4.9	-49
d3	2.6	-40
d3	2.5	-41
d3	4.8	-62
d3	3.6	-69
d3	3.6	-71
d3	3.5	-832
mean d3	3.4	-61

In unfertilized eggs, ooplasmic calcium activity (pCa 4.9 - 6.4, table 1) is somewhat higher than typical intracellular values (pCa 6.9; Yamaguchi, 1986). By d3, ooplasmic calcium levels had increased as high as 4 mM (pCa 2.4, table 1) which is typical of extracellular values in orthoptera (concentration 7 - 17 mM, estimated activity 6 - 15 mM, pCa 2.1 - 1.9; Edney, 1979; Woodring, 1985). The calcium activity coefficient in moth haemolymph (0.85; Carrington and Tenney, 1959) has been used to convert orthopteran haemolymph calcium concentration to calcium activity. In d1 eggs, the calcium activity was equal to or larger than the levels measured in unfertilized eggs. Although interpretation of trends from a small sample size requires caution, the differences between calcium activities in unfertilized eggs and d1 eggs suggest that the increase in calcium may be gradual, beginning after fertilization and continuing after d1.

PD_{egg} became less negative after fertilization and more negative by d3 (table 1), as seen in the results of chapters 2 - 3. Mean PD_{egg} of d3 eggs (-61 mV) was smaller than values reported in chapters 2 - 3 (-78 mV), but the difference is easily explained by the different conditions under which they were measured. Day 3 eggs become more negative when placed in low chloride salines, such as the pCa 5 saline.

4.3 Discussion

Ooplasmic calcium activity in unfertilized eggs (pCa 4.9 - 6.4) is well below extracellular values, but somewhat higher than values measured in unfertilized sea urchin eggs (Poenie *et al.*, 1985), *Xenopus* eggs

(Robinson, 1985), or hamster eggs (pCa 6.5 - 6.9; Igusa and Miyazaki, 1986). Similarly, ooplasmic calcium activity in unfertilized eggs of locusts is somewhat higher than intracellular calcium activity in muscle cells, for example in the toad (pCa 6.9; Yamaguchi, 1986).

Within the ooplasm of the egg, calcium activity increases from typically intracellular values in unfertilized eggs to values which are typical for extracellular fluids by d3. Mean ooplasmic calcium activity by d3 is approximately 0.4 mM (pCa 3.4), lower than the estimated activity range of pCa 2.1 - 1.9 derived from measurements of calcium concentration in orthopteran haemolymph (Woodring, 1985; Edney, 1979), but still well above typical intracellular values. As discussed below, the d3 ooplasmic calcium activity is also well above pCa 7 - 8 which is used experimentally to dissociate cells (Hynes, 1972; Spiegel and Spiegel, 1975; Fink and McClay, 1980).

Ooplasmic calcium activity appears to be regulated because it increases from pCa 4.9 - 6.5 in unfertilized eggs to pCa 4.9 - 2.4 in d3 eggs, whereas the 73 % increase in egg water content over this period would be expected to dilute the initial calcium levels. Moreover, this regulation appears to be independent from regulation of potassium or sodium activities, which peak on d1 - 2 and subsequently decrease on d3, whereas calcium levels do not drop on d3.

For about 16 hours after fertilization, nuclei within the egg are syncytial and are exposed to increasing ooplasmic calcium activities. Calcium is known to play important roles in the control of many cell functions, including the events of the cell cycle. For example, increases in ooplasmic calcium activity have been suggested as signals initiating

anaphase and cleavage in sea urchin eggs (Poenie *et al.*, 1985, 1986). In insects, Wolf (1985) has suggested that each wave of increased calcium activity traversing gall midge eggs acts as a signal to initiate anaphase in syncytial nuclei, thereby causing the nuclei to divide metachronously. Wolf also found that presence of microtubules was a prerequisite for the waves of randomly oriented saltations of yolk particles which accompanied the waves of increased calcium activity and anaphase initiation. Microtubules disassemble in the presence of calcium activities greater than pCa 5, and assembly of microtubules is also inhibited above this level (Margoli, 1983). Calcium activity in locust eggs on d1 (pCa 4.5 - 4.9) is above this level. These data suggest that : 1) metachronous cleavage does not occur in locust eggs just prior to d1, or 2) that cleavage is independent of microtubules (which seems unlikely), or 3) that microtubules in locust eggs can assemble at somewhat higher calcium activities than in the cells considered by Margoli (1983).

Developmental changes in older eggs seem less likely to be controlled by changes in ooplasmic calcium activity. For example, dorsal closure begins on d3 when the ooplasmic calcium level is pCa 3.4, and blastokinesis begins even later, on d4. The control of these processes is poorly understood, but is unlikely to involve increases in ooplasmic calcium for two reasons: 1) very large quantities of calcium would have to be released to effect the 1 - 2 unit change in pCa typical of calcium signals in nerve and muscle cells (Yamaguchi, 1986; Lipscombe *et al.*, 1988; Thayer *et al.*, 1988), and 2) calmodulin and calcium-dependent protein kinases, which mediate many cell functions, are stimulated by changes in calcium activity from pCa 3.9 - 5.1 (Carafoli, 1987). It seems

likely that after d1, ooplasmic calcium is no longer used as a signal or second messenger, but that it functions in the same capacity as extracellular calcium in other cells and tissues. For example, high ooplasmic calcium activity may act to maintain the intregrity of junctions between cells or to maintain a consistent surface potential on the yolk-facing surface of serosal cells. Reduction of external calcium activity increases the negative surface potential of cells due to loss of screening divalent cations, and muscle cells for example react as if they were depolarized and become hyperexciteable (Frankenhauser and Hodgkins, 1957).

Ooplasmic calcium levels in unfertilized eggs (pCa 4.9 - 6.4) approach the levels of calcium used experimentally to dissociate a variety of vertebrate and invertebrate cell types (pCa 7; Hynes, 1972; Spiegel and Spiegel, 1975; Fink and McClay, 1980), and the levels of calcium and magnesium used experimentally to disrupt tight junctions in epithelial cells (pCa 7; Alberts *et al.*, 1983). The increase in calcium in d1 eggs (pCa 4.5 - 4.9) may favour or help to maintain adhesion between adjacent cells as the serosal epithelium forms. By d3 the level of ooplasmic calcium (pCa 3.4) is much closer to the level in haemolymph (pCa 1.8 - 2.2; Edney, 1979; Woodring, 1985) than to the level used to dissociate cells (pCa 7; Hynes, 1972; Speigel and Speigel, 1975; Fink and McClay, 1980).

This study has not addressed the possible mechanisms involved in regulation of calcium activity in locust eggs, nor the means by which increases in calcium activity occur during development. The increase in ooplasmic calcium activity immediately after fertilization could be brought about either by release from an intracellular source or entry from

an extracellular source. The foam secreted around the eggs during oviposition passes through the lateral oviducts which are permeable to proteins (Uvarov, 1966), and presumably are permeable to ions. The ionic composition of the foam may therefore be similar to haemolymph and the foam could act as a calcium source for the ooplasm before d1. Injection of calcium or firing of a calcium-dependent action potential in locust eggs isolated from virgin females increases the percentage of eggs parthenogenically activated from 30% to 70% (Smith and O'Donnell, unpublished results). The latter rate is only slightly lower than the 85% rate of activation in fertilized eggs (Smith and O'Donnell, unpublished results). These experiments suggest that calcium can play a role in egg activation, and that sufficient quantities can enter from the surrounding fluid during an action potential. An alternative and less likely possibility is that electrical activity brings about release from intracellular stores. The increase in ooplasmic calcium activity after d1, however, is more likely to result from release from internal stores. As previously mentioned, locust eggs can develop in low ionic strength solution or even distilled water (Lees, 1976; Hunter-Jones and Lambert, 1961), so uptake of calcium from the surroundings is not necessary for embryo development. Potassium and sodium have been shown to be released from yolk during development of chicken eggs (Davis, *et al.*, 1989), and yolk has been suggested as a store of calcium ions within butterfly eggs (Przelecka *et al.*, 1986). Yolk spheres were suggested as stores for sodium and potassium in Chapter 3, and these spheres may also store calcium. As mentioned previously, however, calcium is released at different times than sodium and potassium. After fertilization, calcium

is released so that there is a relatively continuous rise in activity until d3, whereas sodium and potassium are released on the first day of development and then again after d3. It appears unlikely that the control of sodium, potassium, and calcium release involves the lysis of yolk spheres and general release of all sphere contents, including ions. Calcium may be released selectively from the yolk spheres, independent of the release of potassium and sodium. Alternatively, calcium may be released from other stores such as the endoplasmic reticulum (Albert *et al.*, 1983).

The use of double-barrelled calcium selective electrodes in this chapter raises the possibility of using this technique to address other questions concerning calcium regulation and function in chorionated insect eggs. For example, is ooplasmic calcium activity locally increased near the micropyle of locust eggs during and after a calcium-dependent action potential? Similarly, can waves of increased calcium activity, possibly related to the control of metachronous cleavage, be detected in recently fertilized eggs?

CHAPTER 5

General Discussion

This thesis is the first study to examine membrane properties, ionoregulation, and pH in a chorionated insect egg after fertilization. Until 5 days after fertilization, locust eggs can be impaled reliably with ion-selective double-barrelled electrodes sensitive to potassium, sodium, calcium, chloride and pH. Both electrodiffusive and metabolic components contribute to PD_{egg} of developing locust eggs. Ooplasmic pH and the activities of sodium, potassium, and calcium are regulated. Ooplasmic chloride activity appears not to be closely regulated because measured values were consistent with those expected on the basis of dilution by water uptake.

Measurements of PD_{egg} as negative as -100 mV suggest that the egg membranes seal effectively around the electrodes and that electrical shunts at the impalement site are not a significant source of error. Values of PD_{egg} measured in control saline are not representative of *in vivo* values; the lower ionic strength and chloride content of ground water relative to control saline will tend to depolarize the egg. Although values of PD_{egg} are artificial, comparison of PD_{egg}'s measured under different conditions has provided information about the sources of PD_{egg}, and indicates that the membrane properties, measured under standard conditions (i.e. in control saline), undergo dramatic changes during post-fertilization development.

Two components of PD_{egg} were measured: a metabolic component, possibly a H^+-pump, across the cellular membranes of the egg; and a

chloride-diffusion component across the acellular membranes. Chilling and anoxia make PD_{egg} less negative, implying that part of PD_{egg} is metabolically dependent. Correlation of experimental acidification of the ooplasm with hyperpolarization of PD_{egg} in unfertilized eggs is consistent with the trend predicted by the actions of a hydrogen pump (chapter 2). A chloride-diffusion potential also contributes to PD_{egg}, and in d3 eggs this component is developed across the acellular membranes of the egg. However, more than 40% of PD_{egg} cannot be explained on the basis of chloride diffusion or metabolic pumps, so additional potential sources must exist. Because the serosal cuticles and chorion could not be removed without cell damage, the potential across the surface of the serosal epithelium facing the serosal fluid could not be measured. Ion diffusion across the membranes between the serosal epithelial cells and either the fluid-filled serosal space or the ooplasm, or both, may also contribute to PD_{egg}.

Ooplasm in locust eggs is not a static reservoir for ions. Ion activities in the ooplasm appear to be regulated independently during changes in egg water content, and this regulation may involve release from internal stores. Potassium and sodium are released into the ooplasm before d1 and also from d3 - 5, and calcium activity increases in the ooplasm after fertilization and until d3. Unlike potassium and sodium, the initial increase of ooplasmic calcium may have an external source, i.e. the foam surrounding the egg (see chapter 4), however subsequent increases in ooplasmic calcium activity are more likely the result of release from internal stores. In marked contrast to the measured cation activities, ooplasmic chloride activity in unfertilized eggs is typical

of extracellular values and appears to follow the pattern of dilution predicted by water uptake on the first 3 days after oviposition.

Measurements of pH and pCa are of particular relevance to discussions of egg activation. In marine invertebrates, egg activation triggers a calcium-dependent action potential and a transient increase in ooplasmic calcium. The increase in ooplasmic calcium triggers an alkalinization of the ooplasm and the changes in calcium and pH encourage DNA and protein synthesis and cell division. It is not known if the ionic hypothesis of egg activation is applicable to insects (Jaffe, 1985). However, results of this thesis and other studies in this laboratory suggest that locust eggs may, in fact, be activated by an increase in ooplasmic calcium. Ooplasmic calcium levels in unfertilized locust eggs are in a range appropriate for the use of calcium as a signal or second messenger (see chapter 4), and calcium may enter the egg from an extracellular source through voltage-dependent channels during a calcium-dependent action potential (O'Donnell and Solweij, 1990). An increase in ooplasmic calcium activity occurs within the first day after oviposition, but it is not known when this increase is initiated. Direct testing of the ionic hypothesis of activation requires measurements of an increase in calcium activity during or shortly after fertilization or an action potential. Results of the present thesis suggest that such a direct test is technically feasible. Although increasing ooplasmic calcium activity may act as a signal for events in early development, calcium activity after d1 has surpassed the level at which changes in activity modulate cell processes such as microtubule formation in other cell types.

The increase in calcium activity in sea urchin eggs is thought to activate a Na^+/H^+ exchanger which alkalinizes the ooplasm. Alkalinization of ooplasm in locust eggs by a similar Na^+/H^+ exchanger after activation seems unlikely, however. As discussed in Chapter 2, locust ooplasm pH is sensitive to external pCO_2 before fertilization. The ooplasm, therefore, probably alkalinizes as the eggs pass from a pCO_2 of 4 - 5 % in the body fluids of the female (Harrison, 1988, 1989) to a pCO_2 of 0.03 % in air, during oviposition or experimental collection. In addition, activity of a Na^+/H^+ exchanger in the locust egg would be restricted to the micropyle at the posterior end of the egg where the egg membrane is in contact with sodium in the external fluid and is not covered by the chorion. Moreover, because a metabolically-dependent H^+-pump appears to be present in locust eggs, modulation of pH by Na^+/H^+ exchange would appear to be redundant.

This thesis raises further questions concerning the membrane physiology of locust eggs and ionoregulation during egg development. Quiescence, suspension of embryo development by unfavourable conditions, is initiated on d3 - 4 in locust eggs which are kept dry, and continues until the egg can take up water from the environment (Uvarov, 1966). Development in about 5% of locust eggs under field conditions is also suspended on d3 - 4 without an apparent external reason, and this is called diapause (*ibid*). In most temperate species of orthopterans, most eggs undergo diapause, which is related to overwintering or drought resistance (Uvarov, 1966). Cellular controls of diapause and quiescence in locust eggs are unknown. In brine shrimp embryos, dormancy may be initiated by a >1 pH unit acidification (Busa and Crowe, 1983). Does the onset of diapause and quiescence in locust eggs correlate with an

acidification of egg ooplasm? Diapause and quiescence may also alter PD_{egg}. Does the metabolic component of PD_{egg} decline as the eggs enter diapause and quiescence, and similarly does the chloride permeability of the egg membranes decrease to limit chloride efflux?

Another series of questions relate to the mechanisms of ionoregulation in locust egg ooplasm. Potassium, sodium, and calcium activities in locust egg ooplasm are regulated independently during water uptake, but each ion appears to be released from internal stores on d3. If yolk spheres store these ions, fluorescent dye techniques and radioisotopes might be used to determine if sodium, potassium and calcium are sequestered in yolk spheres in vitellogenic eggs and are released from yolk spheres collected from d3 eggs. An earlier study using fluorescent dyes attempted to examine pH changes within locust egg yolk spheres but was unsuccessful because the dye, acridine orange, was quenched by vitellogenin within the spheres (Cousins, Zuk, and O'Donnell, unpublished results). Yolk spheres have been shown to acidify, however, during proteolysis in developing cockroach eggs (Nordin 1991 cited in Nordin *et al.*, 1990). Other dyes such as 9-amino acridine may be used to estimate pH in yolk spheres from developing locust eggs.

After chorionation, pH in the locust egg may be regulated by a metabolic H^+-pump. Is pH regulated before chorionation, i.e. during vitellogenisis and uptake of other nutrients, with a metabolic H^+-pump? This would permit two functions for a single pump: pH regulation of the outward facing pump in the oolemma, and endosome acidification after endocytosis of vitellogenin.

As mentioned in chapter 1, many cells exploit the sodium gradient (high extracellular Na^+, low intracellular Na^+) to take up amino acids and glucose, or to regulate pH or intracellular calcium activity. In locust eggs, the sodium gradient between serosal cells and ooplasm may be quite small. A larger sodium gradient may exist between the cytoplasm of the serosal cells and the serosal fluid. ISME's might be used to measure the sodium activity in the serosal space after treatment of eggs with bleach to increase the volume of the serosal space (Jones, 1959). Is the sodium activity in the serosal space typical of extracellular levels and might the serosal cells use this source for sodium gradient transport?

The serosal epithelium has been shown to maintain a transepithelial potential difference. Improvements of the internal perfusion technique would facilitate study of the basal or yolk-facing surface of the serosal cells. For example, the effect of putative blockers or inhibitors of ion channels, carrier proteins or transport ATPases on transepithelial potential difference might be measured. Patch techniques might also be used on ooplasm-facing membrane of the serosal epithelium. Treatment of eggs with bleach (Jones, 1959) moves the serosal epithelium away from the serosal cuticles at the posterior end of the egg. If a segment of this monolayer could be excised, patch techniques might also be used on the surfaces of the serosal cells facing the serosal space. Questions to be addressed include: what is the basis of the PD in serosal epithelial cells, and do the basal and peripheral surfaces of the serosal cells have different permeabilities?

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