Investigation of the role of smooth muscle function in egg transport in the mouse oviduct.

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INVESTIGATION OF THE ROLE OF SMOOTH MUSCLE FUNCTION IN EGG TRANSPORT IN THE MOUSE OVIDUCT

Bernadette Lee B.Tech. Hons.

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Abstract

Investigation of the Role of Smooth Muscle Function

in Egg Transport in the Mouse Oviduct

Bernadette Lee

The time course of egg transport along the mouse oviduct is important for successful implantation to occur. It is generally accepted that variations in the innate contractile activity of the oviduct musculature regulates the rate of egg transport but the controlling mechanism behind these changes in muscular activity is unclear.

This study investigated the role of calcium ions in oviduct smooth muscle function and the regulation of egg transport. A variety of methods were utilized including: a new *in vitro* technique, which allowed observation of egg movement and muscle activity in a controlled environment, *in vivo* measurement of transport rates; measurement of oviduct calcium ion content; and the effects of pharmacological agents, ovarian hormones, ovariectomy and ligation and cauterization of the uterus from the oviduct on egg transport and oviduct calcium levels.

The results indicate that the concentration of calcium could play an important role in the regulation of oviduct contractility and egg transport. A definite pattern of calcium ion concentration changes occurred during normal egg transport and a close correlation was found between the effects of pharmacological stimuli on the rate of egg transport and on oviduct calcium concentrations.

There was little evidence to support the concept of control of egg transport by post-ovulatory ovarian hormones, but pre-ovulatory progesterone levels may be important. If this is the case variations in the contractile activity of the oviduct could perhaps be mediated through progesterone induced changes in tissue calcium content or distribution, but further study is needed to clarify such a relationship.

To my parents

who have always given me their

support and help

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Part 1

General Introduction

Egg Transport through the mouse oviduct

Egg transport is the term used to describe the process by which newly ovulated eggs (or ova) are moved through the oviducts from the ovary to the uterus. Fertilization of the eggs takes place during this process. The time course of egg transport is critical for normal fertility, the developmental stage of both the eggs and the uterine endometrium when the two come in contact determines whether or not implantation occurs.

The transport of eggs through the female genital tract has two phases, a rapid phase, the passage of the ovum from the ovarian follicle to the site of fertilization and a much slower phase, the passage of the ovum through the isthmus of the oviducts or Fallopian tubes and into the uterus (Austin, 1963).

The oviducts are paired, convoluted, smooth muscle structures deriving embryonically from the Mullerian ducts and they connect the ovaries to the uterus (Nalbandov, 1964). The degree of convolution varies between species, some are almost completely straight (rabbit) while others are highly coiled (mice and rats).

The anatomy of the mouse oviduct has been described by Burdick, Whitney and Emerson (1942), Humphrey (1968a), Nilsson and Renius (1969) and others, using the terminology of Hartman (1939). The mouse oviduct connects with the ovary by an ovarian bursa and is coiled, consisting of about ten loops. The final section of the oviduct runs intradermally in the uterus ending with the colliculus tubarius (Fischel, 1914;).

The fimbriae and the first two loops comprise the preampulla, here the muscular tissue consists of only two or three layers of cells, mainly arranged longitudinally. The inner mucosal membrane is scanty,

extending into longitudinal folds and the lumen is restricted. The epithelium is mainly ciliated cells with a few non-ciliated cells dispersed amongst them. The third loop is the ampulla, easily distinguished on the first day of the pregnancy when it is distended by fluid and eggs. Again the muscle layers are not well developed, one or two layers of cells and the mucosa forms small longitudinal folds into a wide lumen. Non ciliated cells predominate the epithelium although ciliated cells are numerous.

After the ampulla is the largest section of the oviduct, the isthmus, the fourth to the ninth loop. The isthmus is separated from the ampulla by the ampullary-isthmic junction (AIJ), this is formed by a narrow curve of the loop next to the ampulla, the inner curve displays a massive thickening, mainly of circular muscle, whilst the outer curve shows only a gradual thickening of the muscle wall. At the opening into the isthmus long mucosal folds fill the lumen and protrude through into the ampulla. Externally the AIJ is noticeable for a constriction of the muscle walls and a ramification of the blood vessels. The muscle tissue of the isthmus is well developed, particularly the inner circular layer and in addition an outer longitudinal layer is present. The lumen is occluded by epithelial folds except for local widening when eggs are present. Unlike the preampulla and ampulla the epithelial cells are nonciliated and the mucosal layer less folded.

The final section the utero-tubal junction (UTJ) or junctura consists of the last loop of the extradermal and intradermal portions of the oviduct, it passes into and through the upper wall of the uterus and continues into the cavity of the uterus, ending as a blunt papilla, the colliculus tubarius. The muscular tissue forms a thicker coat than in the isthmus, mostly circularly arranged cells. The

mucosal layer is dense and fibrous and forms a framework of high longitudinal folds along the UTJ but the folds are missing in the last portion of the colliculus.

Transport of eggs along the mouse oviduct can be directly observed, using the technique of Burdick, Whitney and Emerson (1942) and many reviews are available on the time course of egg transport (Burdick, 1942; Burdick, Whitney and Emerson, 1942; Nalbandov, 1958; Humphrey, 1968a). Movement of ova through the initial section of the ampulla (or preampulla) is by ciliary action in the mouse (Humphrey, 1968) as in the rabbit (Bennett and Rowson, 1961; Harper, 1961, 1965, 1966) but progression through the remainder of the ampulla and the AIJ is produced by segmental contractions of the ampulla although the fluid distending the ampulla at the time of fertilization and the epithelial cilia could also be involved (Nilsson and Reinius, 1969). The cilia on the longitudinal folds of the preampulla and ampulla at the time of ovulation cause rotation of the whole cluster of eggs in cumulus but this activity ceases shortly after the eggs reach the ampulla, then only small particles such as dead sperm cells and leucocytes continue to be circulated.

Humphrey (1968) observed that the eggs came to rest in the distended ampulla by six hours post coitum (Humphrey, 1968a) and remained there during the first twenty-four hours during which time fertilization took place. After fertilization the cumulus cells surrounding the ova begin to fall away and the eggs gradually move towards the AIJ under the action of local peristalsis. During Day 1 of pregnancy the AIJ is a block to transport of eggs, not until 18-24 hours after ovulation do the eggs enter the isthmus, then the first segment of the isthmus dilates and peristaltic movement propels the eggs forward.

Movement through the isthmus is fairly rapid, by 30 hours most of the ova are scattered throughout the length of the isthmus and by 42-48 hours they have reached the UTJ. Transport through the isthmus is by a series of pendular movements which cause a constant back and forth motion of the eggs and the lumen of the isthmus widens in the region of the eggs. Once the eggs reach the UTJ they again cluster together and remain there for up to 24 hours. Normally all the eggs have entered the uterus by 84 hours after ovulation.

Physiological mechanisms involved in egg transport

The transport of eggs within the oviducts involves at least three factors: the frequency, force and co-ordination of muscular contractions; the rate and direction of beat of the cilia lining the mucosal folds; and the hydrodynamics and rheology associated with the luminal fluids at the critical times eggs are being transported (Blandau and Verdugo, 1976). It is generally accepted that the primary factor in the accomplishment of egg transport is variations in the contractile activity of the innate musculature of the oviduct (Blandau, Boling, Halbert and Verdugo, 1975). There is however no hard evidence for this and there appears to be significant species differences in the primary roles of muscles and cilia in accomplishing egg transport.

The transportation of freshly ovulated eggs into the oviducts depends on: the anatomical configuration of the fimbria of the infundibulum and its relationship to the surface of the ovary at ovulation; the way in which the cumulus oophorus and its contained eggs is shed from the ovarian follicle; and the physical characteristics of the antral fluids and the fluids that comprise the matrix of the cumulus oophorus. In the mouse, rat and hamster only a small proportion of their small, ciliated, fimbriae come into direct contact with the ovaries, which are completely enclosed by the ovarian bursa or perovarian

sac, into which the fimbriae project. At ovulation the bursa is dilated with fluid lifting it from the ovarian surface and allowing movement of eggs in cumuli within the periovarian space. The eggs in cumulus have been shed from the follicle into the periovarian fluid and here they move randomly as intermittent mesovarian contractions cause displacement of the ovary and hence the surrounding fluid. The cilia covering the fimbriae beat in the direction of the ostium and once the cumulus mass comes within the influence of the fimbriae, fluid currents will direct the eggs towards the ostium. The eggs are then transported rapidly over the surface of the fimbriae and once contact between the cumulus mass and the fimbriae occurs, through the ostium into the ampulla (Gaddum-Rosse and Blandau, 1973; Gaddum-Rosse et al., 1973; Harper, 1961).

In contrast, species such as the rabbit, guinea-pig and cat have fimbriae that surround the ovary forming an almost complete bursae ovarii and at ovulation the eggs come into intimate contact with the ciliated cells lining the fimbriae (Clewe and Mastroianni, 1958). There appears to be a functional relationship between the anatomical configuration of the fimbriae of the infundibulum and the manner of ovulation and transport of eggs into the ampulla. The decisive factors in this stage of egg transport are thought to be whether the eggs are shed completely from the follicles at ovulation and the physical characteristics of the cumulus oophorus. Despite anatomical variations of the fimbriae, this stage of egg transport is an efficient biological phenomenon. The cilia covering the fimbriae beat in the direction of the ostium and transport through the ostium and preampulla is effected by ciliary action in all species studied to date.

Egg transport through the ampulla to the AIJ shows species difference in the relative roles of the cilia and muscles. Ciliary beat is in the

direction of the ampullary-isthmic junction in all animals so far examined e.g. rat, rabbit, cat, cow, sheep, monkey and human and transport through the ampulla has been observed in the living rat, rabbit and monkey (Blandau, 1973).

In the mouse and rat distension of the ampulla, at ovulation, with fluid, renders the cumulus mass relatively small in relation to the volume of the ampulla. Vigorous, peristaltic contractions quickly propel the eggs through to the AIJ. The ampulla of the rabbit is only slightly distended and two mechanisms of transport are exhibited: localized segmental, peristaltic contractions and ciliary action. Once the eggs reach the AIJ they remain there for one or two days (Hafez, 1973a), this region acts as a functional block and its anatomy shows species variation. In mice, rats and hamsters the lumen at the AIJ is very narrow and several physiological mechanisms have been suggested to explain the blocking of eggs here: temporary inactivity of the epithelial cilia; localized oedema of the isthmic region; adovarian activity; constriction or inactivity of the isthmic circular muscle; or tubal locking by a specific sphincteric muscle or muscles in the isthmus (Hafez, 1973b). Histological techniques have failed to demonstrate any isolated sphincteric structure in the isthmus of the rabbit (Greenwald, 1961) or man (Lisa, Gioia and Rubin, 1954) but El-Banna and Hafez (1970) measuring oviduct lumen size in rabbits and cattle found the AIJ to be the smallest at all reproductive stages. In man (Brundin and Wirsen, 1964) and other species (Owman, Rosengreen and Sjoberg, 1967) the isthmus and AIJ have a rich adrenergic plexus intermingled with the circular muscle fibres. The adrenergic innervation of the ampulla is sparse and confined mainly to perivascular areas and to a thin subserosal nerve plexus (Brundin, 1965). Hafez (1973) suggested that at the AIJ the isthmus may be functioning as a physiological, if not anatomical sphincter.

During Day 1 of pregnancy the opening of the isthmus is not wide enough even for single eggs and the AIJ must widen either by a relaxation of the muscle layers or by a diminution of the mucosal folds obstructing the lumen (Nilsson and Reinius, 1969).

After leaving the AIJ the eggs pass fairly rapidly through the isthmus towards the UTJ during Day 2. Anatomically and functionally the oviduct may be regarded as two distinct tubular organs anastomosed at the AIJ (Bourdage, 1978), and transport through the isthmus involves different mechanisms to transport through the ampulla. Burdick et al., (1942) observed peristaltic and antiperistaltic movements of the isthmus in the vicinity of the eggs, producing pendular movements of the ova in the loops of the isthmus. According to Humphrey (1968a) periodic decreases of a sphincter type action at the end of each loop permit progression of eggs towards the uterus. It has been suggested that unlike intestinal peristalsis, oviduct peristalsis instead of transporting the eggs rapidly along the isthmus, tends to slightly delay their progression (Hafez, 1976). If so, this would mean that the oviduct musculature is playing a regulatory role in egg transport.

Passage through the isthmus takes between twelve and eighteen hours and during Day 3 the passage of eggs is again delayed, this time at the UTJ, where the eggs remain for about thirty hours (Humphrey, 1967). The eggs do not enter the uterus until a relaxation of the muscular walls of the intramural or papillary sections occurs and then both eggs and fluid enter the uterus in a peristaltic rush.

The importance of the autonomic nervous system in the control of egg transport through the oviduct is still unclear. Most studies on oviduct function have been carried out in the rabbit (Bodkhe and Harper 1972a, 1972b; Brundin, 1965; Pauerstein, Hodgson, Fremming and Martin, 1974; Thoenen, Tranzer and Hausler, 1970) and humans (Brundin, 1969; Coutinho, Maia and Adeodato Filho, 1970; Marshall, 1970, 1973; Owman et al., 1967) and have concentrated on the adrenergic nervous system.

The adrenergic innervation to the oviduct is extensive and well defined, extrinsically the pattern of adrenergic nerve distribution shows little species variation. "Long" postganglionic fibres arise from the hypogastric, celiac and pelvic ganglia and 'short' neurones from proximal ganglia located in the cervicovaginal region, the latter possessing specific physiological and pharmacological characteristics (Brundin, 1965; Sjoberg, 1967; Owman, Sjoberg and Sjostrand, 1974). The nonvascular intrinsic adrenergic innervation is most dense in the circular muscle layer. The density of nerve endings increases from the ampulla to the uterus, the thin muscularis of the ampulla is sparsely innervated and the isthmus densely throughout its length especially at the AIJ.

Brundin (1965) first suggested that the adrenergic innervation was important for intact reproductive function of the oviduct and it was also thought that the isthmus functions as an adrenergically controlled sphincter, regulating the passage of eggs through the tube to the uterus (Brundin, 1965; Pauerstein, Fremming, Hodgson and Martin, 1973). Physiologically, however, it is difficult to establish a clearcut association between the autonomic nervous system and the reproductive process (Black, 1976). Reserpine, which depletes catecholamine stores has

been shown to retard or arrest egg transport, but this effect is due to hypothermia and not the loss of adrenergic function in the oviduct of the mouse (Bennett and Kendle, 1967; Kendle and Bennett, 1969a, 1969b). Reserpine also causes little or no disruption of ovum transport in the rabbit (Bodkhe and Harper, 1972). 6-hydroxydopamine, which causes irreversible degeneration of peripheral adrenergic nerve terminals (Thoenen and Tranzer, 1968), does not effect the fertility of mice (Johns, Chlumeck, Cottle and Paton, 1975) and rabbits (Eddy and Black, 1974).

Adrenergic agonists have been investigated for their potential as contraceptive agents and for further elucidation of the role of the adrenergic nervous system in the control of egg transport (Bodkhe and Harper, 1972b; Longley, Black and Currie, 1968; Pauerstein, Fremming and Martin, 1970a, 1970b; Polidoro, Howe and Black, 1973; Polidoro, Heilman, Culver and Reo, 1976). Exogenous administration of these drugs, in the rabbit, appears to modify the rate of normal egg transport, but these effects are time, oestrogen and progesterone dependent (Polidoro et al., 1976). Adrenaline, noradrenaline and phenylephrine, and adrenergic agonists, have all been shown to accelerate egg transport into the uterus (Polidoro et al., 1973, 1976) but isoprenaline, a β-receptor agonist, was ineffective. Polidoro et al., (1976) attributes the acceleration of egg transport to an α-stimulatory effect on the smooth musculature but finds this incompatible with the sphincteric effect of noradrenaline postulated by Brundin (1965) and the report of successful pregnancies after autograft transplantation of the oviduct (Winston and Browne, 1974), this procedure would most probably leave the oviduct free of adrenergic innervation, though possibly intrinsic "short adrenergic neurones" might remain functional.

The use of adrenergic agonists and selective inhibition of their responses has demonstrated the presence of both α- and β-adrenoceptors in the oviduct, particularly the isthmus (Longley et al., 1968; Ueda, Mattos and Coutinho, 1973; Howe and Black, 1973). As in most other smooth muscles α-receptors mediate excitatory responses and β-receptors inhibitory or relaxation. The longitudinal muscle of the rabbit oviduct is more sensitive to α-adrenergic agonists than the circular muscle (Hodgson and Pauerstein, 1974; Johns and Paton, 1974). The longitudinal muscle, like cardiovascular smooth muscle, is more sensitive to noradrenaline than adrenaline, while the circular muscle is more sensitive to adrenaline than noradrenaline. The adrenaline response in both circular and longitudinal muscle layers is blocked by phentolamine, an α-receptor antagonist and potentiated by propranolol, a β-receptor antagonist (Kendle and Lam Shang Leem, 1976). Practolol, a specific β_1-antagonist potentiated only the circular muscle contractions and salbutamol, a β_2-stimulant caused inhibition of longitudinal muscle contraction. The results of Kendle and Lam Shang Leem (1976) suggested that the inhibitory receptor in the circular muscle layer is of the β_1 type and of the β_2 type in the longitudinal muscle. No detailed evidence on the relative innervation of the circular and longitudinal muscle layers of the ampulla is yet available, (Kushiya, 1968).

Although adrenergic agents can be shown to effect oviduct motility and egg transport, it must be remembered that pharmacological and not physiological doses have been used and for contraceptive effects the doses needed would be toxic.

The oviduct is only sparsely innervated by cholinergic fibres (Woodruff and Pauerstein, 1969), this may be a reflection of a lack of direct methods of measuring the presence and amount of acetylcholine, the neurotransmitter, but it is generally accepted that the parasympathetic nervous system probably plays little role in the control of tubal transport.

The role of the ovarian hormones in the control of egg transport and oviduct motility

The role of the ovarian hormones in the control of egg transport is still unclear. Early studies in the mouse (Whitney and Burdick, 1939) the rat (Alden, 1942b) and the rabbit (Adams, 1958) have shown that bilateral ovariectomy after ovulation does not alter the rate of egg transport, thus indicating that post-ovulatory ovarian hormone secretion does not control egg transport. However ovariectomy one to four weeks before measurement of the rate of transport of transferred eggs (Noyes, Adam and Walton, 1959; Noyes, 1959) or radioactive spheres (Harper, 1964, 1965) results in an accelerated rate of transport through the oviduct. Treatment of the rabbits with oestrogen or progesterone or a combination of both, before transfer, did not restore normal rates of transport. Low doses of oestrogen produced very variable rates of transport and higher doses caused some retention of eggs in the oviduct but not the uterus. The activity of oestrogen was thought to be modified by progesterone when the two were used in combination, progesterone reducing the activity of the circular muscle layer, which would otherwise expel the spheres into the uterus (Harper, 1965). Hilliard, Archibald and Sawyer (1963) showed an abrupt rise in progesterone and 20α hydroxy-progesterone levels 10 to 25 minutes after mating or an ovulatory injection of gonadotrophins, in the rabbit, i.e. some 16 hours before ovulation. This suggests that a preovulatory secretion of progesterone may play a role in the control of egg transport, ovariectomy after ovulation would not effect this rise in progesterone levels.

The effects of the female sex hormones on egg transport and oviduct motility vary with species, dose given and the time of administration, (Pauerstein et al., 1974). Interruption of pregnancy in the mouse by

"oestrin" was first shown by Parker and Bellerby (1926) and in the rat with "follicular extract" by Smith (1926). A decade later Burdick and Pincus (1935) demonstrated egg retention in the oviduct of mice and rabbits for 14 and 9 days respectively, after copulation, following daily oestrin administration, 5-10 rat units in the mouse and 100-150 rat units in the rabbit. Further studies by Whitney and Burdick (1936, 1938) and Pincus and Kirsch (1936) led to the conclusions that moderate doses of oestrogen retained eggs in the oviduct for longer than the normal period ("tube-locking") but larger doses accelerated transport, prematurely expelling the ova into the uterus. In both cases the consequent effect being infertility.

Greenwald (1967), Humphrey (1968a) and Humphrey and Martin (1968) have reaffirmed the acceleration and "tube-locking" of ova by natural and synthetic oestrogens in the mouse, rabbit and several other species. Greenwald (1967) observed that a single dose of oestradiol cyclopentyl-propionate (ECP) administered shortly after mating caused both acceleration (low doses) and retardation (high doses) of ova in the guinea-pig, hamster, mouse and rabbit, only in the rat did "tube locking" not occur. More detailed experiments in the mouse by Humphrey (1968b) and Yanagimachi and Sato (1962) revealed contradictory results. Humphrey found the time of administration to be the determining factor for accelerative or "tube-locking" effects. Using oestradiol 3:17β, large doses on Day 1 caused ovum retention in the ampulla, whilst low doses on Day 2 caused premature entry of eggs into the uterus and vagina. Treatment on Days, 1, 2 and 3 with a range of doses resulted in a proportion of the eggs being lost from the reproductive tract whilst the remainder were retained. Yanagimachi and Sato observed the dose level to be significant, low doses of ethinyloestradiol, an orally active synthetic oestrogen, on Day 1 was accelerated, whilst high doses were retarding.

It seems likely that the different experimental conditions and the rate of uptake and metabolism of the various oestrogens used are responsible for the contradictory results.

The rabbit has been used extensively for egg transport studies and, as in the mouse, the overall effect of oestrogenic compounds appears dependent upon the oestrogen chosen and the regime of treatment. Greenwald (1957) reported that small doses of oestradiol (5ug) administered on Days 1, 2 and 3 of pregnancy caused retention of nearly half the eggs in the oviduct whilst the same dose administered at copulation and 48 hours later caused retention of only 4% of the eggs. ECP, a long acting oestrogen, (25ug), immediately after copulation caused premature entry of 78% of the eggs into the uterus, but a much larger dose (250ug) had the opposite effect (Greenwald, 1961).

The "tube-locking" effect of oestrogens has been thought to demonstrate the AIJ as the primary oviductal site for sphincteric activity. In all these earlier studies oviductal flushings or autoradiography techniques have been employed to determine egg positions, in the rabbit. More recently Howe (1970) used a freeze-clearing technique and his data differed from those obtained by oviductal flushings or autoradiography and pointed to the UTJ being the primary site for sphincteric activity. Pauerstein et al., (1974) however, using benzyl benzoate clearing techniques, found that oestradiol at the time of ovulation (250ug) caused retention of the eggs at the AIJ.

Acceleration of egg transport, in rabbits, due to administration of progesterone was reported by Black and Asdell (1959) contrary to the findings of Adams (1958) that progesterone immediately after ovariectomy delays egg transport. Greenwald (1961) showed that various doses of progesterone or Delalutin, a long-lasting progestin, soon after mating could accelerate egg transport along the oviduct, but not

necessarily egg transport from the oviduct to the uterus.

Progesterone administration, for three days, prior to ovulation, accelerates egg transport (Chang and Bedford, 1961) and also causes degeneration of eggs, but when administered after ovulation egg transport and development were unaffected (Chang 1961a). Chang concluded that progestin administration before ovulation was similar in effect to that of oestrogen administered after ovulation. He also concluded that endogenous or exogenous oestrogen causes rapid egg transport from the oviduct to the uterus, whilst endogenous or exogenous progesterone counteracts the oestrogenic effect and delays egg transport (Chang, 1966b).

Pauerstein, Anderson, Chatkoff and Hodgson (1974a) confirmed the results of Chang and has consistently obtained similar results (Pauerstein, Fremming and Martin, 1970a, 1970b; Pauerstein, Fremming, Hodgson and Martin, 1973) and Gonzalez De Vargus, Hodgson and Pauerstein (1975) drew the conclusions: that progesterone given at least one day and not more than two days prior to ovulation induces accelerated egg transport; that the progesterone responsive mechanism is dose dependent; and acceleration is partially antagonised if treatment is begun three days prior to ovulation.

Harper's (1966) view that oestrogen accelerates, whilst progesterone retards egg transport by respective stimulation and depression of the oviductal muscle was contradicted by Boling and Blandau (1971a, 1971b). They postulated that withdrawal rather than dominance of oestrogen increases the vigour and orderly programming of muscular contractions as well as the acceleration of eggs. Progesterone alone given to ovariectomized rabbits does not significantly change the rate of transport but given to intact or ovariectomized, oestrogen treated animals it increases the amplitude and decreases the frequency of

contractions. Thus it may, in a sense, be considered facilitatory to egg transport (de Mattos and Coutinho, 1971).

Controversy still exists on the mechanism of hormonal regulation of egg transport. Spillman and Harper (1974) have reiterated the early views of Harper (1966), but an important factor in the different results is probably that in one case the studies were of chronic effects and in the other acute effects of progesterone administration.

Since the attempts of Harper (1964, 1963b) to reproduce normal egg transport in ovariectomized animals, Hilliard and Eaton (1971) have shown, in mated rabbits, that a rapid fall in oestrogen and progestins (progesterone and 20 α-hydroxyprogesterone) occurs after their immediate preovulatory rise. Such hormonal conditions have not been created in previous experiments. It is questionable however, taking these factors into consideration, whether normal transport can be obtained in ovariectomized animals. Sequential treatment of ovariectomized monkeys with fixed doses of oestrogen and progesterone give rise to variation in oestrogen receptors in different parts of the oviduct, with resultant manifestation of different physiological responses (Bremner, Resko and West, 1974).

In vitro oestrogen incorporation by different parts of the rabbit oviduct in oestrus varies, incorporation by the isthmus being significantly higher than by the ampulla (Roy, Roy, Dasgupta, Engineer and Kar, 1972). Earlier work by Kim, Coates and Flickinger (1967) and Martin, Ware, Crosby and Pauerstein, 1970), was contrary to this, but the experimental conditions were not identical. During egg transport, in the rabbit, Karkum (1976) showed that the in vitro capacity of the ampulla, AIJ, isthmus and UTJ all change significantly, but differently. Incorporation by the ampulla and AIJ gradually increased from lowest values at 14 hours post coitum to the highest at 70 hours post coitum, whilst the isthmus and UTJ remained unchanged at the highest level.

In general oestrogens have been regarded as activators of reproductive smooth muscle and progestins as inhibitors. This is in fact contradictory to the findings that tubal excitation is greatest during menstruation, the time when endogenous oestrogens reach their lowest level (Coutinho, 1973).

The response to oestrogen treatment is so varied from species to species and to changes in dose or timing of dose that no one comprehensive explanation of its effect has yet been given. There is indirect evidence in women to suggest that oestrogen-domination during the pre-ovulatory phase establishes a block in the isthmus preventing passage of oviductal contents into the uterus (Pauerstein et al., 1974a; Croxatto, 1974). Oestrogen is known to induce protein synthesis of contractile proteins and it can activate both uterine and oviductal musculature, causing an increase in the frequency of contractions and a decrease in their amplitude (Coutinho and de Mattos, 1968). If exogenous oestrogen is activating the tubal musculature by increasing the frequency of contraction and maintaining a high level of activity, it may be that it is being impeded from contracting or relaxing fully. The position of the eggs in the oviduct may be critical in determining the effect of exogenously administered oestrogen. If the eggs are in the ampulla, then exogenous oestrogen will probably enhance the already present block at the AIJ, thus causing "tube-locking" of the eggs. Alternatively if the eggs are already in the isthmus then an increase in tubal activity is likely to accelerate their passage through the lumen towards the uterus.

Exogenously administered progesterone appears to effect the function of the oviduct if it is given prior to ovulation. This suggests that progesterone needs time to be effective, its action is not direct but triggers other events. The final product of progesterone metabolism

is oestrogen, which suggests that delayed responses to progesterone are oestrogenic effects. However, Megestrol acetate, a synthetic progesterone administered for 3 days before ovulation accelerates egg transport, in the rabbit (Kendle and Telford, 1970) and Megestrol acetate is not converted to oestrogen. Therefore the temporal effects of progesterone and progestins are probably acting through progesterone receptors to trigger other events such as protein synthesis. The concept of hormonally-induced alterations of contractions has been established in both the oviduct and the uterus (Hodgson and Daly, 1976) and it could be that the ovarian hormones act via other events in the smooth muscle cells. Calcium binding or exchange is a likely target area. Intracellular free calcium ions mediate the effects of chemical, mechanical and electrical stimuli, causing either activation or inactivation of contractile proteins and therefore contraction or relaxation of smooth muscle cells. Alterations in calcium binding or exchange by hormones could alter the rate and amplitude of contractions and relaxations and hence affect oviduct motility and egg transport.

Recent work by Batra and Bengtsson (1978) in the rat uterus indicates that oestrogens and progesterone are capable of decreasing calcium entry into uterine cells. Uterine contractility is known to be greatly influenced by ovarian hormones, oestrogenic compounds appear to increase the contractile response of the uterus and progestins mainly decrease uterine contractility (Csapo, 1956; Kumar, 1967).

Tension development in muscles is now considered to be controlled by the concentration of free calcium reacting with the contractile proteins and drug actions on muscular contraction are thought to be mediated through variations in the intracellular free calcium concentration

(Batra and Bengtsson, 1978). Ovarian hormones have been shown to affect the calcium transport in myometrial mitochondria (Batra and Bengtsson, 1972; Batra, 1973) and the effects of ovarian steroids on myometrial contractility may be explainable on the basis of alterations in calcium metabolism. If the ovarian hormones are capable of influencing uterine contractile activity via calcium ions it would seem likely that the ovarian hormones could have similar effects in oviductal smooth muscle.

Further evidence that ovarian hormones may effect oviduct smooth muscle function via calcium metabolism came from Hodgson and Daly (1976) who found that $^{45}Ca^{2+}$ uptake, in the rabbit oviduct, varied under the influence of oestrogen and progesterone and in the different regions of the oviduct. Ovulation was induced in the rabbits with Human Chorionic Gonadotrophin (HCG) and uptake into the AIJ and isthmus was decreased after 24 hours and also in the isthmus after 72 hours and 24 hours after HCG and progesterone. Oestrogen treatment was found to restore isthmic levels to that in oestrus and tended to increase uptake into the AIJ. The fastest exchanging calcium compartment was the extracellular compartment and oestrogen consistently decreased the extracellular compartment.

It does seem possible that calcium binding and/or exchange could represent a site of hormonal control of contractile activity and hence egg transport but more information is still needed on calcium movements and on <u>in vivo</u> motility.

Assessment of methods used to study oviduct motility and egg transport

The mechanism of gamete transport will be clarified only when the respective roles of oviductal muscular activity, ciliary activity, rheology and hydrodynamics of oviductal fluid, contractions of the various tubal mesenteries and the intrinsic activities of the gametes can be fully ascertained and their integration clarified (Blandau et al., 1975).

In vivo studies of egg transport involve various approaches: direct observation of eggs or surrogates; intraluminal pressure sensitive monitors; forcing fluids (gas or liquid) through the oviduct and measuring the back pressure generated; and extraluminal strain gauge transducers.

The rate of in vivo egg transport can be measured accurately in laboratory animals. The oviduct of the mouse and rat is sufficiently thin walled for microscopic examination to reveal egg positions. In the case of the rabbit the cumulus mass can be observed in the ampulla, microscopically, but the thick muscular walls of the isthmus necessitate the use of flushing or clearing techniques. Direct observation of egg transport in anaesthetised rabbits has been described by Harper (1961a, 1961b). He observed ampullary transport of eggs in cumulus by withdrawing the oviducts through an incision in the lateral body wall. His observations indicated roles for both the cilia and smooth muscle of the ampulla. Blandau (1971) carried this technique further with the 'open-dish' preparation where the nerve and blood supply are left intact.

The advantages of these preparations are the lack of interference with transport and, with care, minimal interference to the oviduct and its control systems. The use of anaesthetics however and the opening of the abdomen may affect some control systems and the duration

of observation is limited. This preparation is useful for observing the effects of hormones and drugs on oviductal activity and tubal transport. A further advance is the isolated blood-perfused whole oviduct system which is advantageous in not having to be concerned with changes in the cardiovascular status, endocrine imbalance, or other complications arising from anaesthesia. This preparation is capable of transporting eggs in their cumuli in the ampulla of the rabbit oviduct for up to four hours (Vincenzi and Raess, unpublished data, in Blandau et al., 1975). A disadvantage of these systems is that not all the activity will be observed either visually or photographically and the cause of any egg movement is inferred without sufficient information.

Intraluminal pressure recordings have serious limitations, the probes are usually so large as to distend the oviduct (often variable from experiment to experiment) and interfere with normal egg transport. They may also cause local irritation. Partial or complete blockage of the tip of open ended fluid filled tubes can occur and considerable damping prevents rapid pressure events being accurately followed. Occlusion of the tip is prevented in closed balloon systems but accumulation of air bubbles and damping effects often distort readings. Another problem is the possibility that an artificial closed system is being created, superimposing itself on or replacing the physiological system under investigation. Intraluminal techniques record pressure changes from only one or a few sites, which is inadequate if the oviduct comprises local systems partially or completely independent.

Insufflation techniques using gas or liquid also incorporate a distending stimulus and the flow in these systems is usually from the isthmus to the ampulla, opposite to the movement of eggs. The pressure-volume relations of the delivery and recording systems must be known first, otherwise the recordings are meaningless. Further

experimentation is needed before the site of resistance to flow can be determined.

Nelsen, Nunn and Angell (1973a, 1973b) developed a microminiature pressure-sensitive device for use in small hollow organs which incorporates modern integrated circuit techniques in the hope of avoiding the problems of air bubbles in tiny-fluid catheters and interference with ovum transport. However this type of small transducer, designed specifically for mammalian oviducts is not yet adequately reliable.

Various extraluminal recorders have been employed for monitoring oviductal motility, including: semiconductor strain gauges, mercury strain gauges and mutual inductance transducers. All these devices have serious limitations, the dimensions of most are too large and can cause distortion and limiting of oviductal wall motion and may also produce injury reactions. Again the number which can be applied to the oviduct wall limits the information obtainable. Whether there is motion in all axes and the effects of wall motion on intraluminal pressure and transport is often difficult to determine.

Nelson et al., (1973a, 1973b) and Nunn, Angell and Nelsen (1973) have made improvements in design and size of strain gauge transducers and according to Nelson they are now sufficiently small and sensitive to monitor isthmus deformations for extended periods, applied longitudinally. However, applied across the longitudinal axis to measure circular muscle contractions severe flattening of the isthmic wall occurs.

Conductive elastomer strain gauges (Gallant and Fromm, 1973; Garcia, Fromm, Jeutter, Aller and Harrison, 1974; Jeuther and Fromm, 1971; Jeuther, Fromm and Garcia, 1973) measure a change in resistance of a small carbon-doped silicone rubber rod, the strain-sensitive element.

It is specifically sensitive to longitudinal bending or shortening of the tube, but the significance of these contractions in terms of gamete transport is difficult to elucidate and this also applies to all methods of monitoring muscle activity.

Maistrello (1971) has described a specialized application of mercury strain gauges, which normally tend to lack sensitivity, utilizing a mercury-filled silastic tube mounted against the outer diameter of the oviduct. Unfortunately it has only a short life span and is subject to artifacts if the tubal mensenteries are not monitored simultaneously, there is also difficulty in application which limits the number of points for monitoring.

The advantage of mutual inductance transducers (Duff, Stegall, Nelsen, Blandau and Boling, 1972) is their reduction in irritation and burdening of the oviduct as they are very small with extremely flexible leads. Extraluminal diameter changes are measured as an indication of oviductal contractions likely to be significant in the transport of the luminal contents. Their primary disadvantage is their sensitivity to other movements of the oviduct and other abdominal organs.

Recording oviduct motility presents many problems, including the small size of the organ and the necessity of inferring the effect of changes in wall motion and intraluminal pressure on egg transport. No one of these techniques is sufficient on its own, only a combination of methods is likely to provide sufficient information to deduce the role of oviduct motility in egg transport.

In vitro studies are on the whole simpler and allow far more controlled conditions to exist. However, they necessitate isolating the oviduct from its normal environment and therefore exclude many possible control systems. An essential control system may be missing in vitro but at least new control systems are not being acquired or

superimposed on existing ones. In vitro preparations may be used to evaluate the importance of a supposed in vivo control system.

The cilia lining the luminal surfaces of the mammalian oviduct, particularly in the ampulla are thought to play a role in egg trasnport. Measurement of the strength rate and direction of ciliary beat using a number of in vitro methods, in conjunction with cinematographic recordings, allow evaluation of the effects of hormones, anaesthetics, environmental changes and pharmacological agents.

Gaddum-Rosse and Blandau (1973) described a method for studying the direction and strength of ciliary beat in longitudinally opened oviducts maintained in glass moisture chambers, on blackened paraffin wax. The rate of beat can also be followed microscopically and cinematographically, but this method has the disadvantage of not allowing continuous measurements and it is possible that minor effects of pharmacological agents would pass unobserved.

A method which yields rapid and accurate determinations of ciliary beat under a controlled environment is that of a photo-sensitive probe (Dalhamm and Rylander, 1962). Movement of the cilia across the miniature photo-probe generate a varying electrical signal which is then amplified, filtered and recorded directly.

Studying the behaviour of oviduct smooth muscle in vitro is useful for understanding the biophysical aspects of the electromechanical physiology of the oviductal smooth muscle in different hormonal states and the effects of pharmacological agents. These studies are however limited in determining the importance of oviductal contractions in the regulation of egg transport. One method in use is the in vitro circular muscle preparation to study the oviductal smooth muscle's intrinsic behaviour, its neurohormonal control and electromechanical behaviour (Vinzenzi and Raess, 1972).

Studies on the physiology and function of the oviduct has tended to focus on the role of muscle activity and there is still no clear understanding of how this relates to the control of egg transport The roles of the cilia, oviductal fluids, mesenteries and the cumulus mass deserve more investigation as no one aspect of oviduct physiology is likely to control the process of egg transport alone.

Smooth Muscle Physiology

The area of interest for this study is the control of egg transport in the mouse oviduct, with particular interest in the development of a new *in vitro* technique for studying oviduct function and the role of calcium ions in oviduct smooth muscle function.

Variations in the contractile activity of the oviduct musculature are considered responsible for the transport of eggs along the oviduct to the uterus. The cilia lining the mucosa and the luminal fluid also have their role to play. The controlling mechanisms which govern the pattern of oviductal contractions are uncertain, but hormonally induced alterations of contractions have been established in both the oviduct and the uterus. An obviously important factor in this case would be the regulation of calcium binding and exchange, via alterations of the size of calcium pools, the affinity of binding sites for calcium or the permeability of membranes to divalent ions.

The oviducts like most hollow viscera are composed of smooth muscle layers which can be orientated longitudinally, circularly or spirally. The specific orientation varies along the length of the oviduct and from species to species (Beck and Boots, 1974; Nilsson and Reinius, 1969). The smooth muscle cells do not exist in isolation but in conjunction with secretary, neural and connective tissue cells, elements which must be taken into account when studying the effects of various experimental techniques on the muscle cells. The muscle coat of the oviduct varies in thickness along its length, decreasing from the UTJ to the infundibulum in all species investigated so far. The orientation of the muscularis in the mouse and rat is the reverse of that in the guinea-pig, in the former the outer longitudinal layer is thin compared to the thick, inner, circular layer, whilst in the

guinea-pig the circular muscle is outermost. The rabbit and human display a further variation, a circular layer lying between inner and outer longitudinal layers, which increases in thickness towards the uterus. Anatomical differences between species and within the different regions of the oviduct may imply variations in contractile patterns (Marshall, 1976).

In general, smooth muscle is distinguished from skeletal muscle by a lack of regular transverse alignment of thick and thin filaments and by the absence of T-tubules. Coupling between cell membrane and contractile elements is more direct and consequently the fibre diameters are much smaller. Visceral smooth muscle, including oviductal and uterine smooth muscle, demonstrates myogenic rhythmicity, conduction is from fibre to fibre and they are capable of being stimulated by quick stretch. Although autonomic nerves have a modulating action, they have no triggering action.

Scanning electron microscopy of smooth muscle has revealed rows of small vesicles which are probably a sarcoplasmic reticulum (SR) and under "good" fixation both rough and smooth SR have been identified (Devine, Somlyo and Somlyo, 1973). The cell volume of SR varies with the type of smooth muscle, more tonic muscles e.g. pulmonary artery and aorta have larger amounts and oviductal smooth muscle (turtle) is intermediary between tonic and phasic muscles (Somlyo, Vinall and Somlyo, 1969). Myometrial smooth muscle SR has been shown to increase during pregnancy and oestrogen treatment (Somlyo, Devine, Somlyo and North, 1971). The vesicles comprising the SR have been considered to be regions of calcium storage and release and muscles with high concentrations of SR are able to maintain their contractile responses longer than those with little SR.

Intracellular calcium concentrations and the amount of bound calcium in smooth muscle are low compared to striated muscle, even in the smooth muscles with most SR. It has been postulated that there is an active extrusion of internal calcium, perhaps a sodium-calcium exchange system, dependent on ATP (Casteels, Van Breeman and Mayer, 1972, 1973). By means of calcium washout curves three calcium compartments have been indicated; extracellular calcium, cell and vesicle membrane bound calcium and intracellular calcium, part of which is slowly but measurably exchangeable (Weiss and Goodman, 1969). Smooth muscle contractions utilize calcium from the extracellular space and intracellular and membrane stores, the importance of the particular store varying with the muscle (Potter and Sparrow, 1968; Potter, Sparrow and Simmonds, 1970). Intracellular calcium can be assayed by a method based on displacement of extracellular calcium by lanthanum (Van Breemen, Daniel and Van Breeman, 1972). Lanthanum displaces extracellular calcium, blocks calcium influx and therefore inhibits contraction.

Visceral smooth muscles show several kinds of slow electrical events, one type of spontaneous activity is rhythmic oscillations of the membrane potential or "slow waves", usually occurring in longitudinal muscle. They are prominent in many species and have been studied extensively in the smooth muscle of the intestines (Prosser, 1974). Slow waves in the oviduct and uterus have received little attention (Talo and Hodgson, 1978). Slow waves set the frequency of segmental movement (Bass and Weisbrodt, 1971), in intestinal muscle they are generated in the longitudinal muscle and do not trigger contractions in the absence of spikes, they pass to circular muscle passively via thin connecting muscle strands and bring the membranes of each layer up to threshold for spiking. Tomita and Watanabe (1973) recorded slow waves

from the guinea-pig oviduct and Nelsen et al., (1976) from the rabbit oviduct. The former used the sucrose-gap technique but failed to state the location on the oviduct or the hormonal state and the latter used extracellular needle electrodes and demonstrated slow and fast electrical events as well as pacemaker activity. Talo and Hodgson (1978) have observed slow waves in the postovulatory guinea-pig oviduct, recorded with intracellular microelectrodes and suction electrodes and in the mouse and immature baboon oviduct with suction electrodes. Slow waves in both the guinea-pig and mouse oviducts, recorded with suction electrodes, were either notched on the rising phase or had a single spike on the rising phase. The slow waves coincided with increases in longitudinal tension and were usually propagated in the fimbrial direction in the guinea-pig and in both directions in the mouse. In both cases they were less frequent in the ampulla and decayed more rapidly. In the immature baboon, the slow waves were more difficult to record, but they appeared to be propagated from the AIJ. Whilst Tomita and Watanbe (1973) suggested that the notch on the rising phase of the slow wave was due to two separate components of the slow wave, Talo and Hodgson (1978) suggest that notching is due to the activity of the two muscle layers as it did not occur in the ampulla or during intracellular recordings. Whether the spikes originated in the longitudinal or circular muscle was uncertain, but the spikes and notches appeared to relate to muscle contractions. In some cases, in the mouse, the spikes and slow waves could be dissociated and this has also been observed in the cat intestine (Specht, 1976). From the results in the immature baboon showing the slow waves originating from the AIJ Talo and Hodgson also speculate as to whether this could be a reason for delayed transport at the AIJ. The slow waves probably regulate the period of spikes and hence contractions, as occurs in other smooth muscles.

The electrical spikes of smooth muscle are conducted from cell to cell and it is they that trigger contractions (Prosser and Bartoff, 1968). The spikes of visceral smooth muscles are more sensitive to replacement of external calcium than sodium. In a calcium free medium there is only a slight depolarization and decrease in membrane resistance, no spikes occur and contractions fail (Kuriyama and Tomita 1970). Calcium influx is the main event in the rising phase of the spike and the spikes can be abolished by replacing calcium ions with magnesium ions (Bulbring and Tomita, 1970), inhibiting the entry of the trigger calcium associated with spiking with D-600 (Verapamil) or by blocking all calcium uptake with lanthanum ions, which have a high affinity for extracellular calcium binding sites (Mayer, Van Breemen and Casteels, 1972). High extracellular calcium hyperpolarizes and increases membrane conductance, spike height and rate of rise of spikes (Brading, Bülbring and Tomita, 1969) and increases in spontaneous spiking and amplitude of spikes have been shown in duodenal longitudinal and circular muscle with high calcium.

The ionic mechanism associated with the falling phase of spikes is unclear, but it may be associated with an increase in potassium conductance (Ito, Kuriyama and Sakamoto, 1970).

There appears to be some antagonism between sodium and calcium ions, though the effects of calcium are more pronounced than of sodium. Sodium is necessary for recovery from potassium induced contracture but extracellular calcium is required for the contraction phase, extracellular sodium decreases the permeability to calcium ions. It is not known if the effect of sodium is on the release of membrane-bound calcium or if it is an allosteric type action on the calcium channel. In uterine muscle, Anderson et al., (1971) and Anderson (1972) suggest that there may be a single conductance channel requiring

both sodium and calcium.

Smooth muscle contractions require calcium ions and there is probably a steady state between membrane and intracellular calcium. One theory is that a calcium influx during the spike is a trigger for release of internal calcium for contraction (Mayer et al., 1972). There is no T-tubule system in smooth muscle and the calcium spikes allow a more direct coupling than occurs in striated muscle. The importance of the sodium channels in smooth muscle varies and if there is synergism between sodium and calcium it is uncertain whether sodium is influencing calcium release or if there are parallel channels or both ions interact with a common carrier molecule. Repolarization may involve efflux of potassium ions but in some muscles calcium binding may bring about repolarization.

There is abundant evidence to suggest a close relationship between calcium and contractile coupling. Depending on the particular muscle the relative importance of extracellular membrane bound and intracellular calcium varies. The spike involves an influx of calcium and there is a more direct relation between action potential and contraction than in skeletal muscle. Spontaneous contractions and contractions in response to stretch in the myometrium are both abolished in calcium free mediums. Calcium may enter during the action potential, it may be released from cell membranes or it may be released from internal stores. Measurements of calcium influx and the use of agents blocking contraction provide evidence concerning the role of calcium in contraction.

The cellular calcium of smooth muscle tends to exchange quickly with the extracellular calcium in comparison to skeletal muscle, it appears that this is related to the importance of the sarcoplasmic reticulum i.e. the sarcoplasmic reticulum is of least importance in

smooth muscle (Lullman, 1970). It is thought that calcium which is bound into the endoplasmic reticulum does not exchange with extracellular calcium at least during the resting state. In smooth muscle the surface to volume ratio is much larger than in skeletal muscle and calcium penetration through the cell membrane or transfer of membrane-bound calcium towards the contractile proteins occurs rapidly enough to activate the actomyosin. Smooth muscle cells take up relatively large amounts of calcium compared to other muscular tissues when stimulated electrically, indicating the importance of calcium ions in excitation and contraction.

Relaxation of smooth muscle requires sequestering of calcium as is also the case in striated muscle. The microsomes of the rough SR of myometrial smooth muscle (Christensen, Caprilli and Lunal, 1969) or aorta (Allen and Daniel, 1970; Baudouin-Legros and Meyer, 1973) are capable of taking up calcium ions. Bound microsomal calcium can be displaced by other divalent cations. Mitochondria and microsomes from rat myometrium bind calcium in the presence of adenosine triphosphate (ATP) and the total amount capable of being bound can account for relaxation (Batra and Daniel, 1971). Calcium sequestering is correlated with membrane or intracellular storage sites of varying amounts in different smooth muscles (Prosser, 1974).

The relatively large and rapid movements of calcium ions in smooth muscle would indicate that changes in calcium metabolism could effect the contractility of the muscle and its functioning. The ability of the different sections on the rabbit oviduct to transport ova (Pauerstein et al., 1974) has been shown to have some correlation to calcium exchange in the rabbit oviduct (Hodgson and Daly, 1976).

The aims of the proposed study

The proposed study is an investigation into the process of egg transport in the mouse oviduct and oviduct function with a particular interest in the role of calcium ions in oviduct smooth muscle function. The initial hypothesis for the study was: "The hormone controlled alteration of oviduct musculature, which regulates the rate of egg transport, is mediated by changes in the concentration or distribution of calcium ions in the tissue".

To date egg transport has been studied mainly *in vivo*, involving immediate removal of the oviducts on killing and measuring mean percentage oviduct traversed by the eggs. This gives a critical index of egg movement allowing changes in egg position, either on different days of pregnancy or during drug treatment, to be determined. The disadvantages of this approach are that only one measurement can be taken from each oviduct and there is no control over the oviductal environment.

A substantial proportion of the work was devoted to the development and subsequent use of an *in vitro* technique for studying oviduct function. This *in vitro* method, comprising an air-tight optically transparent chamber, allowed for observation of oviduct motility and egg movement under controlled conditions. It was hoped that the oviducts could be maintained viable for periods up to 24 hours and by choosing the time when the eggs are in the isthmus i.e. Day 2 to Day 3 of pregnancy the period of greatest egg movement, *in vivo*, could be investigated.

Once the operating conditions for the *in vitro* method were established, then the environmental conditions could be regulated and varied to observe effects on oviductal function. The parameter

of most interest was calcium ion levels in the perfusion medium and their importance. Apart from calcium ions many other substances which may play a role in the control of egg transport were studied i.e. biogenic amines, ovarian hormones, prostaglandins, prolactin and thyrotroptin. In addition compounds which may also effect smooth muscle function were studied i.e. lanthanum ions, prostaglandin endoperoxides and a calcium ionophore, X537A.

Another approach in this study was measurement of *in vivo* oviduct calcium levels, using atomic absorption spectroscopy techniques. Calcium levels during early pregnancy were investigated and during various drug treatments which altered the normal patterns of egg transport. Changes occuring in oviduct calcium levels during the *in vitro* perfusion were also studied.

Additional experiments were performed to check the importance of ovarian hormone secretion during egg transport and the role of the uterus in the function of the oviduct.

Part 2

Experimental Methods

Methods

1. Animal Husbandry

Albino mice of the RGIT/SLAC strain were used in all the experiments. The animals were housed under constant conditions of light: 12 hours light 08:00 h - 20:00 h, 12 hours dark 20:00 h - 08:00 h, intensity : 30 lumens, temperature : 21 $\pm$ 1^{o}C, humidity : 50% + RH and diet : Oxoid Pasturised Breeding diet for rats and mice, water *ad lib*.

Mature virgin females were used between 5 and 12 weeks of age and mature males between 6 and 14 weeks of age. For mating purposes two females were caged with one male. The females were examined each morning and the presence of a vaginal plug taken as evidence of successful copulation. The day on which a vaginal plug was found was designated Day 1 of pregnancy. The mated females were replaced daily with fresh females to maintain the two female to one male ratio. At the end of each week all the females were removed and replaced with a new batch of virgin females.

2. Measurement of the rate of egg transport in vivo

The rate of egg transport *in vivo* was determined by measuring the position of eggs in the reproductive tract, at autopsy, on defined days of pregnancy. The position of the eggs was expressed as the mean percentage of the oviduct traversed.

The mice were killed by cervical dislocation and the ovaries, oviducts and uteri exposed by a mid-ventral abdominal incision. To prevent loss of intra-uterine fluid a small artery clamp was placed below the UTJ and the uterine contents gently moved away from the area of the clamp with fine, curved, iris forceps. The

oviduct was freed from the uterus by cutting the uterus 1-2 mm from its tip and the oviduct could then be manipulated by the attached section of uterus, thus preventing displacement of any contained eggs and damage to the intramural region of the oviduct.

Straight iris forceps were used to hold the uterine attachment and fine, pointed iridectomy scissors to remove and straighten the oviducts. The ovarian ligaments were cut first and the oviduct transferred onto fine filter paper under a stereo dissection microscope (Beck Binomax 15329, magnification approximately x10) and illuminated by an angle-poise lamp, situated at a distance to prevent subjecting the oviduct to excessive heat. The mesosalpinx was then carefully sectioned away, starting at the uterine end and following a parallel line to the oviduct, gradually straightening out the oviduct. The straightened oviduct was then placed on a microscope slide and a heavy coverslip placed over the tissue, normal saline (0.9% $^{w}/_{v}$ sodium chloride solution) at room temperature was injected under the coverslip using a Pasteur pipette.

The microscope slide was placed on a microscope (Vickers Instruments, magnification x50) with a mechanical stage and vernier scale. The exact position of the ovarian end, the uterine end and each egg was then read off the vernier scale and the developmental stage of the eggs noted. Mounting the oviducts under the heavy coverslip reduced any peristaltic movements of the oviduct thus preventing changes in egg position. The mean percentage oviduct traversed by the eggs was then calculated.

To locate eggs in the uterus, the already clamped uterine horn was freed by holding the horn, with straight iris forceps, immediately above the cervix and sectioning the mesometrium. Intra-uterine fluid was drained into the well of a cavity slide by relaxing the hold of the forceps. A blunted No. 20 hypodermic needle was inserted into the tubal end of the uterine lumen, clipped into place and 0.2 ml normal saline flushed through the lumen into a second cavity slide. Finally, reverse flushing was carried out via the cervical end. The intra-uterine fluid and washings were examined under the microscope (magnification x50) and the eggs present counted and their developmental stage noted.

This procedure was carried out on both sides of the reproductive tract.

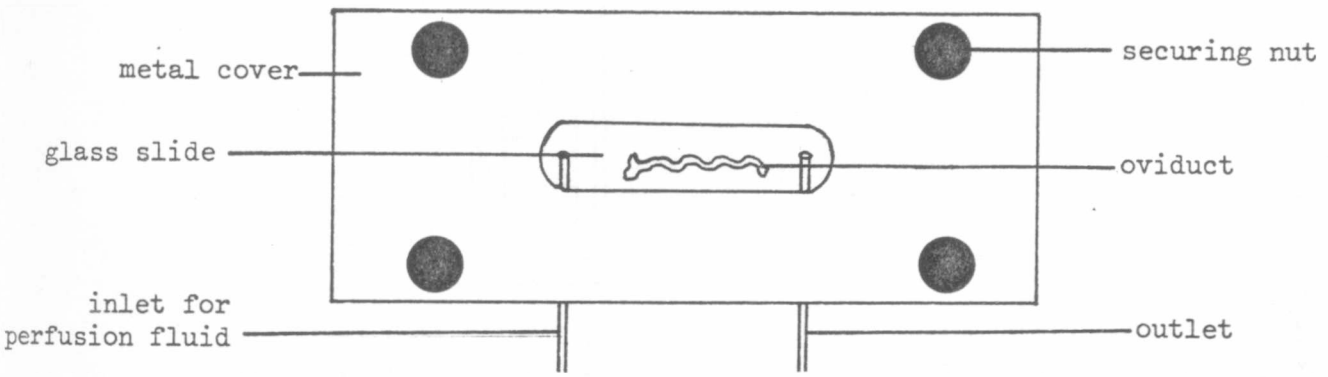
3. In vitro mouse oviduct preparation

In order to keep oviducts viable, in vitro, for periods up to 24 h, an incubation chamber was constructed (Fig. 1). The chamber was designed such that it allowed: adequate circulation of physiological fluid and oxygen; temperature regulation and constancy; and microscopic examination of the oviduct to locate egg position and observe muscle motility.

The air tight, optically transparent chamber consisted of a perspex base (9 x 4 x 0.5 cm) through which two channels, 3 cm apart, were drilled, opening up in the middle of the upper surface. Four screws were drilled through the base, one at each corner. The base was then covered with a sheet of thin rubber (7 x 2.5 x 0.1 cm) from which a section (3.5 x 1 cm) had been cut from the centre. A dissected, straightened oviduct could then be placed between the two channels in the perspex base.

Fig. 1 Diagram of chambers used for perfusion of the oviduct in vitro

A Plan view



B Horizontal view

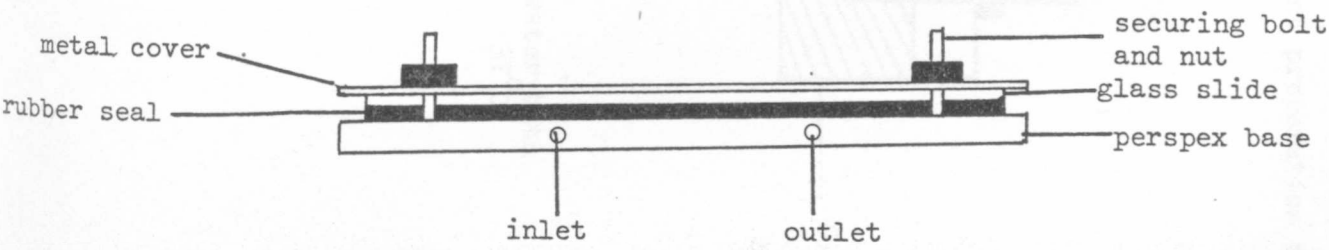
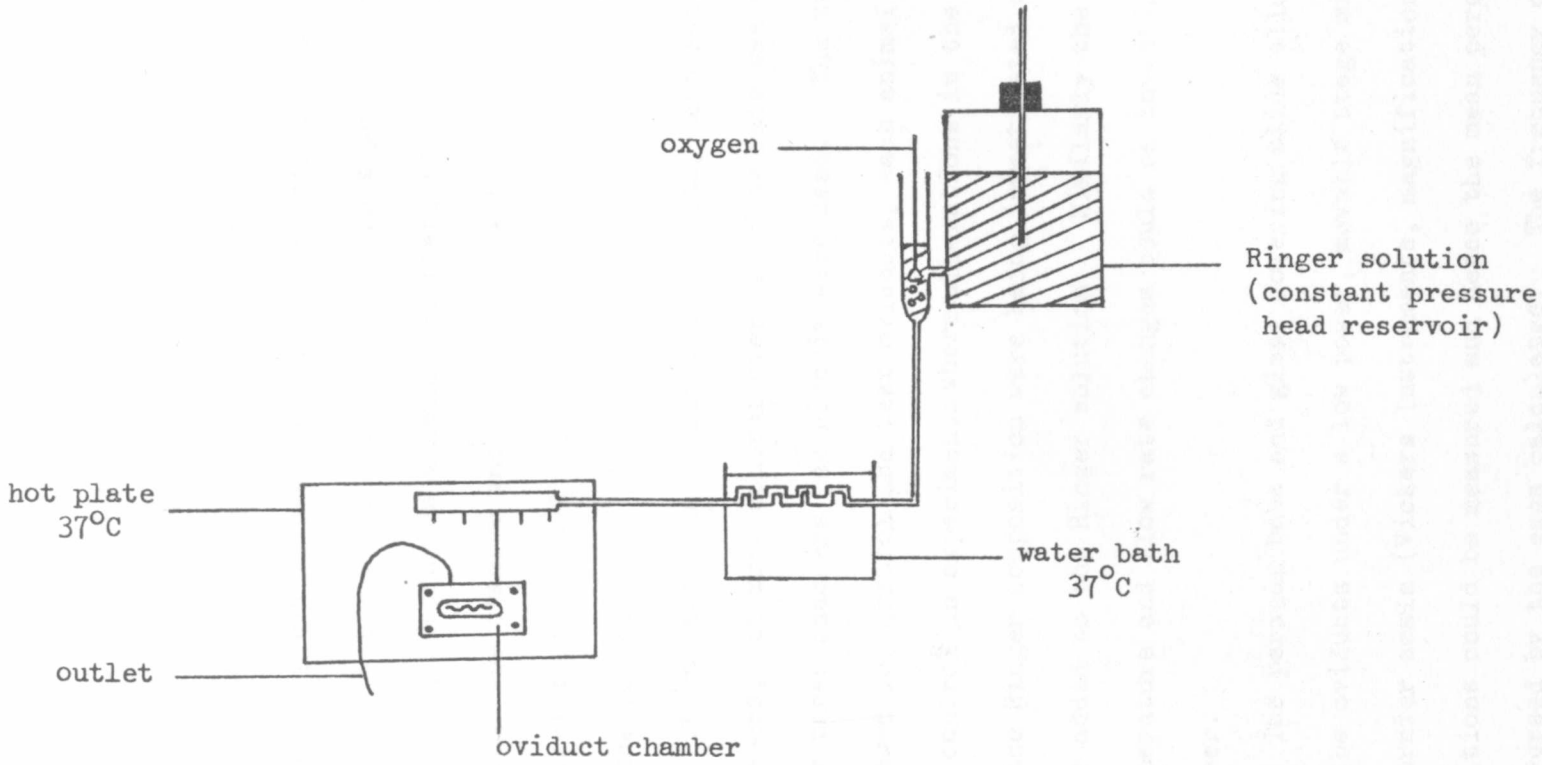


Fig 2. Diagram of apparatus for the perfused mouse oviduct preparation in vitro



A glass microscope slide was then placed over the oviduct and an air tight seal between the slide and the rubber was formed using Silicone high vacuum grease (Edwards, USA). Finally the chamber was secured with a metal cover screwed down to the base. The two channels in the base formed an inlet and outlet for the physiological solution.

Oxygenated Ringer solution of the following composition (mM): NaCl 153.8, KCl 5.6, $CaCl_2$ 4.3, $NaHCO_3$ 5.81, glucose 5; from a constant pressure head reservoir was passed through a heated water bath (37°C) and heating coil and supplied five oviduct chambers, resting on a metal hot plate (37°C). The flow rate through the chamber was controlled at the inlets, the physiological fluid flowed through each chamber and was drained away at the outlet (Fig. 2).

It was possible to run two circuits, each supplying five chambers, but more control over the flow rate was obtained when only three chambers per circuit were used. The two circuits allowed for control and test oviducts, each animal serving as its own control in experiments where alterations in the calcium content of the Ringer composition were being investigated or when drugs were added to the Ringer solution. Similarly the effects of temperature and flow rate changes could be investigated in this manner.

The perspex base and glass covering slide allowed observation of the oviducts under a low power, movable stage microscope with a vernier scale (Vickers Instruments, magnification x50). Egg positions could be measured and hence the mean percentage oviduct traversed by the eggs calculated. The frequency of contraction of the oviduct musculature was counted per minute and the oviductal

muscle motility assessed on a 0-4 scale: (0 - no movement, 1 - very weak, 2 - moderate, 3-strong, 4 - very strong).

4. Ovariectomy

Mice were ovariectomized on Day 1 of pregnancy. They were anaesthetised with pentobarbitone sodium (Sagattal, May and Baker, Dagenham) 10 mls/kg body weight, intraperitoneally. The anaesthetised mice were kept warm until they fully recovered from the operation.

All instruments and suture materials were sterilized by boiling for at least 15 minutes. The surface of the operating table was swabbed down with 5% Cetavlon, in 70% ethanol before use. The animals' fur was shaved on either side, below the rib cage and the skin swabbed with Cetavlon solution. The ovaries were exposed through small lateral incisions and the ovarian bursa ruptured, the ovary was removed by cutting its attached ligaments. The peritoneium was closed with continuous cotton sutures, using a small (No. 11) round bodied curved suture needle and the skin closed with individual cotton sutures. All the instruments and suture cotton were kept in the swabbing solution during the operation.

Sham operated controls were performed by exposing the ovaries in the above manner and rupturing the ovarian bursa then closing up the incision.

5. Analysis of oviduct calcium levels, by atomic absorption spectroscopy

Female mice were killed by cervical dislocation during early pregnancy. The oviducts were immediately removed and cleared of any fat under a dissecting microscope (Beck Binomax 15329), the mesosalpinx was left intact. Each pair of oviducts was weighed in preweighed silica crucibles and ashed overnight at 450°C in a muffler oven.

After ashing the crucibles were allowed to cool, then the tissue ash was dampened with a drop of distilled water from a Pasteur pipette. The ash was dissolved in 1 ml of 50% hydrochloric acid (Analar, B.D.H. Chemicals Ltd, Poole) and evaporated to dryness on a hot plate, in a fume cupboard. The ash was redissolved and evaporated in a further 1 ml of 50% hydrochloric acid and finally dissolved in 4 ml of 2.5% hydrochloric acid and left for 30 min covered by a watch glass, on the cooling hot plate.

All the samples, standards and blank had to contain 0.05%, calcium free, lanthanum chloride (Koch-Light Laboratories Ltd, Bucks) which prevents anionic interference of calcium atomization. The final hydrochloric acid concentration was 1% in all the solutions. The blank, standards and samples were of the following compositions:

Blank: 2.5 ml 5% $LaCl_3$, 100 ml 2.5% HCl, distilled water to 250 ml

Standards: 0.5 ml 5% $LaCl_3$, 20 ml 2.5% HCl, calcium chloride, distilled water to 50 ml

Samples: 0.1 ml 5% $LaCl_3$, 4 ml 2.5% HCl (containing digested ash), distilled water to 10 ml

Calcium ion concentration was measured using an Instrumentation Laboratories 1L151 Atomic Absorption/Atomic Emission Spectrophotometer under the following conditions: Air/acetylene fuel; stochiometric flame; wave length 322.7 nm; slit width 320 μm; hollow cathode lamp; current 5mA.

Absorbance was read out on a digital display, with integration facilities for periods of $^1/_{16}$, $^1/_4$, 1, 4 and 16 seconds, integration was automatic and normally a 4 second period was used.

The instrument was zeroed automatically, using the blank solution, between every standard and sample solution. At least

five readings were taken for every solution.

A standard curve equation for calcium absorbance against calcium concentration was obtained using an analysis of linear regression programme and the readings for each sample were fed into the programme to obtain their calcium concentration. The results were then converted to ug/mg wet weight of tissue.

Sensitivity of assay (detection limits): 0.05 ug/ml. Replication: inter assay variation < 15%, intra assay variation < 1%.

6. Drugs used

Adrenaline acid tartrate (BDH) Isoprenaline sulphate (BDH)	Stock solution stored in 0.1NHCl, in refrigerator. Ascorbic acid, 20 ug/ml added to dilutions.
Endoperoxide Analogue U-46619 (Upjohn)	
Oestradiol benzoate (BDH) Progesterone (BDH) Pregnenolene (BDH)	Stock solution dissolved in absolute ethanol. Dilutions made up with either 0.9% saline (for injections) or Ringer solution (for <u>in vitro</u> oviduct perfusions).
Prostaglandins PGE and $PGF_{2\alpha}$ (Upjohn)	
Reserpine (Courtin and Warner)	Stock solution prepared by dissolving 320 mg reserpine and 375 mg citric acid in 6 ml benzyl alcohol, using very gentle heat. A volume of 15 ml "Tween 80" was added and made up to 100 mls with distilled water. The stock solution was protected from light by storing in a dark bottle

in a cupboard. The stock was diluted when needed, immediately before use, with distilled water.

Reserpine Vehicle

A solution exactly similar to the reserpine stock solution, minus the reserpine, was prepared for use as a vehicle control.

X537A (Roche)

Part 3

Experimemtal Results

Chapter 1: An in vitro mouse oviduct preparation allowing observation of oviduct muscle motility and egg position

Introduction

In order to study the possible factors involved in the control of egg transport a new *in vitro* technique was developed allowing the oviduct to be observed over a period of time, in a controlled environment. The objective was to monitor changes in oviduct motility and measure any resulting egg transport during a specified period of early pregnancy. Most work to date on the study of egg transport has been performed *in vivo*, involving the removal of oviducts, at autopsy, and determining the egg positions in order to follow egg movement on different days of pregnancy or during drug treatment. The disadvantages of *in vivo* work are that only one measurement can be obtained from each oviduct and there is no control over the oviductal environment.

Kendle (1969) incubated mouse oviducts, at 37°C, for 48 hour periods and measured egg position after incubation, but found that egg transport had not occurred. Lack of egg transport was not however thought to be due to failure of the oviduct to survive *in vitro* as other workers had found segmental movement occurring after four days *in vitro* (Biggers et al., 1962). Unlike the method of Kendle (1969) this *in vitro* technique allows for continuous observation of the oviducts and their contained eggs.

The initial experiments were performed to determine the optimum operating conditions for this preparation and involved varying the flow rate of the tissue solution and the chamber temperature. Once these parameters could be established then a more detailed investigation into this preparation could be undertaken. Day 2 to Day 3 of pregnancy

in the mouse oviduct was chosen as the period of investigation, as *in vivo* this is the time of greatest egg movement, along the isthmus from the AIJ to the UTJ.

Methods

The oviducts of the mice were removed on the morning of the second day of pregnancy and quickly straightened by dissection of the mesosalpinx, as described in the methods section. Each oviduct was placed in a chamber through which oxygenated Ringer solution was perfused at the rate of 1 ml per minute. The water bath, through which the Ringer solution was passed before entering the chamber and the hot plate on which the chambers rested, were both at 37°C and this maintained a temperature of 33°C inside the chamber.

The oviducts were observed under the microscope at regular intervals over a period of about 20 hours. During the first hour observations were usually approximately every 15 minutes, then afterwards every hour until 6 hours, then at 8-9 hours and finally the next morning at 20 hours. At each observation, frequency of contraction of the oviduct musculature was counted and expressed as contractions per minute. The strength of oviduct motility was assessed on the 0-4 scale. Finally the positions of the contained eggs were measured in order to calculate the mean percentage oviduct traversed by the eggs.

To determine the importance of the flow rate, slow and fast flow rate experiments were carried out. As the flow rate was difficult to keep constant over the 20 hour period a slow flow rate of 0.2-0.6 ml and a fast flow rate of 0.7-1.4 ml were chosen to allow for variations occurring.

The next parameter studied was temperature, the effect of a 2°C rise and a 2°C fall in chamber temperature. Chamber temperature was altered via

changes in the bath temperature and hot plate temperature. The flow rate was maintained between 0.5 and 1.0 ml per minute.

The results from each group of oviducts subject to the same conditions were compared at 1 hour and 20 hours. The effects of different flow rates and temperatures were compared at 1 hour and 20 hours. Statistical analysis was performed using a Students t test on mean percentage oviduct traversed by the eggs and frequency of contraction to test for any significant differences. Strength of motility assessments give rise to non-parametric data and an analysis of variance was performed to yield the F ratio, which showed any differences in variance between the groups. In all these experiments n is the number of oviducts.

Results

Microscopic examination of the oviducts *in vitro* revealed that the oviduct remained viable over the 20 hour period. Rhythmic contractions of the oviduct musculature were observed and the eggs could be clearly seen in the lumen of the isthmus, moving backwards and forwards as the oviduct contracted and relaxed. Initially the contractions were small and the frequency low but by 15 minutes the musculature was contracting vigorously and remained so for the first 6 to 8 hours. After this time there was a gradual weakening of the strength of contraction and the frequency of contraction began to decrease. By 20 hours the frequency of contraction had significantly decreased from 13.33 $\pm$ 0.66 to 1.83 $\pm$ 0.048 contractions per minute ($P < 0.001$) and the strength of motility dropped from 4 to 1, with no significant difference in variance (Table 1). Although the eggs were continually moving during the 20 hour period they made no significant progression along the isthmus (Table 1).

The effect of variations in the flow rate of the perfusion fluid

Tables 2 and 3 show the results from the slow and fast flow rate groups respectively. As with the initial experimental group (Table 1) frequency of contraction decreases significantly over the 20 hour period and strength of motility falls dramatically, with no significant change in the variance. Egg position again does not vary significantly in either group during perfusion. The two groups were compared at 1 hour and 20 hours and no significant differences were found between any of the parameters investigated (Tables 4 and 5).

As variations in flow rate did not appear to effect the *in vitro* preparation it was decided to combine the results from the two groups (Table 6) to serve as control data for experiments performed at 33°C. This was done to compensate for any changes in flow rate that might occur over the 20 hour period when running two perfusion circuits, with three oviducts per circuit, especially overnight when the oviducts were not observed. Prior to this only one oviduct per circuit had been used and control over flow rate was better.

In the flow rate experiments the discrepancy in n between 1 hour and 20 hours occurred through failures in the experimental technique during the running of the experiment. When combing the data only the oviducts which survived the full 20 hours were used.

The effect of variations in chamber temperature

A 2°C increase and decrease in chamber temperature to 35°C and 31°C respectively, over a 20 hour period produced similar results to the control groups in that strength of motility and frequency of contraction decreased significantly and no significant changes occurred in egg position (Table 7 and 10). Comparing increased temperature (35°C) to the control date (33°C) no significant differences were observed at 1 hour but at 20 hours the frequency of contraction is

much lower in the high temperature group ($P < 0.001$) and the strength of motility is also weaker (Tables 8 and 9). In the low temperature group (31°C) no significant differences occurred from the controls although strength of motility and frequency of contraction appeared not to have decreased to the same extent (Tables 11 and 12).

Discussion

The *in vitro* oviduct preparation was capable of maintaining oviduct muscle motility, at least over a 20 hour period. The design of the chamber made possible a critical assessment of the functional state of the oviduct. At the time of observation eggs were present in the lumen of the isthmus, the lumen being distended in the regions containing eggs. The observed oviductal contractions were capable of moving the eggs backwards and forwards, usually within the one loop of the isthmus but sometimes into the next loop and then back again. The contractions, especially initially were very vigorous and appeared capable of propelling the eggs a considerable distance but no overall forward progression was made.

Egg transport through the isthmus is considered to be brought about by contractions of the oviduct musculature (Blandau et al., 1975) but in the case of this *in vitro* preparation the dynamics of the muscular contractions must vary from those *in vivo*, since transport is absent. This suggests, perhaps that certain vital controlling factors for oviductal muscular contractions are missing *in vitro* or alternatively that the environmental conditions *in vitro* are too far removed from *in vivo* for the oviduct to function normally.

Considering the environmental conditions, the most obvious ones were flow rate of the perfusion fluid and temperature. The perfusion fluid, oxygenated Ringer solution, should be providing the tissue with

an adequate supply of oxygen, and glucose for its energy requirement and should maintain the oviduct in an ionically correct state. The experiments investigating the effects of changes in flow rate showed that the frequency of contraction and strength of motility of the oviduct did not change with flow rate. This would suggest that the energy requirements of the tissue are being met and that the variations performed were not critical to the survival of the tissue.

In these experiments, however, the concentrations of the various ionic components of the Ringer solution are always constant. They are obviously allowing muscle contraction and relaxation to occur but this is not necessarily of the same pattern as *in vivo*. It is possible that membrane conductance is being changed and hence the pattern of ionic movements which govern the intrinsic myogenic rhythm, if the correct ionic gradients are not being established. This would apply particularly to calcium ions which are intimately involved in both the electrical and mechanical events of smooth muscle contractions and possibly also to sodium and potassium ions.

Changes in the environmental temperature were without affect on the oviduct muscle function. The most noticeable effect was that a $2^{o}C$ increase in temperature tended to impair muscle function, which is probably a direct action on the muscle cells themselves, as temperature changes can be shown to effect both smooth and skeletal muscle.

The oviductal environment must be further investigated to elucidate the controlling factors for egg movement along the oviduct. *In vitro* the oviduct is removed from its normal hormonal background and its autonomic nervous control or modulation. It is possible that the ovarian hormones play a role in controlling oviduct function, although from experiments in ovariectomised animals (Alden, 1942b;

Noyes, 1959; Noyes et al., 1959) the secretory levels of the ovarian hormones after ovulation do not appear important in the control of egg transport. Pre-ovulatory hormone secretion is a more likely candidate for influencing oviduct function, possibly through protein synthesis, but the hormonal background during egg transport must not be ruled out.

The undoubted importance of the oviduct smooth muscle function for egg transport makes it necessary to consider the physiology of smooth muscle in general. As already stressed both the electrical and mechanical events occurring have an absoluted requirement for calcium ions. This being the case, it was decided to further investigate the role of calcium ions both *in vivo* and *in vitro*. It was therefore decided to look *in vivo* at calcium levels and any changes occurring in them *in vivo* and the influence of changing calcium levels in the perfusion fluid of the *in vitro* preparation.

Table 1 - Mouse oviduct perfusion preparation, in vitro, initial experiments, comparison between 1 and 20 hour readings

Temp. 33°C Flow Rate 1 ml/min	1 Hour	20 Hours
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	32 ± 4.47 (6)	32.03 ± 5.14 (6)
strength of motility	4 ± 00 (6)	1 ± 0 (6)
frequency of contraction	13.33 ± 0.66 (6)	***1.83 ± 0.48 (6)

Tables 1-12 * $P < 0.05$

*** $P < 0.001$

n number of oviducts

Table 2 - Effects of a slow flow rate (0.2-0.6 ml/min) comparison of 1 and 20 hour readings on the oviduct perfusion preparation in vitro

Temp. 33°C Flow Rate 0.2-0.6 ml/min	1 Hour	20 Hours
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	43.94 ± 6.49 (9)	38.96 ± 7.06 (7)
strength of motility	3.67 ± 0.17 (9)	2.29 ± 0.36 (7)
frequency of contraction	13.11 ± 1.16 (9)	***6.43 ± 0.68 (7)

Table 3 - Effects of a fast flow rate (0.7-1.4 ml/min) 1 and 20 hour readings on the oviduct perfusion preparation in vitro

Temp. 33°C Flow Rate 0.7-1.4 ml/min	1 Hour Mean ± S.E. (n)	20 Hours Mean ± S.E. (n)
% oviduct traversed	44.93 ± 5.05 (10)	49.18 ± 8.63 (6)
strength of motility	3.6 ± 0.16 (10)	1.5 ± 0.34 (6)
frequency of contraction	11.6 ± 1.19 (10)	***6.66 ± 0.8 (6)

Table 4 - 1 hour comparison of slow and fast flow rate on the oviduct perfusion preparation in vitro

Temp. 33°C 1 Hour Readings	Flow rate 0.2-0.6 ml/min	Flow rate 0.7-1.4 ml/min
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	43.94 ± 6.49 (9)	44.93 ± 5.05 (10)
strength of motility	3.56 ± 0.18 (9)	3.6 ± 0.16 (10)
frequency of contractions	13.11 ± 3.48 (9)	11.6 ± 3.75 (9)

Table 5 - 20 hour comparison of slow and fast flow rate on the oviduct perfusion preparation in vitro

Temp. 33°C 20 Hour Readings	Flow rate 0.2-0.6 ml/min	Flow rate 0.7-1.4 ml/min
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	38.96 ± 7.06 (7)	49.18 ± 8.63 (6)
strength of motility	2.43 ± 0.37 (7)	1.5 ± 0.34 (6)
frequency of contraction	6.43 ± 0.68 (7)	6.66 ± 0.8 (6)

Table 6 - Combined flow rates 1 and 20 hour comparisons on the oviduct perfusion preparation in vitro

Temp. 33°C Flow Rate 0.2-1.4 ml/min	1 Hour Mean ± S.E. (n)	20 Hours Mean ± S.E. (n)
% oviduct traversed	46.08 ± 5.25 (13)	45.68 ± 5.69 (13)
strength of motility	3.7 ± 0.13 (13)	1.7 ± 0.24 (13)
frequency of contraction	12.92 ± 0.98 (13)	***7.46 ± 1.21 (13)

Table 7 - Effect of an increased temperature on the oviduct perfusion preparation in vitro

Temp. 35°C Flow Rate 0.5-1.0 ml/min	1 Hour	20 Hours
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	49.18 ± 8.63 (8)	47.69 ± 8.1 (8)
strength of motility	4 ± 0 (8)	0.38 ± 0.26 (8)
frequency of contraction	11.75 ± 0.8 (8)	***1.25 ± 0.84 (8)

Table 8 - Comparison of increased temperature to controls at 1 hour on the oviduct perfusion preparation in vitro

1 Hour Readings Flow Rate 0.5-1.0 ml/min	Controls (Temp. 33°C) Mean ± S.E. (n)	Temp. 35°C Mean ± S.E. (n)
% oviduct traversed	46.08 ± 5.25 (13)	49.18 ± 8.63 (8)
strength of motility	3.7 ± 0.13 (13)	4 ± 0 (8)
frequency of contraction	12.92 ± 0.98 (13)	11.75 ± 0.8 (8)

Table 9 - Comparison of increased temperatures to controls, 20 hours, on the oviduct perfusion preparation in vitro

20 Hour Readings	Controls	Temp. 35°C
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	45.68 ± 5.69 (13)	47.69 ± 8.1 (8)
strength of motility	1.7 ± 0.24 (13)	0.38 ± 0.26 (8)
frequency of contraction	7.46 ± 1.21 (13)	***1.25 ± 0.84 (8)

Table 10 - Effect of a decreased temperature on the oviduct perfusion preparation in vitro

Temp. 31°C Flow Rate 0.5-1.0 ml/min	1 Hour	20 Hours
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	51.92 ± 6.0 (10)	53.03 ± 5.35 (10)
strength of motility	3.9 ± 0.1 (10)	2.3 ± 0.15 (10)
frequency of contraction	11.4 ± 0.95 (10)	*8.6 ± 0.85 (10)

Table 11 - Comparison of decreased temperature to controls, 1 hour, on the oviduct perfusion preparation in vitro

1 Hour Readings	Controls (Temp. 38°C)	Temp. 31°C
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	46.08 ± 5.25 (13)	51.92 ± 6.0 (10)
strength of motility	3.7 ± 0.13 (13)	3.9 ± 0.1 (10)
frequency of contraction	12.92 ± 0.96 (13)	11.4 ± 0.95 (10)

Table 12 - Comparison of decreased temperature to controls, 20 hours, on the oviduct perfusion preparation in vitro

20 Hour Readings	Controls	Temp. 31°C
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	45.68 ± 5.69 (13)	53.03 ± 5.35 (10)
strength of motility	1.7 ± 0.24 (13)	2.3 ± 0.15 (10)
frequency of contraction	7.46 ± 1.21 (13)	8.6 ± 0.85 (10)

Chapter 2: The effect of varying the calcium content of the perfusion fluid on the oviduct preparation in vitro

Introduction

Intracellular free calcium is the mediator of contraction and relaxation of smooth muscle cells to electrical, mechanical or chemical stimuli. Exchange between cellular and extracellular calcium is rapid, smooth muscle cells are capable of taking up relatively large amounts of calcium compared with other muscular tissues (Lullman, 1970). The calcium content of smooth muscle is known to be dependent on extracellular calcium levels (Schatzmann, 1961) therefore it seemed possible that changes in the calcium content of perfusion fluid could effect oviduct muscle motility and contraction and hence egg transport. Modification of external calcium levels could indicate whether the oviduct is susceptible to changes in its local environment.

Methods

The oviducts of the mice were removed on the second day of pregnancy and set up in the chambers as described previously. Two perfusion circuits were utilized with up to three oviducts perfused from each circuit, one circuit contained normal Ringer solution and the second circuit contained the Ringer solution with a modified calcium content. The experiment was designed such that each animal could serve as its own control, one oviduct going to each circuit. The oviducts were observed over a period of up to 22 hours throughout which time egg position and frequency of contraction was measured and the strength of motility assessed.

The calcium content of normal Ringer solution (4.3 mM) was varied in the modified solutions to 0% (0 mM), 25% (1.075 mM), 50% (2.15 mM),

200% (8.6 mM), 300% (12.9 mM) and 400% (17.2 mM) of the normal concentration.

In a further experiment lanthanum ions (1.5 μM) were added to the perfusion fluid in the presence of normal concentrations of calcium ions (4.3 mM).

Results

In all the experiments with variable calcium concentrations no overall egg transport occurred *in vitro*.

To compare the effect of changing the calcium concentration of the Ringer solution on oviduct muscle function, frequency of contraction was chosen as the parameter most suitable for statistical analysis. As each animal was serving as its own control a paired t-test was used for statistical analysis.

The effect of zero calcium levels in the perfusion fluid

When no calcium was present in the perfusion fluid the frequency of contraction of the oviducts quickly decreased and was significantly lower after one hour ($P < 0.05$). The decrease in the rate of contraction continued and by 4 hours muscle movement had almost stopped altogether (Table 13; Fig. 3).

The effect of reducing the calcium concentration of the perfusion fluid to 25% (1.075 mM)

In 25% of the normal calcium concentration the oviducts survived much longer than when no calcium was present at all, although the rate of contraction was significantly lower from 1 hour onwards. By 6-8 hours the rate of contraction was half that of the controls and all movement had ceased before the end of the 18-22 hour period (Table 14; Fig. 4).

The effect of reducing the calcium concentration of the perfusion fluid to 50% (2.15 mM)

The effect of 50% calcium was not as dramatic as 0 or 25%, no significant decrease in contraction rate occurred until 4 hours ($P < 0.05$) although the rate of contraction was always lower from the beginning. Motility was maintained over the 18-22 hour period but by the end of the experiment muscle activity was very weak (Table 15; Fig. 5).

The effect of increasing the calcium concentration of the perfusion fluid to 200% (8.6 mM)

During the first 2 hours, 200% calcium appeared to increase the frequency of contraction of the oviduct, though this was not significant. Throughout the 18-22 hours the 200% calcium group showed a tendency to a higher frequency of contraction (Table 16; Fig. 6) but this was never significant.

The effect of increasing the calcium concentration of the perfusion fluid to 300% (12.9 mM)

No significant differences were seen between the controls and the 300% groups throughout the experiment but the 300% group consistently showed a slightly lower frequency of contraction (Table 17; Fig. 7).

The effect of increasing the calcium concentration of the perfusion fluid to 400% (17.2 mM)

Over the first 4 hours 400% calcium significantly reduced the frequency of contraction ($P < 0.05$) and this decreased motility was evident for the rest of the 18-22 hour period, though not significant (Table 18; Fig. 8).

The effect of adding lanthanum ions (1.5 μM) to the perfusion fluid

Lanthanum ions in the presence of normal concentrations of calcium had a dramatic effect on the frequency of oviductal contractions. Within 15 minutes the contraction rate was half that of the controls

and after 1 hour there was a significant difference ($P < 0.05$). The contraction rate continued to decrease over the next hour and all motility had ceased by 2 hours (Table 19; Fig. 9).

Discussion

Variation of the concentration of calcium ions in the perfusion fluid produced changes in oviduct muscle motility, the frequency of contraction being the most easily observable feature to be affected. Both low calcium levels and high calcium levels reduced the frequency of contractions, although zero and very low levels (25%) also reduced the length of time oviduct motility was retained.

These results show the ability of the external calcium levels to influence muscle contraction, the oviduct must be capable of utilizing the calcium ions in the perfusion fluid. Smooth muscle membranes are depolarized by increasing external calcium levels and polarized when external calcium is decreased (Kuriyama, 1971; Liu, Prosser and Job, 1969).

Low external calcium levels will have their most direct effect on the extracellular calcium compartment, which is the fastest exchangeable compartment (Lullman, 1970). Extracellular levels would change through diffusion gradients, calcium being lost to the perfusion fluid. Low extracellular calcium levels effect both the electrical and mechanical events associated with contraction, membrane conductance would be decreased and there would be a loss of the ability of spiking. A decrease in extracellular levels would also lead to changes in internal calcium levels, calcium bound to cell and vesicle membranes is exchangeable as is a proportion of intracellular calcium. All smooth muscles require calcium for contraction, but the requirements vary with the muscle reflecting differences in calcium stores (Potter and Sparrow,

1968; Potter et al., 1970). In some cases calcium for contraction enters during the action potential or in others it may be released from cell membranes or from internal stores. If calcium influx is reduced then excitation-contraction coupling will also be impaired. In zero calcium contractile activity ceases within 4 hours, the oviduct must therefore initially utilize its own calcium stores and once these are exhausted then the muscle ceases to contract.

High external calcium levels tend to increase membrane conductance and accordingly spike height and the rate of rise of spikes (Brading, Bulbring and Tomita, 1969). The muscle cells behave as though they were hyperpolarized. The 200% calcium perfusion fluid shows an increased frequency of contraction whilst the 300 and 400% calcium perfusion fluid have decreased frequencies. This probably reflects that within a finite range increases in calcium improve the performance of smooth muscle but beyond a certain point hyperpolarization is occurring and the muscle cells are losing their ability for rapid contractions and relaxations.

Lanthanum ions block calcium uptake and therefore inhibit contraction, they bind to extracellular calcium binding sites, thus preventing calcium entry (Mayer, van Breeman and Casteels, 1972). This was demonstrated in vitro, lanthanum's effect on contraction rate was immediate and all activity soon ceased thus demonstrating the necessity of calcium ion movements for oviductal muscle contraction.

External calcium levels therefore are capable of modifying the contractile pattern of the oviductal musculature. This suggests that in vivo, where presumably calcium levels are under some controlling factors, the maintenance of the various calcium stores can govern the pattern of contractile activity. The controlling factors for in vivo levels are however unclear. As mentioned earlier, ovarian hormones

are known to affect calcium transport in myometrial mitochondria (Batra and Bengtsson, 1972; Batra, 1973), suggesting their ability to alter calcium metabolism. It is highly likely that ovarian hormones have similar effects on the oviduct especially considering the findings of Hodgson and Daly (1976) who found $^{45}Ca^{2+}$ uptake to be influenced by oestrogen and progesterone in the rabbit oviduct. If oviduct calcium levels are under hormonal influence this is probably through induction of protein synthesis and consequently changes in calcium binding, exchange and permeability. Changes of this nature would tend to have a time delay rather than be an immediate response to a change in oestrogen and/or progesterone levels again favouring the importance of preovulatory ovarian secretion rather than postovulatory.

If postovulatory ovarian hormone secretion is not vital for egg transport the lack of forward egg movement *in vitro* is unexpected. The role of calcium ions must be further investigated, there is an obvious need for calcium exchange and the oviduct has been shown to be sensitive to changing levels of external calcium. Further investigation of oviduct calcium levels *in vivo*, is necessary to determine whether they are constant or if they vary during egg transport.

Table 13 The effect of reducing the calcium concentration of the perfusion fluid to 0% (0mM) on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min mean ± S.E.	
	Controls	0% calcium (n)
1	12.33 ± 1.67	6 ± 1.93 (6)
2	11.67 ± 0.95	*4.17 ± 1.56 (6)
4	11 ± 1.69	***1 ± 1.41 (6)

Table 14 The effect of reducing the calcium concentration of the perfusion fluid to 25% (1.075mM) on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min mean ± S.E.	
	Controls	25% calcium (n)
1	11.33 ± 0.99	*9 ± 0.68 (6)
2	12.33 ± 0.8	*8.33 ± 0.95 (6)
4	14 ± 2.13	**8.67 ± 1.69 (6)
6-8	13 ± 1.24	**7.67 ± 1.2 (6)
18-22	5 ± 0.97	***0 ± 0 (6)

Tables 13 - 19 comparison of results by paired t test

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

n number of oviducts per group

Fig 3 The effect of reducing the calcium concentration of the perfusion fluid to 0% (0mM) on the frequency of contraction of the oviduct preparation in vitro

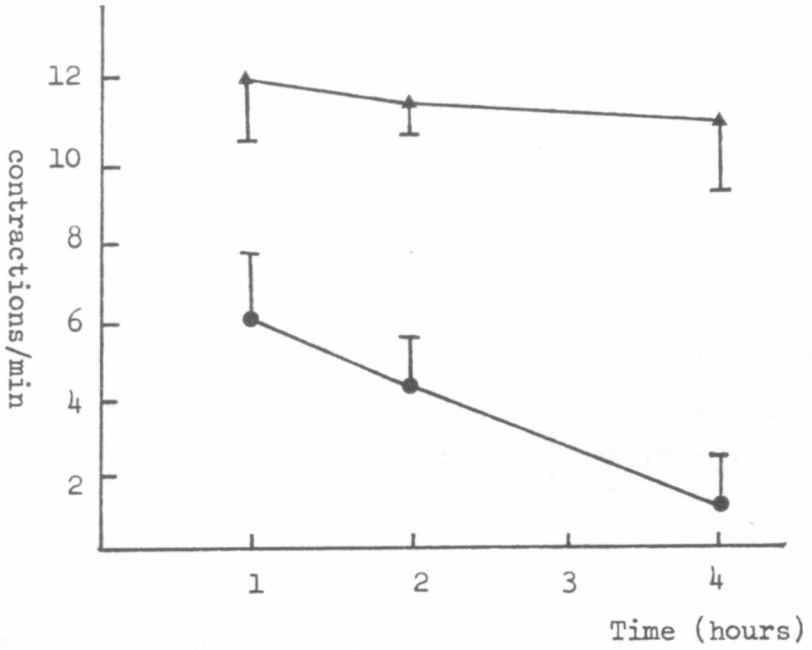


Fig 4 The effect of reducing the calcium concentration of the perfusion fluid to 25% (1.075mM) on the frequency of contraction of the oviduct preparation in vitro

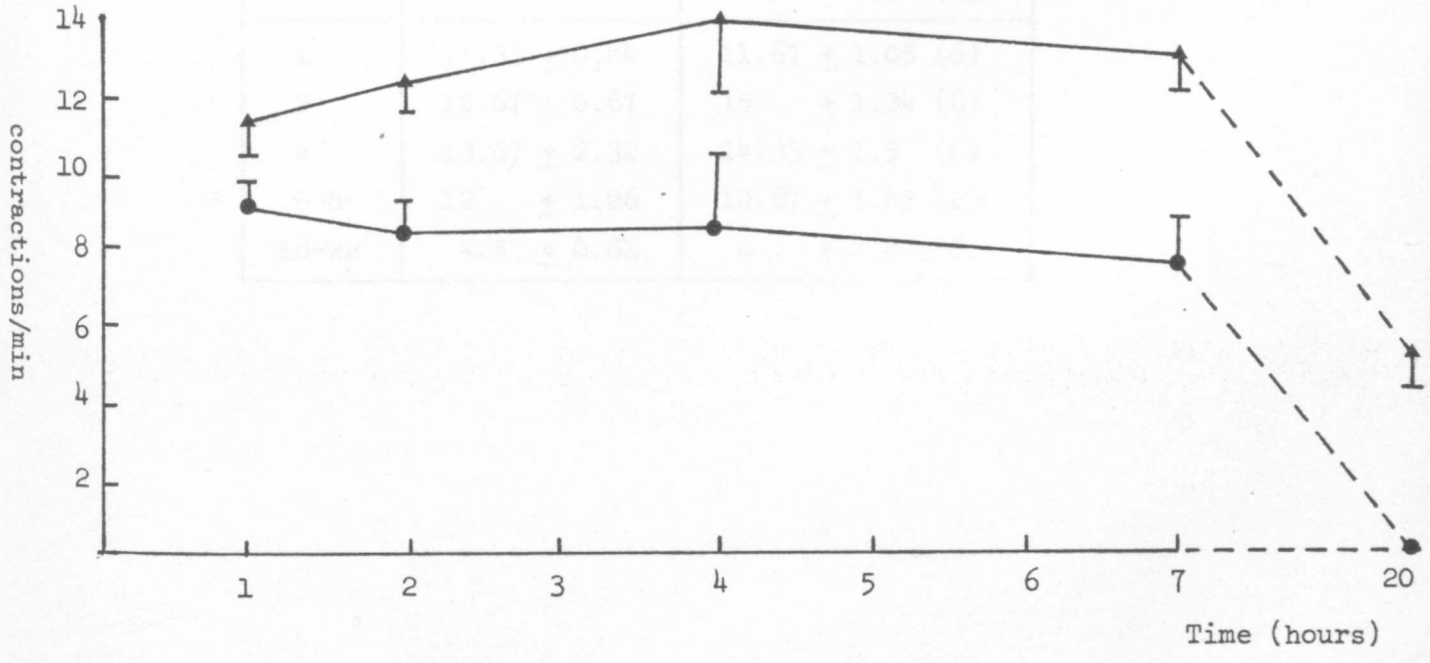


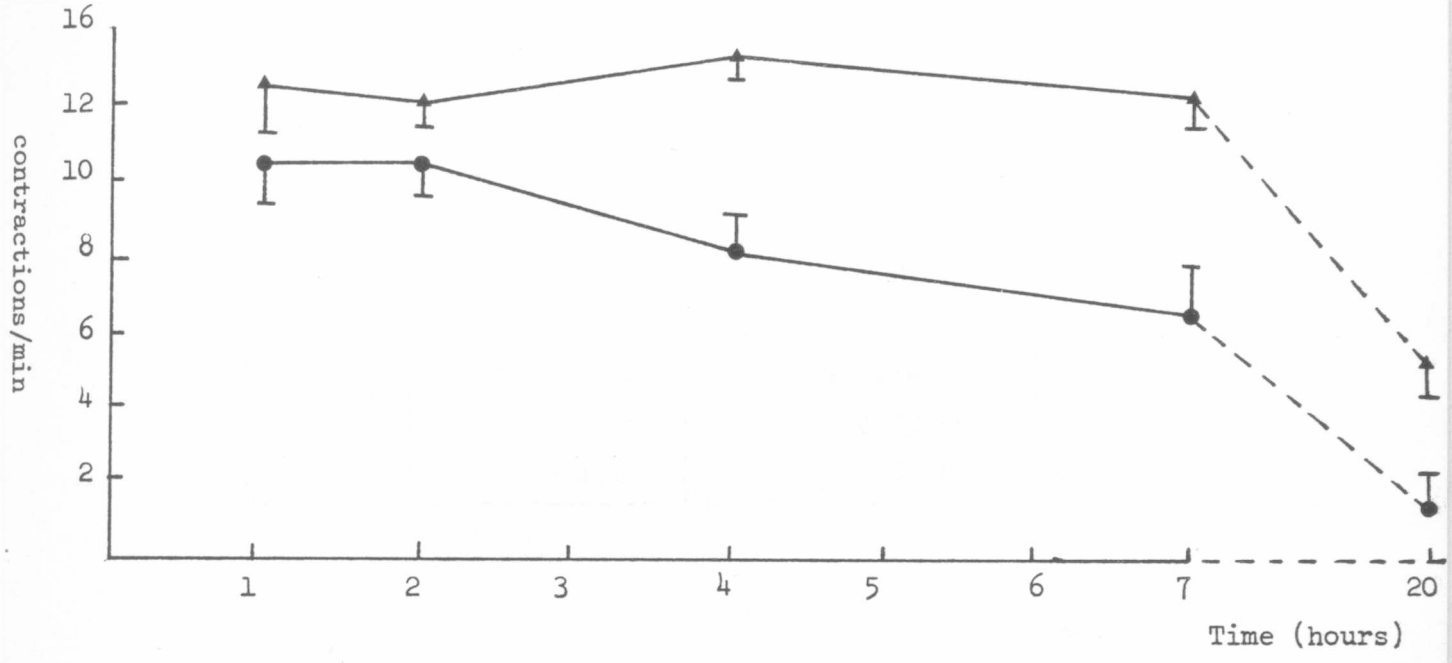
Table 15 The effect of reducing the calcium concentration of the perfusion fluid to 50% (2.15mM) on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min mean ± S.E.	
	Controls	50% calcium (n)
1	12.33 ± 1.09	10.33 ± 0.95 (6)
2	12 ± 0.73	10.33 ± 0.95 (6)
4	13 ± 0.63	*8 ± 0.73 (6)
6-8	12 ± 0.73	*6.33 ± 1.31 (6)
18-22	4.83 ± 0.98	*1.17 ± 0.98 (6)

Table 16 The effect of increasing the calcium concentration of the perfusion fluid to 200% (8.6mM) on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min mean ± S.E.	
	Controls	200% calcium (n)
1	11.33 ± 0.84	11.67 ± 1.05 (6)
2	12.67 ± 0.67	15 ± 1.34 (6)
4	13.67 ± 2.32	14.33 ± 1.5 (6)
6-8	12 ± 1.26	12.67 ± 1.43 (6)
18-22	4.5 ± 0.81	6 ± 0.89 (6)

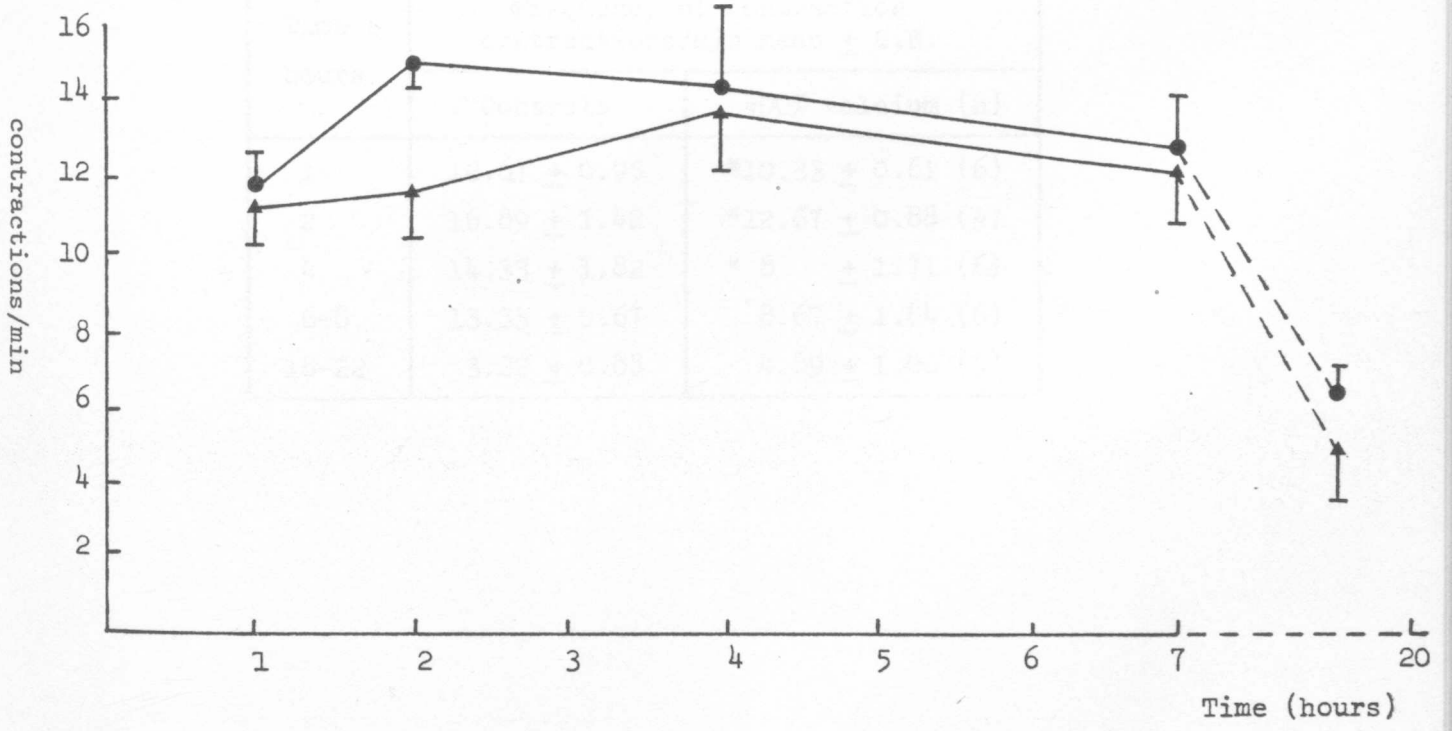
Fig 5 The effect of reducing the calcium concentration of the perfusion fluid to 50% (2.15mM) on the frequency of contraction of the oviduct preparation in vitro



▲ controls

● 50% calcium

Fig 6 The effect of increasing the calcium concentration of the perfusion fluid to 200% (8.6mM) on the frequency of contraction of the oviduct preparation in vitro



▲ controls

● 200% calcium

Table 17 The effect of increasing the calcium concentration of the perfusion fluid to 300% (12.9mM) on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min mean ± S.E.	
	Controls	300% calcium (n)
1	12.5 ± 1.78	10.33 ± 0.83 (6)
2	13.5 ± 1.15	13.5 ± 1.2 (6)
4	11.83 ± 1.38	12.5 ± 1.54 (6)
6-8	13.33 ± 1.05	12.8 ± 0.73 (6)
18-22	4.2 ± 1.2	3.1 ± 0.8 (6)

Table 18 The effect of increasing the calcium concentration of the perfusion fluid to 400% (17.2mM) on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min mean ± S.E.	
	Controls	400% calcium (n)
1	12.67 ± 0.95	*10.33 ± 0.61 (6)
2	16.89 ± 1.42	*12.67 ± 0.88 (9)
4	14.33 ± 1.82	* 8 ± 1.71 (6)
6-8	13.33 ± 0.67	8.67 ± 1.84 (6)
18-22	3.22 ± 0.88	4.89 ± 1.06 (9)

Fig. 7 The effect of increasing the calcium concentration of the perfusion fluid to 300% (12.9mM) on the frequency of contraction of the oviduct preparation in vitro

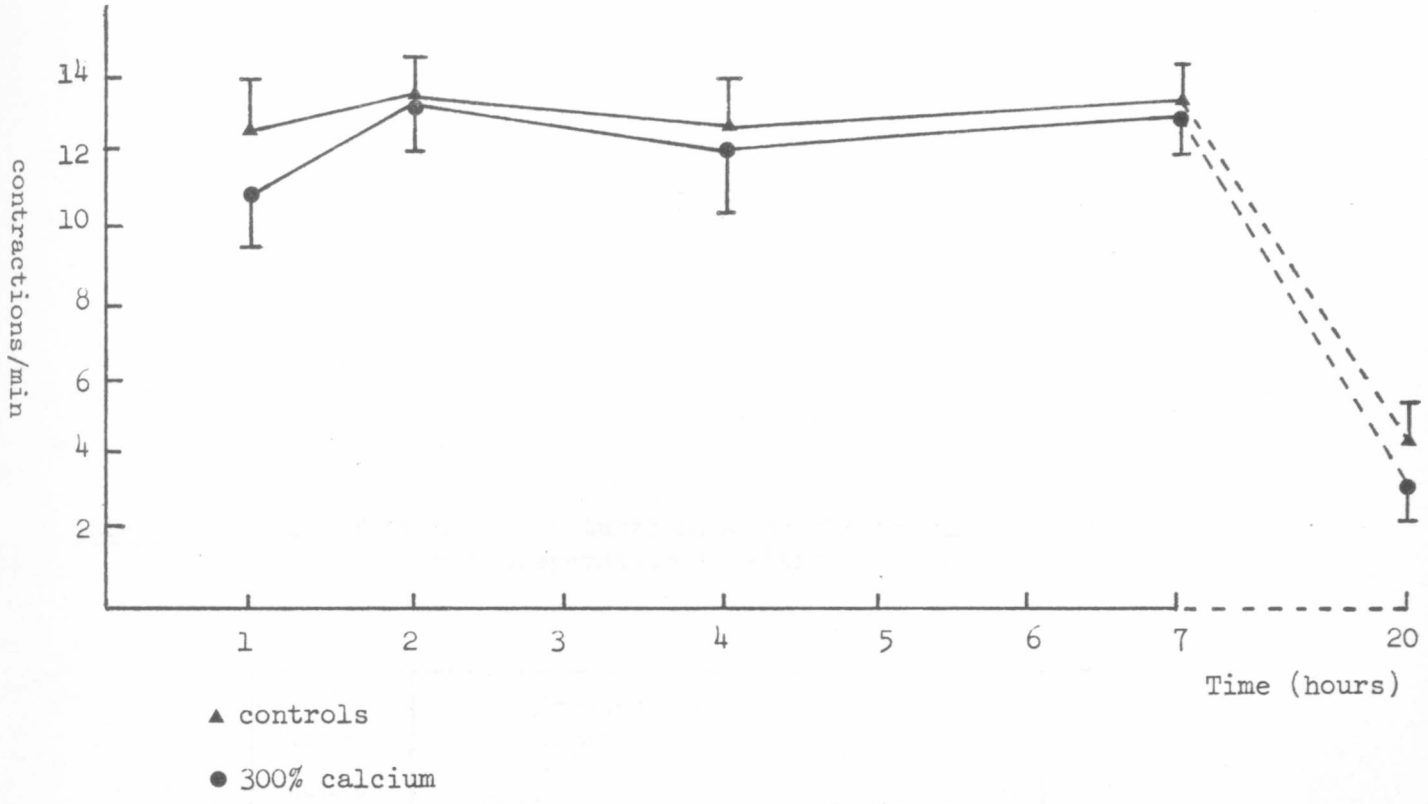


Fig. 8 The effect of increasing the calcium concentration of the perfusion fluid to 400% (17.2mM) on the frequency of contraction of the oviduct preparation in vitro

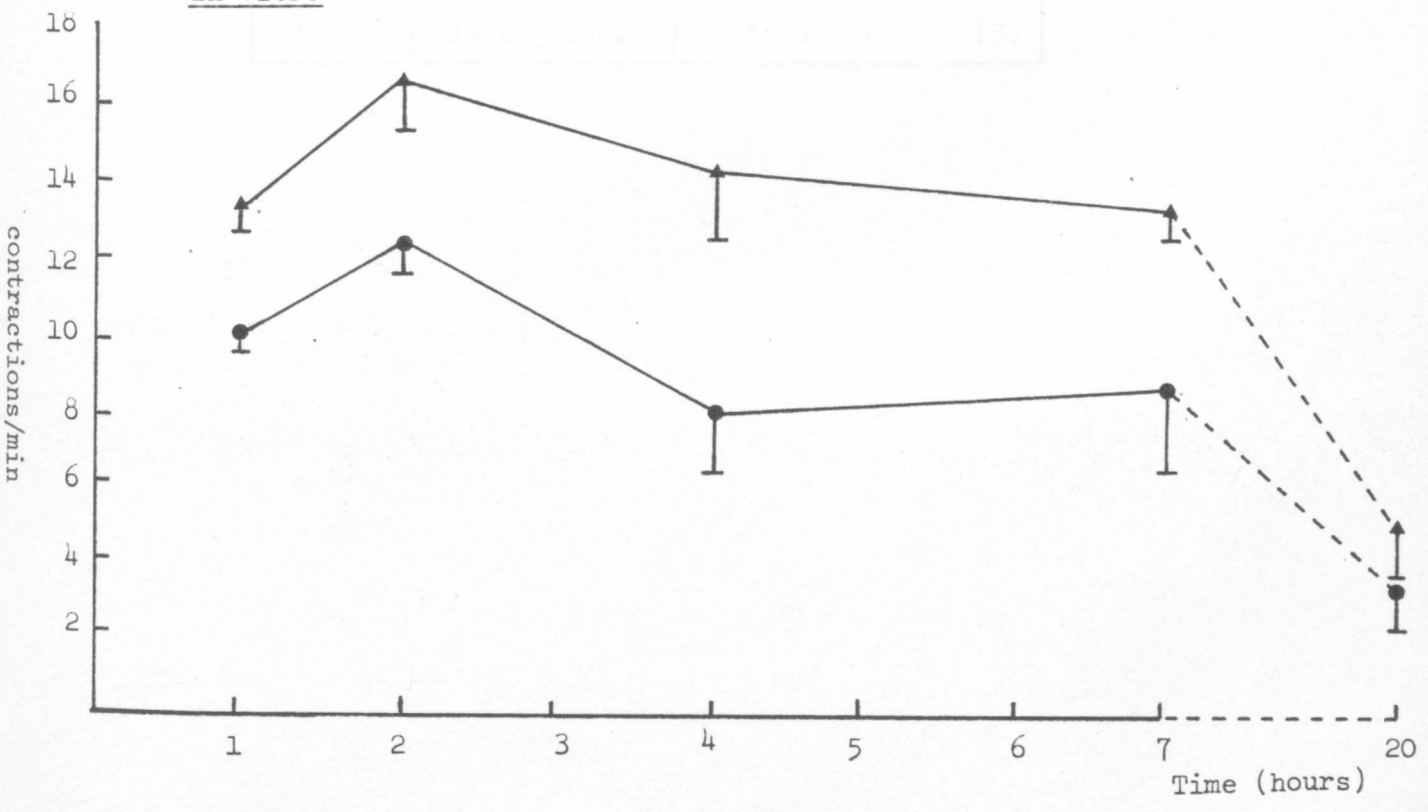
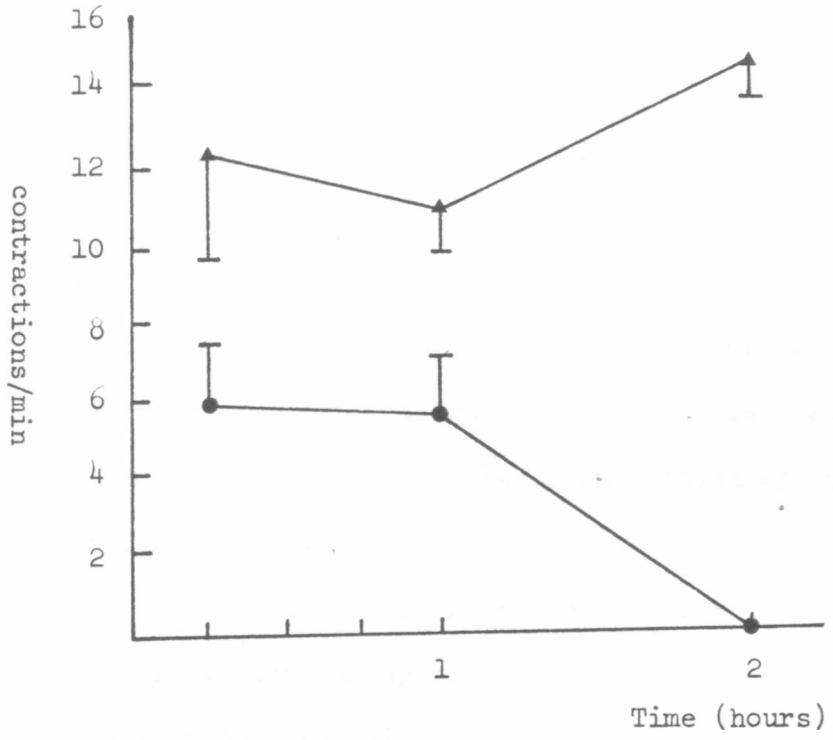


Table 19 The effect of 1.5μM lanthanium on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min mean ± S.E.	
	Controls	1.5μM lanthanum (n)
0.25	12.4 ± 2.79	6 ± 1.67 (5)
1	10.8 ± 0.8	*5.6 ± 1.72 (5)
2	14.8 ± 1.02	***0 ± 0 (5)

Fig. 9 The effect of 1.5μM lanthanum on the frequency of contraction of the oviduct preparation in vitro



▲ controls

● 1.5μM lanthanum

Chapter 3: The measurement of in vivo oviduct calcium levels during egg transport, oviduct calcium levels during in vitro perfusion and in vivo calcium levels during reserpine retardation of egg transport

Introduction

In the *in vitro* experiments described in Chapter 2 variation of external calcium concentrations changed oviductal contractility while lanthanum ions inhibited oviductal contractions in the presence of calcium ions. This shows that calcium movement and exchange is important for oviduct contractility, variations in which are considered to be the primary factor in accomplishing egg transport (Blandau and Verdugo, 1976).

To assess the importance of calcium ions *in vivo* the calcium content of the oviduct during early pregnancy, when the eggs are being transported along the oviduct, was determined. Oviduct calcium levels during *in vitro* perfusion between Days 2 and 3 of pregnancy were measured to determine any differences occurring in calcium levels under conditions which do not result in egg movement.

A further experiment was performed *in vivo*, to investigate the effect of pharmacological retardation of egg transport on oviduct calcium levels. Reserpine was chosen as a compound known to retard egg transport in mice.

Methods

To measure oviduct calcium levels, groups of control, reserpine treated and reserpine vehicle treated mice were killed on each day of Days 1-5 of pregnancy. Each pair of oviducts was ashed and analysed by atomic absorption spectroscopy for calcium content.

In vitro oviduct perfusion was started on Day 2 of pregnancy at 34hpc. One group of oviducts was perfused for 24 hours, until

Day 3 (58hpc) and a further group for 6 hours only (until 40hpc). The oviducts were observed during perfusion to ensure they remained viable and at the end of the specified period they were removed from the perfusion chambers and blotted on fine filter paper. Each original pair of oviducts was then weighed, ashed and their calcium content measured. A further three groups of mated mice were killed at 34, 40 and 58hpc to provide control oviduct calcium levels.

Comparisons of egg position and calcium concentrations between the different groups were performed using a students t test and a multiple range t test respectively.

Results

The effect of reserpine on in vivo egg transport

Reserpine retarded the rate of egg transport by approximately 24 hours compared to the control and vehicle treated groups (Table 20). The eggs had all entered the uterus by Day 5 of pregnancy after reserpine treatment compared to Day 4 in the other groups. Retardation started within the first 24 hours after reserpine administration and egg development was arrested, at the two cell stage. There were no significant differences between the control and vehicle treated groups in egg position and development.

In vivo oviduct calcium levels

Oviduct calcium levels were not constant during early pregnancy (Table 21), a distinctive pattern emerged. Between Days 1 and 2 a significant fall in calcium levels occurred ($P < 0.05$) followed by a rise between Days 2 and 3 ($P < 0.001$). The rise continued on Day 4, although the levels were not significantly higher than on Day 3 and started to fall again on Day 5. The Day 4 and Day 5 levels were still both significantly higher than the Day 2 levels ($P < 0.001$).

The effect of reserpine on in vivo oviduct calcium levels

Reserpine altered the pattern of oviduct calcium levels (Table 21). In the control groups Day 2 gave the lowest calcium values, but after reserpine treatment the Day 2 fall and the Day 3 rise were abolished. Comparing Days 1, 3, 4 and 5 to Day 2 no significant change in calcium levels occurred until a rise on Days 4 and 5 ($P < 0.01$). In the vehicle treated groups the actual levels of calcium ions were higher from Days 1-5 but the results followed the same pattern as the control groups, a significant fall on Day 2 ($P < 0.05$) followed by a significant rise on Day 3 ($P < 0.05$). The different pattern of calcium levels after reserpine treatment can be seen in Fig. 14.

In vitro oviduct calcium levels during perfusion

During in vitro perfusion oviduct calcium levels rose significantly within 6 hours ($P < 0.01$) compared to in vivo levels at 34hpc, they then decreased over the next 18 hours but were still significantly higher ($P < 0.01$) than at the start of the perfusion (Table 22). During the same time period *in vivo* a rise occurred at both 40 and 58hpc but only at 58hpc was this significant ($P < 0.01$).

Discussion

During normal egg transport in the mouse oviduct a distinctive pattern of calcium levels emerges. Oviduct calcium levels fall between Days 1 and 2 of pregnancy and then rise again between Days 2 and 3. Day 2 of pregnancy is the day of most rapid egg movement *in vivo*, the eggs traversing from the AIJ to the UTJ. Days 1 and 3 are periods of relative inactivity of the musculature, the eggs being more or less stationary at the AIJ and UTJ respectively and here oviduct calcium levels are comparatively high.

High calcium levels might be having an inhibitory effect on muscle motility. During reserpine treatment the absence of the Day 2 fall in calcium levels is accompanied by a retardation of egg transport. Reserpine is known to deplete adrenergic neurones, but its retardation of egg transport is thought to be due, not to this, but to its hypothermic effects (Bennett and Kendle, 1967; Kendle and Bennett, 1969a, 1969b). A dose of 2mg/kg on Day 1 of pregnancy causes a significant fall in oesophageal temperature within the first 24 hours after administration and returns to control levels by Day 4 (Kendle, 1969).

One obvious consequence of hypothermia will be a decrease in metabolic rate. If calcium levels are being altered during egg transport via control mechanisms such as hormone induction of protein synthesis and consequent changes in ionic movements and exchange then these processes will also be slowed down. No significant changes are occurring from Days 1 to 3 under the influence of reserpine but a significant rise is occurring between Days 3 and 4 when the effects of reserpine on body temperature will be becoming less marked. It may be that by this time any effects on calcium metabolism are also ending and normal processes returning.

An absence of low Day 2 calcium levels after reserpine treatment and a corresponding delay in transport does suggest the involvement of calcium ions in the regulation of egg transport.

In vitro, where there is a total lack of forward progression of the eggs during Day 2, there is again an alteration in oviduct calcium levels. In vitro there appears a much more rapid increase in tissue calcium levels which then decreases whereas the rise in vivo is more gradual and sustained. It may be that in vitro the levels of calcium in the perfusion fluid are such that the oviduct is taking up calcium

from its environment thus altering tissue levels, particularly extracellular levels, from normally occurring *in vivo* levels, as the calcium content of smooth muscle is known to be dependent on extracellular calcium levels (Schatzman, 1961). Consequently this may lead to changes in the muscle contractility either in the pattern or force of the contractions and relaxations or both, thus impairing egg transport. Eventually, *in vitro*, the oviduct appears to be losing calcium, this may be the result of a loss of function, if the tissue is beginning to break down then its cell regulatory mechanisms will be impaired and there will be a loss of ability for maintenance of ionic concentrations.

From these experiments there are indications that a changing pattern of calcium ions is involved in the regulation of egg transport. *In vivo* calcium shows a fall at the time when the AIJ relaxes and the eggs enter the isthmus, this does not occur after reserpine treatment where transport is retarded. Significantly increased calcium levels are found when transport of the eggs is impeded by the UTJ. After reserpine the significant increase is delayed but occurring when the eggs are in the UTJ region and *in vitro* premature increases in calcium levels are associated with lack of transport.

The significance of high and low calcium levels and their possible effects on smooth muscle function are interesting. If calcium levels are high then the musculature could be in a state of tension, not relaxing adequately. On Day 1 the lumen of the oviduct is restricted at the AIJ and on Day 3 the lumen at the uterine end of the isthmus and at the UTJ is also very narrow. This obviously impedes egg movement and suggests that the musculature of the isthmus, particularly the circular muscle layer is not able to relax. During Day 2 the lumen is always distended in the region of the eggs allowing them to be forced backwards and forwards as the isthmus contracts and relaxes within one loop and also forwards into the next loop (Humphrey, 1968a).

The work of Hodgson and Daly (1976) which tried to define calcium distribution and exchange in the oviduct and the source of calcium for contractions, did demonstrate some correlation between the ability of different segments of the oviduct for calcium exchange and transport of eggs. They found $^{45}Ca^{2+}$ uptake varied in the different regions of the oviduct in rabbits after treatment with HCG (Human Chorionic Gonadotrophin) to induce ovulation. Twenty-four hours after HCG $^{45}Ca^{2+}$ uptake was significantly decreased in the isthmus and decreased at the AIJ. The uptake levels had risen again by 72 hours in both the isthmus and the AIJ, significantly in the latter. The decreased uptake occurring at 24 hours after HCG in the rabbit oviduct may be related to total oviduct calcium levels and a decreased calcium uptake could be occurring on Day 2 in the mouse oviduct. The use of pharmacological agonists on 24 hour isthmic tissues resulted in very poor responses, suggesting a poor ability of these tissues to utilize calcium for contraction. The decreased uptake at the AIJ at 24 hours indicates a possible relationship between uptake and delayed transport (Pauerstein et al., 1974a) and the apparent temporary reduction in the activity of egg retaining mechanisms which occurs at this time (Gómez and Croxatto, 1977). Relaxation of the AIJ to allow entry of the eggs into the isthmus involves a decrease in the tension of the muscular tissue in this region which would be brought about by a decreased uptake of calcium ions or a loss of calcium ions from the muscle cells.

Table 20 - The effect of reserpine 2mg/kg administered on Day 1 of pregnancy (9hpc), on egg transport in vivo

	Mean % oviduct traversed by eggs		
Hours post coitum	Controls Mean ± S.E. (n)	Vehicle Mean ± S.E. (n)	Reserpine Mean ± S.E. (n)
12	17.1 ± 2.1 (14)	18.4 ± 0.9 (10)	18.7 ± 1.1 (10)
36	43.9 ± 3.1 (16)	47.5 ± 6.0 (10)	***27.6 ± 2.8 (12)
60	75.2 ± 3.4 (14)	84.9 ± 4.1 (16)	***63.9 ± 4.9 (11)
84	101 ± 0.0 (14)	101 ± 0.0 (10)	***80.8 ± 5.0 (11)
106			101 ± 0.0 (12)

Reserpine and vehicle treated groups compared to control groups using a Students t-test

n number of oviducts

*** $P < 0.001$

Table 21 - The effect of reserpine 2mg/kg administered on Day 1 of pregnancy (9hpc), on oviduct calcium levels in vivo

	Calcium content, ug/mg wet weight of tissue		
Hours post coitum	Controls Mean ± S.E. (n)	Vehicle Mean ± S.E. (n)	Reserpine Mean ± S.E. (n)
12	*0.81 ± 0.06 (8)	*1.28 ± 0.15 (5)	0.89 ± 0.08 (8)
33	0.65 ± 0.04 (8)	0.96 ± 0.10 (6)	1.03 ± 0.07 (9)
57	***1.10 ± 0.11 (11)	*1.33 ± 0.14 (5)	1.01 ± 0.05 (6)
81	***1.24 ± 0.09 (11)	***1.40 ± 0.06 (5)	**1.40 ± 0.11 (6)
105	***1.08 ± 0.05 (8)	***1.64 ± 0.15 (5)	**1.57 ± 0.03 (6)

Each group within a treatment compared to the Day 2 (33hpc) value for that treatment, using a multiple range t-test. This procedure was used as the oviducts from mice receiving different treatments were not assayed at the same time and some variation was found in the sensitivity of the assay.

n number of mice

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

Table 22 - The effect of in vitro perfusion on oviduct calcium levels

Day of pregnancy	Calcium content, ug/mg wet weight of tissue			
	In vivo		In vitro	
	Mean ± S.E.	n	Mean ± S.E.	n
2 (34hpc)	0.41 ± 0.01	6		
2 (40hpc)	0.46 ± 0.03	6	0.58 ± **0.05	6
3 (58hpc)	0.52 ± **0.03	6	0.49 ± **0.05	6

Each group compared to Day 2 (34hpc) control group,

Students t-test

** P < 0.01

n number of mice

hpc hours post coitum

Chapter 4: The effect of oestradiol during in vitro perfusion, on in vivo egg transport and on oviduct calcium levels

Introduction

The effect of oestradiol in the perfusion fluid of the *in vitro* oviduct preparation was investigated to determine whether the presence of oestrogen would effect oviduct motility or initiate egg transport *in vitro*. Low concentrations of oestradiol were added to the perfusion fluid in an attempt to reproduce a physiological situation rather than observing pharmacological effects of oestradiol.

Administration of oestrogens after ovulation can prevent pregnancy by disturbance of egg transport and endometrial development (Chang, Casas and Hunt, 1971). The effects of oestrogens are dependent on compound, dosage, species and time of administration (Chang, 1967a). A low dose of oestradiol administered on Day 2 of pregnancy was chosen for study as this is known to accelerate egg transport in mice, when the eggs have entered the isthmus, resulting in premature entry of the eggs into the uterus (Humphrey, 1968b).

In vivo oviduct calcium levels during oestradiol treatment were measured to determine whether any corresponding changes occur.

Methods

The effect of oestradiol on the *in vitro* oviduct perfusion preparation was investigated during Day 2 of pregnancy for periods up to 20 hours. Two perfusion circuits were used, a control circuit of normal Ringer solution and the other with oestradiol added, either 0.01 or 0.1 ug/ml. Each animal was used as its own control, one oviduct to each circuit and the preparations were set up as described in the methods section.

A stock solution of oestradiol (Oestradiol-3-17β) was prepared in absolute ethanol, 500 ug/ml, dilutions were made immediately before use, with Ringer solution and the volume of ethanol added to the perfusion fluid never exceeded 1ml/5l. An equivalent volume of ethanol was added to the control circuit.

Mean percentage oviduct traversed by the eggs, strength of motility and frequency of contraction were measured throughout the experiment. Egg position and frequency of contraction data were analysed using a paired t-test and strength of motility by an F-test as this was non-parametric data.

Oestradiol, 2ug/mouse i.p., was administered to three groups of mated female mice on Day 2 of pregnancy at 09:00 hours (33hpc). A further four groups received normal saline, 0.1ml/mouse at the same time. At 36, 40 and 60hpc an oestradiol treated group was killed and the control groups at 36, 40, 60 and 84hpc. Additional groups of untreated, mated, female mice were killed at 12 and 33hpc. The oviducts from each mouse were removed, the egg positions measured and the mean percentage oviduct traversed calculated.

The same procedure of oestradiol and saline administration was carried out to investigate the effect on oviduct calcium levels. Oestradiol and saline groups were killed at 36, 40, 60 and 84hpc and untreated groups at 12 and 33hpc. The oviducts were analysed for their calcium content as previously described in the methods section.

Oestradiol stock solution was prepared in absolute ethanol, 2mg/ml and diluted 1:100 in normal saline for injection. The injection volume was 0.1ml/mouse.

Students t-test was used to compare the oestradiol treated to the control treated groups in both experiments.

The effect of oestradiol on the in vitro oviduct perfusion preparation

Oestradiol, 0.01ug/ml in the perfusion fluid, had no significant effects on egg position, strength of motility and frequency of contraction (Tables 23-25). One hour after the start of perfusion both strength of motility and frequency of contraction were slightly lower than in the controls. Frequency of contraction rose slightly between 6 and 12 hours and fell again with strength of motility between 17 and 20 hours.

0.1ug/ml oestradiol also had little effect on the in vitro preparation (Tables 26-28). Strength of motility was slightly higher at 1 hour and 6-12 hours but frequency of contraction was the same. At 17-20 hours strength and frequency were both slightly lower, but none of these effects was significant. No changes in egg position were observed.

The effect of oestradiol on in vivo egg transport

Oestradiol accelerated the rate of egg transport in vivo (Table 29). Within 3 hours of administration (36hpc) the mean percentage oviduct traversed had increased. This increase became significant ($P < 0.05$) after 7 hours (40hpc), most of the eggs being located near the UTJ. By 60hpc all the eggs were found to have left the oviduct and entered the uterus, whilst the control eggs were still in the isthmus or at the UTJ.

The effect of oestradiol on in vivo oviduct calcium levels during egg transport

The control calcium levels showed a slight decrease from Day 1 to Day 2 (12 to 33hpc) and then started to rise significantly during Day 2 (36 and 40hpc), the rise continuing on Days 3 and 4. The oestradiol treated groups initially showed the same rise between 33 and

36hpc but after this calcium levels fell again to pretreatment levels and remained so until 84hpc. At 40, 60 and 84hpc the calcium levels of the oestradiol treated groups were significantly lower than the control levels (Table 30).

Discussion

Oestradiol, *in vitro*, appeared to have no effect on oviduct function at the doses investigated. Oestrogens are generally considered activators of oviductal and uterine smooth muscle (Coutinho, 1973). If oestradiol was to have had an effect on the oviduct *in vitro* an increased frequency of contraction and a decreased amplitude of contraction would have been expected as occurs *in vivo*, after oestrogen administration (Coutinho and De Mattos, 1968). However, the time span of the *in vitro* experiment might not have been sufficient for these types of effects to occur as they are considered the result of an induction of synthesis of contractile proteins. Another factor to consider is the metabolism of oestradiol *in vivo* and *in vitro*, this could perhaps be different, although the rabbit oviduct *in vitro* is known to be capable of incorporating oestrogen (Roy et al., 1972).

However the lack of activity of oestradiol *in vitro* could be indicating that post ovulatory oestrogen secretion is not a requisite for normal egg transport to occur as has been demonstrated in the rat (Alden 1942b) and rabbit (Adams, 1958) where postovulatory ovariectomy did not alter egg transport. There is the possibility however that the balance of oestrogen and progesterone levels may be important and further investigation is needed.

Accelerated egg transport, *in vivo*, was demonstrated with Day 2 administration of oestradiol, the effect was rapid and highly significant.

Corresponding changes also occurred in the pattern of oviduct calcium levels. Normally calcium levels drop by Day 2 and then rise again by Day 3, but after oestradiol treatment a significant increase in the rate of transport correlates with a significant fall in calcium levels. Calcium levels remained low through to the fourth day.

In the human there is evidence that the smooth muscle of the reproductive tract is under oestrogen domination during the preovulatory phase (Pauerstein et al., 1974a; Croxatto, 1974). The oestrogen domination is thought to establish an isthmic block before ovulation which is overcome two to three days after ovulation (Coutinho, 1974a). In the rabbit a decline in the influence of oestrogens is considered a possible factor in allowing eggs through to the isthmus to resume their passage towards the oviduct. If this is the case in the mouse then administration of oestradiol on Day 2 pregnancy would be after any oestrogen block had been overcome and the eggs had reached the isthmus, the accelerating effect on transport observed must be due to an influence on isthmic contractility.

Oestrogens are known to be capable of decreasing calcium entry into uterine cells (Batra and Bengtsson, 1978) and they probably have similar effects on oviductal smooth muscle cells. The decrease in oviduct calcium levels after oestradiol treatment would support this phenomenon. If calcium entry is decreased then alterations in the development of tension in the muscle would occur as the concentration of free calcium available to react within the contractile proteins is thought to control the development of muscle tension (Batra and Bengtsson, 1978).

The close correlation between the onset of accelerated egg transport and oestradiol and the decrease in oviduct calcium levels

does suggest that oestradiol is altering oviductal contractility via alterations in calcium metabolism. The target area for changes in calcium metabolism is probably the mitochondria as ovarian hormones have been shown to alter calcium transport in myometrial mitochondria (Batra and Bengtsson, 1972; Batra, 1973).

Additional evidence for altered calcium metabolism by oestrogens comes from the work of Hodgson and Daly (1976), they demonstrated the ability of oestrogens to consistently decrease the extracellular compartment in the rabbit oviduct. The extracellular compartment is the fastest exchanging calcium compartment, exchange between cellular and extracellular calcium is very rapid in smooth muscle cells, which are capable of taking up relatively large amounts of calcium compared to other muscular tissues (Lullman, 1970). The calcium content of smooth muscle is known to be dependent on extracellular levels (Schatzmann, 1961), so if oestrogens are capable of effecting the size of the extracellular calcium compartment then this constitutes another level at which oestrogen can alter calcium metabolism.

If exogenous oestrogens are capable of altering oviduct smooth muscle contractility via changes in calcium ion metabolism it is probable that their role in the control of normal egg transport is through similar mechanisms. In the oviduct, oestrogens enter the cytoplasm and form complexes with cytoplasmic oestrogen receptors. The complex is then able to enter the nucleus and interact with chromatin. The reaction with chromatin is thought to be achieved by complexing with the acid protein fraction, the resultant events are an increased protein synthesis (Pauerstein et al., 1973). Thus in vivo pre-ovulatory oestrogen secretion could be initiating a chain of events which determines the pattern of oviduct motility and contractility during egg transport which then regulates the passage of eggs through the oviduct.

Oestrogen alone, however, cannot be considered as the only controlling factor, progesterone secretion is likely to influence the role of oestrogens and interaction of oestrogen with the adrenergic system is also possible. Pauerstein et al., (1973) have shown antagonism of oestrogen induced retardation of transport in rabbits by a combination of progesterone and phenoxybenzamine, an α adrenergic antagonist.

The effect of oestradiol, 0.01 ug/ml, on the in vitro oviduct perfusion preparation

Table 23

1 Hour Readings	Controls	Oestradiol 0.01ug/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	48.02 ± 7.3 (5)	32.22 ± 7.4 (5)
strength of motility	3.2 ± 0.37 (5)	2.8 ± 0.09 (5)
frequency of contraction	15.2 ± 1.36 (5)	13.6 ± 0.98 (5)

Table 24

6-12 Hours Readings	Controls	Oestradiol 0.01ug/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	47.96 ± 7.3 (5)	34.02 ± 7.52 (5)
strength of motility	2.6 ± 0.24 (5)	2.6 ± 0.24 (5)
frequency of contraction	11.6 ± 1.67 (5)	15.6 ± 1.54 (5)

Table 25

17-20 Hours Readings	Controls	Oestradiol 0.01ug/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	44.46 ± 8.19 (5)	36.5 ± 8.18 (5)
strength of motility	1.8 ± 0.2 (5)	1.2 ± 0.52 (5)
frequency of contraction	5.2 ± 1.36 (5)	3.6 ± 1.63 (5)

% oviduct traversed and frequency of contraction, comparison by paired t-test

Strength of motility comparison, analysis of variance

n number of oviducts

The effect of oestradiol, 0.1ug/ml, on the in vitro oviduct perfusion preparation

Table 26

1 Hour Readings	Controls	Oestradiol 0.1ug/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	30.91 ± 2.4 (6)	35.5 ± 5.08 (9)
strength of motility	2.83 ± 0.167(6)	3.56 ± 0.18 (9)
frequency of contraction	12 ± 1.15 (6)	12.22 ± 1.02 (9)

Table 27

6-12 Hours Readings	Controls	Oestradiol 0.1ug/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	34.43 ± 3.05 (6)	29.8 ± 4 (6)
strength of motility	2.16 ± 0.17 (6)	3.1 ± 0.43 (6)
frequency of contraction	9.33 ± 1.51 (6)	10 ± 1.71 (6)

Table 28

17-20 Hours Readings	Controls	Oestradiol 0.1ug/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	32.83 ± 3.05 (6)	29.4 ± 6.46 (6)
strength of motility	1.33 ± 0.42 (6)	0.5 ± 0.34 (6)
frequency of contraction	4 ± 1.37 (6)	1.5 ± 1.31 (6)

% oviduct traversed and frequency of contraction, comparison by paired t-test

Strength of motility comparison, analysis of variance

n number of oviducts

Table 29 The effect of oestradiol, 2ug/mouse, administered 33 hours post coitum on in vivo egg transport

Hours post coitum	Mean % oviduct traversed by eggs	
	Controls	Oestradiol
	Mean ± S.E. (n)	Mean ± S.E. (n)
12	17.8 ± 2.2 (14)	
33	43.9 ± 3.1 (16)	
36	46.9 ± 3.9 (13)	59.9 ± 8.7 (13)
40	57.7 ± 4.2 (10)	*73.7 ± 7.1 (13)
60	81.9 ± 2.7 (10)	***101 ± 0.0 (10)
84	101 ± 0.0 (10)	

Oestradiol treated groups compared to control groups, Students t-test

* $P < 0.05$

*** $P < 0.001$

n number of oviducts

Table 30 The effect of oestradiol, 2ug/mouse, administered 33 hours post coitum, on in vivo oviduct calcium levels

Hours post coitum	Calcium content, µg/mg wet weight of tissue	
	Controls	Oestradiol
	Mean ± S.E. (n)	Mean ± S.E. (n)
12	0.65 ± 0.06 (7)	
33	0.56 ± 0.05 (7)	
36	0.90 ± 0.01 (7)	0.86 ± 0.06 (6)
40	1.05 ± 0.10 (8)	**0.67 ± 0.04 (7)
60	0.98 ± 0.11 (7)	*0.60 ± 0.06 (6)
84	1.03 ± 0.07 (6)	**0.65 ± 0.07 (7)

Oestradiol treated groups compared to control groups, Students t-test

* $P < 0.05$

** $P < 0.01$

n number of mice

Chapter 5: The effect of pregnenolone and progesterone with oestradiol on the mouse oviduct perfusion preparation, in vitro. The effect of preovulatory administration of progesterone on egg transport and oviduct calcium levels in vivo

Introduction

Progesterone has been generally assumed to inhibit the smooth musculature of the genital tract (Allen and Reynolds, 1935; Bickers and Main, 1941; Csapo, 1956). The activity of oestrogen was thought to be modified by progesterone when the two were used in combination, progesterone reducing the activity of the circular muscle layer, in the oviduct of ovariectomized, oestrogen treated rabbits (Harper, 1965).

The effect of progesterone in the presence of oestradiol on the *in vitro* oviduct perfusion preparation was studied. *In vivo*, at the time of egg transport both progesterone and oestrogen are present in the oviductal environment, the concentration of progesterone being much higher than oestrogen. To simulate the *in vivo* situation, the concentrations of progesterone in the perfusion fluid exceeded those of oestradiol by either a thousand or a hundred fold.

Exogenous administration of progesterone produces effects on egg transport that are both time and dose dependent (Gonzalez de Vargas and Pauerstein, 1976). Black and Asdell (1958) and Greenwald (1961) reported acceleration of eggs, in the rabbit by postovulatory administration of progesterone. Chang (1966a) however found postovulatory progesterone to have no effect on egg transport but preovulatory progesterone administered on the day of ovulation or the preceeding day, accelerated egg transport. An accelerating dose of progesterone administered on the day of pro-oestrus was chosen for study. The dose needed for a significant acceleration of egg transport was determined by preliminary studies on egg transport rates *in vivo*

In vivo oviduct calcium levels were measured during progesterone treatment for determination of any corresponding changes.

In addition to the effect of progesterone on the oviduct preparation in vitro the effect of pregnenolone was also investigated. Pregnenolone is a precursor of progesterone.

Methods

The effect of combinations of oestradiol and progesterone and the effect of pregnenolone on the mouse oviduct in vitro were investigated on Day 2 of pregnancy. The preparations were set up as described in the methods section, two perfusion circuits were used, an oviduct from each animal to each circuit. Oestradiol and progesterone stock solutions were prepared in absolute ethanol, this was then diluted in the perfusion fluid of one circuit, the volume of stock solution added to 5l of the Ringer perfusion fluid never exceeded 1ml. The ratio of oestradiol to progesterone was 1:100 (0.001 µg/ml) and 1:1000 (0.0001 µg/ml) respectively. The oviducts were observed over a period of 20 hours and egg position, frequency of contraction and strength of motility were measured throughout this period. Egg position and frequency of contraction were compared to the controls using a students t-test. Strength of motility was compared by an F-test as these are non-parametric data and only differences in variance between test and control groups can be demonstrated.

Pregnenolone, 1 µg/ml, was dissolved in the perfusion fluid and the above parameters observed over 20 hours. The effect on frequency of contraction was investigated by use of the students t-test.

To study the effect of progesterone administration on in vivo egg transport and oviduct calcium levels only mice with regular oestrus cycles were used. Vaginal smears were performed daily on virgin

female mice and after 3 consecutive regular oestrus cycles were established the animals were treated with either progesterone or the vehicle. Progesterone was suspended (8 mg/ml) in an aqueous solution of carboxymethylcellulose (0.25% v/v) and administered subcutaneously, 20 mg/kg (2.5 ml/kg), on the morning of pro-oestrus, 00:09h. The female mice were then caged with males (2:1 respectively) for mating and the presence of a vaginal plug the next morning was taken as evidence of copulation. Control mice were administered the carboxymethylcellulose vehicle, 2.5 ml/kg, subcutaneously, at 00:09h on pro-oestrus and mated in the same manner as the progesterone treated mice.

The effect of progesterone on egg transport was determined by killing a progesterone treated and a vehicle treated group of mated mice on each day of Days 1 to 4 of pregnancy and measuring egg positions. Comparisons of egg positions were performed using a students t-test.

In vivo oviduct calcium levels were determined on Days 1-5 of pregnancy in progesterone and vehicle treated mated mice by atomic absorption spectroscopic analysis, as described in the methods section. Statistical analysis of oviduct calcium levels was carried out using a multiple range t-test.

Results

The effect of combinations of oestradiol and progesterone on the in vitro oviduct perfusion preparation

Oestradiol (0.001 µg/ml) plus progesterone (0.1 µg/ml) in the perfusion fluid produced no significant changes in egg position, strength of motility or frequency of contraction over a 20 hour period (Tables 31-33).

Reduction of the oestradiol concentration to 0.001 mg/ml had little effect on the in vitro oviduct preparation (Tables 34-36). A

slight decrease in strength of motility and frequency of contraction occurred at 6-12 hours but this was not significant. At 1 hour and 17-20 hours no differences could be seen.

The effect of pregnenolone, 1 µg/ml, on the in vitro oviduct perfusion preparation

Pregnenolone, 1 µg/ml, in the perfusion fluid was ineffective in altering the frequency of contraction of the oviduct (Table 37). There was also no significant change in egg position occurring.

The effect of preovulatory administration of progesterone on in vivo egg transport

Progesterone, 20 mg/kg, administered on the morning of pro-oestrus caused an acceleration of egg transport along the oviduct (Table 38). Acceleration had started to occur by the afternoon of Day 1 of pregnancy (12hpc), the eggs had traversed a significantly larger ($P < 0.01$) percentage of the oviduct than the vehicle controls, approximately 40% of the eggs had left the ampulla on Day 1 and entered the isthmus. On Days 2 and 3 the acceleration continued and the eggs were still significantly further along the oviduct ($P < 0.01$ and $P < 0.001$ respectively). By Day 3 approximately 90% of the eggs had entered the uterus in the progesterone treated mice whereas in the vehicle controls the eggs do not enter the uterus until Day 14.

The effect of progesterone administration on in vivo oviduct calcium levels during early pregnancy

Progesterone, 20 mg/kg, administered on the morning of pro-oestrus altered the pattern of oviduct calcium levels during egg transport in vivo (Table 39). The vehicle controls showed the characteristic Day 2 fall followed by a rise on Day 3 ($P < 0.05$) with calcium levels still increasing on Day 4 and falling off on Day 5. Following progesterone treatment calcium levels are significantly lower on Day 1 than in the controls ($P < 0.01$). After Day 1 the calcium levels

start to rise and continue rising until Day 4 when they become significantly higher than Day 1 ($P < 0.01$).

Discussion

In vivo administration of progesterone to intact or ovariectomized, oestrogen treated rabbits has been shown to increase the amplitude and decrease the frequency of oviductal contractions (Boling and Blandau, 1971a, 1971b; Coutinho, 1973) and because of this De Mattos and Coutinho (1971) have considered progesterone facilitatory to egg transport. Controversy still exists however and Harper (1966) and Spilman and Harper (1974) postulate that progesterone retards egg transport by depression of oviductal muscle activity. The results obtained *in vitro* give little indication of progesterone being active on the smooth muscle of the mouse oviduct, in the presence of oestradiol. The only exception being when the ratio of oestradiol to progesterone was 1:1000 respectively and a slight decrease in frequency of contraction and strength of contraction occur. Coutinho (1973) observed that *in vivo* when oestradiol and progesterone were given in combination, unless the respective ratio is greater than 1:1000 respectively then oestrogenic effects predominate. The purpose of the experiments *in vitro* was to try and provide the oviducts with a hormonal background similar to that *in vivo*, rather than look for pharmacological effects of progesterone and in this case the presence of progesterone and oestradiol do not influence muscular contraction or the movement of eggs within the oviduct.

Pregnenolone was added to the *in vitro* perfusion fluid alone, further demonstrating the insensitivity of the mouse oviduct to hormonal agents during egg transport.

The ability of the oviduct to bind progesterone has been demonstrated in the human fallopian tube (Kumra, Sen, Hingorani and Tawar, 1974; Fuentealba, Escudero and Swaneck, 1976). Kurma et al., (1974) demonstrated that progesterone binds to a thermolabile molecule of protein nature, which appears to be different from corticosteroid binding globulin (CBG) as determined by ammonium sulphate precipitation. Progesterone receptors were found in cytosol from ampullary and isthmic segments and from the mucosal layer but not the muscular layer. The largest concentration of binding sites was found in the ampulla where the mucosal layer is predominant. As yet whether there is cyclic variation in the concentration of binding sites is unknown. ^{3}H-progesterone uptake *in vitro*, at midcycle has shown only the distal ampulla capable of retaining ^{3}H-progesterone, although receptors were detected in ampulla and isthmic segments in experiments with cytosol (Fuentealba, 1976).

If the isthmus of the mouse oviduct is not capable of uptake and retaining of progesterone then this would explain its lack of activity on the *in vitro* preparation.

Preovulatory exogenous administration of progesterone in mice causes an acceleration of egg transport beginning on Day 1 of pregnancy. Pauerstein et al., (1974a) and Gonzalez de Vargas et al., (1975) have also demonstrated this acceleration in the rabbit. The onset of acceleration on Day 1 and the finding that on Day 1 some of the eggs have already entered the isthmus indicates that an early relaxation of the AIJ is occurring. Pauerstein et al., (1974a) concluded that progesterone exerted its influence at both the AIJ and UTJ resulting in early entry of the eggs into the uterus. The time of administration and dose of progesterone are critical in determining the

final effect on egg transport. A progesterone-sensitive mechanism becoming operative prior to ovulation has been postulated (Gonzalez de Vargas et al., 1975). In the rabbit this mechanism comes in to play about 60 hours before ovulation and remains sensitive until at least 36 hours prior to ovulation, sensitivity at 36 hours being greater than at 60 hours. Once 'operational' the mechanism is dose dependent and once progesterone is administered the life span of the mechanism is only about 3 days. The existence of this mechanism suggests a preovulatory role for progesterone in the control of egg transport, perhaps overcoming oestrogenic dominance which is thought to delay eggs at the AIJ.

Investigation of oviduct calcium levels during egg transport after progesterone treatment reveal a change in pattern from the controls. Acceleration of egg transport starts to occur on Day 1 and calcium levels on this day were found to be at their lowest, gradually rising through to Day 4 when they were significantly higher than Day 1 levels.

It appears that progesterone is shifting the pattern of calcium levels forward by approximately 24 hours. The eggs are starting to enter the isthmus during Day 1 instead of Day 2 and calcium levels are at their lowest on Day 1 rather than on Day 2. As in the controls passage of the eggs through the isthmus is associated with rising calcium levels, but after progesterone administration the rise is more gradual, no significant change is observed until Day 4 by which time the eggs had entered the uterus about 24 hours earlier.

From these results the relaxation of the AIJ appears to be associated with low calcium levels, the day of lowest calcium levels corresponds to entry of eggs into the isthmus. After progesterone the 24 hour delay of eggs at the UTJ is not occurring either, the UTJ must be already relaxed allowing the passage of eggs through to the

uterus. Pauerstein et al., (1974a) in their study of the effects of oestrogen and progesterone on egg transport in rabbits postulated that progesterone exerted its influence at both the AIJ and UTJ allowing rapid passage of the eggs.

Batra and Bengtsson (1978) found progesterone capable of having an inhibitory effect on the contractile activity of uterine smooth muscle probably by decreasing the entry of calcium ions into the uterine cells, from the extracellular compartment. Hodgson and Daly (1976) in their studies on calcium uptake in the rabbit oviduct found that progesterone treatment decreased calcium uptake in the isthmus 24 hours after administration with HCG and increased the efflux of calcium.

Prior to ovulation progesterone appears to influence egg transport, exogenous administration at this time is capable of altering egg transport rates and oviduct calcium levels. Alteration of the rate of transport is brought about by changes in contractile activity of the oviduct and calcium binding and/or exchange may represent a site of hormonal control of contractile activity (Hodgson and Daly, 1976).

The effect of combination of oestradiol (0.001µg/ml) and progesterone (0.1µg/ml) on the in vitro oviduct perfusion preparation

Table 31

1 Hour Readings	Controls	+ Oestradiol 0.001µg/ml progesterone 0.1µg/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	30.1 ± 1.72 (8)	35.2 ± 4.85 (8)
strength of motility	2.75 ± 0.21 (8)	3.25 ± 0.31 (8)
frequency of contraction	12.75 ± 1.75 (8)	13.25 ± 0.65 (8)

Table 32

6-12 Hours Readings	Controls	+ Oestradiol 0.001µg/ml progesterone 0.1µg/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	28.2 ± 2.69 (8)	34.7 ± 4.35 (6)
strength of motility	2.6 ± 0.4 (8)	2.7 ± 0.61 (6)
frequency of contraction	10.8 ± 0.85 (8)	9.2 ± 2.14 (6)

Table 33

17-20 Hours Readings	Controls	+ Oestradiol 0.001µg/ml progesterone 0.1µg/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	30.3 ± 2.75 (8)	36.2 ± 4.72 (6)
strength of motility	1.75 ± 0.22 (8)	2 ± 0.46 (8)
frequency of contraction	6.5 ± 1.05 (8)	5.8 ± 1.32 (8)

% oviduct traversed and frequency of contraction, comparison by paired t-test

Strength of motility comparison, analysis of variance

n number of oviducts

The effect of combination of oestradiol (0.0001µg/ml) and progesterone (0.1µg/ml) on the in vitro oviduct perfusion preparation

Table 34

1 Hour Readings	Controls	+Oestradiol 0.0001µg/ml progesterone 0.1µg/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	51.5 ± 9.64 (5)	42.2 ± 6.88 (6)
strength of motility	3.4 ± 0.24 (5)	3.17 ± 0.17 (6)
frequency of contraction	13.2 ± 2.02 (5)	12 ± 1.37 (6)

Table 35

6-12 Hour Readings	Controls	+Oestradiol 0.0001µg/ml progesterone 0.1µg/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	61.53 ± 12.6 (5)	49.4 ± 12.13(5)
strength of motility	3.33 ± 0.67(5)	2.67 ± 0.33(5)
frequency of contraction	18.67 ± 3.53(5)	14 ± 4.62(5)

Table 36

17-20 Hour Readings	Controls	+Oestradiol 0.0001µg/ml progesterone 0.1µg/ml
	Mean ± S.E. (n)	Mean ± S.E. (n)
% oviduct traversed	53.2 ± 7.88 (5)	45.8 ± 6.38 (6)
strength of motility	1.6 ± 0.51 (5)	1.67 ± 0.24 (6)
frequency of contraction	7.2 ± 2.06 (5)	7.5 ± 1.45 (6)

% oviduct traversed and frequency of contraction, comparison by paired t-test

Strength of motility comparison, analysis of variance

n number of oviducts

Table 37 The effect of pregnenolone, 1µg/ml, on the in vitro oviduct perfusion preparation

Time hours	Frequency of contraction	
	Contractions/min Mean ± S.E.	
	Controls	Pregnenolone 1µg/ml (n)
1	12 ± 1.26	*10.8 ± 1.74 (5)
2	14.8 ± 0.77	15.2 ± 0.48 (5)
4	13.6 ± 1.8	14 ± 1.10 (5)
20	3.8 ± 0.86	3.6 ± 0.54 (5)

Comparison paired t-test

* $P < 0.05$

n number of pairs of oviducts

Table 38 The effect of preovulatory progesterone, 20mg/kg, administered on the day of pro-oestrum, 00:09h, on in vivo egg transport

Hours post coitum	Mean % of oviduct traversed by eggs	
	Controls Mean ± S.E. (n)	Progesterone Mean ± S.E. (n)
12	16.0 ± 1.47 (14)	26 ± 2.89 (16)
36	47.8 ± 4.27 (16)	**66.9 ± 5.35 (16)
60	65.4 ± 3.9 (16)	***91.3 ± 3.98 (16)
84	101 ± 0.0 (16)	101 ± 0.0 (16)

Progesterone treated groups compared to control groups, Students t-test

** $P < 0.01$

*** $P < 0.001$

n numbers of oviducts

Table 39 The effect of progesterone, 20mg/kg, administered on the day of pro-oestrus (09:00h), on in vivo oviduct calcium levels during early pregnancy

Day of pregnancy	Oviduct calcium content ug/mt wet weight of tissue	
	Controls Mean ± S.E. (n)	Progesterone Mean ± S.E. (n)
1	+0.59 ± 0.05 (8)	**0.28 ± 0.04 (9)
2	0.35 ± 0.02 (10)	0.43 ± 0.04 (7)
3	+0.59 ± 0.07 (7)	0.46 ± 0.02 (7)
4	++0.62 ± 0.02 (8)	++0.63 ± 0.08 (8)
5	++0.57 ± 0.02 (8)	0.45 ± 0.06 (6)

Comparisons by multiple range t-test.

** $P < 0.01$ comparison between control and progesterone group

+ $P < 0.05$)
++ $P < 0.01$) comparison to Day 2 within treatment

n number of mice

Chapter 6: The effect of various pharmacological agents on the in vitro oviduct preparation

Introduction

Various physiological and anatomical mechanisms have been implicated in the control of egg transport through the mammalian oviduct. The sympathetic nervous system and prostaglandins have received much attention and are thought to influence the muscular activity of the oviduct (Brundin, 1965; Spilman and Harper, 1975).

Brundin (1965) first suggested the adrenergic innervation was important in the control of egg transport and proposed that the isthmus acts as an adrenergically controlled sphincter, under hormonal influence, Pauerstein et al., (1974b) reiterated this view. Adrenergic receptors, both α-excitatory and β-inhibitory, have been demonstrated in a number of species (Brundin, 1969; Pauerstein et al., 1974). Pharmacological alteration of egg transport and oviduct contractility can be shown with adrenergic agents (Longely et al., 1968; Marshall, 1973; Polidoro et al., 1973).

Prostaglandins (PGs) are known to effect oviduct motility (Spilman and Harper, 1975), *in vitro* studies, on the human fallopian tube, have shown that the PGE series tend to be inhibitory and the PGF series, stimulatory. There is some variation in their effects depending on the segment of oviduct chosen and the stage of the menstrual cycle. Ingleman-Sundberg (1971) suggests that there might also be differential effects on the longitudinal and circular smooth muscle layers. These *in vitro* effects have also been observed *in vivo*, in rabbits and primates and Spilman and Harper (1975) have proposed that: PGEs might promote transport of eggs into the uterus by stimulation

of longitudinal and inhibition of circular smooth muscle activity; and PGFs might retain eggs by a reverse action i.e. inhibition of longitudinal and stimulation of circular smooth muscle.

The in vitro oviduct perfusion preparation presents an opportunity to observe the effects of adrenergic agonists: adrenaline, which stimulates both α and β receptors and isoprenaline which has almost exclusively β receptor activity; and PGE_1 and $PGF_{2\alpha}$, on oviductal smooth muscle contractility. If they are capable of influencing muscle motility any consequent effects on egg transport could be observed.

In addition to PGE_1 and $PGF_{2\alpha}$ the prostaglandin endoperoxide, U-46619, was also investigated. Prostaglandin endoperoxides are thought to be intermediates in the enzymic conversion of certain polyunsaturated fatty acids into prostaglandins (Samuelsson, 1972). They are potent smooth muscle stimulants and are considered to be more effective than their degradation products i.e. PGs, in some systems (Hamberg, Hedgvist, Strandberg, Svensson and Samuelsson, 1975).

To elucidate further the influence of calcium ions on oviduct function a calcium ionophore, X537A, was added to the perfusion fluid. Ionophores are mobile carriers of ions, those of biological origin are usually products of the genus Streptomyces. X537A is a linear molecule with a single negative charge due to the presence of a carboxyl group. It cyclizes by hydrogen bonding between groups at either end of the molecule to form a cavity lined with bonding atoms which have an affinity for the carried ions. X537A is a carrier for divalent cations, it is able to dissipate ion gradients by acting as an exchange diffusion carrier and capable of introducing the particular ion into the cell. Ionophores have been used to mobilise calcium in single muscle fibres (Hainaut and Desmedt, 1974)

and to increase cardiac contractility (Schaffer, Safer, Scarpa and Williamson, 1974; de Guzman and Pressman, 1974; Holland, Steinberg and Armstrong, 1975). In addition to observing X537A's effect on oviduct muscle function its effect on the calcium content of the oviduct was measured.

Methods

The effects of adrenaline, isoprenaline, PGE_1 and $PGF_{2\alpha}$, U-46619 and X537A on the *in vitro* oviduct preparation were investigated on Day 2 of pregnancy. The oviduct preparations were set up as described in the methods section, using two perfusion circuits an oviduct from each animal going to each circuit, a control and a test circuit.

1, 10 and 20 μM concentrations of adrenaline were added to the perfusion fluid and 20μg/ml ascorbic acid to prevent oxidation of the adrenaline. As further precaution against oxidation the reservoir of Ringer solution was cooled, in a fridge, for a few hours before adding the adrenaline and during the experiment it was protected from the light. Observations on egg position oviduct motility and frequency of contraction were made for up to 22 hours.

Isoprenaline, 1 μM, was investigated in the perfusion fluid, 20μg/ml ascorbic acid was added to prevent oxidation of the isoprenaline and the reservoir cooled and protected from the light as for adrenaline. Egg position, oviduct motility and frequency of contraction were observed for 6 hours.

Prostaglandins E_2 (2.5, 20, 25, 50 and 100ng/ml) and $F_{2\alpha}$ (2.5, 5 and 50ng/ml) were only investigated on small numbers of oviducts, owing to limited supplies, for indications of any alterations of oviduct function.

The prostaglandin endoperoxide, U-46619, was not added to the perfusion fluid in the reservoir, because of its instability, an infusion pump was used which injected the U-46619 solution into the perfusion circuit near the oviduct chambers. A 100ng/ml solution was infused at the rate of 30ng/hour into the perfusion fluid which was flowing at the rate of 0.5ml/min. For these experiments only one oviduct per circuit was utilized and the observations were carried out for 4 hour periods.

X537A was added to the perfusion fluid in the reservoir at concentrations of 0.1 and 1 μM. The effect of 0.1 μM X537A was observed for a 3 hour period and 1 μM X537A for 18 hour periods.

Oviduct calcium levels after perfusion with 1 μM x537A for 18 hours were measured by atomic absorption spectroscopy and compared to the control perfusions.

Comparisons of the effects of these pharmacological agents on frequency of contraction of the oviduct in vitro was by a paired t-test and comparison of oviduct calcium levels by a students t-test.

Results

The effect of adrenaline on the oviduct perfusion preparation in vitro

All three concentrations of adrenaline, 1, 10 and 20 μM, had no significant effect on egg position. Frequency of contraction was used as the parameter for comparison of adrenalines effects on the oviduct (Tables 40-42). For the first hour 1 μM adrenaline appeared to slightly decrease the frequency of contraction but for the remainder of the experiment no differences could be seen from the controls (Table 40). 10 μM adrenaline significantly increased the frequency of contraction within the first 15 minutes ($P < 0.05$) almost doubling the rate and this was also the case after 30 minutes

($P < 0.001$). The increased contraction rate was observed at 1 hour ($P < 0.01$) and 2 hours ($P < 0.05$) and for the remainder of the experiment the frequency was always greater than the controls but not significantly (Table 41). 20 μM adrenaline had no effect on the oviduct (Table 42).

The effect of isoprenaline on the oviduct perfusion preparation in vitro

Only one trial experiment was performed with isoprenaline at 1 μM for six hour periods. No effects were observed on egg position and although the frequency of contraction appeared to be slightly lower this was not significant (Table 43).

The effect of prostaglandins E_1 and $F_{2\alpha}$ on the oviduct perfusion preparation in vitro

PGE_1 and $PGF_{2\alpha}$ at the various concentrations investigated showed no indications of any effects on the oviduct preparation *in vitro*.

The effect of prostaglandin endoperoxide analogue U-46619 on the oviduct perfusion preparation in vitro

Over the 4 hour period U-46619 had no effect on egg position and the frequency of contraction was not significantly different from the controls (Table 44).

The effect of calcium ionophore X537A on the oviduct perfusion preparation in vitro

Initially 0.1 μM X537A was added to the perfusion fluid and the oviducts observed for 3 hour periods. No discernable effect was seen on the frequency of contraction (Table 45) and no change in egg position occurred. A higher concentration of X537A (1 μM) was then investigated over a period of 18 hours. Again no change in egg position occurred and the frequency of contraction resembled that in the controls (Table 46).

In vitro oviduct calcium levels showed no significant difference from control perfusions after perfusion with 1 μM X537A (0.70 ± 0.13 compared to 0.79 ± 0.16 ug/mg wet weight of tissue, $n = 5$ and 6 pairs of oviducts respectively, $t = 1.21$).

Discussion

The autonomic nervous system has been regarded as a regulatory mechanism in the contractile pattern of mammalian oviductal smooth muscle and consequently considered important in egg transport (Bell, 1972; Marshall, 1973; Black, 1974). The hormonal state of the animal also seems important in determining the response of the smooth muscle to autonomic stimulation and the concentration of neurotransmitter in the tissue. However, the majority of the work in this field has been done in the rabbit and it is possible that this species is atypical, the adrenoceptors of the cat have been shown to differ from the rabbit (Kendle and Lam Shang Leem, 1976). 10 μM adrenaline caused an increased frequency of contraction of the mouse oviduct, this stimulatory effect on the oviductal smooth muscle suggests the presence of α-adrenoceptors in the mouse oviduct. Why this effect was only significant for 2 hours is uncertain, the tissue may not be capable of maintaining such a high frequency of contraction for long periods or the energy supply i.e. oxygen and glucose might not have been sufficient to sustain the increased activity. Another possibility is oxygenation of the adrenaline in the perfusion fluid especially during passage through the heating coils. 1 μM adrenaline must have been too low a concentration to stimulate the tissue. 20 μM adrenaline might have been expected to show a potentiated effect compared to the 10 μM response, unless the effects of β-adrenoceptor stimulation were now being observed, as these would be inhibitory and would antagonise the α-adrenoceptor responses.

Although adrenaline was capable of increasing the frequency of contraction this did not result in progression of the eggs along the isthmus. This could indicate that adrenergic activity is not a controlling factor in the transport of eggs unless the hormonal background is also important for control of adrenergic response. Johns

et al., (1975) have shown, using chemical sympathectomy techniques, that an intact adrenergic innervation is not necessary for fertility in mice. It is possible that the importance of the adrenergic system varies from species to species.

At the concentration of isoprenaline used (1 μM) there is only an indication of an inhibitory effect which would be through β-adrenoceptor stimulation, but this was not significant.

The physiological role of prostaglandins in the control of oviduct function is as yet unclear. Spilman (1976) postulated that pre-ovulatory increases in ovarian steroid secretions could be stimulating PGF synthesis in the oviductal isthmus in a sequential fashion and post-ovulatory increases in progesterone secretion may then cause a decrease in tissue concentrations of PGF and a decrease in the response of the isthmus to $PGF_{2\alpha}$, allowing a progressive movement of the eggs.

The rabbit oviduct is capable of binding PGE_1 and $PGF_{2\alpha}$ during oestrus and pregnancy (Spilman, 1976) and the changes in specific binding of prostaglandins by the oviduct (Wakeling and Spilman, 1973) correlate with functional changes during the time of egg transport and with observed changes in the response of the oviduct to prostaglandin administration (Spilman, 1974b). Spilman (1976) proposed that a high binding of $PGF_{2\alpha}$ relative to PGE_1, in the isthmus of oestrus rabbits, may be important in the maintenance of oviductal tone and the retention of eggs in the oviduct. A decrease in $PGF_{2\alpha}$ binding and an increase in PGE_1 binding, found 72 hours after mating, may then reduce oviductal occlusion allowing passage of eggs into the uterus. However it is not clear how this mechanism operates physiologically.

The ability of prostaglandins to alter oviduct function may depend on time of administration relative to ovulation. Temporal effects to subcutaneous administration in rabbits have been observed (Aref, Hafez and Kamar, 1973; Chang, Saksena and Hunt, 1974).

Prostaglandins showed no alteration of oviduct function in the mouse *in vitro* oviduct preparation and neither did the prostaglandin endoperoxide, U-46619, which would possibly have been expected to be more potent. Recent work in the guinea-pig (Maia, Salinas, Fernandez and Pauerstein, 1977) showed $PGF_{2\alpha}$ and PGE_2 to be uneffective in altering *in vivo* tubal transport rates, suggesting that the role of prostaglandins is subject to species variation although prostaglandins may play a role in the control of egg transport in the rabbit this might not be true for other species. The doses of PGE_1, $PGF_{2\alpha}$ and U-46619 used in this *in vitro* preparation are capable of causing responses in other smooth muscle tissues i.e. rat stomach strip and rabbit aorta. The mouse oviduct might therefore not be sensitive to prostaglandins, at least at the time investigated.

The ionophore X537A has a broad range of specificity, it will act as a carrier for all the alkali metal cations, some divalent cations e.g. calcium ions and primary amines (ethanolamine, noradrenaline and dopamine). At the concentrations used (0.1 and 1 μM) X537A did not affect oviductal smooth muscle activity. This could be due to its lack of specificity or too low a concentration. From the measurements of oviduct calcium levels X537A is not causing calcium entry into the oviduct smooth muscle cells. Ionophores do not exactly mimic the physiological process of ion entry and there must also be a counter cation to effectively 'load' the ionophore, this is probably magnesium which is the major divalent cation found intracellularly, if calcium is the cation entering the cell.

The results from this chapter indicate that the adrenergic innervation in the mouse is not a major factor in the control of egg transport, which agrees with the findings of Johns et al., (1975). Prostaglandins

were also ineffective in altering oviduct function, as is the case in the guinea-pig (Maia et al., 1977). X537A failed to reveal more information on the role of calcium ions in the control of egg transport.

Table 40 The effect of 1μM adrenaline on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min, mean ± S.E. (n)	
	Controls	1μM adrenaline
0.5	14 ± 2.45	10 ± 1.26 (5)
1	18 ± 2.45	10 ± 1.26 (5)
2	17.6 ± 1.6	18.8 ± 1.2 (5)
3	16 ± 2.19	18.4 ± 2.4 (5)
4	15.6 ± 1.83	14.8 ± 1.49 (5)
6-8	15.2 ± 1.62	15.6 ± 1.47 (5)
18-22	4 ± 0.84	4.2 ± 0.92 (5)

Adrenaline perfusion compared to controls paired t-test

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

n number of pairs of oviducts

Table 41 The effect of 10μM adrenaline on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min, mean ± S.E. (n)	
	Controls	10μM adrenaline
0.17	10 ± 1.26	17 ± 3.68 (6)
0.25	11 ± 1.98	*20.33 ± 4.72 (6)
0.5	11.45 ± 1.18	***22 ± 2.17 (11)
1	15 ± 1.44	**21.67 ± 2.09 (6)
2	15.77 ± 1.35	*20.67 ± 1.91 (9)
3	17.33 ± 0.99	21 ± 2.46 (6)
4	18.29 ± 0.68	21 ± 1.87 (7)
18-22	4.8 ± 2.06	8.8 ± 1.02 (5)

Adrenaline perfusion compared to controls paired t-test

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

n numbers of pairs of oviducts

Fig.10 The effect of 1μM adrenaline on the frequency of contraction of the oviduct preparation on in vitro

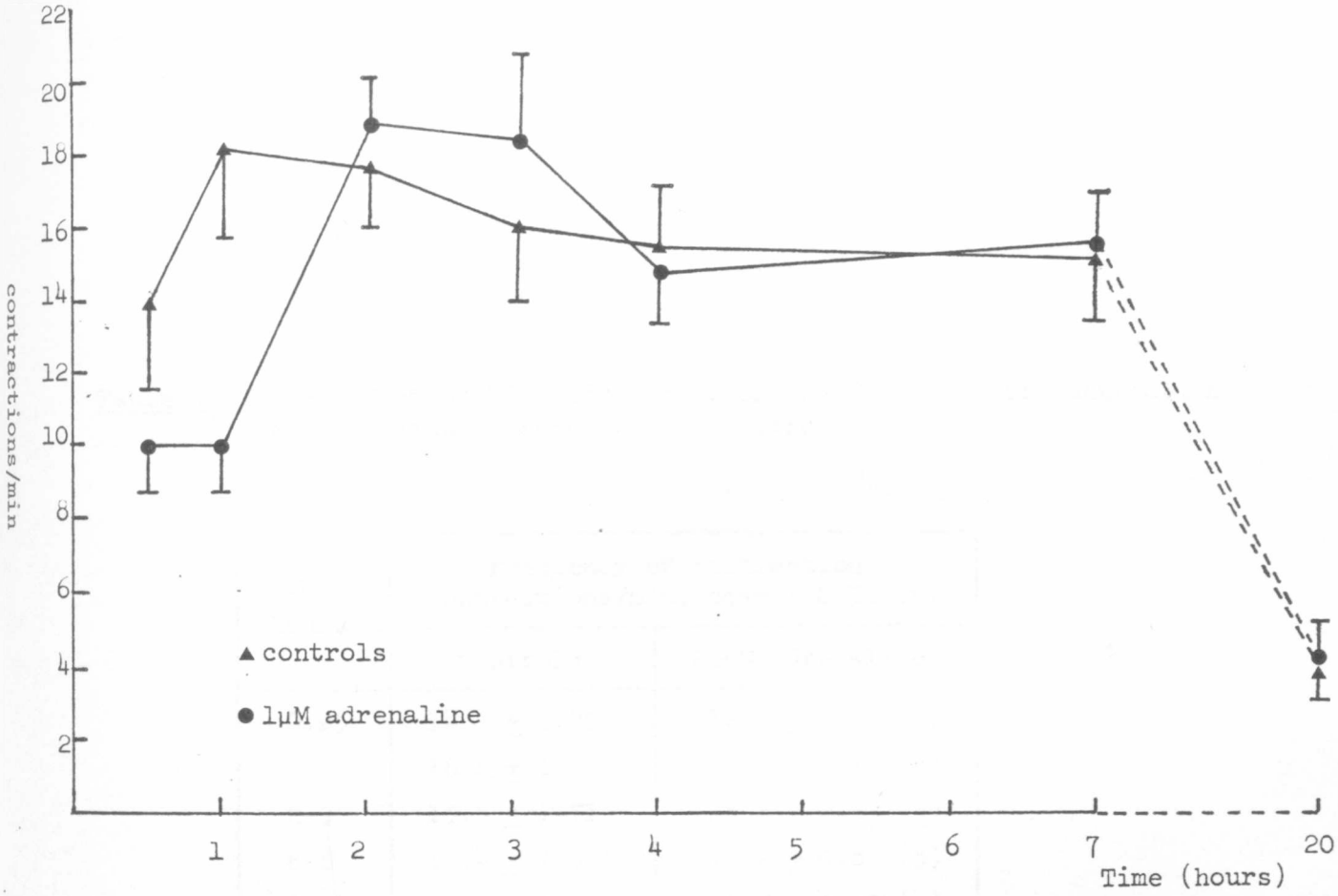


Fig. 11 The effect of 10μM adrenaline on the frequency of contraction of the oviduct preparation in vitro

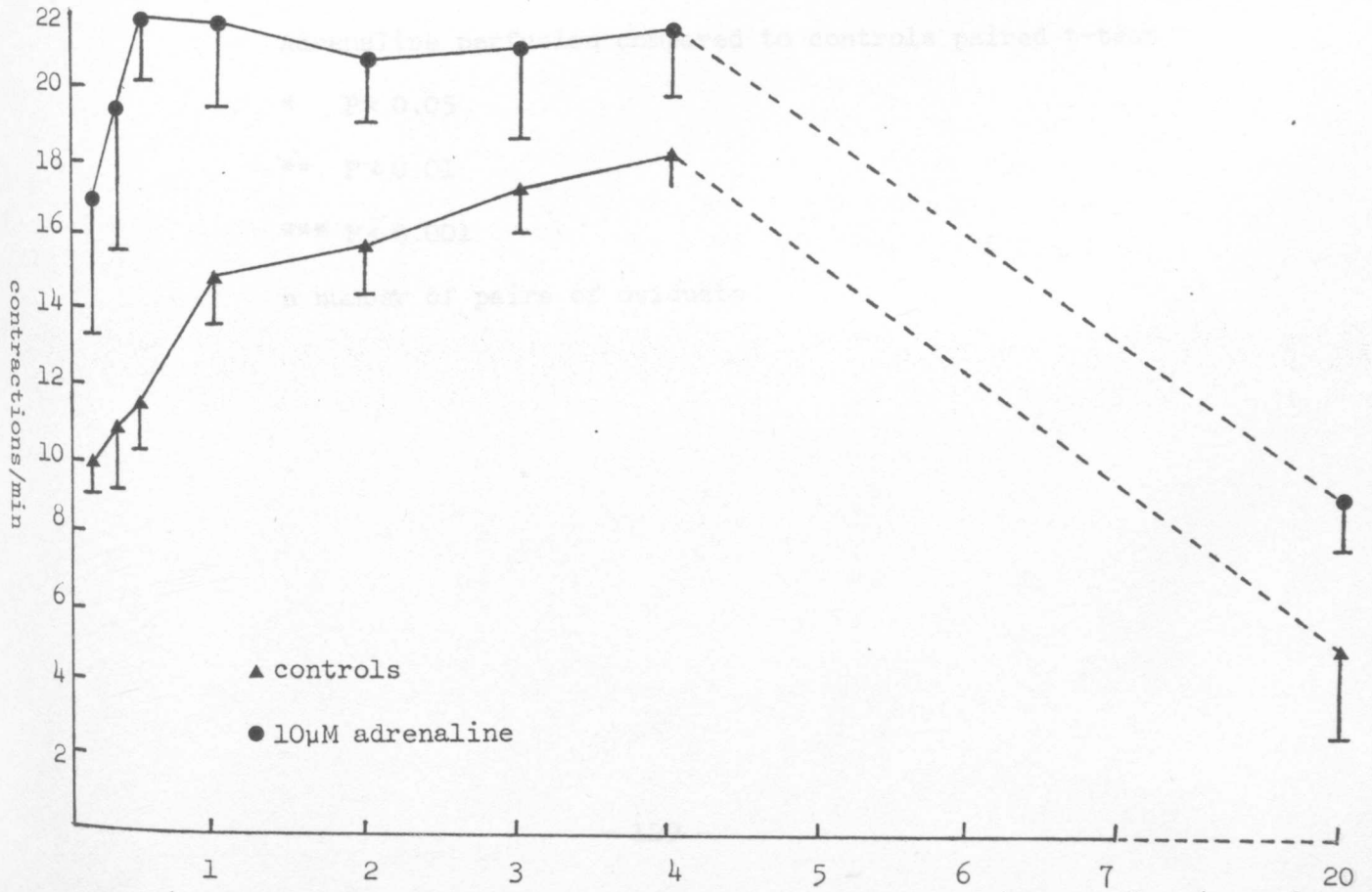


Table 42 The effect of 20μM adrenaline on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min, mean ± S.E. (n)	
	Controls	20μM adrenaline
0.25	14 ± 2.25	12 ± 3.44 (5)
1	16.8 ± 1.5	18 ± 1.79 (5)
2-3	15.5 ± 1.71	16.5 ± 2.63 (4)
6-8	13.2 ± 1.85	13.2 ± 0.8 (5)
18-22	3.25± 1.25	4.5 ± 2.22 (4)

Adrenaline perfusion compared to controls paired t-test

* $P < 0.05$

** $P < 0.01$

*** $P < 0.001$

n number of pairs of oviducts

Fig. 12 The effect of 20μM adrenaline on the frequency of contraction of the oviduct preparation in vitro

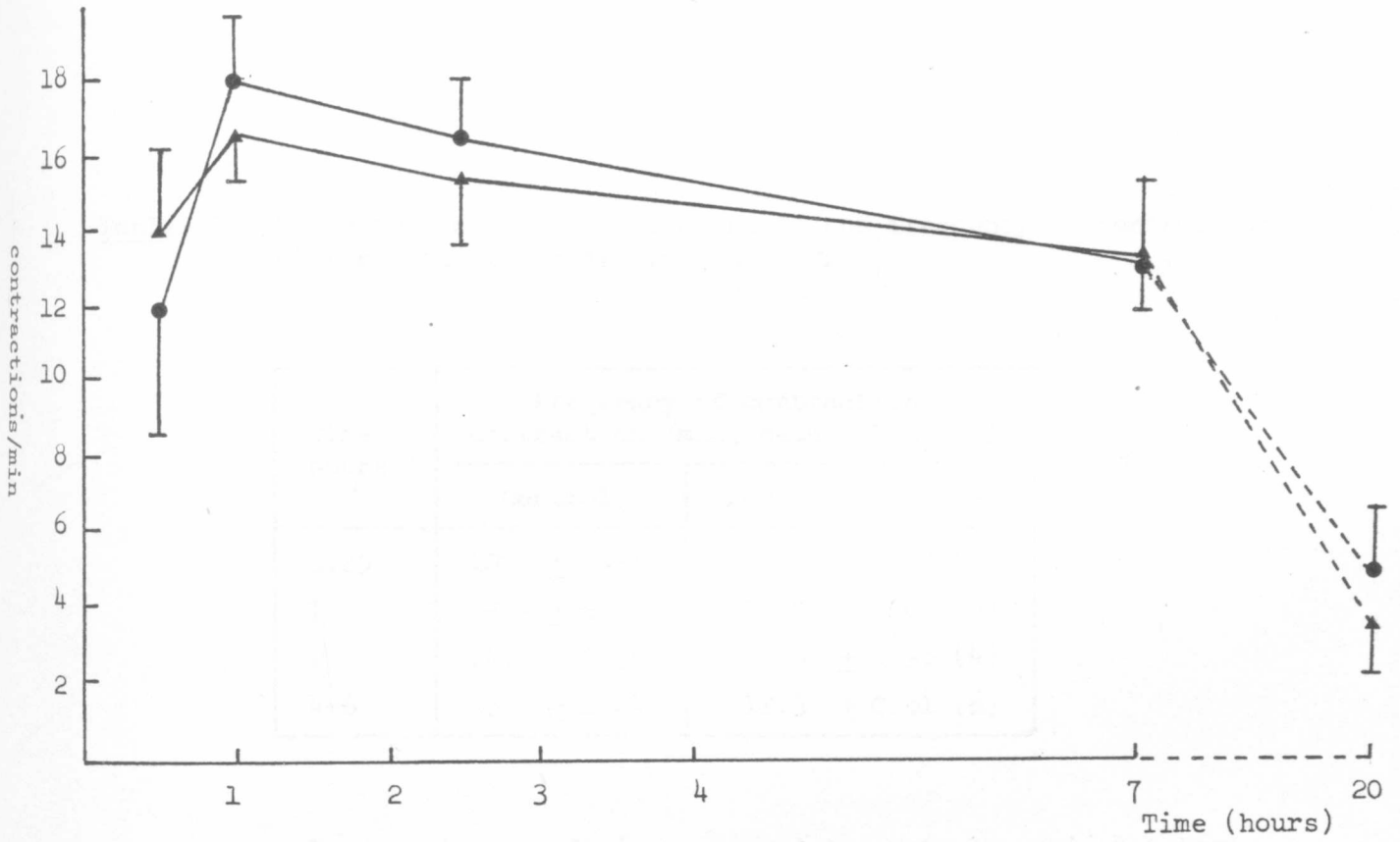


Table 43 The effect of 1μM isoprenaline on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min, mean ± S.E. (n)	
	Controls	1μM isoprenaline
0.25	10 ± 1.41	8.4 ± 1.17 (5)
1	12.4 ± 1.6	10.8 ± 0.8 (5)
2	14.5 ± 2.36	12.5 ± 0.96 (4)
4-6	13 ± 1.24	12.3 ± 0.61 (6)

Isoprenaline perfusion compared to controls, paired t-test

n number of pairs of oviducts

Table 44 The effect of endoperoxide analogue U-46619 (1ng/ml) on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min, mean ± S.E. (n)	
	Controls	U-46619, 1ng/ml
0.25	9 ± 1.73	9.5 ± 1.55 (4)
1	14.8 ± 1.02	14.8 ± 1.2 (5)
4	14.4 ± 0.75	15.6 ± 1.33 (5)

U-46619 perfusion compared to controls paired t-test

n number of pairs of oviducts

Table 45 The effect of calcium ionophore X537A (1μM) on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min, mean ± S.E. (n)	
	Controls	1 X537A
0.5	9.6 ± 0.98	10.4 ± 0.75 (5)
1	15.2 ± 1.36	16 ± 0 (5)
3	16.4 ± 1.6	16.8 ± 0.49 (5)

Table 46 The effect of calcium ionophore X537A (1μM) on the frequency of contraction of the oviduct preparation in vitro

Time hours	Frequency of contraction contractions/min, mean ± S.E. (n)	
	Controls	1 X537A
0.5	8.8 ± 1.11	8.6 ± 1.52 (6)
1	13.4 ± 1.29	13.7 ± 1.27 (7)
2	15.1 ± 1.56	14.3 ± 1.19 (7)
4	12 ± 0.98	13.7 ± 0.81 (7)
6-8	8.3 ± 0.96	7.9 ± 1.23 (8)
18	6.6 ± 1.47	5.6 ± 0.79 (5)

X537A perfusion compared to controls, paired t-test

n numbers of oviducts

Chapter 7: The role of the ovaries and the uterus during egg transport in the mouse oviduct

Introduction

Croxatto (1974) in a review of the physiological regulation of egg transport stated that the classic concept is that a characteristic pattern of transport is activated by the hormonal changes accompanying ovulation resulting in the timely arrival of the eggs into the uterus. The role of the ovaries in egg transport has been studied since the beginning of this century but since the works of Alden (1942) and Whitney and Burdick (1939) in the rat and mouse respectively, little attention has been paid to the effects of ovariectomy in these species.

Whitney and Burdick (1939) observed that ovariectomy after ovulation in the mouse caused a retardation of egg transport and egg degeneration, oestrogen treatment failed to reverse this effect. In the rat, however, Alden (1942) found the presence of the ovary not to be vital to normal egg transport and development. Ovariectomy in the rabbit (Adams, 1958) after ovulation does not alter transport rates but in chronically ovariectomised rabbits transferred eggs are subject to accelerated transport (Noyes, 1959; Noyes et al., 1959).

As the effect of ovariectomy in the mouse differed from the results found in the rat and rabbit, reinvestigation of the effect of post ovulatory ovariectomy on egg transport in the mouse was carried out.

The oviduct and uterus are both smooth muscle structures, the oviduct running intramurally into the uterus at the UTJ. The oviduct shows various kinds of contractile activities, always associated with spike discharges, which are analogous to other visceral smooth muscle and there is no barrier between the oviduct and the uterine horn to

block transmission of excitation (Nishimura, Nakajima and Hayashi, 1969). The oviduct of rabbits shows multipacemaker activity with changing pacemaker location although the highest frequency of generation is the distal isthmus (Talo, 1974). To what extent the oviduct and uterus influence the activity of each other is not clear. If there is no block to electrical transmission between the two organs then their pacemaker activities could influence each other.

The lumen of the oviduct is fluid filled, the volume of fluid varying during egg transport, on Day 1 of pregnancy in the mouse and rat the ampulla is swollen with fluid and this swelling subsides by Day 2. As the lumen of the oviduct is continuous with the lumen of the uterus the latter could influence oviduct intraluminal pressure.

The effect of both ligating and cauterizing the uterus just below the UTJ was investigated. Simple ligation would only close the lumen but cauterization would destroy any electrophysiological connection between the oviduct and the uterus.

Methods

Ovariectomy was carried out as described in the Methods section. Three groups of mated female mice were ovariectomised on the afternoon of Day 1 pregnancy (12-14hpc). A further four groups of mated mice were sham operated, at 12-14hpc, i.e. the ovaries were exposed and the ovarian bursa broken. Initially the operations were carried out on the morning of Day 1 (9hpc) but at this time the ovarian bursa was found to be very distended with fluid and the eggs were often lost along with the fluid.

An ovariectomised and a sham operated group was killed on each day of Days 2-4 of pregnancy at 36, 60 and 84hpc. The oviducts were removed, straightened and the position of the eggs measured and the

uteri were flushed out and the flushings examined microscopically for the presence of eggs.

Comparisons of egg positions were carried out using a Students t-test.

Ligation and cauterization of the uterus was carried out in mated female mice on Day 1 of pregnancy (12-14hpc). The mice were anaesthetised as described in the methods section and the oviductal end of the uterus exposed through a small lateral incision. Each animal served as its own control i.e. the uterine horn on one side was merely exposed and then returned to the abdomen whilst the other uterine horn was either ligated or cauterized. Ligation involved tying off the uterus just below the UTJ, with a strong cotton ligature, to restrict the lumen but not damage the uterine muscle. Cauterization involved exposing the uterus and destroying the muscle wall below the UTJ with a fine plantinum wire through which an electric current of 10 volts was passed. This also resulted in sealing off the lumen of the uterus at this point.

The mice were killed at either 36, 60 or 84hpc and the oviducts examined for the position of their contained eggs.

Comparisons of egg positions were carried out using a paired t-test, as each animal was serving as its own control.

Results

The effect of post ovulatory ovariectomy on egg transport

Examination of egg positions after ovariectomy detected no alteration in the rate of egg transport compared to the sham operated control mice (Table 47). The sham operated mice showed no differences in egg transport rates from previous data in untreated mice. After ovariectomy

the eggs enter the isthmus on Day 2, are found at the UTJ on Day 3 and have entered the uterus by Day 4.

The effect of ligation and cauterization of the uterus on egg transport

Ligation and cauterization of the uterus on Day 1 of pregnancy produced retardation of egg transport (Table 48). On Day 2 in both cases the ampulla of the oviducts were found to be still swollen and nearly 50% of the eggs were found in the ampulla, whereas in the controls the eggs had all entered the uterus. The mean percentage oviduct traversed by the eggs on Day 2 was significantly smaller after ligation or cauterization compared to the controls ($P < 0.05$).

On Day 3 after ligation and cauterization the swelling in the ampulla had subsided but egg transport was still retarded (Table 48). The eggs were found scattered through the isthmus whereas in the controls the eggs were found all to be at the UTJ. The mean percentage oviduct traversed by the eggs on Day 3 after ligation or cauterization of the uterus was still significantly smaller than in the controls ($P < 0.05$).

Unfortunately on Day 4 after ligation or cauterization location of the eggs was very difficult. The UTJ area had become opaque as a result of the operation, but as the eggs were not found in the isthmus they were considered to be at the UTJ.

In most of the oviducts after ligation or cauterization the area of the uterus above the operation area became swollen with fluid on Days 3 and 4 and the swollen ampulla found on Day 2 had subsided.

Discussion

Ovariectomy after ovulation was found to have no effect on egg transport along the mouse oviduct. These findings are at variance with those of Whitney and Burdick (1939) who found egg retention

occurring after postovulatory ovariectomy and postulated that this was the result of hormonal deficiency. However, their experiments were performed only on a small number of animals and the timing of ovariectomy and autopsy lacked precision, no two animals were investigated at the same time. The exact locations of the eggs were not specified simply their presence in either the oviduct or the uterus.

The results obtained here are in agreement with the finding of Alden (1942) and Adams (1958) in the rat and rabbit respectively. The hormonal output of the ovaries during egg transport does not appear essential for normal egg transport to occur. Ovariectomy was performed 12-14 hours after copulation had occurred and ovulation is occurring before this, therefore the influence of oestrogen and progesterone secreted immediately after ovulation cannot be ignored, but changing levels of oestrogen and progesterone during the time course of egg transport are not responsible for controlling the rate of transport.

Oestrogens and progesterone are capable of effecting oviduct muscle motility (Coutinho, 1973). The majority of work carried out to investigate the effects of exogenous ovarian steroids on oviduct motility has been performed in the rabbit and human and really only demonstrates pharmacological effects of these compounds, not their role *in vivo*. Preovulatory ovarian hormone levels have been postulated as being responsible for controlling the contractile activity of the oviduct during egg transport (Boling and Blandau, 1971a). An increased secretion of progesterone and 20α-hydroxypregn-4-en-3-one is known to occur in the rabbit prior to ovulation (Hilliard et al., 1963) and in the rat (Hori, Ide and Miyake, 1968; Miyake, 1968) and ewe (Moore, Barret, Brown, Schindler, Smith and Smyth, 1969) oestrogen secretion in ovarian venous blood decreases rapidly a few hours prior to ovulation. Preovulatory hormone secretion is thought to be more important in the

control of oviduct contractility and hence egg transport than post-ovulatory secretion which has been shown not to control egg transport.

If preovulatory ovarian steroid output controls egg transport this would be through effects on the pattern of oviduct contractility possibly by changes in smooth muscle physiology i.e. ionic permeability and distribution and protein synthesis.

The role of the uterus in the control of egg transport through the oviduct has received little attention. There is no barrier to the transmission of excitation between the two organs (Nishimura et al., 1969) therefore it is possible that the pacemaker activity in one could influence the other. Both these organs are hollow, the lumens fluid filled and the UTJ is the only barrier between the two lumens. Intraluminal oviductal pressure and luminal fluid movements are both factors that will influence egg transport (Blandau and Verdugo, 1976). In the mouse the ampulla is dilated with fluid on Day 1 of pregnancy and by Day 2 when the eggs are entering the isthmus the ampulla has lost its fluid, either by reabsorption, flow into the isthmus or loss into the peritoneal cavity.

Ligation and cauterization of the uterus prolonged the dilation of the ampulla with fluid until Day 2 and by Day 3 the initial portion of the uterus is swollen with fluid. Associated with the prolonged swelling of the ampulla is retardation of egg transport, implying that for the eggs to move normally into the isthmus on Day 2 the associated fluid must also move into the isthmus. Ligation or cauterization of the uterus will prevent free flow of fluid from the oviduct to uterus and increase the intraluminal back pressure which must be impeding the movement of the eggs. The ampular fluid is gradually lost and fluid builds up below the UTJ, indicating movement of fluid down the oviduct accompanies egg transport.

Cauterization was undertaken to determine whether block of electrical transmission between the oviduct and uterus effects egg transport. As the effect of cauterization was the same as that of ligation, the retardation of egg transport in this case must be the result of impeding the flow of luminal fluid through the isthmus to the uterus. Unfortunately the technique used for cauterization resulted in sealing off the uterus, where it had been hoped to leave the uterine lumen patent, to study the effect of blocking electrical transmission.

Table 47 The effect of postovulatory ovariectomy, at 12-14hpc, on the rate of egg transport through the mouse oviduct

Time of autopsy hpc	Mean percentage oviduct traversed by eggs	
	Sham operated Mean ± S.E. (n)	Ovariectomy Mean ± S.E. (n)
36	42.8 ± 5.61 (12)	42.6 ± 5.35 (12)
60	78.5 ± 4.89 (12)	73.6 ± 6.35 (10)
84	101 ± 0 (10)	101 ± 0 (10)

Statistical comparison by Students t-test

n number of oviducts

Table 48 The effect of uterine ligation or cauterization, at 12-14 hpc, on the rate of egg transport through the mouse oviduct

Time of autopsy hpc	Mean percentage oviduct traversed by eggs			
	Sham operated mean ± S.E.(n)	Ligation mean ± S.E. (n)	Sham operated mean ± S.E.(n)	Cauterization mean ± S.E.(n)
36	51.5±4.87 (10)	*31.8±2.93 (10)	47.9±3.59 (10)	*32.7±3.64 (10)
60	82.2±4.47 (10)	*52.8±5.33 (10)	89.3±3.09 (10)	*59.2±7.20 (10)
84	101 ± 0 (10)	- (10)	101 ± 0 (10)	- (10)

Statistical comparison by paired t-test

* $P < 0.05$

n number of oviducts

Chapter 8: The effect of prolactin on the mouse oviduct preparation in vitro

Introduction

The experiments described in this chapter were undertaken at Beecham Pharmaceutical's Medical Research Centre, in conjunction with Dr. J.D. Flack. Dr. Flack and his research team run a radioimmunoassay for prolactin release from rat anterior pituitary cells.

The *in vivo* secretion of prolactin by the anterior pituitary gland is normally inhibited by the hypothalamus (MacLeod and Lehmeyer, 1974). Dopaminergic neurones have been identified in the hypothalamus and they are involved in the regulation of prolactin release (Fuxe and Hokfelt, 1969; Kamberi, Mical and Porter, 1971; Hornykiewicz, 1974). Prolactin secretion is predominantly under inhibitory control, pituitary stalk section leads to excessive prolactin secretion (Meites, Nicoll and Talwalker, 1963; Meites and Nicoll, 1966).

Originally, dopamine was thought to cause the release of a prolactin-inhibiting factor (P.I.F.) similar to other hypothalamic regulatory hormones (Besser and Mortimer, 1974). However, P.I.F. has since been found to be predominantly catecholamine in nature (Schally, Arimura, Takahara, Redding and Dupont, 1974) and is now thought to be dopamine itself.

The preovulatory surge of lutineizing hormone (LH) occurring in the afternoon of pro-oestrus in the rat and at midcycle in women is thought to be due to an increased sensitivity to lutineizing hormone releasing factor (LHRH) (Gordon and Reichlin, 1974; Ferland, Borgent, Labrie, Bernard, De Lean and Raynaud, 1974) and oestrogens play a crucial role in this increased pituitary response to the neurohormone (Ferland, Drouin and Labrie, 1976; Drouin, Lagace and Labrie, 1976). The rate of prolactin secretion also increased during the afternoon of

pro-oestrus (Wuttke and Meites, 1970; Neill, Freeman and Tillson, 1971) and oestrogens have a stimulatory effect on prolactin secretion (Chen and Meites, 1970; Yen, Ehara and Siler, 1974).

As prolactin secretion is involved in the events leading up to ovulation in the rat it was decided to investigate whether prolactin could have any post-ovulatory effect on oviduct motility and egg transport, in vitro. This was carried out by linking perfused rat anterior pituitary cells with the mouse oviduct preparation in vitro.

Methods

Five male, Hooded Lister, rats (OLAC), weighing approximately 300g, were killed by a blow to the back of the neck and decapitated to avoid haemorrhaging. The brain was then exposed and carefully removed leaving the pituitary gland exposed but intact. The posterior pituitary, which sits on top of the two lobes of the anterior pituitary was removed first. The anterior pituitary was then removed, weighed, manually chopped and incubated in a siliconized beaker with 20 ml of trypsin solution (0.25% Krebs solution containing 2g/l glucose) at 37°C for 20-30 minutes. The trypsin solution was aerated (not bubbled) with 95% oxygen and 5% carbon dioxide and continually stirred. After incubation, the dispersed pituitary cells were poured into siliconized, plastic, centrifuge tubes and the remaining clumps of pituitary were reincubated with a further 20 ml trypsin solution before being poured into centrifuge tubes. The pituitary cells were spun at 1000 r.p.m. for 30 minutes, at 4°C and the resulting pellets were rehomogenized in 1 ml lima bean trypsin inhibitor (LBTI) solution (0.04g LBTI, 0.4g bovine serum albumen (BSA) and 10^{-8}M dopamine in 80 mls Krebs solution).

The pituitary cells were layered onto a pre-swollen biogel column (Biogel P-2, Bio-RAD CABS), after aerated LBTI solution had been first run through the column for a few minutes, to ensure the system was working.

Perfusion of the column continued with the LBTI solution after the addition of the pituitary cells at a rate of 1 ml per minute.

A Day 2 mouse oviduct in vitro preparation was set up as previously described in Chapter 1, using the modified Krebs solution (LBTI solution) instead of Ringer solution. Perfusion of the oviduct (1 ml per minute) commenced at least 30 minutes before pituitary cell perfusion started, to ensure viability of the oviduct before connecting the oviduct chamber to the perfusate from the pituitary cells. Oviduct motility and egg position were monitored every five minutes both before and after perfusion with the pituitary perfusate.

The addition of dopamine during both the preparation and perfusion of the pituitary cells prevents the secretion of prolactin. In order to release the prolactin either thyrotrophin releasing hormone (TRH), 25ng/ml or metaclopramide, 10^{-4}M, were added to the perfusion fluid. Perfusion of the pituitary cells last for a maximum of one hour thus only allowing prolactin release to be performed only one or two times.

Once the perfusate had passed through the oviduct chamber it was collected serially for prolactin radioimmunoassay, which was carried out using the reagents and protocol supplied by the Hormone Distribution Program (National Institute of Arthritis and Metabolic Diseases).

Results and Discussion

As the time spent performing these experiments was limited, insufficient results were obtained, which could be statistically evaluated. However, the in vitro oviduct preparation contracted as was previously observed in earlier experiments and the eggs moved backwards and forwards within the lumen of the oviduct. The use of a Krebs solution rather than Ringer solution did not appear to have any detrimental effect on the function of the oviduct.

When the pituitary perfusate was connected to the oviduct chamber, no apparent changes in oviduct muscle motility or egg position were seen. Both TRH and metaclopramide were used to cause prolactin release and again, after their addition, no oviduct or egg changes were observed.

In order to confirm prolactin release, radioimmunoassay of the serially collected perfusate was carried out. These assays were performed by the research team at Beechams, routinely and their results confirmed the successful release of prolactin.

Although no positive results were obtained from these experiments to suggest a role for prolactin in influencing oviduct motility and egg transport, this cannot be taken as conclusive.

The purpose of this work was also to gain an insight into biological research in an industrial environment and a chance to observe and participate in unfamiliar experimental techniques. This purpose at least, was fulfilled and beneficial.

General Discussion

The establishment of an *in vitro* oviduct preparation provided an opportunity to study the functioning of the mouse oviduct. The thin walls of the mouse oviduct allow observation of the contained eggs and the contractile activity of the smooth muscle walls. Removal of the oviduct and transferring it to a controlled environment made it possible to study the influence of ovarian hormones, pharmacological agents and calcium ions.

The oviduct preparation was viable *in vitro* for periods of up to 24 hours after the removal from the animal. Contractile muscular activity can be clearly seen and the muscle contractions are accompanied by back and forth movement of the eggs in the lumen of the isthmus, but no forward progression of the eggs occurs.

Alterations of the external calcium ion concentration were shown to produce changes in the frequency and strength of oviductal contractions. This effect would be expected in all smooth muscle preparations, as smooth muscle is capable of taking up relatively large amounts of calcium and there is a very rapid exchange between cellular and extracellular calcium (Lullman, 1970). The absence of a T-tubule system in smooth muscle necessitates rapid calcium exchange, spike activity involves calcium influx and there is a more direct relationship between action potentials and contraction than occurs in skeletal muscle (Prosser, 1974).

The failure of varying the external calcium concentration to produce forward egg movement suggests that the correct pattern of oviduct contractility is not obtained *in vitro*. It may be that this problem is intrinsic to the setting up of the preparation. The mesosalpinx is dissected from the oviduct, *in vitro*, to straighten it and allow

* It is perhaps also worth considering that the functional integrity of the entire oviduct may be impaired *in vitro* if the correct physiological environment is not provided to maintain viability of the mucosal layers, which may exert important modifying influences on muscular activity.

good observation of the eggs. Takeda and Doteuchi (1976) considered the mesosalpinx of the rabbit oviduct to be important during egg transport. They found that the mesosalpinx contracted spontaneously *in vitro* and responded to noradrenaline in a similar manner to the oviduct. Contraction of the mesosalpinx caused the oviduct to bend which they thought might occasionally obstruct or contribute to ovum transport. Therefore removal of the mesosalpinx could alter the pattern of oviduct contractility.

Another factor to consider is the severance of the oviduct from the uterus, just below the UTJ. This may effect the intraluminal pressure within the oviduct and/or cause loss of the luminal fluid to the perfusion medium. Kendle (1969) incubated mouse oviducts *in vitro* for 48 hours from Day 1 of pregnancy and in this case the mesosalpinx was left intact but the oviducts were separated from the uterus and no egg transport occurred. The absence of the mesosalpinx is not therefore the sole reason for lack of forward progressive movement of the eggs.*

For further investigation of the oviduct *in vitro* a preparation which consisted of the entire reproductive tract might provide more information on the importance of the anatomical arrangement of the oviduct in egg transport. The difficulty would be observation of the eggs during the experiment, measurement of egg positions would only be possible after the incubation period by dissection of the oviduct.

The various hormonal and pharmacological agents investigated *in vitro* provided little positive evidence to suggest that these compounds i.e oestrogen, progesterone, adrenergic agonists and prostaglandins are the controlling factors for egg transport *in vivo*. The possibility that they have contributing roles cannot be ruled out altogether.

The contractile activity of the smooth muscle of the oviduct is one of the primary factors in the movement of eggs along the oviduct (Blandau and Verdugo, 1976). How the contractile activity is controlled is unclear and the ovarian hormones have long been considered to play a role in the control of oviductal muscular activity (Harper, 1966; Boling and Blandau, 1971a, 1971b; Spilman and Harper, 1974). Evidence to date has demonstrated that the secretion of oestrogen and progesterone during the period of egg transport is not vital for normal transport to occur in the rat (Alden, 1942b) and rabbit (Adams, 1958). Postovulatory ovariectomy was shown to have no effect on egg transport in the mouse oviduct (Chap. 7). The role of the ovarian hormones appears therefore to be preovulatory, chronic ovariectomy causes acceleration of transferred eggs or radioactive spheres (Noyes, 1959; Noyes et al., 1959). If pre-ovulatory ovarian hormone secretion is involved in controlling the rate of egg transport by influencing the pattern of oviductal contractility this would then be through changes in the physiology of the smooth muscle.

Calcium ions play a vital role in the functioning of smooth muscle both in the electrical and mechanical events associated with contraction and relaxation (Lullman, 1970). The smooth muscle of the oviduct, like most visceral smooth muscle is spontaneously active and the electrical slow wave and spike activity is triggered by movement of calcium ions into the cell (Lui et al., 1969; Mayer, et al., 1972). The mechanical process of contraction requires calcium, varying with the type of smooth muscle, calcium may enter during the action potential, be released from cell membranes or be released from internal stores (Prosser, 1974). The availability of calcium ions for interaction with the contractile proteins influences the development of

tension in the smooth muscle cells (Batra and Bengtsson, 1978). Therefore calcium ion permeability, exchange, binding and concentration will all influence the contractile activity of the oviduct.

During normal egg transport in the mouse oviduct changes in the concentration of calcium ions occurred. Entry of the eggs into the isthmus through the AIJ on Day 2 was associated with a fall in the calcium concentration, isthmic transport was occurring when calcium levels were at their lowest and the block to transport at the UTJ on Day 3 came when calcium levels had risen again. This pattern of changes also occurred in all the vehicle treated controls. Figs. 13-14 graphically show the correlations of egg transport with the pattern of calcium ion concentrations during the various experiment procedures. The actual concentration of calcium varied between the different experiments and to overcome this and analyse the pattern of change rather than the actual concentrations the values were expressed in terms of percentage of the Day 2, 33hpc reading which in the untreated and vehicle treated controls is always the lowest value.

Progesterone treatment given on the day of pro-oestrus produced an acceleration of egg transport which has been shown to occur in the rabbit (Gonzalez de Vargas et al., 1975). The acceleration involved early entry of the eggs into the isthmus, more rapid transport along the isthmus and no prolonged block to transport at the UTJ. The dose of progesterone used (2µg/mouse) was not causing a significantly early ovulation as the stage of development of the eggs did not differ from the controls. After progesterone treatment the eggs started to enter the isthmus on Day 1, indicating an early relaxation of the AIJ and on Day 1 the calcium ion concentration was found to be at its lowest, a day earlier than in the controls.

Fig. 13 Graphical representation of the effects of oestrogen, progesterone and reserpine treatment on egg transport through the mouse oviduct in vivo

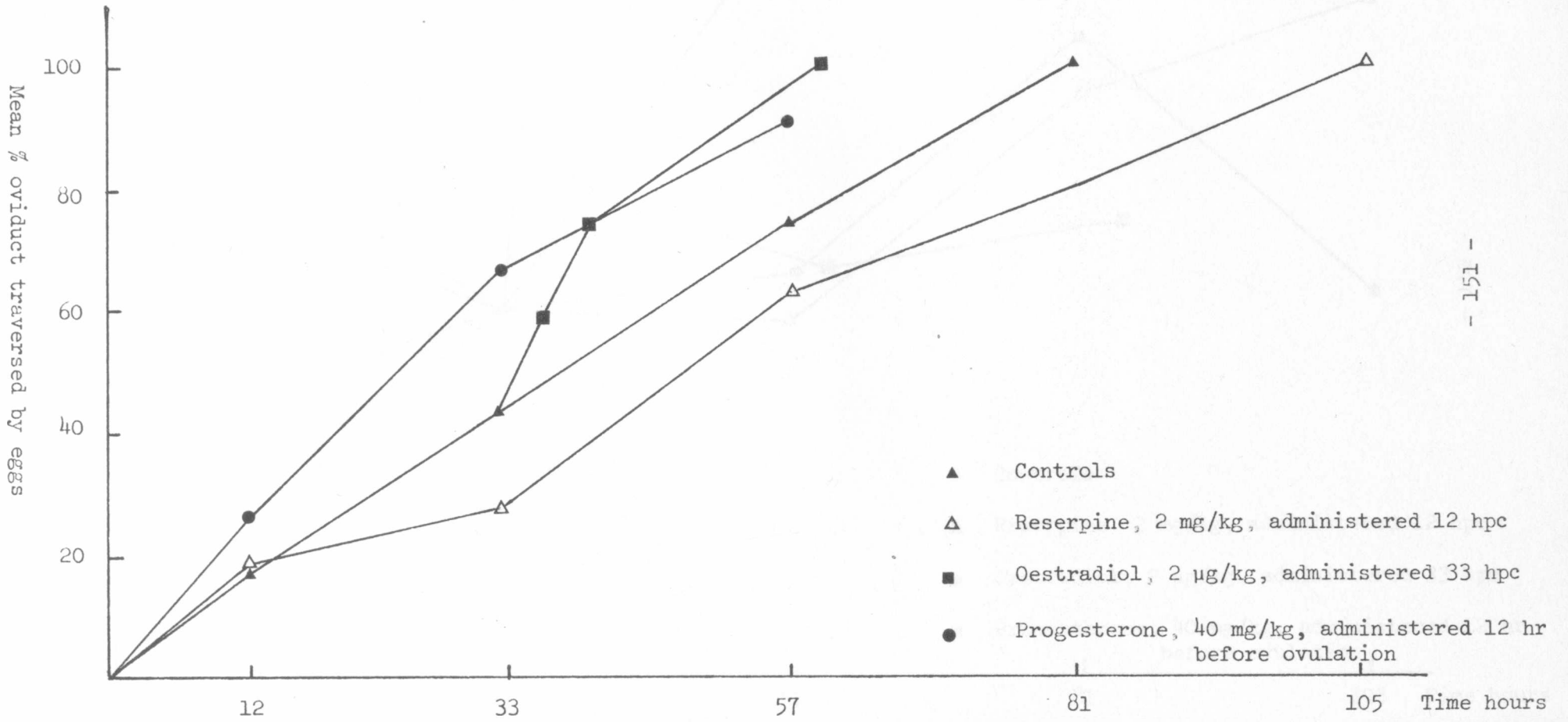
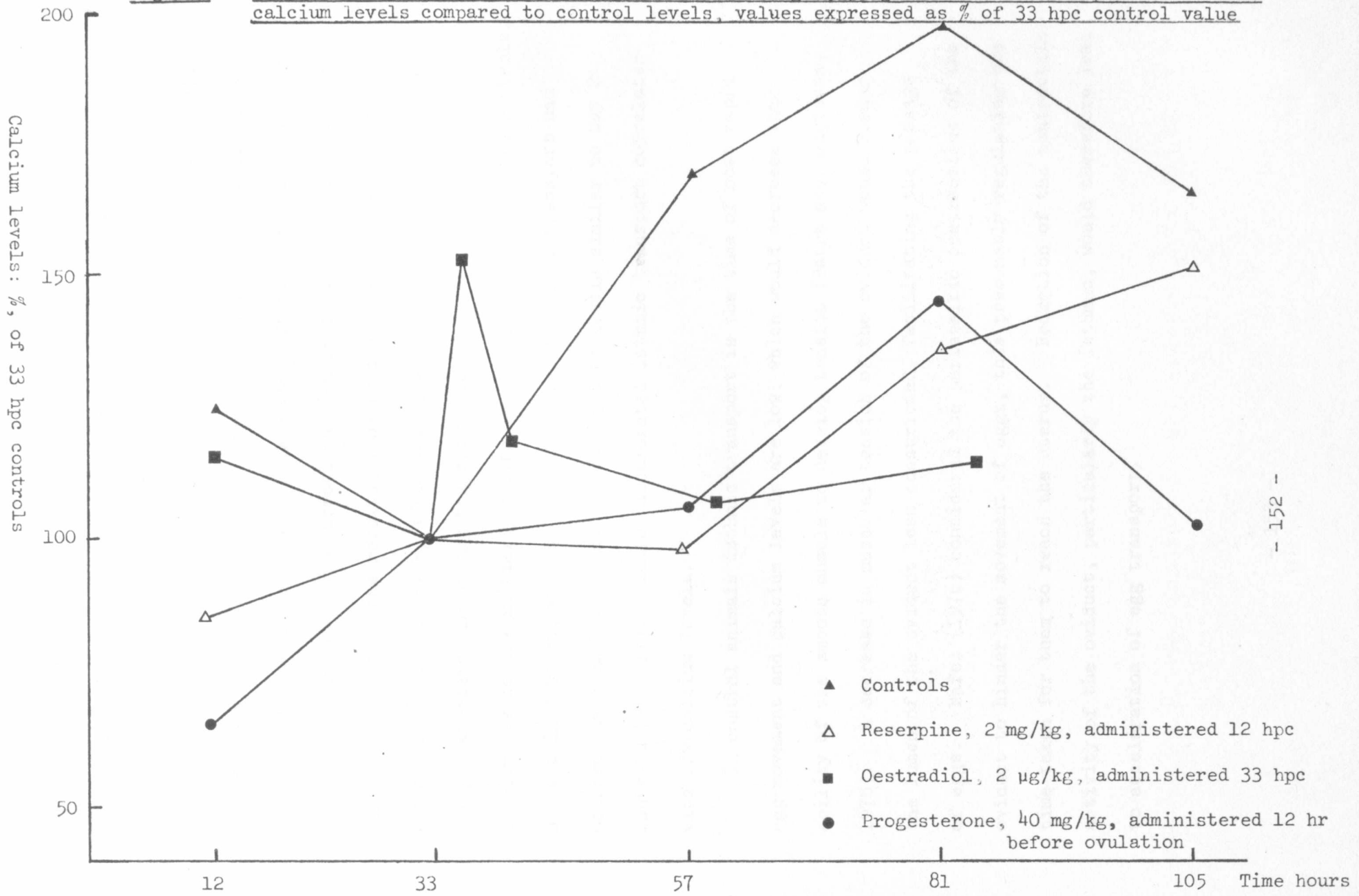


Fig. 14 Graphical representation of the effect of oestrogen, progesterone and reserpine on oviduct calcium levels compared to control levels, values expressed as % of 33 hpc control value



A decrease in oviductal calcium levels appears to be associated with relaxation of the AIJ, indicating either loss of calcium from the muscle cells or a decrease in calcium uptake brought about by permeability changes in the cell membranes or alterations in the functioning of active carrier mechanisms for calcium ions.

Isthmic transport after preovulatory progesterone treatment is more rapid than in control animals (Fig. 11). In both cases calcium levels are rising during isthmic transport but in the control animals the rise is more pronounced. Accelerated isthmic transport is associated with lower than normal calcium levels. Further evidence for this comes from the results of oestradiol treatment. Oestradiol was administered on Day 2, after the eggs had entered the isthmus and a rapid acceleration of egg transport occurs. After oestradiol calcium levels are initially rising in a similar manner to the controls, but within 7 hours, when significant acceleration of transport had occurred calcium levels had fallen again to levels similar to Day 2 controls (Fig. 14). Hence, accelerated isthmic transport correlates with low calcium levels.

In control animals isthmic transport is the time of most rapid egg movement and calcium levels are low, which would decrease the ability of the smooth muscle to develop tension (Batra and Bengtsson, 1978). A decrease in muscular tension of the oviduct would render the lumen of the oviduct less constricted, facilitating the passage of eggs. Hafez (1976) considered the peristaltic contractions of the oviduct to hinder the movement of eggs, thus presumably regulating the time taken for them to reach the uterus. Reduction of the contractile activity of the oviduct, particularly the isthmus, would therefore lead to acceleration of egg transport.

The acceleratory effects of oestrogen and progesterone could therefore be being brought about by alterations in calcium metabolism. Both these hormones have been shown to decrease entry of calcium ions into the rat myometrium (Batra and Bengtsson, 1978), oestrogen more effectively than progesterone, which could explain the latter's more dramatic effect on isthmic transport and oviduct calcium levels. Oestrogen has also been shown to decrease the extracellular calcium compartment in the rabbit oviduct (Hodgson and Daly, 1976). The extracellular calcium compartment is the fastest exchanging calcium compartment, a decrease in this would reduce the amount of calcium available for uptake into the cells for the propagation of electrical activity. In the rabbit oviduct Seki, Rawson and Hodgson (1978) have shown that the extracellular space in the isthmus 24 hours after HCG is significantly smaller than after 68 hours, 24 hours being around the time of isthmic entry of the eggs. The electrical conduction velocity of visceral muscles has been reported to be correlated with the magnitude of the extracellular space, the magnitude was found to be greater in slower or nonconducting muscles than in faster conducting muscles (Prosser, Burnstock and Kahn, 1960).

Seki et al., (1978) studied postovulatory morphological changes in the circular smooth muscle layer of the rabbit oviduct to try and find correlations with the electrical activity of the smooth muscle. Propagation of electrical activity in smooth muscle is via regions of low resistance. The nexus junction is one type of site of electrical transmission commonly found in smooth muscle (Dewey and Barr, 1962, 1964; Barr, Berger and Dewey, 1968) but nexal type connections are rare or absent in some smooth muscles and in these cases simple appositions or interdigitations have been postulated to provide low resistance pathways (Henderson, Duchon and Daniel, 1971; Garfield

and Daniel, 1974). In the human oviduct no nexuses have been found and simple appositions are thought to provide contacts (Daniel, Posey and Paton, 1975) and in the rabbit oviduct simple appositions and interdigitations (Kushiya, 1968; Henderson, Johns and Paton, 1976). Seki et al., (1978) found that the number of cell contacts in the ampulla and AIJ of the rabbit oviduct was greater than in the isthmus, at 24 and 68 hours after an ovulation inducing dose of HCG, and in the ampulla there were more interdigitations at 24 hours than at 68 hours and the mean percentage of cell membranes in contact was also greater. These results correlate with the findings of Talo and Hodgson (1977) that a greater proportion of action potentials were propagated in the ampulla than in the isthmus.

The presence of membrane caveolae were also investigated by Seki et al., (1978). Caveolae have been considered to have various functions in smooth muscle cells: an excitatory function similar to the T-tubule system of skeletal muscle; sites for absorption of ions and metabolites; sites for controlling cellular volume by binding or extruding sodium ions; and sites of an active calcium pump (Nagasawa and Suzuki, 1967; Rangachari, Daniel and Paton, 1973; Prosser, 1974). In the rabbit oviduct the number of caveolae appeared to be inversely related to the frequency of spontaneous electrical activity (Talo and Hodgson, 1977) and to the responsiveness of the muscle cells to calcium ions and drugs (Hodgson and Daly, 1976).

The results of these investigations suggest that the smooth muscle cells of the oviduct are subject to ultrastructural changes after ovulation, particularly at the times of isthmic and uterine entry of the eggs. Changes such as these would not be instantaneous, they would involve processes such as protein synthesis and enzyme induction. The

triggering of morphological changes would therefore be likely to occur prior to and around the time of ovulation.

In the rabbit a preovulatory progesterone sensitive mechanism has been proposed (Gonzalez de Vargas et al., 1975). In vivo there is a preovulatory rapid rise in progesterone levels to a high level which falls to a very low level just before ovulation (Hilliard et al., 1963; Hilliary, Endröczi and Sawey, 1961; Hilliard, Hayward and Sawyer, 1964) and oestrogen levels are at their lowest just prior to ovulation (Hori et al., 1968; Miyake, 1968; Moore et al., 1969). Gonzalez de Vargas et al., (1975) consider progesterone to control the opening of the AIJ and UTJ, which, if the case, must be through changes in the smooth muscle physiology, perhaps via the morphological changes described and the resultant changes in the electrical activity of the smooth muscle. Oestrogen is thought to be responsible for the block to transport occurring at the AIJ on Day 1 and the administration of exogenous oestrogen at this time will prolong the block, retaining the eggs in the ampulla (Greenwald, 1967). The increased progesterone levels occurring before ovulation are thought to be responsible for overcoming the oestrogen block.

If prevoulatory ovarian hormone levels are responsible for initiating the pattern of oviduct contractility, occurring during egg transport, through morphological changes which lead to changes in the electrical and mechanical activity of the oviductal smooth muscle, this implies that the functioning of the oviduct is "programmed" before ovulation. Perfusion of the oviduct in vitro must disrupt the normal pattern of contractility if the oviduct, either by a disturbance of its anatomical arrangement, as mentioned earlier or by alterations of its ionic environment and composition, particularly relevant to smooth muscle contractility would be changes in calcium metabolism, i.e. calcium exchange, permeability and binding. High calcium levels would result

in an increased muscular tension, impeding egg movement and too low a concentration of calcium would weaken the muscular contractions needed to propel the eggs forward. Measurement of the calcium content of the oviduct *in vitro* shows a more rapid increase occurring during Day 2 than *in vivo* (Table 22). This is the opposite effect to oestrogen and progesterone where accelerated transport is associated with less rapid rises in calcium levels (Figs. 13, 14), *in vitro* a more pronounced rise is associated with lack of forward progressive movement of the eggs.

Treatment of the mice with reserpine on Day 1 of pregnancy causes retardation of transport, the passage of eggs through to the uterus takes approximately 24 hours longer. Each stage of the transport process is affected, the eggs remain longer in the ampulla, isthmic transport is slower and the block to transport at the UTJ prolonged. Corresponding changes also occur in oviduct calcium levels (Fig. 14). There is no fall in calcium levels to indicate a relaxation of the AIJ and isthmic entry. Calcium levels show no significant changes for the first 3 days, though possibly the fall is being missed due to the timing of the experiment. A sharp rise in calcium levels occurs after Day 3 continuing until Day 5, steeper than in the control animals and during this period isthmic transport is slower and the eggs remain longer at the UTJ.

Reserpine retardation of egg transport is thought to be the result of its hypothermic action and retardation can be reversed by maintenance of normal body temperature (Kendle, 1969). Hypothermia would cause a decrease in metabolic rate, thus slowing down or inhibiting any changes in oviductal muscle physiology occurring during normal egg transport. The hypothermic effect of reserpine wears off around

Day 3 to Day 4 (Kendle, 1969) and at this time calcium levels start to rise, as happens in the controls after Day 2, when the eggs are in the isthmus and at the UTJ.

The patterns of calcium ion changes show correlations with the rate of egg transport, pharmacological modifications of the rate of egg transport produce differing patterns of calcium levels from control animals. A fall in calcium levels occurs at the time of AIJ relaxation and isthmic entry and high calcium levels when transport is blocked at the UTJ. Isthmic transport corresponds to the period of lowest calcium levels, accelerated isthmic transport is accompanied by a suppressed rise in calcium levels and retardation (by reserpine) or complete inhibition (in vitro) of transport by more pronounced rises in calcium levels.

The alterations in calcium levels are probably the result of ultrastructural changes occurring in the oviductal smooth musculature after ovulation i.e. the size of the extracellular space, the extent of cell-to-cell contacts for electrical transmission and the number of caveolae. These events must be initiated prior to or around the time of ovulation most likely by the ovarian hormones oestrogen and progesterone.

To further investigate the role of calcium ions in the regulation of the rate of egg transport pharmacological modification of transport could be extended by the use of anti-oestrogens and antiprogestins and by varying the time of administration of oestrogens to produce retardation of transport. Studying the accompanying changes or absence of changes of oviduct calcium levels may produce further evidence of the importance of calcium ion changes during egg transport.

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Appendix I - Postgraduate Courses

No appropriate systematic courses were available and a programme of guided reading followed by tutorial sessions was substituted. Tutorial time was:

Physiology of reproduction	40 hours	1st year
Pharmacology of reproduction	21 hours	2nd year
Principles of experimental design and analysis of results	6 hours	2nd year
Principles and philosophy of industrial pharmacology	3 hours	2nd year

These programmes were satisfactorily completed.

Additional advanced study included extensive reading of relevant research literature and attendance at and participation in meetings of the Society for the Study of Fertility.

Appendix II - Communications and Publications

Winter Meeting of the Society for the Study of Fertility, December 1977, London.

Investigation of the functional significance of the oviduct calcium content, B. Lee & K.E. Kendle.

Summer Meeting of the Society for the Study of Fertility, July 1979, Glasgow.

Investigation of the role of progesterone in the control of egg transport through the mouse oviduct, K.E. Kendle & B. Lee.

Lee & Kendle, 1979. The effect of reserpine, oestradiol and *in vitro* perfusion on oviduct calcium levels in the mouse during egg transport. J. Reprod. Fert. 55, 489-493.

Kendle & Lee. Investigation of the influence of progesterone on mouse embryo transport by using antiprogestational steroids. J. Reprod. Fert. in press.

J. Reprod. Fert. (1979) **55**, 489–493

The effect of reserpine, oestradiol and in-vitro perfusion on oviduct calcium levels in the mouse during egg transport

Bernadette Lee and K. E. Kendle

School of Pharmacy, Robert Gordon's Institute of Technology, Schoolhill, Aberdeen AB9 1FR, U.K.

Summary. Mouse oviduct calcium content, determined by atomic absorbance after ashing of the tissue, showed a significant fall on Day 2 of pregnancy followed by a significant rise on Day 3. This pattern was altered by administration of reserpine and oestradiol in doses which were shown to alter the rate of egg transport. In-vitro perfusion of the oviduct, capable of maintaining muscular activity and back and forth movement of eggs for 24 h, was associated with lack of forward progressive motion of eggs and by a more rapid increase in tissue calcium levels during incubation than occurred *in vivo*.

Introduction

The transport of ova through the oviduct is thought to be effected primarily by the contractile activity of the oviduct musculature (Blandau, Boling, Halbert & Verdugo, 1975). Since smooth muscle activity depends upon calcium ions (Lullman, 1970), changes in contractile activity in the oviduct during egg transport may be mediated through changes in tissue calcium content or distribution. In the present study oviduct calcium content in mice during normal, delayed and accelerated egg transport *in vivo* and during perfusion *in vitro* has been determined. Reserpine (Bennett & Kendle, 1967) and oestradiol (Humphrey, 1968) were used to produce retardation and acceleration of egg transport respectively.

Materials and Methods

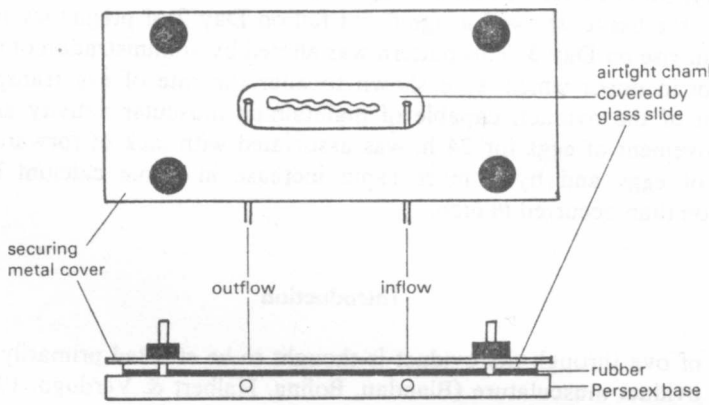
Albino mice of the RGIT/SLAC strain were housed under constant conditions of 12 h light (08:00–20:00 h) and 21 ± 1°C. Water and Oxoid Pasturised Breeding diet for rats and mice were always available. Mature virgin females, 6–12 weeks old, were paired with mature males and examined each morning for the presence of a vaginal plug which was taken as evidence of successful copulation (Day 1 of pregnancy), and copulation was assumed to occur at 24:00 h on the previous day. The mice were killed at the times indicated in 'Results'.

Egg transport in vivo. The position of the eggs in the oviduct and the mean percentage oviduct traversed by the eggs were determined by the methods of Bennett & Kendle (1967) and Kendle & Bennett (1969).

Oviduct calcium levels. Oviducts removed at autopsy were blotted on filter paper, cleaned of fat, and then ashed in pairs in silica crucibles at 450°C. The ash was dissolved in 1 ml 6 M-hydrochloric acid (Analar: B.D.H. Chemicals Ltd, Poole), evaporated to dryness, redissolved and evaporated, and then dissolved in 4 ml 0·3 M-hydrochloric acid. The final volume was adjusted to 10 ml with distilled water and calcium concentrations were measured by atomic absorption spectroscopy. The results were converted to μg/mg wet weight of tissue.

The detection limit of the assay was 0·05 μg/ml with linearity up to 1·5 μg/ml and the inter- and intra-assay variations were <15% and <1%, respectively. Blank readings automatically zeroed. All samples, standards and the blanks contained 0·1% (w/v) calcium-free lanthanum chloride (Koch-Light Laboratories Ltd, Bucks) to prevent anionic interference.

Oviduct perfusion in vitro. The oviducts were removed from animals killed on Day 2 of pregnancy (34 h *post coitum*) and straightened. Each oviduct was placed in an air-tight, optically transparent chamber (Text-fig. 1). The chamber was perfused with a Ringer solution of the following composition (mM): NaCl, 153·8; KCl, 5·6; $CaCl_2$, 2·16; $NaHCO_3$, 5·81; glucose, 5; which was gassed with pure oxygen and maintained at 33°C. Microscopic examination of the oviducts in the chambers enabled location of the eggs, calculation of the mean percentage oviduct traversed by the eggs, and assessment of oviduct contractions, counted per minute, and motility, based on a 5-point scale (0 = no movement, 1 = very weak, 2 = moderate, 3 = strong, 4 = very strong).



Text-fig. 1. Perfusion chamber for in-vitro oviduct preparation.

Untreated mice were killed at 10:00 h on Days 1, 2, 3, 4 or 5 for determination of egg positions and oviduct calcium levels. The effects of oestradiol and reserpine on egg transport and oviduct calcium levels were then investigated and compared with vehicle-treated controls.

The reserpine (Courtin and Warner, Lewes) stock solution consisted of 300 mg reserpine + 375 mg citric acid in 6 ml benzyl alcohol + 15 ml Tween 80, made up to 100 ml with distilled water (see Bennett & Kendle, 1967). Dilutions were made with distilled water. Mice were treated intraperitoneally with 2 mg reserpine/kg or with vehicle (10 ml/kg) at 09:00 h on Day 1 of pregnancy (i.e. 9 h *post coitum*) and killed at 10:00 h on Days 1, 2, 3, 4 or 5 for determination of egg positions and oviduct calcium values.

Oestradiol (B.D.H.) was prepared as a stock solution (8 mg in 10 ml absolute alcohol (B.D.H.)) and dilutions were made with 0·9% (w/v) NaCl. Mice were given an intraperitoneal injection of 2 μg oestradiol at 33 h *post coitum* (Day 2, 09:00 h). Control animals were treated with saline (10 ml/kg). Oestradiol-treated animals were killed at 36, 40 and 60 h and controls at 12, 33, 36, 40, 60 and 84 h *post coitum*. The oviducts were removed for determination of egg transport and calcium content.

Results

Egg transport and calcium levels in vivo

The results are shown in Tables 1 and 2. Reserpine retarded the rate of egg transport by approximately 24 h (Table 1), all the eggs not entering the uterus until Day 5. Oestradiol accelerated egg transport (Table 2). Calcium levels in the untreated mice were lowest on Day 2 of pregnancy (Table 1) with a significant rise on Day 3. As precision within calcium assays was

Table 1. The effect of reserpine (2 mg/kg), administered on Day 1 of pregnancy, on egg transport and oviduct calcium levels in the mouse

Time of killing (h *post coitum*)	Mean % oviduct traversed by eggs			Calcium content (μg/mg wet weight tissue)		
	Experiment 1	Experiment 2		Experiment 1	Experiment 2	
	Untreated animals	Vehicle controls	Reserpine	Untreated animals	Vehicle controls	Reserpine
10	17·1 ± 2·1 (14)	18·4 ± 0·9 (10)	18·7 ± 1·1 (10)	†0·81 ± 0·06 (8)	†1·28 ± 0·15 (5)	0·89 ± 0·08 (8)
34	43·9 ± 3·1 (16)	47·5 ± 6·0 (10)	*27·6 ± 2·8 (12)	0·65 ± 0·04 (8)	0·96 ± 0·10 (6)	1·03 ± 0·07 (9)
58	75·2 ± 3·4 (14)	84·9 ± 4·1 (16)	*63·9 ± 4·9 (11)	†1·10 ± 0·11 (11)	†1·33 ± 0·14 (5)	1·01 ± 0·05 (6)
82	101 ± 0·0 (14)	101 ± 0·0 (10)	*80·8 ± 5·0 (11)	†1·24 ± 0·09 (11)	†1·40 ± 0·06 (5)	†1·40 ± 0·11 (6)
106			101 ± 0·0 (12)	†1·08 ± 0·05 (8)	†1·64 ± 0·15 (5)	†1·57 ± 0·03 (6)

Values are mean ± s.e.m., no. of oviducts (egg transport) or paired oviducts (calcium content) in parentheses.
* Significantly different from control group, $P < 0·001$ (Students t test).
† Significantly different from Day 2 value of same treatment, $P < 0·05$ (Multiple range t test).

Table 2. The effect of oestradiol (2 μg/mouse), administered 33 h *post coitum*, on egg transport and oviduct calcium levels in the mouse

Time of killing (h *post coitum*)	Mean % of oviduct traversed by eggs		Calcium content (μg/mg wet weight of tissue)	
	Controls	Oestradiol	Controls	Oestradiol
12	17·8 ± 2·2 (14)		0·65 ± 0·06 (7)	
33	43·9 ± 3·1 (16)		0·56 ± 0·05 (7)	
36	46·9 ± 3·9 (13)	59·9 ± 8·7 (13)	†0·90 ± 0·01 (7)	†0·86 ± 0·06 (6)
40	57·7 ± 4·2 (10)	*73·7 ± 7·1 (13)	†1·05 ± 0·10 (8)	0·67 ± 0·04 (7)
60	81·9 ± 2·7 (10)	*101 ± 0·0 (10)	†0·98 ± 0·11 (7)	0·60 ± 0·06 (6)
84	101 ± 0·0 (10)		†1·03 ± 0·07 (6)	0·65 ± 0·07 (7)

Values are mean ± s.e.m., no. of oviducts (egg transport) or paired oviducts (calcium content) in parentheses.
* Significantly different from control group, $P < 0·001$ (Students t test).
† Significantly different from 33 h control group, $P < 0·05$ (Multiple range t test).

much greater than between assays, only within-experiment comparisons could be made. The calcium level on Day 2 was arbitrarily selected as the reference level to which the other values were compared using the multiple range *t* test. The same pattern was observed after reserpine vehicle but there were no changes in calcium levels after reserpine treatment until the increase observed on Day 4 (Table 1). In the saline-treated controls calcium levels rose significantly between 33 h and 36 h *post coitum* and the rise was sustained at 40, 60 and 84 h while in the oestradiol-treated mice at 40, 60 and 84 h levels had fallen and were not significantly different from the 33 h control (Table 2).

Calcium levels during in-vitro perfusion

The oviduct musculature remained viable for at least 20 h, showing rhythmic contractions although the frequency decreased significantly ($P < 0{\cdot}001$, paired *t* test) from $12{\cdot}9 \pm 0{\cdot}98$ to $7{\cdot}5 \pm 1{\cdot}21$ contractions/min ($n = 13$). The eggs moved backwards and forwards within the oviduct lumen but made no forward progression along the isthmus (mean % oviduct traversed at 1 h and 20 h was $46{\cdot}1 \pm 5{\cdot}25$ and $45{\cdot}7 \pm 5{\cdot}69$ respectively, $n = 13$).

Table 3. Effect of incubation *in vitro* on oviduct calcium levels in the mouse

	Time (h *post coitum*)		
	34	40	58
In vivo	0·41 ± 0·01 (6)	0·46 ± 0·03 (6)	0·52 ± 0·03 (6)*
In vitro		0·58 ± 0·05 (6)*	0·49 ± 0·03 (6)*

Values are mean ± s.e.m., no. of paired oviducts in parentheses.
* Significantly different from the 34 h control group, $P < 0{\cdot}05$ (Multiple range *t* test).

The calcium levels of 6 oviducts were measured at 6 and 24 h after the start of perfusion (40 and 58 h *post coitum* respectively) and comparisons were made with the values obtained for the oviducts from 6 untreated animals killed at 34, 40 and 58 h *post coitum*. The results are shown in Table 3. Calcium levels rose *in vivo* after copulation but were lower than the values represented for similar tissue in Table 2. The values obtained *in vitro* were comparable to those found *in vivo* but rose earlier.

Discussion

Normal egg transport rates were observed in untreated mice and in mice given saline or reserpine vehicle. The mean percentages of the oviducts traversed by the contained eggs on Days 1, 2, 3 and 4 of pregnancy showed close correlation between experiments and with previous investigations (Kendle & Bennett, 1969). Although the absolute concentrations of calcium showed considerable variation between experiments a consistent pattern was observed during normal egg transport. Levels fell between Days 1 and 2 then rose sharply between Days 2 and 3 and were subsequently maintained at the higher level. Thus Day 2 of pregnancy, when egg movement is most rapid as the eggs are transported from the isthmo-ampullary to the utero-tubal junction, was consistently the time at which calcium levels were lowest.

Pharmacological alteration of the rate of egg transport was associated with alteration of the pattern of calcium levels. Reserpine, which delayed egg transport, abolished the Day 2 fall and delayed the significant rise by 24 h, while oestradiol accelerated egg transport and prolonged the low Day 2 levels.

The in-vitro mouse oviduct preparation allowed observation of oviduct muscle motility and although forward and backward movement of the contained eggs occurred the eggs did not

progress. The more rapid rise of calcium levels *in vitro* than *in vivo* might be inhibiting egg transport via calcium-induced changes in the pattern of oviduct contractility, as the calcium content of smooth muscle is known to be dependent on extracellular calcium levels (Schatzman, 1961). The apparently later rise in calcium levels *in vivo* in this experiment compared with the data in Table 2 is probably due to variation in the actual time of mating between experiments as this was assumed to be constant for convenience in describing the data but would in fact be subject to variation of undetermined magnitude both within and between experiments.

The hypothesis that the concentration of calcium ions could play an important role in the regulation of oviduct contractility and egg transport is supported by the findings in this study of a definite pattern of concentration changes during normal egg transport and of a close correlation between the effects of pharmacological stimuli on the rate of egg transport and on oviduct calcium concentrations. Correlation has been shown between the ability of segments of the oviduct to transport ova (Pauerstein, Anderson, Chatkoff & Hodgson, 1974) and to exchange calcium (Hodgson & Daly, 1975) in the rabbit, indicating the importance of calcium distribution in oviduct function. Further study is therefore needed to clarify the relationship between calcium content and distribution in the tissue.

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Investigation of the influence of progesterone on mouse embryo transport by using antiprogestational steroids

K. E. Kendle and Bernadette Lee

School of Pharmacy, Robert Gordon's Institute of Technology, Schoolhill, Aberdeen AB9 1FR, U.K.

Summary. The rate of embryo transport through the mouse oviduct was unaltered by ovariectomy after ovulation, essentially unaltered by administration of progesterone after ovulation but increased by preovulatory administration of progesterone. The antiprogestational steroid RMI 12,936 caused an arrest of embryo movement when given on Day 1, 2 or 3 of pregnancy and similar, though less marked, delay was caused by R2323, another progesterone antagonist. The effects of RMI 12,936 given on Day 1 were reversed by progesterone administration after a latent period of 24–48 h. These results indicate that the egg transport process in the mouse is triggered by progesterone and requires continued progesterone activity for its maintenance.

Introduction

Effects of oestrogens on egg transport in various species have been the subject of numerous investigations from the classic studies of Burdick & Pincus (1935) to the definitive experiments of Humphrey (1968). The action of progesterone, however, has received much less attention and most of the available data is from work in rabbits. This work, reviewed by Chang (1976), indicates that progestagens given after ovulation do not produce marked or consistent changes in the rate of egg transport but when given before ovulation cause acceleration and premature entry of embryos into the uterus. Preovulatory progesterone might be active by virtue of its metabolic conversion to oestrogen, but Kendle & Telford (1970) showed similar acceleration following administration of megestrol acetate, a progestagen which does not have oestrogenic activity and is not easily aromatized metabolically (David, Edwards, Fellows & Plummer, 1963). Acceleration of egg transport was also observed in these experiments when administration of megestrol acetate was continued up to the day of autopsy (Day 1, 2 or 3 of pregnancy), indicating that the effect was due to progestagen administration and not to progestagen withdrawal. Similar effects of preovulatory progestagen administration have not to date been reported in the mouse.

In the rat post-ovulatory ovariectomy does not modify the rate of egg transport (Allen, 1942), suggesting that the process was not controlled by post-ovulatory secretion of ovarian hormones in this species. Similar experiments have not, however, been reported in the mouse.

The control of oviduct function may be explained by the hypothesis that the sequence of muscular activities which result in the normal process of egg transport is initiated by the preovulatory secretion of progesterone and is subsequently independent of further hormone secretion. Administration of progesterone at a time earlier than the preovulatory peak would therefore cause premature activation of oviduct function and accelerated embryo transport.

The present work was undertaken to see whether this hypothesis could be evaluated by using antiprogestational steroids in the mouse. Since no entirely specific agents of this type are available two steroids with different mechanisms of antiprogestational activity and different spectra of other biological activities were used. RMI 12,936 (17β-hydroxy-7α methyl androst-5-

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en-3-one) is an antiprogestational steroid with weak oestrogenic, antioestrogenic and androgenic activities; it inhibits progesterone secretion and may be metabolized to a further compound which antagonizes the effects of progesterone (Kendle, 1975, 1976, 1978; Grunwell, Benson, Johnson & Petrow, 1976; Geddes, Kendle, Shanks & Stephen, 1979). R2323 (13-ethyl-17-hydroxy-18,19-dinor-17α-pregna-4,9,11-trien-20-yn-3-one) has weak oestrogenic, weak androgenic and antioestrogenic activities and is a competitive antagonist of progesterone (Sakiz & Azadian-Boulanger, 1971; Sakiz, Azadian-Boulanger & Raynaud, 1974; Raynaud *et al.*, 1974).

Materials and Methods

Albino mice of the RGIT/SLAC strain were housed as described previously (Lee & Kendle, 1979). For post-ovulatory treatments mature virgin females were paired with mature males and examined each morning for the presence of a vaginal plug which was taken as evidence of successful copulation (Day 1 of pregnancy). For preovulatory treatment vaginal lavages were examined daily and only animals showing at least 2 regular 4-day oestrous cycles were used. Treatments were given on the day of pro-oestrus and the animals were then paired with mature males and examined for the presence of a vaginal plug the following morning; any unmated females were rejected.

Ovariectomy was carried out under sodium pentobarbitone anaesthesia (60 mg/kg i.p.) through bilateral flank incisions. Steroids (progesterone purchased from BDH (Chemicals) Ltd, Poole; RMI 12,936, a gift from Merrell National Laboratories; and R2323, a gift from Roussel Uclaf) were suspended in water containing 0·25% (w/v) sodium carboxylmethylcellulose and 1% (w/v) 'Tween 80'. Doses were administered subcutaneously in a dose volume of 0·1 or 0·2 ml.

Four experiments were carried out as detailed in the 'Results'. At autopsy the position of embryos within the reproductive tract was determined as described previously (Bennett & Kendle, 1967; Kendle & Bennett, 1969). Results were compared, when appropriate, using Student's *t* test. When variances could not be assumed to be equal and were not proportional to the mean, the Welch test was used (Welch, 1947). Differences were considered significant if $P < 0·05$ for 2 sample tests or $<0·05$/no. of contrasts for multirange tests.

Results

Ovariectomy at 12:00–14:00 h on Day 1 of pregnancy did not alter the rate of embryo transport when compared with that in sham-operated controls (Table 1). Administration of progesterone at 09:00 h on the day of pro-oestrus produced significant acceleration (Table 2). Embryo

Table 1. The effect of ovariectomy, performed at 12:00–14:00 h on Day 1 of pregnancy, on embryo transport in the mouse

Day of pregnancy when killed	% of oviduct traversed by embryos: Sham-operated mice	Ovariectomized mice
2	42·8 ± 5·61 (12)	42·6 ± 5·35 (12)
3	78·5 ± 4·89 (12)	73·6 ± 6·35 (10)
4	100 (10)	100 (10)

Values are mean ± s.e.m., no of oviducts in parentheses, with 100% representing the uterus.

Table 2. The effect of progesterone (0·8 mg/mouse), administered at 09:00 h on the day of pro-oestrus, on embryo transport in mice

Day of pregnancy when killed	% of oviduct traversed by embryos	
	Control mice	Progesterone-treated mice
1	16·3 ± 1·47	*26·0 ± 2·89
2	47·8 ± 4·27	*66·9 ± 5·35
3	65·4 ± 3·90	**91·3 ± 3·98
4	100	100

Values are mean ± s.e.m. for 16 oviducts/group; 100% represents the uterus.

Significantly different from control group (Student's *t* test): $*P < 0·01$, $**P < 0·001$.

Table 3. The effect of progesterone (0·8 mg/mouse) and RMI 12,936 (0·4 mg/mouse), administered at various times after ovulation, on embryo transport in mice

Day of pregnancy when killed	% of oviduct traversed by embryos					
	Control	RMI 12,936			Progesterone	RMI 12,936 + progesterone
	—	Day 1	Day 2	Day 3	Day 1	Day 1
1	16·8 ± 0·66	—	—	—	—	—
2	44·9 ± 2·59	24·3 ± 2·46	—	—	42·1 ± 2·77	24·5 ± 1·84
3	69·4 ± 3·20	34·4 ± 5·73	54·8 ± 5·56	—	81·2 ± 2·00	78·3 ± 5·36
4	100	27·2 ± 4·74	61·3 ± 5·57	82·4 ± 3·30	100	86·0 ± 5·54

Values are mean ± s.e.m. for 16 oviducts/group; 100% represents the uterus.
Statistical analysis of data used the Welch test; the results are given in the text.

transport through the oviduct is a discontinuous process and there is no theoretical justification for drawing straight lines through plots of mean percentage oviduct traversed against time. In practice, however, such plots are reasonably close to a linear relationship and afford a convenient means of roughly estimating the extent of acceleration or retardation of embryos. After progesterone treatment the transport was thus estimated as being accelerated by approximately 14 h.

The results given in Table 3 show that administration of RMI 12,936 caused arrest of embryo transport when the drug was given on Days 1, 2 or 3 of pregnancy. The positions of embryos in the reproductive tracts of control animals killed on Days 1 to 4 of pregnancy were significantly different from each other ($P < 0·05$/no. of contrasts). Following administration of RMI 12,936 on Day 1 the position of embryos at autopsy on Day 2 was significantly less than in control animals killed on Day 2 and was not significantly different from the positions in animals treated on Day 1 and killed on Day 3 or 4. Similarly when RMI 12,936 was given on Day 2 the mean percentage of the oviduct traversed by autopsy on Day 3 was less than in control animals, although the difference was not statistically significant, but by Day 4 the embryo position was significantly different from that in control animals killed on Day 4. Treatment with RMI 12,936 on Day 3 also led to a significant difference on Day 4. Administration of progesterone caused no change in the pattern of embryo transport except for a small but significant increase in the percentage of the oviduct traversed at autopsy on Day 3. When RMI 12,936 and progesterone were given together on Day 1 the position of embryos at autopsy on Day 2 was not significantly different from that in animals given RMI 12,936 alone. At autopsy on Days 3 and 4, however,

the positions of embryos in animals given RMI 12,936 and progesterone were not significantly different from those in the appropriate control groups. Thus it is shown that progesterone, which has little effect on the rate of embryo transport when given alone after ovulation, reverses the effect of RMI 12,936 after a delay of 24 h. Mean numbers of embryos (given as mean ± s.e.m., $n = 8$, throughout) recovered in all these experimental groups, except that given RMI 12,936 and progesterone and killed on Day 4, were within the range 10·3 ± 0·8 to 13·8 ± 1·28 and there were no significant differences between groups. In the animals given RMI 12,936 and progesterone and killed on Day 4 there was significant loss ($P < 0.01$, multiple range t test) of embryos from the reproductive tract (mean number recovered 5·6 ± 1·40), compared with all other groups.

The results given in Table 4 show that the effect of R2323 on embryo transport was similar to the effect of RMI 12,936 but was less marked. Administration of the drug on Day 1 or 2 of pregnancy resulted in a reduction in the mean percentage of the oviduct traversed which was significant compared with the appropriate control at autopsy 24 h after administration but not at later times. The apparent slight retardation following administration of the drug on Day 3 was not significant. Administration of R2323 and progesterone together on Day 1 resulted in a pattern of embryo transport not significantly different from that in animals given R2323 alone. The mean numbers of embryos recovered were within the range 9·5 ± 3·7 to 14·1 ± 1·0 with no significant differences between groups (multiple range t test).

Table 4. The effect of R2323 (0·8 mg/mouse) administered alone or with progesterone (0·8 mg/mouse) at various times after ovulation, on embryo transport in mice

	% of oviduct traversed by embryos				
Day of pregnancy when killed	Control	R2323			R2323 + progesterone
	—	Day 1	Day 2	Day 3	Day 1
1	15·4 ± 0·80	—	—	—	—
2	38·0 ± 3·42	22·4 ± 1·12	—	—	28·2 ± 2·24
3	71·8 ± 2·80	62·1 ± 3·98	54·2 ± 4·01	—	69·9 ± 4·80
4	100	78·8 ± 7·10	70·7 ± 10·31	98·6 ± 0·98	81·9 ± 7·71

Values are mean ± s.e.m. for 16 oviducts/group; 100% represents the uterus.
Statistical analysis of data used the Welch test; the results are given in the text.

Discussion

Determination of the mean percentage of the oviduct traversed by the contained embryos is a method capable of detecting very slight changes in the rate of embryo transport. It is estimated from unpublished data that acceleration or retardation of less than 4 h can produce significant changes in the index. However, ovariectomy after ovulation does not alter the rate of embryo transport in the mouse, thus showing that the control of the process does not involve post-ovulatory steroid secretion by the ovary. Therefore suggestions that, for example, the opening of the isthmo-ampullary junction is effected by a change in the ratio of ovarian oestrogen : progesterone secretion are clearly untenable in the mouse. Investigation of the effect of preovulatory progesterone shows that in mouse, as in rabbit, embryo transport is accelerated by this treatment. This finding is compatible with the hypothesis that the process of embryo transport is triggered by preovulatory progesterone secretion and may be prematurely triggered by exogenous progesterone. The mechanisms by which preovulatory progesterone regulate muscular activity in the oviduct during early pregnancy require further investigation. One

possibility in view of previous findings (Lee & Kendle, 1979) is that the hormone alters calcium uptake by the oviduct, possibly by modification of the synthesis of calcium-binding proteins. Further work to investigate the possibility will be undertaken. The present study, however, was aimed at establishing whether the continued action of progesterone was necessary during the embryo transport phase for normal functioning of the oviduct.

Since administration of RMI 12,936 on Days 1, 2 or 3 of pregnancy caused arrest of embryo transport, continued activity of progesterone during this period would appear to be essential if it is assumed that RMI 12,936 is acting as a specific antiprogestational agent. This assumption is justified since the effects of RMI 12,936 are (1) mimicked by R2323 and (2) reversed by the administration of progesterone. The failure of exogenous progesterone to reverse the effects of R2323 was not unexpected as there was a latent period of 24 h when progesterone was given with RMI 12,936 and the significant effects of R2323 were only apparent by 24 h even in the absence of exogenous progesterone.

These results indicate that progesterone activity is essential throughout the embryo transport period although the level is not critical because embryo transport is not altered by ovariectomy or markedly changed by administration of exogenous progesterone. The requirement for progestational activity throughout the embryo transport phase may be fulfilled by the persistence of progesterone secreted earlier at the receptor sites or by continued extra-ovarian secretion of progesterone. Work in the rat (Kendle, 1978) indicates that RMI 12,936 inhibits progesterone secretion and that a metabolite with a different mechanism of antiprogestational effect may be formed after approximately 48 h. These findings cannot be applied directly to the mouse without considerable caution as there is no evidence to show that the rates or even the routes of metabolism are similar in the two species.

If there are similarities between the two species, however, and the effects of RMI 12,936 in the first few hours after administration are due to inhibition of progesterone synthesis, this would indicate that normal embryo transport may be maintained by extra-ovarian secretion of progesterone, possibly of adrenal origin.

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