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# DEVELOPMENT OF A NOVEL MEANS OF DRUG ADMINISTRATION TO THE FEMALE GENITAL TRACT

By James Reynolds

## ABSTRACT

The treatment of menopausal symptoms by combined hormone replacement therapy (HRT) utilises drug formulations whereby a progestin is added to the oestrogen component to decrease the risk of endometrial carcinoma produced by the oestrogen. However, administration of large doses of progestins can increase low density lipid cholesterol, as well as reduce some of the beneficial effects of oestrogens. Local administration of progestins to the uterus may allow a much smaller dose to be used reducing the antagonism produced by progestins on desired oestrogenic effects. The simplest way to administer drugs to the uterus is by inserting a device through the cervix, however, this procedure may be associated with bacterial contamination of the uterus leading to pelvic infection. This study has therefore set out to develop a device for the delivery of antimicrobial agents to the uterus to reduce microbial contamination associated with transcervical insertion, and also the delivery of progestins which could be used as an adjunct to oestrogen HRT by targeting the progestin locally to the endometrium.

Hollow nylon and polyethylene fibres were filled with solutions or suspensions of chlorhexidine acetate (CA), levonorgestrel (LNG) or progesterone (Prog.) in various solvents and their release into water monitored by UV spectroscopy. The fibre showing the optimal rate of release of CA, and both of the steroids, was then used to demonstrate the bactericidal activity of CA after release from the fibre. Nylon fibres loaded with CA were placed into phosphate buffered saline (PBS) inoculated with *E. coli* and the bactericidal activity of the CA monitored by viable counting. After 6 hours, bactericidal activity was exhibited which was significantly different from vehicular controls ( $0.001 > p$ ). A similar experiment was performed using purified porcine gastric mucus in the place of PBS however. After 72 hours, the CA had exerted an effect which was statistically significant compared with vehicular controls ( $0.01 > p$ ), such that bacterial counts were lower in the case of CA loaded tubes, but the viable count with the CA loaded fibre was not significantly different from that at time 0 hours.

Nylon fibres loaded with the LNG, Prog. or with CA were inserted into guinea-pigs to determine the effect of the steroids on the white blood cell (WBC) count, endometrial histology and vaginal cytology, and also to establish whether the CA releasing fibre would reduce the amount of bacterial contamination in the uterus following transcervical insertion of the device.

Results showed that the steroid releasing devices had no significant effect upon the WBC count, the endometrial histology or the vaginal cytology. Devices releasing CA did not show a decreased amount of bacterial contamination compared to vehicular controls. Since both the steroids and CA are mucospissic, this may have contributed to the inability of the drugs being released from the fibres and reaching the target site.

The fibre used in these studies did not produce the desired effects with the steroids or with the CA. Modification of the vehicle allowing a greater amount of drug to be loaded into the fibres may be one solution which may allow a greater initial rate of release. Alternatively, if the mucospissic action of the steroids and the CA is the problem by causing increased viscoelasticity of the mucus, and therefore producing a diffusional barrier surrounding the fibre, incorporation of a mucolytic into the fluid loaded into the fibres may reduce the viscoelasticity of the mucus decreasing the ability of the mucus to act as a diffusional barrier.

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# **1 INTRODUCTION**

## 1 INTRODUCTION

There are many types of drug treatments that can be administered which produce an action at a specific site. They may be as simple as applying an ointment to a grazed knee to prevent infection, and may be as sophisticated as the attachment of a drug molecule to a monoclonal antibody to act at a specific site in the treatment of cancer. The advantage of such local administration of drugs enables much smaller doses to be used which will inevitably decrease any side effects associated with the drug. Another situation which may benefit from local drug delivery, is the alleviation of post-menopausal symptoms by hormone replacement therapy where steroids may be directly administered to the uterus. Menopausal symptoms such as hot flushes, osteoporosis and dyspareunia may be treated by oestrogens. Use of oestrogens alone, however, causes proliferation of the endometrium leading to endometrial hyperplasia and/or endometrial carcinoma. The effects of the oestrogens on the endometrium can be limited by cyclical co-administration of progestogens. The major drawback of the systemic administration of progestogens however, is the adverse effect on plasma lipid profiles which, in turn, increases the risk of cardiovascular disease. If the progestogen could be delivered directly to the endometrium the endometrial effects could be controlled without eliciting these adverse cardiovascular effects.

Unfortunately, transcervical introduction of devices into the uterus is associated with bacterial contamination (Mishell *et al* 1966). In the case

of intrauterine contraceptive devices (IUCDs) this is linked with a transient increase in the incidence of pelvic inflammatory disease (PID) for the first month after insertion, which may be of the order of four-times the risk of succumbing to PID compared with the time thereafter (Population Report, 1988). PID has been defined as the 'ascending spread of microorganisms into the uterus and adjoining structures,' and it can have several sequelae ranging from mild pelvic infection to death. Treatment of PID is normally by the systemic administration of antimicrobial agents, however, when an IUCD is present, the use of such a regimen is debatable since bacteria may become embedded within the mucus biofilm adherent to the device. Since systemic use of antibiotics may be unable to combat bacteria within such a biofilm, removal of the device itself is often recommended (Clarke, 1990). In a case such as this, the delivery of antimicrobial drugs directly to the uterus may be of use in the treatment or prophylaxis of PID.

Before embarking upon a description of the current status of research concerning the treatment of uterine drug delivery, it is first necessary to consider the normal anatomy and physiology of the female genital tract.

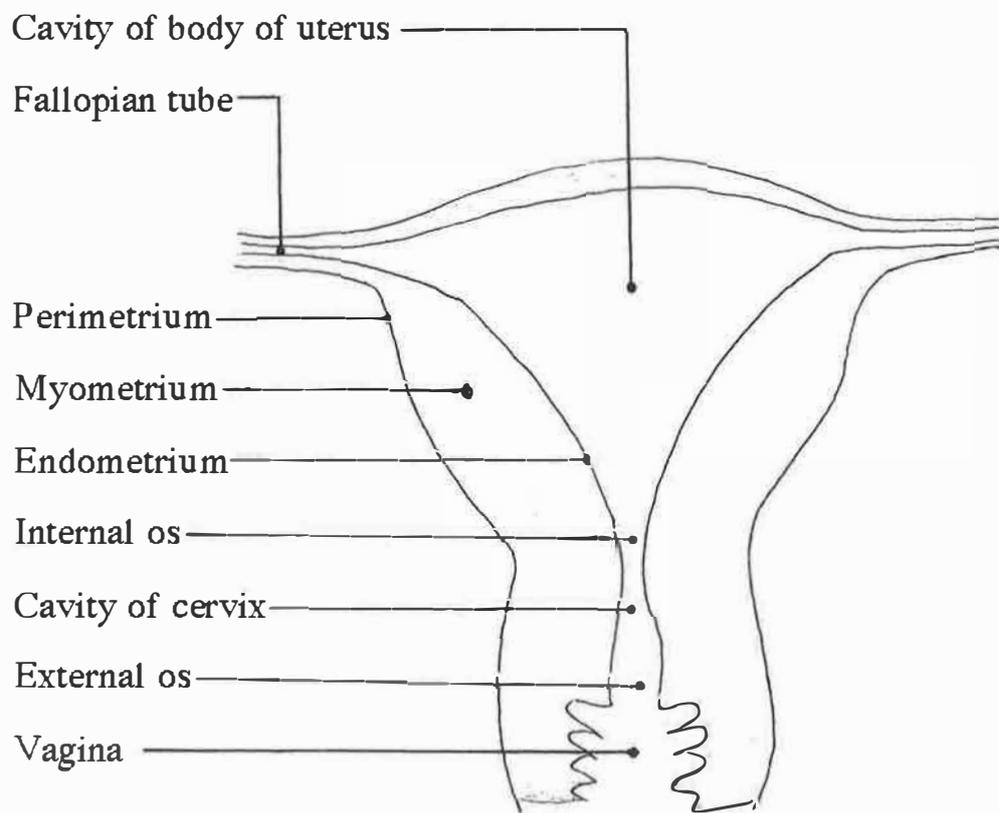
## **1.1 Human female reproductive anatomy and physiology**

### **1.1.1 Anatomy**

The internal genital organs of the human female are the ovaries, the Fallopian tubes, the uterus, the cervix and the vagina (Figure 1.1). The Fallopian tubes convey the ova from the ovaries into the uterus, which is that part of the genital system where the foetus develops. The upper part of the uterus is the fundus, (or body), the constricted middle part is the isthmus and the lower part is the cervix, through which runs the narrow cervical canal connecting the lumen of the uterus with the vaginal passage. The inner layer of the uterus, the endometrium, contains glands, blood vessels and lymphatics in a loose connective tissue stroma and is lined by epithelial cells. The endometrium undergoes cyclic changes throughout the menstrual cycle (see later) and is the structure responsible for the protection and 'nurturing' of the fertilised ovum during the initial stages of pregnancy.

### **1.1.2 Human reproductive endocrinology**

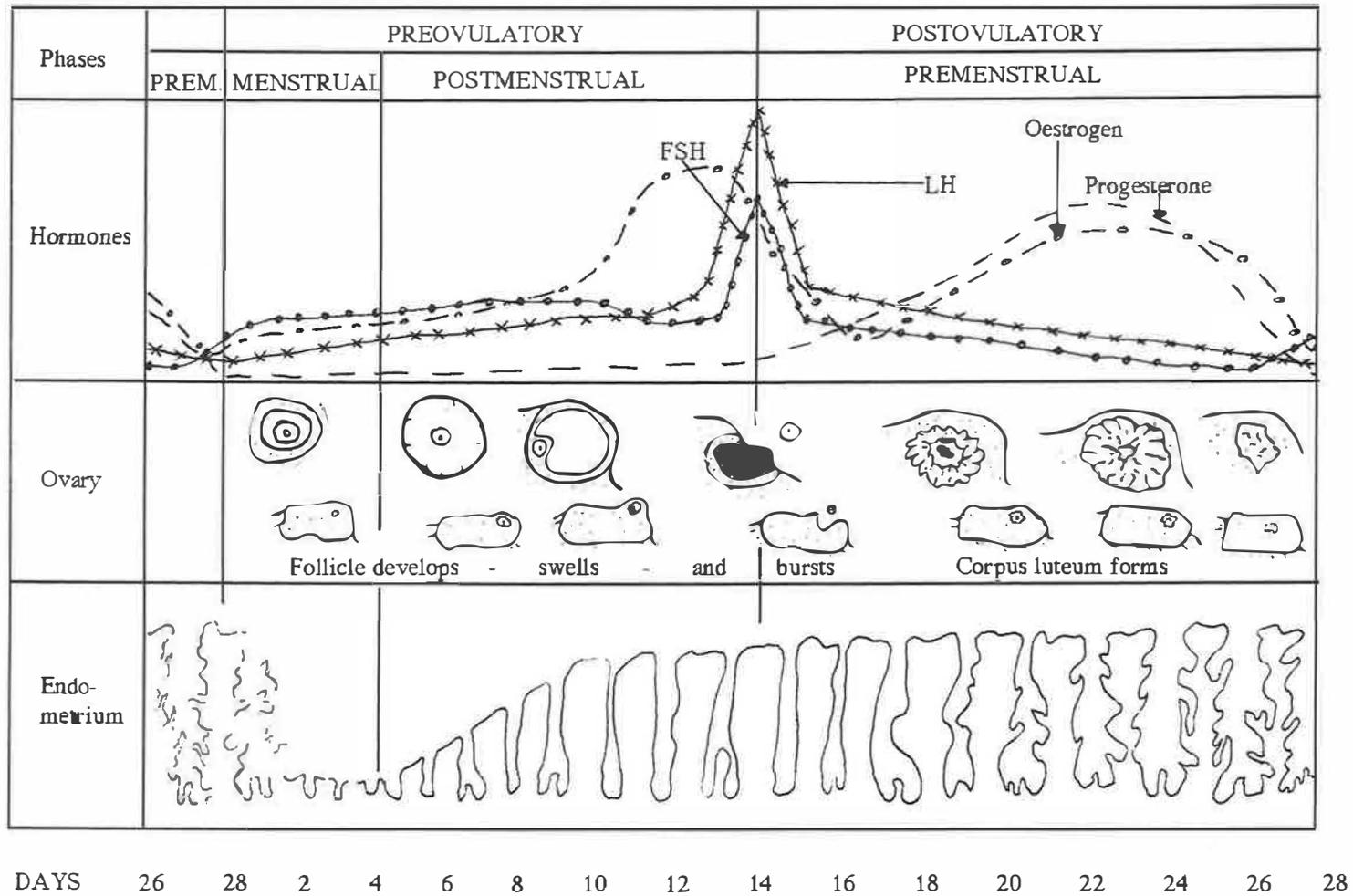
At birth, the secretion of the gonadotrophic hormones follicle stimulating hormone (FSH) and luteinising hormone (LH) from the anterior pituitary gland is elevated, decreasing to low levels within a few months and remaining low through the prepubertal years. During this time the hypothalamic-pituitary unit is very sensitive to the extremely low levels of circulating steroids and therefore negative feedback acts to keep the



**Figure 1.1 The human female reproductive system**

hormone secretion at its low levels (Merck, 1992). Early in puberty a decrease in the sensitivity of the hypothalamus to the gonadal steroids decreases the influence of the negative feedback and thus causes an increase in gonadotrophin secretion, which in turn results in an increase in gonadal steroid production and development of secondary sexual characteristics.

Once puberty is reached in human females there are cyclic variations in the secretion of gonadotrophins and the female sex hormones, progesterone and oestrogens. This cycle recurs monthly and has thus been labelled the menstrual cycle (Latin *mensis* = month). The cyclic phenomenon is largely under the control of the anterior pituitary gonadotrophins, FSH and LH, which are responsible for the development of the follicle and their subsequent secretion of oestrogens and progesterone (Murphy, 1990). As the level of oestrogens in the blood increases, it exerts negative-feedback on the hypothalamico-pituitary axis. When oestrogen secretion from the maturing follicle reaches a peak, shortly before the expected time of ovulation, it causes the anterior pituitary to become much more sensitive to the pulses of gonadotrophin-releasing hormone (GnRH) that are coming to it from the hypothalamus (Murphy, 1990). With this increase in pituitary sensitivity to GnRH, the output of LH from the pituitary is dramatically enhanced. When this high concentration of LH (the so-called LH surge) enters the circulation, it brings about ovulation and the conversion of the ruptured follicle into a corpus luteum. The corpus luteum secretes oestrogens and progesterone for 10 to 12 days. Reduction in the concentration of oestrogen and



**Figure 1.2. The cycle of menstruation and ovulation in women**

(After J. E. Crouch (1965) *Functional human anatomy*, Lea and Febiger, London.)

progesterone, caused by corpus luteum regression, removes the necessary hormonal support of the endometrium (see later), and menstruation ensues (Figure 1.2). The drop in ovarian hormone levels also reduces negative feedback and a new wave of follicular growth begins (Lamb *et al.* 1980). This cycle of events normally continues (except during pregnancy) until the menopause is reached.

The menopause is the cessation of menstruation and is considered complete after one year of amenorrhoea (Merck, 1992). The cause of the menopause is a decline in ovarian function. As the ovaries age, there is a gradual atresia, a decrease in oestradiol production and a concomitant reduction in corpus luteal activity (Glenister *et al.* 1985). This decline of ovarian function accounts for the breakdown of the negative feedback system that controls ovulation and menstruation. Because oestrogen and sequential progesterone production gradually decreases throughout the normal aging process, neuroendocrine stimulation is increased (Glenister *et al.* 1985). This combination of factors results in increased secretion of the pituitary hormones, FSH and LH, as the pituitary attempts to stimulate the ovaries to secrete more oestrogen and progesterone (DeVane, 1983). The loss of inhibition of the gonadotrophins creates a constant state of endometrial proliferation. The ovaries eventually lose their ability to respond to this hyperactive state, leading to a gradual and finally permanent anovulation, the menopause. About 50 % of women experience the menopause between 45 and 50 years of age, 25 % before 45, and 25 % above 50 years of age. The climacteric which refers to the general physiological changes which occur perimenopausally and includes the

menopause, is the prolonged failure of ovulation and the decreasing output of ovarian hormones and may last as long as 20 years (Hafez, 1980). In some cases menstruation may cease abruptly, but more usually menstrual flow becomes gradually reduced, and the interval between periods is increased. As ovarian function further declines the cycle may become irregular. Anovulation may cause missed or shorter and less profuse periods, or functional uterine bleeding with prolonged and grossly irregular cycles (Hafez, 1980).

The symptoms of the climacteric lie in five major areas, i.e. menstrual, vasomotor, musculoskeletal, psychological and sexual.

Menstrual symptoms are characterised by irregular periods which may be heavy and frequent, but more normally exhibit cycles of increasing length with a reduced menstrual blood flow (Studd *et al*, 1977). The mechanisms that may facilitate these menstrual changes include lack of ovulation and corpus luteum development, and failure of feedback in the endocrine system.

Hot flushes, night sweats, palpitations and headaches are due to vasomotor instability (Read and Sturdee, 1993), which allows vasomotor tone and peripheral vessel dilation to be less controlled, and allows rapid vasodilation to occur at minor stress; low oestrogen levels, elevated gonadotrophins and the vasodilator effects of bradykinin and histamine may all contribute (Hafez, 1980).

Loss of muscle strength and muscle mass is also a feature of the climacteric (Siddle and Knight, 1991) as is osteoporosis (Albright, 1941). Together these musculoskeletal symptoms lead to poor posture, altered gait, arthritis, backaches, shortening of the spinal column and an increased incidence of hip fracture. These symptoms are initiated mainly by an increase in bone resorption without a corresponding increase in bone formation (Raisz, 1982).

Psychological symptoms exhibited perimenopausally include increased anxiety, mild depression, emotional outbursts, paranoia, lack of concentration, impaired memory, nervousness, irritability, aggression, fatigue, exhaustion and decreased work performance. It is possible that the reduced free tryptophan level as a result of low oestrogens which are needed to displace 1-tryptophan from its plasma protein binding sites may explain at least some of these psychological disturbances (Coppen, 1972).

Sexual symptoms are characterised by psychosexual problems related to loss of libido and irritability. These symptoms may be linked with development of atrophic vaginitis (Gardener and Kaufman, 1969), dyspareunia and lack of vaginal lubrication during intercourse (Chakravati *et al* 1979).

The severity of these symptoms depends on the amount and rate of oestrogen depletion, the inherited and acquired ability of the women to succumb to or withstand the aging process and the psychological impact of the emotional implications of the change of life (Studd et al, 1977).

The use of hormone replacement therapy (HRT) has been advocated for the treatment of these symptoms, where oestrogen only therapy is generally prescribed to women who have had the uterus removed, and a combined oestrogen/progestin therapy is prescribed to women where the uterus is intact (Harper, 1990). Such treatment regimens are adopted since oestrogen only therapy is associated with endometrial hyperplasia leading to endometrial cancer. However, when a progestin is used in conjunction with the oestrogen, the risk of endometrial hyperplasia is no greater than in women who are not receiving hormones (Voigt *et al*/1991)

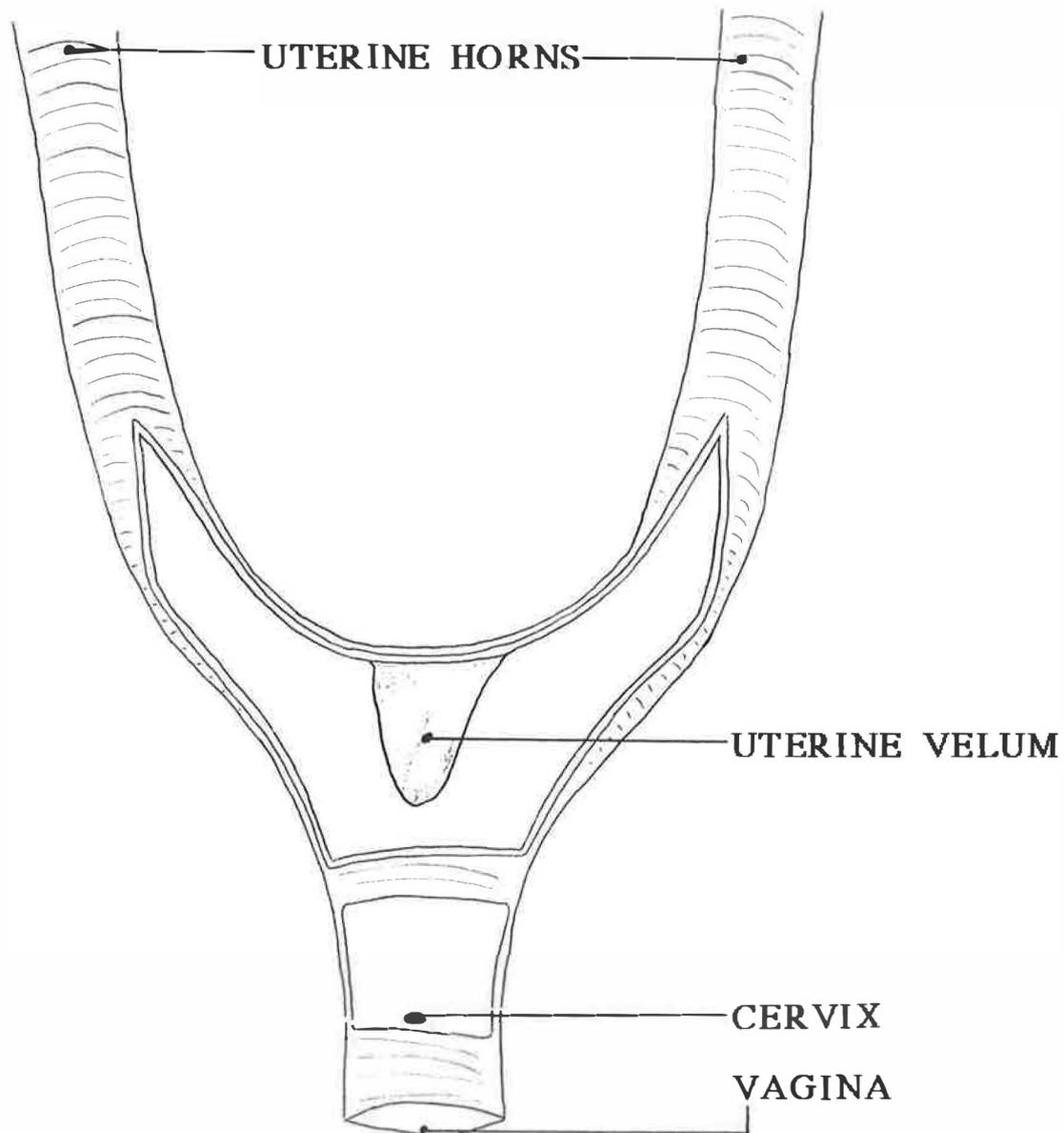
## **1.2 Guinea-pig female reproductive anatomy and physiology**

### **1.2.1 Anatomy**

As in humans, the internal organs of the female guinea-pig are the ovaries, the Fallopian tubes, the uterine horns, the cervix and the vagina (Figure 1.3), but unlike the human uterus the guinea-pig uterus is bicornate. The ova from the ovaries are conveyed directly to the uterine horns where fertilisation occurs and the foetuses develop. At their lower ends the horns connect in a small fundus which is separated from the vagina by the cervix. In guinea-pigs the site of insemination is the uterus (Spector, 1956). The guinea-pig parallels the human reproductive system very well because like humans, it possesses only one cervix, it ovulates spontaneously, and has comparable endocrinology (see below).

### **1.2.2 Guinea-pig reproductive endocrinology**

The guinea-pig cycle of hormone secretion is similar to that found in humans recurring every 18 to 21 days, and is known as the oestrus cycle. It has been observed that during ovulation the guinea-pig vagina is open when compared with the rest of the cycle when it may become less accessible (Stockard and Papanicolau, 1917). However, the fact that the vagina is open does not unmistakably indicate that ovulation is taking place (Stockard and Papanicolau, 1917). Traditionally, the day on which a lordosis response could be elicited was designated day 1 of the cycle. Modern histological techniques have replaced this largely subjective test,



**Figure 1.3 The guinea-pig female reproductive system**  
(Adapted from G. Cooper and A. L. Schiller (1975): *Anatomy of the guinea-pig*, Harvard University Press, Cambridge Massachusetts)

<b>Stage</b>	<b>Duration</b>	<b>Vaginal cytology and secretions</b>
Proestrus	6 - 12 hours	Accumulation of desquamated epithelial cells and presence of polymorphonuclear leucocytes.
Oestrus	2 - 4 hours	Cornified non-nucleated cells present.
Metooestrus	4 - 6 hours	Vaginal fluid appears reddish due to the ingress of traces of blood into the vaginal lumen. Fewer cornified cells are present vaginal secretion becomes serous.
Dioestrus	1 - 2 hours	A large number of desquamated vaginal epithelial cells are present.
Anoestrus	15 - 17 days	Very little fluid is present in the vagina which is leucocyte free. This is the dominant phase of the guinea-pig oestrus cycle.

**Table 1.1 The various stages of the guinea-pig oestrus cycle and their associated characteristics (After Stockard and Papanicolau, 1917).**

whereby the stage of oestrus may be determined by taking a vaginal swab from the guinea pig and examining the residue from the swab microscopically to ascertain the the numbers and types of cells removed from the vagina. The stage of the oestrus cycle determines which cells will be present on the swab (Table 1.1).

Figure 1.4 shows the progesterone levels during the oestrus cycle of virgin female guinea-pigs. Immediately after ovulation plasma progesterone increases from very low levels, to  $2.8 \text{ ng mL}^{-1}$  by day 5. Plasma progesterone levels decrease after day 9 and are less than  $0.5 \text{ ng mL}^{-1}$  by day 12. There is a paucity of data concerning plasma oestrogen, due, primarily to the very low levels of circulating oestrogen where a maximum level of only  $10\text{-}15 \text{ pg mL}^{-1}$  has been reported (Feder *et al*, 1968; Bland and Donovan, 1970; Challis *et al*, 1971).

The peak circulating levels of progesterone and oestradiol in humans are approximately  $10 \text{ ng mL}^{-1}$  and  $200 \text{ pg mL}^{-1}$  respectively (Bell *et al*, 1976; Young and Jaffe, 1976)), which means that circulating progesterone levels in humans are 4 to 5 times those observed in the guinea-pig. In both humans and guinea-pigs the fluctuations in hormone secretion induce changes in cellular activity, notably the cells of the vagina, cervix and endometrium.

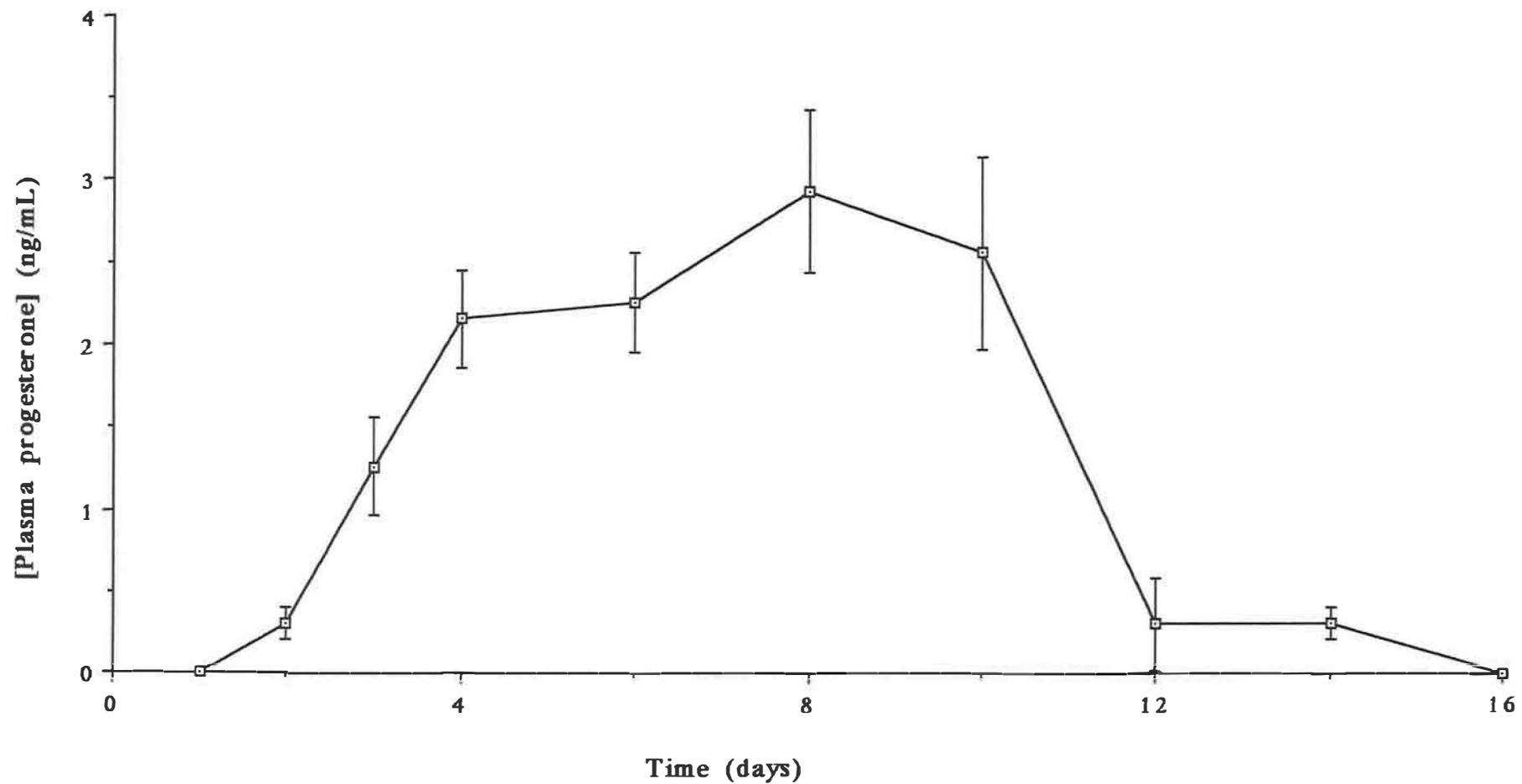


Figure 1.4 Plasma progesterone concentration in virgin female guinea-pigs during the oestrus cycle (Data adapted from Feder et al., 1968)

### **1.3 Variation of vaginal physiology during the ovarian cycle**

In humans, the desquamated epithelial cells of the vagina reflect the level of the ovarian hormones and thus exhibit cyclic variation. The effects of the changing pattern of steroid secretion during the ovarian cycle on the vaginal epithelium are readily assessed by examination of the exfoliated cells. During the follicular phase, with increasing secretion of oestrogens more keratinized cells are shed. After ovulation the number of such cells falls, and an increased number of leucocytes appears. Oestrogen activity is reliably indicated by this pattern, but progestational activity much less so (Glenister, 1985)

The guinea-pig vaginal epithelial cells similarly exhibit cyclic changes, enabling the stage of oestrus to be determined by examination of the vaginal epithelia (Section 1.2.2).

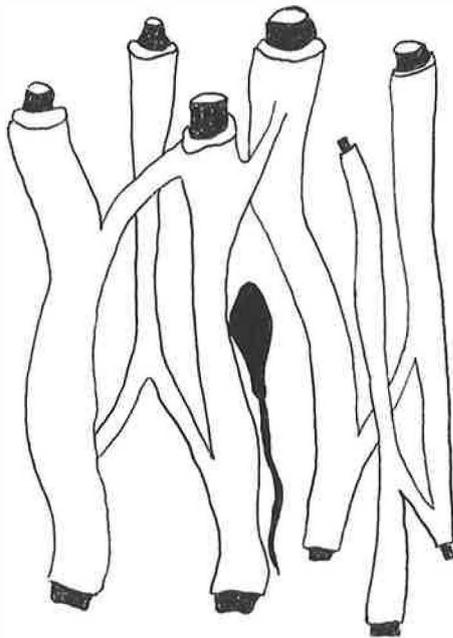
#### 1.4 Variation of cervical physiology during the ovarian cycle

The human cervix secretes mucus the physicochemical properties of which exhibits a cyclic variation during the menstrual cycle dependent upon hormonal status. Oestrogen dominance, at the time of ovulation, results in the secretion of copious quantities ( $600\text{mg day}^{-1}$ ) of low viscoelasticity mucus with a high water content which is penetrated readily by spermatozoa. In contrast, between  $20$  and  $60\text{ mg day}^{-1}$  is present during the luteal phase when progesterone is dominant (Enhorning *et al*, 1970; Elstein, 1974). Luteal phase mucus characteristically exhibits high viscoelasticity and resists penetration by spermatozoa (Chantler, 1982). The essential biochemical component of the cervical mucus gel is a network of thread-like flexible glycoprotein macromolecules - mucins (Gibbons, 1959; Gibbons and Roberts, 1963) and it has been suggested that the rheological properties exhibited by cervical mucus are dependent upon the orientation of these filamentous macromolecules (Odeblad, 1968). Nuclear magnetic resonance techniques were employed by Odeblad (1973) to propose a structure which would explain the unique rheological properties of cervical mucus. Odeblad (1973) proposed that cervical mucus was composed of a high viscosity component (insoluble gel phase) and a low viscosity component (plasma or sol phase), such that the high viscosity component was made up of long flexible macromolecules (Figure 1.5) which were grouped together in micelles averaging  $0.5\ \mu\text{m}$  in diameter. Under a mainly oestrogenic influence, (*e.g.* at the time of ovulation), these micelles would increase in size, whereas under a mainly progestogenic influence (*e.g.* during the

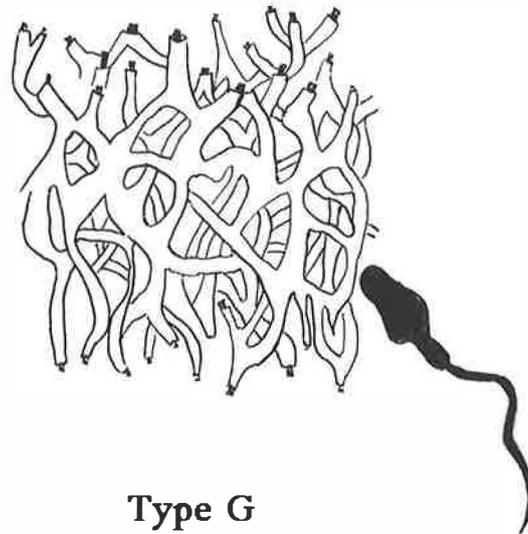
luteal phase of the menstrual cycle), they become very much smaller. Thus under the influence of progestogens the bonding between the glycoprotein molecules alters to form a fine mesh which resists penetration by spermatozoa (type G (gestogenic) mucus). However, under oestrogenic influence, when the micelles are forming, there are free channels between the high viscosity component and this facilitates the transmission of spermatozoa (type E (oestrogenic) mucus) (Figure 1.5). However, the results obtained by other workers (Singer and Reid, 1970; Elstein *et al*, 1971) are not in complete agreement with the description of the ultrastructure of mucus given by Odeblad (1973), although they clearly demonstrated the fibrillar meshwork with interlacing fibres. Elstein (1978) has suggested that many inconsistencies may have arisen by the preparation of the mucus, and the staining itself which can cause artifacts.

The dimensions of the cervical os in humans also exhibit cyclic variations which favour the passage of spermatozoa during the periovulatory phase of the menstrual cycle. During the proliferative phase there is a progressive increase in the diameter of the external os which reaches a maximal width at ovulation and narrows following ovulation (Mann *et al*, 1961). At ovulation the internal os measures about 3 mm in diameter compared with a minimum diameter of 1 mm seen post-menstrually (Hafez, 1980).

Since only very small quantities of cervical mucus are produced by the guinea-pig, cyclical changes in the characteristics of guinea-pig cervical



**Type E**



**Type G**

**Figure 1.5 Types E and G cervical mucus (after Odeblad,1973).**

The macromolecular cores (consisting of several long molecules side by side) are shown in black, together with surrounding hydration cells (white). A sperm moving in the cervical plasma between the micelles in type E and a non-invading sperm outside type G, are also shown

mucus could not be determined (Bilbruck, 1991). However, as the guinea-pig undergoes variation in the amount of circulating sex hormones, it is assumed that such changes would occur.

## 1.5 Variation of uterine physiology during the ovarian cycle

The uterus is a muscular organ lying centrally within the pelvis, anterior to the rectum and superior and posterior to the bladder. It resembles an inverted pear in shape, and comprises, ( from top to bottom), the fundus, the corpus, the isthmus and the cervix.

The uterus is made up of 3 main concentric layers, which from outer to inner are;

- i) The perimetrium. This is a thin peritoneal covering.
  
- ii) The myometrium. This is a very large layer of smooth muscle and connective tissue. There are actually 2 layers of smooth muscle, a thin outer longitudinal layer which is continuous with that of the Fallopian tubes and the vagina, and an inner layer arranged in oblique spirals. Between these 2 layers, blood and lymph vessels, nerves and connective tissue may be found (Bowman and Rand, 1980).
  
- iii) The endometrium. This is composed of epithelium and stroma, with a single layer of columnar cells lining the uterine lumen. It varies in thickness from 1 mm to 6 mm depending upon the stage of the menstrual cycle (Reame *et al*, 1980), and is divided into 3 zones. The zona compacta which is narrow and composed of compact hypertrophied cells, is found uppermost. The zona

spongiosa is much wider and composed of more loosely arranged cells and is found in the middle. The zona basalis is the deepest layer and contains the bases of the glands, and adjoins the myometrium.

Figure 1.2 shows the main hormonal changes and endometrial events over the 28 day menstrual cycle, where day 1 is taken as the day of onset of menstruation. Menstrual fluid is composed of red blood cells and leucocytes, desquamated epithelium, cervical mucus, vaginal cells and bacteria (Reame *et al* 1980). The menstrual cycle is usually divided into three phases despite the fact that it is a continuous process.

Menstruation: (days 1-6). This is considered as phase one. Progesterone secretion declines as the corpus luteum begins involution. The walls of the capillaries and some of the coiled arterioles of the endometrium break down and blood escapes into the stroma of the superficial layer of the endometrium. Fragments of the superficial layer break away and other blood channels are opened. The same contractions of the coiled arterioles which earlier caused break down, now prevent excessive haemorrhage. The basal portions of the endometrium with remnants of the uterine glands, having a conventional blood supply, remain intact and ready to start repair with replacement of the outer layers (Bell *et al* 1983).

Proliferation: (days 6-14). With increased production of oestrogen by the ovarian follicles, the growth of the endometrium accelerates. The uterine glands lengthen and produce a thin secretion; connective cells multiply

and a new meshwork of reticular fibres appear. The endometrium approaches 2 mm in thickness. Ovulation takes place and the endometrium develops progesterone receptors under the influence of the oestrogens.

Secretion: (days 14-28). In this phase the oestrogenic influence is gradually given over to that of progesterone from the corpus luteum. Because of the drop in oestrogen, the thickening of the endometrium may temporarily be stopped and there is sometimes intermenstrual bleeding. The endometrium more than doubles in thickness during this period, reaching 4-5 mm. Its glands become long, swollen and tortuous and produce abundant quantities of a thick, mucoid, glycogen-rich secretion. The uterus is now ready to receive a blastocyst. If fertilisation occurs, a blastocyst is generated and implantation takes place, the endometrium continues its development and the corpus luteum persists. In the absence of a blastocyst, the corpus luteum begins to degenerate, which is the signal for the endometrium to break down. The coiled arterioles of the outer endometrium contract and deprive the superficial layers of blood and oxygen initiating destruction which is characteristic of the next phase which is menstruation.

As observed by scanning electron microscopy, the endometrial surface is composed of ciliated cells covered with kinocilia or solitary cilia and secretory cells covered with microvilli (Jaszczak and Hafez, 1978). The directional beat of the kinocilia suggests that their action is to facilitate the release and distribution of the endometrial secretions and propel fluid currents within the uterus. The surface ultrastructure varies with region

of uterus, the phase of the menstrual cycle, onset of implantation, aging, administration of steroid hormones and presence of IUCDs (Hafez and Ludwig, 1977). Cyclic changes have been reported in cell shape, distribution and number of apical microvilli, ciliation and secretory activity (Hafez, 1980).

The surface of the endometrium in the secretory phase appears to be densely covered by secretory cells with bulging apices stippled with microvilli, and numerous ciliated cells concentrated mainly around gland openings. Secretory particles adhere to cilia and may aggregate to form clumps of secretions which are dispersed across the endometrial surface. Occasionally collapsed cells are seen following release of the secreted material. At midcycle a peak is reached in the ratio of ciliated cells to secretory cells of approximately 1:15 (Hafez, 1980).

## **1.6 Microbiology of the human female reproductive system**

The female genital tract is not only subject to variations in 'endogenous' cellular activity, but also subject to variations in the normal, commensal microbial population. These microorganisms are a normal feature of the genital tract, and only rarely become pathogenic.

### **1.6.1 The vagina**

Any variations in the microbial status of the vagina may have consequences on the microbial status of the endocervix and the uterus. Thus it is important to understand the changes in the microbial status that occur throughout the reproductive system.

The physiological condition of the vagina changes dramatically at different ages, and this causes major changes in the microecosystem which resides there. The conditions of the vagina affecting the microbial population are age related and can be divided into three periods.

#### **1.6.1.1 Pre-pubertal period**

At birth, under normal circumstances the vagina is microbiologically sterile. However, immediately upon parturition circulating maternal oestrogen may result in a microbial flora in neonates which is similar to that found in the mother (Wilson and Miles, 1975). This high level of oestrogen results in the presence of glycogen in the vaginal epithelium

which is broken down to lactic acid resulting in an acidic milieu in the vagina. This pseudomature neonatal vagina changes after 2 to 3 weeks as the oestrogens are metabolised (Brown, 1978), so that throughout childhood the vaginal epithelium lacks glycogen leading to a rise in the pH. During this period, gram-negative bacilli, gram-positive cocci and bacilli (not lactobacilli) predominate, (Brown, 1978), although a scanty background flora of skin organisms and upper respiratory tract commensals are often present during this period (Davies and Jephcott, 1989).

#### **1.6.1.2 Post-pubertal period**

The vaginal epithelium has a high glycogen concentration due to the influence of circulating oestrogens. This is metabolised to lactic acid, the precise mechanism of which is uncertain although 4 proposed mechanisms for the production of lactic acid in the vagina were made by Rakoff *et al* (1944). These are

- (i) Doderlein's bacilli (see later) produce enzymes which convert glycogen to lactic acid.
- (ii) glycogen is broken down to a monosaccharide by a vaginal enzyme which is then converted to lactic acid by Doderlein bacilli.
- (iii) bacteria other than Doderlein bacilli may breakdown carbohydrate to lactic acid.
- (iv) the glycogen is converted to lactic acid solely by vaginal

enzymes.

Doderlein's bacilli are the predominant bacteria found in the vagina during the post-pubertal period, and are known as such due to Doderlein's early description of large Gram-positive bacilli (Doderlein, 1892). However, this term has become a general term referring to any acidophilic Gram-positive bacilli found in the vagina, although Rogosa and Sharpe, (1960), have equated Doderlein's bacilli with *Lactobacillus acidophilus*. Other bacteria which are commonly isolated from the vagina include anaerobic or facultatively anaerobic lactobacilli and, anaerobic *Bacteroides* spp. The commonest aerobic or facultatively anaerobic bacteria isolated from the human vagina include diphtheroids and coagulase-negative staphylococci (Davies and Jephcott, 1989).

Pregnancy has little effect on this flora, although an increase in the glycogen content due to raised oestrogen levels may make the vagina more favourable to acidophilic bacteria (Hurley *et al* 1974).

#### **1.6.1.3 Post-menopausal women**

With a decrease in the oestrogen levels at the menopause, the amount of glycogen in the vagina is also reduced, causing an increase in the vaginal pH such that acidophilic organisms will no longer predominate (Brown, 1978). The microflora of the vagina then resembles that of the pre-pubertal vagina. The variety of bacteria may increase during this period, but the quantity is reduced and becomes similar to that found in the pre-

pubertal vagina (Meisels, 1968). If vaginal discharge occurs which is due to bacterial overgrowth in later life, it usually responds to local oestrogen therapy which will alter the pH of the vaginal environment (Davies and Jephcott, 1989).

### 1.6.2 The cervix

Cultures from the cervix yield fewer organisms than from the vagina, due to the different epithelia and pH values of the two sites (Davies and Jephcott, 1989). The commonest organism isolated from the cervical canal is the lactobacillus (Sparks *et al* 1977). Other organisms that are also commonly isolated included *Staphylococcus epidermidis* and *Bacteroides* spp (Sparks *et al* 1977). The vast majority of microorganisms isolated from the cervix are found in the ectocervix and the first 10 mm of the cervix continuous with the ectocervix (Sparks *et al* 1977). Several mechanisms for this decline in microorganisms with depth into the cervix have been suggested.

- (i) Cervical mucus which is constantly produced and passes into the vagina by both gravity and ciliary action, may wash out invading microorganisms (Sparks *et al* 1977).
- (ii) The alkaline pH of the mucus may inhibit growth of bacteria normally resident in the acid vagina (Sparks *et al* 1977).
- (iii) Immunoglobulin A which is produced in the endocervix will destroy bacteria in the presence of lysosyme and complement, blocking bacterial adhesion to mucosal cells and promoting

agglutination and phagocytosis (Rebello *et al* 1975).

### 1.6.3 The uterus

Determination of the microbial status of the uterus is far more difficult to achieve than that of the vagina, since any transcervical sampling is very likely to introduce microorganisms into the uterus. Samples taken by this route showed bacterial growth in 45 to 67 % of cases (Willson *et al* 1964; Mishell *et al*, 1966; Golditch and Huston, 1973). Direct sampling from the uterine cavity when women are undergoing a gynaecological operation overcomes this potential route of contamination, and shows bacteria in up to only 8 % of cases (Mishell *et al*, 1966; Ansbacher *et al* 1967; Spore *et al* 1970). The most reliable information, has been determined from hysterectomy patients where the uterus was shown to be sterile in all cases (Sparks *et al* 1977). In this study isolation of microorganisms was investigated along the cervix. It was found that compared to the ectocervix, where microorganisms were isolated from 49 of 49 women, no bacteria could be isolated from the same women at a point along the cervix greater than 30 mm from the ectocervix. This suggests that a mechanism or mechanisms exist to prevent bacterial access into the uterus (see later). In a small percentage of cases where the isolation of microorganisms from the uterus was possible it was associated with carcinoma or recent insertion of an IUCD (Ansbacher *et al*, 1967; Mishell *et al* 1966).

Since the association between the IUCD and bacterial colonisation of the

uterus has been well documented, and the development of an intrauterine device is to be addressed in this thesis, it is relevant to discuss the IUCD itself, its association with pelvic infection and how this has been debated.

## **1.7 The intrauterine contraceptive device**

### **1.7.1 History of the intrauterine contraceptive device**

Intrauterine contraception is reported to have been applied firstly to camels, where camel traders taking the animals on long journeys, wanted to prevent conception and did this by placing pebbles into the reproductive tract (Schmidt, 1982). The first mention of intrauterine pessaries, which were used for causing abortion, occurs in Hippocratic writings where a hollow sound was passed into the uterus and used for their insertion as cited by Southam (1965).

The first well documented account of a device being placed in the human uterus, specifically for the purpose of contraception, was a ring of silk worm gut, which was described by Richter in 1909. Other devices that were developed after Richter (1909) included the Grafenburg ring (Grafenburg, 1930), a ring device consisting of a core of silk worm gut encircled with German silver, and the Ota ring (Ota, 1934), another ring device consisting of a silver, or gold plated silver ring with spokes. All of these earlier devices were associated with an unacceptable incidence of infection, which made their use unpopular. In 1960 the so called "second generation" devices were introduced, made entirely from plastic and were relatively inert and inexpensive. Since at this time the rate of population growth was very high, the need for an effective and simple means of contraception necessitated reevaluation of this old technique (Southam, 1965). The first of these was the Margulies spiral (Guttmacher, 1965)

which was coated with barium sulphate to make it radio opaque. The second one of note was the Lippes Loop (Lippes, 1962), which was the first intrauterine contraceptive device (IUCD) to have a marker thread attached, and was withdrawn from the market in 1985, due to commercial and financial considerations connected with the manufacturers inability to obtain liability insurance, possibly because of an alleged connection with PID (MacKenzie, 1987).

Since 1962 modifications to the plastic device have been made, the most notable of which were the incorporation of copper into the device and the design of plastic devices with slow releasing steroid reservoirs.

#### **1.7.1.1 Copper bearing devices**

Zipper (1974), reported that the plain 'T' carrier without copper was associated with a pregnancy rate of 18 per 100 women years of use. When copper was added in the form of a wire, having a surface area of 200 mm<sup>2</sup> a decrease in the rate of pregnancy was observed (down to 1 pregnancy per 100 women years of use). This reduction in the pregnancy rate appeared to be directly proportional to the surface area of the copper attached to the device. These copper bearing IUCDs also caused a reduction in menstrual blood loss (Shaw *et al*, 1980) and frequency of expulsion (Zipper, 1974) compared with non-medicated devices such as the Lippes Loop. Neither vaginal nor cervical cytology appear to change in the presence of the copper 'T' device when compared with the inert 'T' device (Zipper, 1974).

### 1.7.1.2 Hormone releasing devices

Scommenga and co-workers (1970), pioneered a plastic device with a slow releasing reservoir of progesterone. Progesterone was released at a rate of  $65 \mu\text{g day}^{-1}$ , and was shown to be as effective at preventing pregnancy as copper bearing IUCDs. This 'T' shaped device which is the same size as the copper 'T' is fitted with a permeable polymer membrane that is able to release progesterone at a constant sustained rate for a period of one year (Alza Pharmaceuticals, 1976). Although intermenstrual spotting increased with these devices (Rowe *et al* 1975), a reduction in menstrual blood loss as compared with pre-insertion values was noted (WHO, 1980).

Another steroid which is used in a sustained release device is levonorgestrel. This has been incorporated into a device, the shape of which is based upon the Nova-T IUCD releasing  $20 \mu\text{g day}^{-1}$  of levonorgestrel from a polymethylsiloxane 'collar'. This device gives a very low pregnancy rate (0.3 per 100 women years of use) (Sivin *et al* 1984), and a reduction in menstrual blood loss compared to pre-insertion control menstrual blood loss (Nilsson, 1977). However, a higher discontinuation rate is observed due to oligomenorrhoea or amenorrhoea (Sivin *et al* 1987). This amenorrhoea is associated with a local effect of levonorgestrel on the endometrium, but is not always associated with anovulation (Bilian *et al* 1990).

At present there are many types and designs of IUCD being used by

approximately 90 million women world wide, of whom, 59 million are in China, where it is the method of contraception for 30% of married women of reproductive age (% MWRA) (Population report, 1988). Western observers may easily attribute the high levels of IUCD usage in China to a rigidly enforced population control policy coupled with a low regard as to the real or supposed drawbacks (Bromham, 1993). However, few would ascribe such factors to countries as Finland and Norway when it is considered that 30% of female contraceptors use the IUCD. It is possible that the variable uptake of an IUCD as a method of contraception derives from the way in which it is presented to the potential user and the provision each country makes for the support of those choosing the method (Bromham, 1993).

### **1.7.2 Mechanism of action of IUCDs**

There does not appear to be a single mechanism of action associated with the antifertility effect of the IUCD, the mechanisms involved varying from one type of IUCD to another. The inclusion of copper on an IUCD introduces further actions to the otherwise inert IUCD, as does the addition of a steroid reservoir. Actions of the IUCD which may explain the overall mechanism of the antifertility effect are given below.

#### **1.7.2.1 Morphological changes**

The morphological changes in the human endometrium and the biochemical composition of the uterine fluid during the normal menstrual

cycle are both important factors in reproduction. These changes may play a role in the capacitation of spermatozoa as well as the implantation of the blastocyst. Any agent that modifies the endometrial morphology or the composition of the uterine fluid may therefore interfere with the physiology of reproduction (WHO Technical report, 1987a).

Whenever a foreign body is introduced into the uterus, specific changes in the endometrial tissue result causing an increase in vascular permeability, oedema and stromal infiltration of leucocytes, including neutrophils, mononuclear cells and macrophages (Shaw *et al*, 1979). Although all IUCDs will increase leucocyte infiltration into endometrial tissue it is not specific to IUCD use, and is seen 24 - 48 hours prior to menstruation in the normal menstrual cycle (Bartelmez, 1931).

#### **1.7.2.2 Endometrial inflammatory cells**

Insertion of an IUCD may result in small blisters filled with oedema fluid beneath the superficial epithelium. These blisters may be seen in rare endometrial biopsies but it is likely that they remain only for a short time, rupturing to leave a superficial ulceration. With the release of oedema fluid, a migration of neutrophils takes place in the area of ulceration (Moyer and Mishell, 1971). In some patients the superficial epithelium undergoes reepithelialisation whereas in others the ulceration continues for a greater length of time. Although the concentration of neutrophils (polymorphonuclear leucocytes) and macrophages is greatest at the ulcerated areas, these cells are present in smaller numbers in the

surrounding tissue and uterine fluids. This increase in intrauterine leucocyte numbers may be directly related to the antifertility action of IUCDs especially with regard to macrophages (Sagiroglu and Sagiroglu, 1970a). Users of IUCDs show increased intrauterine protein levels which are possibly due to degradation of leucocytes thus further contributing to the antifertility effect (Kar *et al* 1968).

The influx of inflammatory cells into the endometrium is qualitatively and quantitatively related to the time following IUCD insertion (Moyer and Mishell, 1971). Neutrophils and a few mononuclear cells migrate into the endometrium during the first week after insertion. The number of these cells increases greatly during the second week to the third month following insertion. Plasma cell infiltration may be seen in large numbers in some patients during the first few months after insertion, and usually small numbers of plasma cells are present in nearly all endometrial samples during this time (Mishell and Moyer, 1969). Plasma cells are a transient invader of the endometrial tissue and respond to the bacteria introduced from the cervical mucus and possibly to products of the traumatised endometrial tissue as well. Plasma cells are usually absent from the endometrium by six months after IUCD insertion although in some cases they persist for years. Even though the mononuclear cells, such as macrophages and lymphocytes are reduced in numbers six months after insertion, these cells remain in the stroma the entire time that the IUCD remains in the uterine cavity (Moyer and Mishell, 1971).

The primary cell types in the uterine tissues and fluids in response to the

IUCD are neutrophils, macrophages, lymphocytes and plasma cells. Large numbers of macrophages have been shown to adhere to Lippes loops that have been in the uterine cavity for 24 to 36 months (Sagiroglu and Sagiroglu, 1970a, 1970b). These authors, have suggested that the foreign-body reaction may be instrumental in preventing pregnancy by one or more of the following mechanisms:

- (i) The macrophages interfere with implantation by spreading over the superficial surface of the endometrium, isolating the blastocyst from the endometrial tissues.
- (ii) Phagocytosis of spermatozoa by macrophages may reduce the number of viable spermatozoa in the endometrial cavity.
- (iii) The macrophages may destroy the blastocyst by phagocytosis during the period prior to implantation.
- (iv) The macrophages act as secretory cells, elaborating cytoplasmic products that diffuse into the uterine secretion. These cellular products enter the uterine fluid either by secretion of intracellular substances or by the cytolysis of the macrophages.

The macrophages are present in greater numbers close to or adhering to the IUCD. In contrast, the neutrophils are distributed diffusely throughout the uterine cavity. During the first few days after insertion the neutrophils predominate in the uterine fluid, in contrast to the macrophages which increase during the ensuing weeks (Moyer and Mishell, 1971).

### **1.7.2.3 Endometrial mast cells**

Mast cells are not a prominent element in the normal endometrium, although their numbers are reported to increase in the IUCD-exposed endometrium (Mathur and Choudhury, 1968). These could play an important role in the inflammatory response to IUCDs by releasing histamine.

### **1.7.2.4 Biochemical responses to IUCDs**

Many biochemical substances may affect the metabolism of the preimplantation blastocyst as well as the implantation phenomenon. The increased amounts of proteases that occur in response to the IUCD-stimulated inflammatory reaction probably represent one of the most important groups of chemical substances to be associated with the antifertility action (Sagiroglu and Sagiroglu, 1970b). The presence of numerous macrophages wandering free in the uterine cavity and adhering to the surface of the IUCD is a source for these proteases. The increased concentration of these proteases within the uterine cavity as a result of macrophage secretion may have a profound effect on the preimplantation blastocyst and the implantation mechanism.

As well as the increase in protease levels, it has been shown that the activities of lysosomal enzymes and lysozyme are increased in the luminal fluid of rat uterine horns containing an IUCD, in comparison to uterine horns without IUDs. These increases may also exert an antifertility effect

(Schulten *et al* 1975).

Circulating levels of immunoglobulins G and M in the serum were found to increase significantly after insertion of an IUCD in humans (Holub *et al* 1971). When serum IgG and IgM were studied in women having the IUCD in place for less than 3 months or longer than 20 months, the levels were significantly higher in those women having the IUCD in place for longer periods of time. In the case of rats which were given an immunosuppressant drug such as cyclophosphamide, the number of implantations in the horns in which IUCDs were placed was greater in the drug treated group than in the non-drug treated group (Holub *et al* 1971).

A gradient of IUCD-induced substances and cells occurs in the tubular uterus of the rabbit with the highest concentrations in the area closest to the IUCD and the lowest concentrations several centimetres distal to the IUCD. A positive correlation was observed between the quantity of leucocytes and the distance between the IUCD and the implantation site (El Sahwi and Moyer, 1971).

#### **1.7.2.5 Prostaglandins and allied compounds**

IUCDs may increase PGF<sub>2</sub> production in the uterus and induce luteolysis (Spillman and Duby, 1972). Many cellular and vascular changes due to IUCDs may in principle be induced by arachidonic acid metabolites, and cells which attach themselves to either inert or copper bearing IUCDs can

produce PGE<sub>2</sub> and PGF<sub>2</sub> (Myatt *et al* 1975).

Leucotrienes and lipotoxins produced from arachidonic acid via the lipoxygenase pathway are a product of human polymorphonuclear leucocytes (Samuelsson, 1985). These may cause cytotoxicity, chemotaxis and increased vascular permeability (Samuelsson, 1985). The possible relevance with respect to IUCDs remains unexplored.

Three studies that looked at endometrial prostaglandin levels with an IUCD in place showed :-

(i) with respect to PGF<sub>2</sub> and 13,14-dihydro-15-keto PGF<sub>2</sub>, there were no significant changes before and after insertion of the device (Green and Hagenfeldt, 1975).

(ii) PGF<sub>2</sub> before and after insertion showed no change although an increase in PGE<sub>2</sub> levels with both copper bearing and inert IUCDs was noted in women during the luteal phase (Hillier and Kasonde, 1976).

(iii) the Lippes Loop and progesterone releasing IUCDs showed no change in levels of PGF<sub>2</sub>, but after 8 months with a dydrogesterone-releasing IUCD reduced PGF<sub>2</sub> levels were shown (Scommenga *et al* 1978).

Prostaglandins are synthesised and metabolised locally and are not stored in tissues. Therefore, the technique of obtaining endometrial specimens by biopsy or after hysterectomy would invariably evoke a trauma which in

itself triggers the release of arachidonic acid from cell membranes. Hence the studies discussed above do not report on *in vivo* levels of the prostaglandins but rather measure the efficacy of the cyclooxygenase pathway (Hagenfeldt, 1987).

Copper appears to cause metabolism of prostaglandins in the endometrium in favour of a lower PGF<sub>2</sub>:PGE<sub>2</sub> ratio (Kelly and Abel, 1983), and this may contribute to the antifertility action of the device. Increased prostaglandin production may increase uterine activity and/or tubal motility, which have been proposed as mechanisms of action for IUCDs but remain unsubstantiated (WHO Technical Report, 1987a).

#### 1.7.2.6 Embryo specific substances

Twenty four hours after fertilization, when the zygote is in one of the Fallopian tubes, an immunosuppressive protein, early pregnancy factor (EPF) may be detected in the serum (Morton *et al*, 1982). EPF has been found in the serum of IUCD users, (Smart *et al*, 1982) but less often than is found in non-users (Rolfe, 1982), suggesting that the probability of producing embryos with an IUCD in place is less than without an IUCD in place, however, these conclusions have been challenged (Croxatto, 1983). There is no antiserum to EPF as it has not yet been purified and so the more usual approaches to enzyme assay, eg. ELISA tests, are not possible. It is quantified by its ability to decrease or inhibit spontaneous *in vitro* rosette formation between lymphocytes and heterologous red blood cells with complement present. Since this assay procedure is difficult and

fraught with pitfalls (Sinosich *et al* 1985), early production of EPF has not always been confirmed (Thompson *et al* 1980).

Human chorionic gonadotrophin (hCG) is seen in the circulation 9 - 11 days post - fertilization (Kosasa *et al* 1973). Beling (1976) showed a positive hCG result in a urinary assay of 32 out of 73 IUCD users in the luteal phase, suggesting conception. Other workers have failed to confirm this (Klein and Mishell, 1977; Sharpe *et al* 1977) although when hCG was found, levels were the same or less than normal implantation (Nilsson and Lahteenmaki, 1977).

Because of the structural similarity between hCG and human luteinising hormone, analytical problems present themselves (Ortiz and Croxatto, 1987). An immunoradiometric assay specific to the carboxy group on the terminal peptide of the  $\beta$ -hCG chain did not show any transient increase in hCG levels in IUCD users, suggesting that the IUCD interferes with the reproductive process before the embryo produces enough hCG to be detected in the maternal body fluids (Wilcox *et al* 1985).

#### **1.7.2.7 Embryotoxic activity in the uterus**

The intrauterine milieu is greatly modified by the increasing numbers of neutrophils and macrophages migrating from the uterine tissues into the uterine fluid in response to an IUCD and is associated with a decrease in the numbers of blastocysts that implanted. Both homogenates and intact cells when introduced into the uteri of rabbits during the preimplantation

phase markedly reduced the number of pregnancies in a concentration dependent manner (El Sahwi and Moyer, 1977). Homogenised endometrial cells, liver cells or thyroid cells induced the same response. There was no evidence that inflammatory cells specifically produced the embryotoxic effect, but rather the concentration of all cells tested in the cellular homogenates showed direct correlations with embryo toxicity.

In a study using cultures of rat morula (the post-fertilization stage between zygote and blastocyst), inhibition of embryo development to the blastocyst stage was noted with the use of various cell extracts (Parr, 1969). These cellular extracts included rat and rabbit polymorphonuclear leucocytes, macrophages, liver cells, HeLa cells and thyroid cells. Fractionation of the rat leucocytes showed that the embryotoxic effect did not reside in the lysosomal nor the dialysate fractions nor the remainder of the leucocyte extract. However, a recombination of these fractions restored the embryotoxic activity. Again it was the concentration of the cells that was the most important factor in arresting embryo development. This suggests that it is the concentration of the cells rather than the type of cells which inhibit embryonic development.

#### **1.7.2.8 Sperm migration**

Spermatozoa are often reduced in numbers or absent from the upper female genital tract of IUCD users, especially when copper bearing IUCDs are employed ( Croxatto *et al*, 1973). However, sperm can migrate to the Fallopian tubes, but appear to be less likely to reach

normal fertilization sites compared to control women (Sagiroglu, 1971). The IUCD's effect upon sperm, dramatically reducing their numbers, normality and hence fertilization capacity, has been suggested to be their major mechanism of action particularly in the case of copper bearing IUCDs (Bromham, 1993). In a large proportion of women using IUCDs incorporating copper, it was found that the heads of the sperm had separated from the tails (Croxatto *et al* 1973).

#### **1.7.2.9 Transport and development of ova**

Ova are virtually absent from the uteri of IUCD users (Croxatto, 1974), and there is a much lower rate of recovery from the Fallopian tubes as compared with non-users (Ortiz and Croxatto, 1987). Microscopic examination of the majority of fertilized ova recovered from the Fallopian tubes of IUCD users after intercourse showed no signs of development, whereas 50 % of fertilized ova recovered from the control group exhibited features characteristic of a normal healthy embryo (Ortiz and Croxatto, 1987).

As indicated previously it is unlikely that the action of IUCDs is predominantly due to their interference with implantation, but it is more likely that they exert their effect beyond the uterus affecting steps before the ova reach the uterine cavity. IUCDs may alter uterine and tubal fluids, impairing gamete viability and therefore reduce the chances of union and hence fertilization. Copper bearing IUCDs appear to potentiate this effect (WHO Technical Report, 1987a).

### 1.7.2.10 Actions of the copper IUCD

In the presence of a copper IUCD, rodent embryos die just before implantation (Chang *et al*, 1970). The exposure of these embryos to a copper IUCD for relatively short periods of time around the time of implantation will completely suppress their growth and render the embryos non-viable.

Physiological and biochemical changes occur in endometrial tissues as a result of close contact with a copper-containing IUCD (Oster, 1971, 1972). Carbonic anhydrase, a zinc-containing enzyme, undergoes significant modification as the result of its association with copper ions. Carbonic anhydrase is thought to be essential for the adhesion of the blastocyst to the superficial endometrium. Proteolytic enzymes necessary for implantation are inhibited by the action of copper. Brinster and Cross (1972), have shown that metallic copper can cause a disruption of the sulphhydryl bonds in protein which may explain the inhibition of proteolytic enzymes, and this in turn will contribute to the death of the embryo. Copper inactivates the cytoplasmic progesterone receptors and locally inhibits the action of progesterone (Nutting and Mueller, 1975) and appears to affect the amount of DNA in endometrial cells, glycogen metabolism and oestrogen uptake by the uterine mucosa (Hagenfeldt *et al*, 1972).

Hysterectomy specimens taken from women who had used either an inert or copper IUCD for 3 - 6 months showed an inflammatory reaction in the

endometrium when studied with scanning electron microscopy. Increased numbers of inflammatory cells were seen in the lumen of endometrial glands during a 12 month period after insertion of the IUCD and an increase in the number of inflammatory cells in the uterine flushings was noted in association with the T Cu 200 (Hagenfeldt *et al* 1972). This increase was of lesser magnitude than that reported by other investigators studying inert IUCDs, although, it has been shown by Cuadros and Hirsch, (1972), that copper bearing IUCDs will increase the foreign body reaction. Hagenfeldt *et al* (1972), have therefore suggested that it is likely that the copper IUCD exerts its effect on fertility primarily as the direct effect of copper on the blastocyst and only secondarily through a foreign body reaction. This is in contrast to inert IUCDs in which the sterile inflammatory reaction is the basis of its mode of action.

#### **1.7.2.11 Steroid releasing IUCDs**

The progesterone-impregnated IUCD produces marked changes in the endometrial tissue in the majority of women wearing these devices, although the foreign body response itself is minimal with an IUCD constructed of polyethylene with vinyl acetate co-polymer (Moyer and Shaw, 1980). The predominant effect of intrauterine progesterone is its ability to cause atrophy of the glands and to stimulate a pseudodecidual stromal reaction. Secondarily, progesterone in uterine fluid may alter significantly the metabolism of the blastocyst. The endometrial response, when fully developed, creates an environment that is not conducive to implantation or to nourishment of the preimplantation embryo (Lifchez

and Scommenga, 1970). A diffuse pseudodecidual stromal reaction occurs in the functional layer of the endometrium, usually without affecting the basalis. This change is characterised by a marked enlargement of the stromal cells, resulting in eosinophilic cytoplasm and vesicular nuclei. The endometrial glands are small, non-tortuous and non-secretory. The ultimate expression of this change takes place over a period of several months. The progesterone device that releases 65 µg of progesterone each day produces characteristic endometrial changes which were present in approximately 90 % of the women 3 months after insertion of the device. By 6 and 12 months following insertion the endometrium did not show normal cyclic activity, and the predominant state of the endometrium was a pseudodecidual stromal reaction with small inactive glands. Progesterone IUCDs that released only 10 and 25 µg of progesterone each 24 hours showed a typical suppressed endometrial reaction (pseudodecidual stromal reaction with inactive glands) in less than 50 % of the patients. In contrast, a progesterone IUCD releasing 120 µg of progesterone daily showed a suppressed type of endometrium in all patients within a short period after insertion (Martinez-Manautou *et al* 1975).

The metabolism and capacitation of human and rabbit spermatozoa are affected by the progesterone IUCD (Rosaldo *et al* 1974). Uterine washings from women with a progesterone IUCD in place showed a significant decrease in oxygen uptake and glucose utilisation and an inhibition of the peptidase activity.

A decrease in fucose, sialic acid, zinc and calcium concentrations were noted in the endometrial tissue of women wearing a progesterone IUCD during the proliferative phase. At the same time of the cycle, there was an increase in the concentration of sodium and potassium. The concentration of endometrial zinc is significantly decreased in the luteal phase under the influence of a progesterone IUCD (Hagenfeldt and Landgren, 1975).

The levonorgestrel-releasing IUCD was able to produce much the same effects as the progesterone IUCD, but since it is not metabolised by the endometrium as quickly, it produces more profound actions with a smaller daily release of steroid (Nilsson *et al* 1978).

### **1.7.3 Intrauterine contraceptive devices and pelvic inflammatory disease**

The transcervical insertion of devices into the uterus has been associated with the development of PID. This is therefore an important consideration in the development of a device for delivery of drug to the uterus. As with all forms of contraception, certain adverse reactions and complications are associated with the IUCD. Those which are the most cause for concern include infertility, (Sandvei *et al* 1987; Aral *et al* 1987; Population Report, 1988; Struther 1987 and Russel 1987), complications associated with pregnancy (Sandvei *et al* 1987; Population Report, 1988; Herxheimer,1988; Ricci *et al* 1988 and Russell, 1987), complications associated with menstruation (Herxheimer,1988), uterine perforation (WHO technical report, 1987a; Herxheimer,1988), pain

(Population report, 1988), expulsion (WHO technical report, 1987a; Herxheimer, 1988) and pelvic inflammatory disease (PID), which is to be discussed here.

It is estimated that 10 - 15 % of women of reproductive age in the United States have at least one episode of PID during their life, with sexually active 15 - 19 year olds having a 1 in 8 risk of getting PID (National Institutes of Health, 1991).

Two main types of microorganism can be distinguished when analysing microbial isolates from the upper genital tract of women in cases of PID.

(a) Exogenous agents, which are most often transmitted during sexual intercourse and include salpingitis-producing microorganisms such as *Neisseria gonorrhoea*, *Chlamydia trachomatis* and *Mycoplasma hominis*

(b) Endogenous microorganisms commonly isolated from the vagina, the vulva and perineum of healthy women.

PID is defined by the Centre for Disease Control as the acute clinical syndrome associated with the ascending spread of microorganisms (unrelated to pregnancy or surgery) from the vagina and cervix to the endometrium, the Fallopian tubes and/or contiguous structures. Factors which may influence such a spread include direct active transport of bacteria during procedures when instruments, IUCDs, gases or fluids are

forced through the cervical canal (Westrom, 1987). Other factors which may contribute to the susceptibility of women to become infected may be variation in their genital tract physiology. For example, the cervical mucus plug, which generally offers an impenetrable barrier for spermatozoa and microorganisms, is able to be compromised at the times of ovulation and menstruation. Similarly, uterine myometrial activity which causes fluid movement through the genital tract, has been shown to exhibit a retrograde flow during menstrual bleeding (Westrom, 1987). Both these factors could contribute to colonization of the upper genital tract with microorganisms.

Signs and symptoms of PID include low abdominal pain, pelvic tenderness, cervical motion tenderness, elevated erythrocyte sedimentation rate and fever (Kahn *et al*, 1991). These symptoms are not a definitive indication that such patients have PID and in cases where such criteria have been used the prevalence of PID varied from 33 to 74 % (Kahn *et al*, 1991).

The risk of PID has been reported to be higher with an IUCD in place than in those women who use no method of contraception (Sheth *et al*, 1987; Aral *et al*, 1987; Population Report, 1988 and Ricci *et al*, 1988). It has been reported that the rate of PID increases for a few months after insertion of the IUCD (Kessel, 1989; Population Report, 1988; Herxheimer, 1988 and Grimes 1987), when bacteria which colonise the vagina are introduced into the normally sterile uterus (Sparks *et al*, 1977). The risk of PID after insertion of the IUCD is reported as being

four times greater than non-contraceptors after the first month; 1.7 times greater 2-4 months after insertion; and not at a significantly greater risk four months after insertion (Population Report, 1988). These results were achieved provided the Dalkon Shield was excluded, since this particular IUCD, which has now been removed from the market, was associated with a much higher incidence of PID than other IUCDs (Kessel, 1989; Population Report, 1988; Method *et al.*, 1987; Herxheimer, 1988 and Grimes, 1987). The Dalkon Shield's higher incidence of infection has been blamed upon its multifilamentous locating thread enclosed within a plastic sheath, which is reported to act as a wick, drawing microorganisms up into the uterus (Tatum *et al.*, 1975). Any infection that occurs more than four months after insertion of an IUCD (again excluding the Dalkon Shield) has been thought to be primarily due to sexually transmitted pathogens (Population Report, 1988; Herxheimer, 1988, and Grimes, 1987). It has been suggested that the main risk factor for PID is not the use of an IUCD, but sexually transmitted diseases (STDs), most common in women less than 25 years of age with multiple sexual partners (Population Report, 1988 and Edelman, 1988). This finding has been confirmed by other workers (Lee *et al.*, 1988 and Herxheimer, 1988) who showed that women under 25 years of age are less likely to be married and in a mutually faithful monogamous sexual relationship, and therefore at an increased risk from STDs, whereas older women who are within such a relationship are much less likely to be at a risk from sexually transmitted pathogens. This would of course give an increased risk for PID whether the woman was fitted with an IUCD or not.

#### 1.7.4 The IUCD locating thread and its association with PID

Although STDs have now been accepted as the main risk for PID, the IUCD may still play some part in assisting the ascent of sexually transmitted pathogens into the uterus. Lee and co-workers (1988) hypothesise that the IUCD may facilitate ascension of lower genital tract sexually transmitted pathogens, without giving any assistance to any of the endogenous microflora. However they have no supportive evidence of this idea, and do not suggest how the organisms are assisted by the IUCD. Other authors have suggested that the tail of the IUCD, when present, may facilitate the ascension into the uterus of micro-organisms whether the tail is monofilamentous or multifilamentous (Sparks *et al*, 1981). However, Tatum and co-workers (1975) have shown that the tail of the Dalkon Shield, which is the only multifilamentous locating IUCD thread, will more often culture bacteria than monofilamentous locating threads. Using an *in vitro* model, Wilkins *et al* (1989) have demonstrated that microorganisms may migrate through a gel along a monofilament, but were unable to do so when the monofilament was absent. Galvez and co-workers (1985) have shown from a randomised trial that the PID rates between tailed and tailless devices do not differ significantly. Such is the ambiguity of the findings, it would appear that further studies need to be performed in order to clarify the role of the IUCD locating monofilament in the development of pelvic infection.

### 1.7.5 Protection offered by IUCDs

Even though the time immediately after insertion is the time when most episodes of PID materialise, perhaps because micro-organisms are introduced into the uterus during the insertion procedure, the vast majority of women fitted with an IUCD do not become overtly infected. This has led Kessel (1989) to suggest that undiagnosed sexually transmitted pathogens in the lower genital tract, especially gonococci and chlamydia, which are frequently asymptomatic (Cave *et al.*, 1969; Rosenfeld *et al.* 1983), may be responsible for many of the early incidences of PID after transcervical insertion of an IUCD. However, although this has been suggested as a possible method of contamination of the uterus, and as the Population Report (1988) has pointed out, non-bacterial inflammation of the Fallopian tubes may reduce their resistance to micro-organisms introduced by IUCD insertion, several workers have shown that women with an IUCD are at no greater risk from cervical chlamydial infection than non-contraceptors (Edelman, 1987 and 1988). Another study, which compared the risk for chlamydial infection in IUCD users with oral contraceptive (OC) users, found that OC users were at 8.8 times greater risk for contracting a chlamydial infection than IUCD users (Avonts *et al.*, 1990). It should be noted, however, that although Avonts and co-workers (1990) showed OC users to be at an increased risk to chlamydial infection than IUCD users, they state that this increased risk was not due to a protective effect of the IUCD. They suggest that this apparent reduction of chlamydial infection associated with the IUCD may be due to the cervical epithelium of OC users becoming more susceptible

to *C. trachomatis* when transmitted during sexual intercourse or perhaps OCs make detection of chlamydial infection easier. (Unfortunately, for this particular study, a suitable control group of women who practiced no method of contraception could not be found, although the authors did stratify for sexual lifestyle). However, if this were the case, where an IUCD was able to protect against chlamydial infection, the risk for PID with an IUCD in place would have to be re-evaluated, especially when it is considered that in the United States and Scandinavia, chlamydial infections account for greater than 20% and 40% of all cases of PID, respectively (Edelman, 1987).

#### 1.7.6 *Actinomyces israelii* PID and the IUCD

The organism *Actinomyces israelii*, is a normal inhabitant of caried teeth, tonsillar crypts, and the colon (Sheth *et al*, 1987) and has been associated with PID and IUCD use (Sheth *et al*, 1987; Stringer *et al*, 1987; Hsu *et al*, 1988; Mohanty, 1989; O'Connor *et al*, 1989). Different studies have shown the incidence of colonisation of the lower genital tract by this organism to range from 1.6 % to 13 % (Sheth *et al*, 1987; Stringer *et al*, 1987). *A. israelii* itself is not a normal commensal of the lower genital tract (Sheth *et al*, 1987), and it may infect the uterus by transmission from the bowel, via the vagina and cervix, where the IUCD tail may help compromise the cervical mucus plug. It has been postulated that the foreign body reaction in the uterus caused by IUCDs may predispose the uterus to colonisation by *A. israelii* (O'Connor *et al*, 1989). Hsu and co-workers (1988) point out that although *A. israelii* cannot penetrate the

intact endometrium, long term use of an IUCD will facilitate infection or colonisation of the uterus due to pre-existing inflammation or traumatic or mechanical injury resulting from the IUCD, possibly facilitated by calcium deposits on the IUCD itself.

PID caused by *A. israelii* is rare (Sheth *et al*, 1987; Herxheimer, 1988; O'Connor *et al*, 1989), but the probability of contracting an infection via *A. israelii* increases with increased duration of IUCD use (Sheth *et al*, 1987; Stringer *et al*, 1987; Herxheimer, 1988; Maenpaa *et al*, 1988). However, when this does occur, there is cause for concern, since episodes associated with *A. israelii* can give rise to some of the most severe cases of PID (Shurbaji *et al*, 1987; Population Report, 1988). One study noted that with increased duration of IUCD use there was increased risk of contracting severe PID (Kozuh-Novak *et al*, 1988), and yet with an increase in duration of use the risk of contracting PID which is not severe was reduced (Population Report, 1988; Wright *et al*, 1989). This suggests that certain women are more prone to infection by *A. israelii* after long duration of IUCD use. This is confirmed by another study which has shown that the presence of *A. israelii* in Papanicolau smears was not necessarily associated with sickness and symptoms of PID (Surico *et al*, 1987). It seems likely, therefore, that colonisation precedes infection, although infection does not always occur (Surico *et al*, 1987).

### 1.7.7 Biases introduced in case-control studies involving the IUCD

In the early 1970s, the Dalkon Shield was the focus of much publicity, implicating it with not only PID, but also serious pregnancy complications (Kessel, 1989). At the same time, many womens' magazines in the United States reported the dangers associated with the Dalkon Shield with such a degree of alarm that sales of IUCDs in the United States reduced dramatically (Kessel, 1989). It has been suggested (Kessel, 1989; Kramer, 1989) that if two women were to approach their physician with signs and symptoms of PID, where only one of these women was fitted with an IUCD, PID is far more likely to be diagnosed in the woman with the IUCD. Given the seriousness of PID, such diagnosis could jeopardise the future fertility of the women without an IUCD, should the diagnosis be proven to be incorrect. In the light of this potential bias, some authors believe that PID should be confirmed by laparoscopy or at laparotomy (Kessel, 1989; Kramer, 1989 and Method *et al* 1987). This is because clinical diagnosis of PID has been known to be incorrect up to 38% of the time (Method *et al* 1987), and inaccuracy of diagnosis may have been one of the factors that so often implicate IUCDs in their association with PID. The majority of case-control studies showing an increased risk for PID associated with IUCD use were conducted after publicity with regard to the Dalkon Shield had peaked (Kessel, 1989). This may suggest that these investigations were biased and therefore expected to find a higher incidence of PID associated with IUCD use.

Epidemiologic studies in the 1970s and early 1980s frequently included users of the Dalkon Shield in the sample group (Population Report, 1988). This would show an overestimation of the risk for PID from IUCD use. Studies from this era failed to screen the sample population sufficiently well, such that women were included who had a previous history of PID (Population Report, 1988), and as Westrom (1987) has said, after the first episode of PID women are at an increased risk of contracting it again, which, (if such women were included in the group) would show an increased risk of contracting PID from IUCD use.

Grimes (1987) looked at 25 different studies and concluded that even the most objective of these were possibly overestimates of the risk of PID from IUCD use, due to selection bias and confounding (two parameters are confounded if they cannot be estimated separately; this may be intentional or due to poor experimental design). With respect to selection bias, other authors have noted that many of the studies performed in the 1970s and early 1980s frequently did not take into account age, number of sexual partners and sexual behaviour (Population Report, 1988 and Method *et al*, 1987). Although differing reports have been made with respect to the age factor, either decreased risk from PID as age increases (Kozuh-Novak *et al*, 1988) or the risk of PID to be spread evenly across all age groups (Darj *et al*, 1987), it seems more likely (as previously mentioned), that unmarried women less than 25 years of age with multiple sex partners, are at an increased risk from PID.

Use of barrier methods of contraception, such as the condom or

diaphragm, or use of OCs, are known to protect against PID (Population Report, 1988). Barrier methods simply prevent access of potential pathogens into the uterus while OCs may protect against PID by one of several mechanisms, or by a combination of these mechanisms, which include,

- (i) decreasing menstrual blood, reducing the availability of this rich medium for incubation and growth of bacteria,
- (ii) thickening cervical mucus thereby preventing access of potential pathogens into the uterus,
- (iii) reducing dilation of the cervical os at the time of menstruation and midcycle, inhibiting upward migration of pathogens,
- (iv) reducing the strength of uterine contractions decreasing the possibility of infection spreading from the uterine cavity to the Fallopian tubes (Clarke, 1990).

When women who use such methods of contraception are included in the control group, and their incidence of PID is compared against the incidence of PID in IUCD users, the risk of contracting PID is of course much greater with the IUCD users (Kessel, 1989; Population Report, 1988; Herxheimer, 1988). It has been suggested (Kessel, 1989; Population Report, 1988) that the only true control group would be non-contraceptors who are sexually active and have no previous history of PID. However, although it is believed that OC use can protect against PID in general, it has been reported that OCs may not protect against PID that is due to *Chlamydia trachomatis* (Washington *et al* 1985). This may be

because the cervical epithelium of OC users is more susceptible to colonization by *C. trachomatis* when transmitted during sexual intercourse, or the OC may improve detection of a cervical infection with the organism (Washington *et al* 1985; Avonts *et al* 1990).

Despite the findings of most studies that the risk for PID is increased through use of an IUCD, it still appears that incomplete elimination of biases may be responsible for this apparent increased risk. Grimes (1987) cites the most objective studies that he looked at as showing a relative risk of contracting PID with an IUCD in place as between 1.5 and 2.6 and believes that even in these studies there still may be selection bias and confounding. Method and co-workers (1987) have shown that IUCD users are at between 1.5 and 3.0 times higher risk of contracting PID with respect to non-users, but they could not conclude that there was a cause/effect relationship. It has been suggested by Fugere (1990), that if the usual criteria for the use of the Nova-T IUCD were adhered to, (i.e. women over 25, ideally multiparous and within a mutually stable monogamous relationship), the risk of contracting PID is no greater than that with any other type of contraception. Toivonen *et al* (1991) when looking at PID rates between the Nova-T copper releasing IUCD and the levonorgestrel releasing IUCD noted that the PID rate associated with the levonorgestrel releasing device was significantly lower ( $p < 0.013$ ), and suggested that levonorgestrel may protect against PID by thickening of the cervical mucus, suppression of the endometrium or decreased bleeding.

With these findings and the results of one study, (Russell, 1987), showing that IUCDs reduce the risk for chlamydial infections, it still seems uncertain whether or not IUCDs are a risk factor for PID.

It has been suggested that IUCD-related infections may be prevented by prophylactic administration of systemic antibiotics at the time of IUCD insertion (Jovanovic *et al*, 1988). However, evidence suggests that microorganisms associated with such devices are resistant to antibiotic treatment (Nickel *et al*, 1985). One possible explanation for this is the presence of adherent bacterial biofilm surrounding the IUCD when *in situ* (Marrie and Costerton, 1983).

## 1.8 Bacterial biofilms

When devices, for example, drug-releasing devices, are placed into a bacterially rich environment, it has been shown that they become covered by a biofilm. Biofilms are thus an important consideration in the development of a device to deliver drugs to the female genital tract.

### 1.8.1 Introduction

Many bacteria which adhere to surfaces in a natural environment are capable of producing an exopolysaccharide (the glycocalyx). This exopolysaccharide surrounds the bacterium securing it to the surface, and may offer some protection against external factors, such as attack by antibacterial agents or phagocytosis (Ruseska, 1982; Costerton, 1987; Costerton, 1988). The bacterium is free to multiply within the exopolysaccharide to form microcolonies. Should this microcolony merge with other microcolonies which have undergone similar processes, a biofilm is formed. The biofilm may be defined as a functional consortium of microorganisms enveloped within an extensive exopolymer matrix composed either of protein and/or polysaccharide (Gilbert, 1992).

It has long been recognised that antibacterial agents appear to be more effective *in vitro* when administered to planktonic microorganisms raised in broth cultures replete in nutrients, than when applied directly to the same strain of microorganism from a natural or pathogenic environment (Costerton & Marrie, 1983; Millward & Wilson, 1989; Nickel *et al*, 1985;

Anwar *et al.*, 1989). This difference is often attributed to the bacterial biofilm, which, it appears, most bacteria are capable of producing given a suitable environment (Costerton, 1988). Bacterial biofilms have been found associated with many forms of implanted medical devices. Some of these devices and the materials from which they are made are shown in table 1.2, along with infections associated with them.

### 1.8.2 Bacterial adhesion to surfaces

Prior to production of a biofilm, the bacterial cells must firstly adhere to a surface. This is believed to be a two stage process, an initial reversible phase explained in terms of the DLVO theory (see later) followed by an irreversible phase involving polymeric bridging between the bacterial surface and the substrate surface (Marshall *et al.* 1971; Peters *et al.* 1982; Chu & Williams, 1984; Costerton *et al.* 1987; Wilkins *et al.* 1990). However, in any natural environment, it has been said that 'clean' surfaces for microbial colonization do not exist, since adsorption of naturally occurring organic materials would have taken place prior to any adhesion by bacteria (Fletcher, 1991). This being the case, true prediction of the events that occur between a bacterium and the substrate interface is difficult. Depending upon the organic molecules attached to the surface, bacteria may either be assisted in adsorbing to the surface, or repelled from it. This section sets out to summarize the events that occur at the bacteria-substrate interface, simplifying these events by assuming the substrate surface is free of adsorbed molecules prior to bacterial colonization (Fletcher *et al.* 1979).

<b>Indwelling Device</b>	<b>Material(s)</b>	<b>Associated Infection</b>
Suture	Polypropylene Polyamide Silk	Septicaemia
Urinary catheter	Latex Teflon coated latex Siliconised rubber	Bacteriuria Epididymitis Septicaemia
Intravenous cannulae	Polyethylene Polypropylene Polyvinylchloride	Septicaemia
Intrauterine contraceptive device	Polyethylene Stainless steel	Pelvic inflammatory disease
Ocular prostheses (contact lenses)	Polymethylmethacrylate Hydrogel polymer /copolymer	Conjunctivitis

**Table 1.2. Infections associated with some indwelling devices, along with common materials from which the devices are made**

### 1.8.2.1 The DLVO theory

The DLVO theory (derived independently by Derjaguin and Landau, 1941, and by Verwey and Overbeek, 1948), addresses the stability of aqueous dispersions of lyophobic particles with respect to mutual aggregation in the presence of simple electrolytes. Although this theory relates to colloid science, it may easily be applied to the initial stages of bacterial-surface interactions (Lips and Jessup, 1979; Olivera, 1992). The interaction energies involved are considered to be solely of the electrostatic and van der Waals type, the total energy of interaction being given by their summation, such that,

$$V_T = V_R + V_A$$

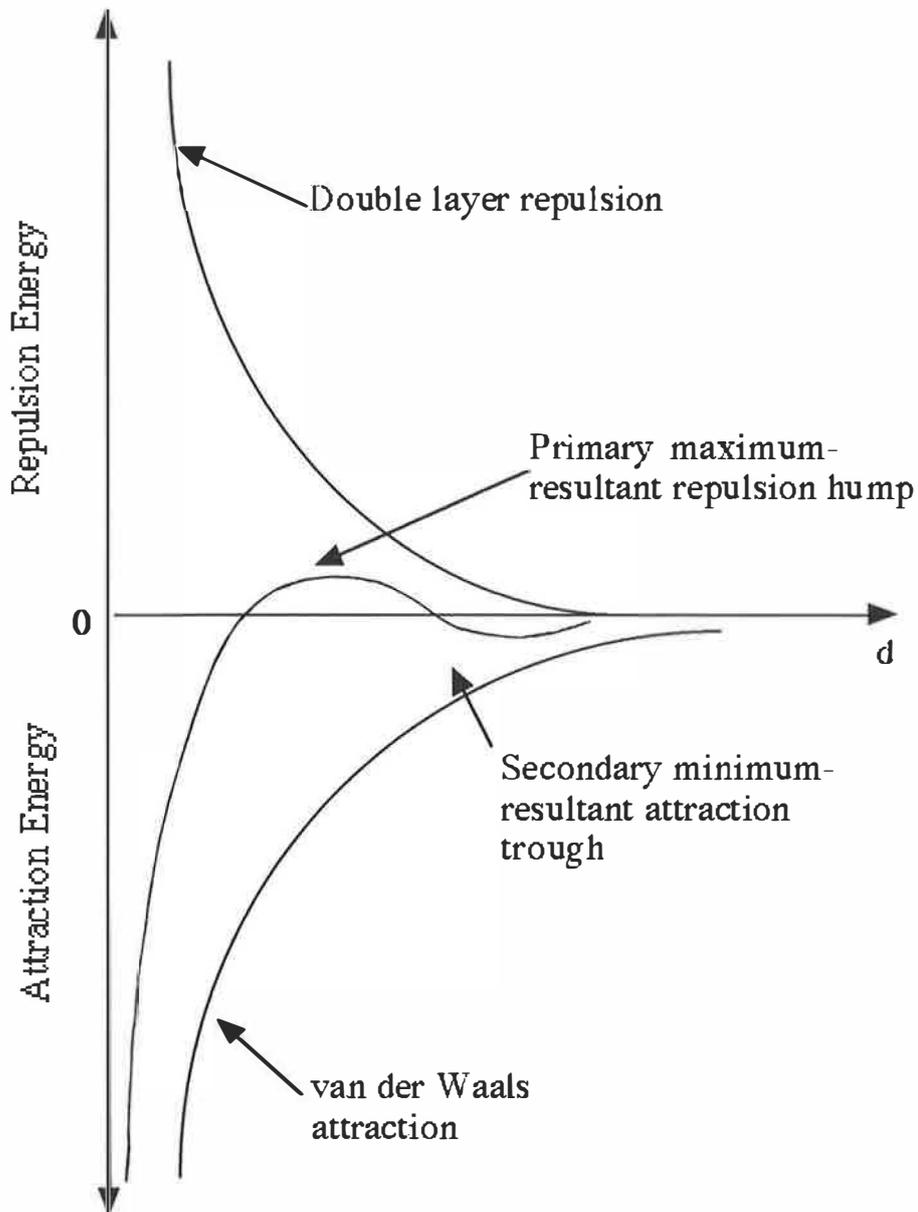
The potential energy at any given distance ( $V_T$ ), is the sum of the electrostatic repulsive energy ( $V_R$ ) and the attractive dispersion force (van der Waals force) ( $V_A$ ) at that distance between the bacterium and the substrate surface. Figure 1.6 shows a typical energy-separation profile of the adhesion of a microorganism to a planar hydrocarbon substrate. It is noted that there are two regions of attraction, separated by a repulsion barrier. The most distant region of attraction is typically found at a separation of 5 - 8 nm, and is termed the secondary minimum, which is amenable to relatively accurate theoretical prediction. The primary minimum occurs at a much shorter range, which is very difficult to predict to any degree of accuracy.

On immersion in an aqueous environment, the solid surface and the bacterium usually acquire a negative charge, by adsorption of ions or ionisation of existing surface groups. These surface charges will then attract oppositely charged ions from the surrounding aqueous environment resulting in a diffuse double layer of counter ions associated with each surface. As the two surfaces are drawn together by the van der Waals forces, a repulsive force comes into effect due to the overlapping ionic atmospheres around the two surfaces. This repulsion is dependent upon the ionic potentials of the two surfaces, the ionic strength and dielectric constant of the surrounding medium and the distance between the bacterium and the substratum. With relatively large bacteria (approximately 1  $\mu\text{m}$ ) and with low surface charges, significant secondary minimum interactions may be expected allowing weak adhesion to the substratum (Olivera, 1992). Even though the secondary minimum is of sufficient depth to effect substantial deposition in its own right, it is possible that in some cases the primary minimum is the major locus of interaction, as the interaction barriers may not always be high enough to prevent kinetically the establishment of the primary minimum contacts. These weak bonds occurring at the secondary minimum can easily be sheared by simply rinsing off the bacteria from the substrate surface (Marshall *et al* 1971; Lips and Jessup, 1979; Olivera, 1992).

#### **1.8.2.2 Polymer bridging**

At a distance of approximately 1 nm, there is a very high potential energy barrier, the primary repulsion barrier, and if interaction between the

Figure 1.6. Diagram showing the variation between total interaction energy (the summation of the van der Waals attraction curve and the double layer repulsion curve) with 'd' the separation between a colloid particle and a solid surface.



bacterium and the surface is to occur this barrier must be overcome. Calculations of the energy sufficient to drive a pseudomonad forward at  $33 \mu\text{m sec}^{-1}$  have proved insufficient to do this (Marshall *et al* 1971), as has molecular bombardment, imparting Brownian movement to bacteria (Brooks *et al* 1967). At approximately 5 - 10 nm between the bodies a secondary minimum exists (see above), where the bacterium may be held in place long enough for events to occur that can more securely hold the bacterium in place. One mechanism that has been shown to do this is for the bacterial cell to form an extracellular material, a polysaccharide, such that it is able to approach the substrate surface forming a bond with it (Rogers, 1979). This is possible since the potential energy barrier is related to the radius of the surface and the bacterium, and if polymer fibres are produced which have a relatively smaller radius, little resistance will be encountered and the bacterium may gain a 'foothold' on the surface (Weiss and Harlos, 1977). Appendages such as flagella (very fine threads protruding from the bacterial cytoplasm through the cell wall and responsible for motility), pili (very fine threads shorter than flagella not involved with motility), stalks and prostechae (filiform or blunt extensions of the cell wall and membrane not involved with motility) that have relatively small radii will lower the potential energy barrier and may also enable bacteria to gain a 'foothold' on the substratum surface (Olivera, 1992). Pili, stalks and prostechae are thought to be involved with this aspect of adhesion as they have no function with respect to motility (Handley *et al* 1991). This latter phase of bacterial adhesion to a surface is considered to be irreversible, and occurs when the bacterial cell lays down a polymeric substance which is able to bridge the gap between

the cell and the adherent surface, overcoming any electrical repulsion between these surfaces. Further evidence for this process of adhesion consisting of an initial reversible, and final irreversible stage has been shown by Wilkins *et al*(1990), who visualised intrauterine contraceptive device (IUCD) monofilament threads using scanning electron microscopy (SEM) which had been incubated in the presence of *E.coli* and *S.aureus*. Initially the bacterial cells were seen to adhere to the monofilaments via no obvious attachment, but after 20 hours or longer, distinct polymeric material was observed attaching both cell types to the monofilament thread. The strength of this attachment was also noted to increase with time (Wilkins *et al* 1990). Similarly, Chu and Williams (1984) who studied adhesion of *E.coli* and *S.aureus* to suture materials, noted that reversible attachment occurred more often with motile bacteria, which reversibly adhere to the suture surface only to move off again, whereas a less motile organism would be more likely to adhere reversibly, then secrete polysaccharide material to form a glycocalyx, which would make the adhesion irreversible. Clearly, there is a time lag prior to irreversible adhesion, which is the time between reversible adhesion and polysaccharide secretion. This polymeric substance appears to adhere more strongly to the adherent surface than to the bacteria, since it has been observed that after cells have been forcibly removed from the surface, "foot-prints" of this polymeric substance remain (Marshall *et al* 1971).

As well as the influence of van der Waals and electrostatic forces on bacterial adhesion the influence of hydrophobicity and hydrophilicity of

both the bacteria and the surface play a crucial role in bacterial adhesion to surfaces. General features which have been identified by Skvarla (1993) are listed below.

- (i) Hydrophobic bacteria adhere to hydrophobic surfaces irreversibly in the so-called primary minimum even at strong electrostatic repulsion.
- (ii) Moderately hydrophobic bacteria adhere reversibly in the so-called secondary minimum irrespective of the electrostatic interaction when the surfaces are hydrophobic.
- (iii) Hydrophilic bacteria adhere to both hydrophilic as well as hydrophobic surfaces in the secondary minimum. Adhesion of these bacteria (as well as of any bacteria to hydrophilic surfaces) is relatively weak and reversible and mostly requires electrostatic attraction.

### 1.8.3 The Bacterial Glycocalyx

In almost all natural and pathogenic environments an extracellular polymeric substance (EPS) is associated with adherent bacterial cells (Costerton *et al.* 1981). It consists mainly of polysaccharides (exopolysaccharides) with varying amounts of protein within it depending upon the bacteria from which the EPS is secreted (Cooksey, 1992). This polysaccharide/protein case is known as the glycocalyx, within which the organism is free to replicate, forming micro-colonies, causing spread of the glycocalyx, that may merge with similar micro-colonies enclosed

within glycocalyces, and ultimately forming a bacterial biofilm (Costerton *et al.* 1987).

It has been observed (Fletcher and Floodgate, 1973) that more than one type of polymer may be secreted by a single strain of bacteria, depending upon the circumstances. These workers proposed the involvement of two polysaccharides in the adhesive process of *Pseudomonas* strain NCIMB 2021. The first of these was thought to be involved in the initial adhesion and was presumed to exist on the surface of all bacterial cells. The second polymer was produced subsequent to the adhesive event and possibly caused a firmer association with the substratum. An alternative explanation was that the secondary polymer was produced only by cells in the "resting phase," which are cells deep within the EPS and are effectively dormant in as much as they are not actively replicating.

As well as those substances that are secreted by the bacteria themselves and found within the glycocalyx, other substances may become entrapped within the secretion. Such substances include leucocytes and cellular debris as found associated with IUCDs (Marrie and Costerton, 1983) and very small invertebrates and algae as may be found in a marine environment (Fletcher, 1991).

Bacterial cultures when grown on solid media or within broth replete in nutrients do not produce a biofilm (Costerton, 1988). In such situations the bacteria do not require protection from antibacterial agents, nor do they need to adapt to an environment which is lacking suitable nutrients.

It appears, therefore that the biofilm is an example of the phenotypic plasticity which is exhibited by bacterial cells. The plasticity of bacteria is also exhibited by Gram negative microorganisms that are deprived of iron (Costerton, 1988 ; Brown and Williams, 1985). Iron depletion is a general host defence mechanism, where the iron sequesterers lactoferrin and transferrin 'mop up' any free serum iron. Bacterial cells respond to this by producing iron-chelators, or 'siderophores', which compete with the hosts iron sequesterers, and then attach to siderophore receptors, which are produced by changes in composition of the outer membrane proteins (Brown and Williams, 1985). Phenotypic changes induced by iron depletion in host organisms can be mimicked by raising bacteria in an iron depleted environment *in vitro*. Other demonstrations of the effects of altering the *in vitro* environment to mimic *in vivo* situations and cause changes in the bacterial cell envelope have been provided by Denyer *et al* (1992) and Anwar *et al* (1984). Denyer *et al* (1992) were able to change the character of the cell envelope of *Staphylococcus epidermidis* by growing the organism in an atmosphere of 5 % carbon dioxide and 95 % air within human peritoneal dialysate obtained from the dialysed peritonea of uninfected patients which resembled that of the same organism found *in vivo* in the peritonea of patients infected with *S. epidermidis*. Anwar *et al* (1984) showed that 3 outer membrane proteins induced in cells of *Pseudomonas aeruginosa* taken directly from the sputum of cystic fibrosis (CF) patients could also be seen in the same organism grown under iron depleted conditions *in vitro*. They also showed that the presence of antibiotics did not influence expression of these iron regulated membrane proteins (IRMPs). These IRMPs are

immunogenic, and cause production of immunoglobulin G (IgG) which effectively reacts with these proteins when the cells are in the serum, but is unable, despite large amounts of IgG, to work in the lung of a CF patient. This may be because IgG or other classes of immunoglobulins cannot function effectively in the lungs of CF patients, possibly due to the presence of highly viscous materials such as the glycocalyx surrounding the bacteria (Anwar *et al* 1984). Another way in which the bacterial glycocalyx may interfere with host immune responses has been proposed by Peterson *et al* (1978), who has suggested that encapsulation of the bacterial cell within a polysaccharide substance interferes with opsonisation and thereby depresses phagocytosis.

#### **1.8.4 Bacterial adhesion to indwelling devices**

The presence of indwelling devices in the body, whether permanent or transient, increases the risk of infection by bacteria which are primarily part of the skin microflora. These devices provide a surface to which microorganisms may adhere and form a bacterial biofilm. Presumably the reason for any increase in infection rate, is the fact that when bacteria are adhered to a structure within a biofilm, they are proffered a great deal of protection, not only from host immune responses such as the oxidative burst response by polymorphonuclear leucocytes (Jenson *et al* 1990), but also from anti-bacterial agents such as anti-septics (Millward & Wilson, 1989; Marrie & Costerton, 1981), antibiotics (Nickel *et al* 1985), and bacteriophage. Chu and Williams, (1984), have noted how the presence of foreign materials in a wound greatly increases the susceptibility of the

surrounding tissues to infection. Paterson-Brown *et al*, (1987), who introduced bacteria into sutured guinea-pig wounds noted that all of the suture types that they studied, increased the rate of infection in such wounds compared to when bacteria were introduced into wounds where suture material was absent. Wilkins *et al* (1990), who were looking at the adhesion of microorganisms to threads similar to those attached to IUCDs, suggested that the IUCD may contribute towards PID by allowing its surface to be colonised by microorganisms on insertion. Wilkins *et al* 1989, have also demonstrated the ability of the microorganisms *E. coli* and *S. aureus* to migrate through an SCMC gel along a thread, (analogous to the locating monofilament of an IUCD passing through the cervical mucus plug), which is one of the possible mechanisms by which contamination of the uterus occurs after insertion of an IUCD (Sparks *et al* 1981).

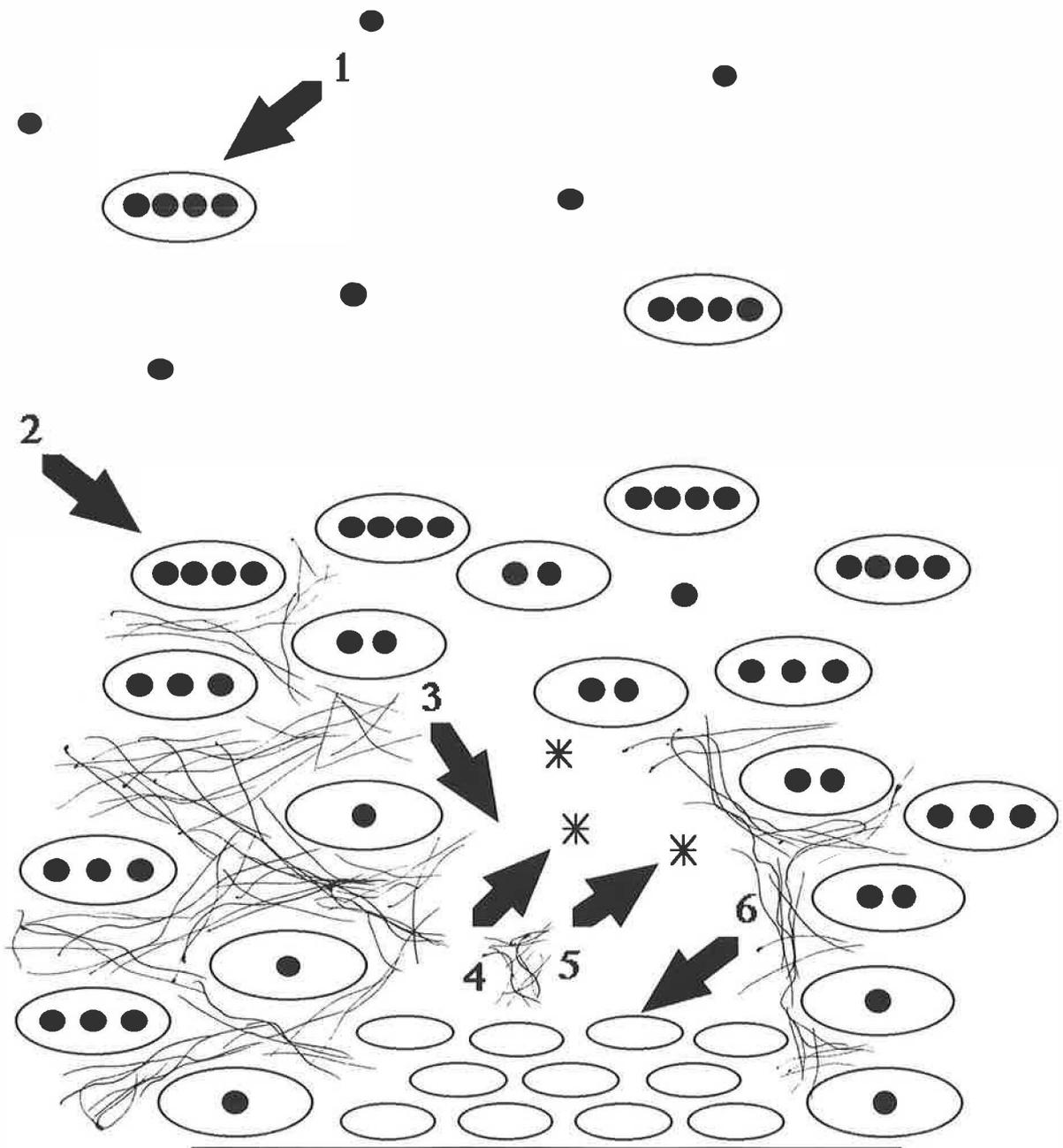
It has been suggested that colonisation of any indwelling device should be dealt with as early as possible, (Anwar *et al*, 1989), since, when young biofilms (2 days old) were exposed to the antibiotic tobramycin, it was found that they were far less resistant than old biofilms (7 days old), although they were more resistant than the same species in its planktonic form. Furthermore, when bacteria in either young or old biofilms are resuspended in broth, and then exposed to tobramycin, the microorganisms are found to be as sensitive to the same concentration of the antibiotic as was a planktonic culture of the same species of microorganism. It must be assumed that the microorganisms that are deep within the biofilm matrix and unable to acquire nutrients are at a dormant

stage and therefore not actively dividing. As such, the physiological properties of the cell membrane will have altered and become far less permeable to antibiotics (see section 1.8.5). Once resuspended, however, they have ample access to nutrients and the cell membrane will return to its pre-dormant state, and antibiotics will be able to enter and kill the cells.

### 1.8.5 Protection provided by the bacterial biofilm

Because of the protection offered to bacteria by the biofilm matrix, they are frequently involved with recurrent infections which are considered as one of the characteristics of an indwelling device infection (Costerton *et al* 1987). This biofilm protection has been demonstrated by Nickel *et al* (1985), where planktonic *Pseudomonas aeruginosa* exhibited a minimum inhibitory concentration of  $< 1\mu\text{g mL}^{-1}$  and an minimum bactericidal concentration of  $50\mu\text{g mL}^{-1}$ , when exposed to the antibiotic tobramycin, yet was able to survive exposure to  $1000\mu\text{g mL}^{-1}$  of tobramycin when living within thick biofilms. The mechanism by which the biofilm may protect these bacteria from antibiotics has been reduced to 6 steps by Anwar *et al* (1992) (Figure 1.7). Step 1 involves planktonic bacteria which are large in size and surround those bacteria within the biofilm. These cells possess a cell membrane that is permeable to nutrients and antibiotics, and the number of antibiotic molecules entering them is likely to be greater than that required to kill the cells. Step 2 involves the surface biofilm cells. These cells resemble planktonic bacteria, being large in size and possessing a cell membrane which is permeable to

Figure 1.7. Proposed model for describing the complex nature of the resistance of bacteria in biofilm to antibiotics (Stars indicate antibiotic degrading enzymes).



nutrients and antibiotics. Again, the number of antibiotic molecules that enter these cells is likely to be greater than that required to kill the cells. This phenomenon of absorbing more molecules of antibiotic than is actually required to kill the cells greatly reduces the amount of antibiotic available to inactivate the biofilm cells deeply embedded within the glycocalyx matrix. Step 3 involves the binding of antibiotic molecules to the exopolysaccharide produced by the biofilm cells. The glycocalyx produced by the biofilm cells is negatively charged, and is known to function as an ion-exchange resin which is capable of binding a very large number of antibiotic molecules (Anwar *et al*, 1992) . Step 4 involves immobilisation of antibiotic-degrading enzymes on the glycocalyx matrix. These antibiotic-degrading enzymes include  $\beta$ -lactamases (Costerton *et al*, 1985). Step 5 involves inactivation of antibiotic molecules by antibiotic-degrading enzymes immobilised on the glycocalyx matrix. Step 6 relates to embedded biofilm cells which are at a stage of dormancy because of a lack of accessibility to essential nutrients. They are smaller in size, and the cell membrane has been physiologically adjusted to be less permeable to antibiotic molecules. The events described in steps 1-6 significantly reduce the rate and the number of antibiotic molecules that reach the embedded biofilm cells, These cells will therefore have sufficient time to switch on the production of antibiotic-degrading enzymes to facilitate the inactivation of antibiotic molecules.

Due to the resistance of biofilm bacteria to antimicrobial agents, it has been suggested by Anwar and Strap, (1992), that antibiotic treatment needs to be early before bacteria within the aging biofilm become

resistant. However, an alternative approach that may overcome the problems posed by bacterial biofilms is to treat the substrate itself, such that the antimicrobial agent is released directly into the biofilm. A device to release an antiseptic agent directly into the biofilm itself has been developed by Bilbruck *et al*(1991). This device, which takes the form of a monofilament thread coated with hydrogel impregnated with chlorhexidine has proved very effective at reducing bacterial numbers *in vitro* and may be attached to an IUCD to reduce bacterial numbers introduced into the uterus on transcervical insertion of the IUCD.

## 1.9 Aim of the thesis

Symptoms of the climacteric are generally treated by administration of oestrogens. However, because this may increase the risk of developing endometrial cancers, a progestin is normally given concomitantly to those peri- and post-menopausal women who still possess a uterus. It appears, however, that this concomitant administration of a progestin can increase low density lipid cholesterol, as well as reduce some of the beneficial effects of oestrogens. A local drug delivery system whereby a progestin is administered directly to the endometrium would require a much smaller dose to be used so that oestrogenic effects are reduced less. Unfortunately, as can be seen from section 1.7.3, transcervical insertion of a device into the uterus appears to be associated with an increase in the risk of uterine infection for a short period after insertion. This is generally explained by the fact that the uterus is normally sterile compared with the vagina which has a dense polymicrobial population and transcervical insertion of a device will inevitably introduce bacteria into the uterus.

The aims of the current studies were therefore:

- (a) To develop separate devices capable of releasing an antimicrobial agent and progestins for a prolonged period of time, yet small enough to be passed through the human cervical canal.
- (b) To characterise the profile of drug release from these devices.

(c) To study the antimicrobial effect of the antimicrobial agent when released *in vitro* and *in vivo* in a mammalian model.

(d) To study the effect of progestins when released *in vivo* in a mammalian model.

## **2 MIGRATION OF VAGINAL BACTERIA INTO THE UTERUS**

## 2.1 Introduction

The cervical mucus plug is a very effective means of preventing bacteria from passing from the vagina, which has a dense polymicrobial population, into the uterus which is normally sterile (Sparks *et al* 1977). Malhi *et al*(1987) have demonstrated that by use of mucolytic agents, the integrity of the cervical mucus plug may be compromised such that bacteria normally isolated from the vagina may be found in the uterus. It has been suggested that the uterus may also become infected after insertion of an intrauterine contraceptive device by bacteria growing along the locating monofilament traversing the cervix and thereby gaining access to the uterus. This migration of bacteria has been demonstrated *in vitro* (Wilkins *et al* 1989), but has not been shown *in vivo*. However, Tatum *et al* (1975), who investigated a spate of septic spontaneous abortions linked with the Dalkon Shield, discovered that the tail of this device allowed microorganisms to migrate along its length indicating a possible relationship between the locating string of this device and pelvic sepsis.

Sparks *et al* (1981), who demonstrated the presence of commensal bacteria in the uterus of women fitted with an IUCD long after insertion of the device, have suggested that these bacteria gain entry into the uterus by migrating along the locating monofilament of the IUCD. Jovanovic *et al* (1988) and Sinei *et al* (1990) have suggested the use of prophylactic antibiotics at the time of insertion of an IUCD, since at this time cervical and vaginal contaminants, including bacteria may become introduced into

the uterus and cause infection. However, the mucus that will coat the IUCD and the attached monofilament on transcervical insertion, is likely to prevent the access of antibiotics to any bacteria embedded within it (Kearney and Marriott, 1987). Tetracyclines themselves have been shown to exert a mucospissic action which will further decrease the diffusion rate of antibiotic through the mucus (Marriott and Kellaway, 1975) reducing access of antibiotic to the bacteria. Concurrent administration of a mucolytic agent with antibiotic therapy has been suggested as a means of increasing the concentration of antibiotics within cervical mucus to bactericidal levels (Malhi *et al* 1988).

The purpose of this part of the study was to determine whether microorganisms are able to compromise the cervix when a fibre which crosses from the uterus to the vagina is in place. Since microorganisms which are located in the vagina will inevitably be introduced into the uterus during transcervical insertion, an easily recognisable, non-pathogenic, non-commensal motile organism was introduced into the vagina after insertion of the fibres. If these microorganisms could then be isolated from the uterus, it would appear that they are able to cross the cervix and gain access to the uterus when a fibre is present.

## 2.2 Materials

### Bacteria

*Serratia marcescens* NCTC 4618 was obtained from The National Collection of Type Cultures, Central Public Health Laboratory, Colindale Lane, London, England. It was maintained on Nutrient Agar at 4°C, and subcultured at approximately 3 month intervals.

### Media

Nutrient Broth (C. M. 1) and Nutrient Agar (C. M. 3) were obtained from Oxoid Ltd., Basingstoke, Hampshire. Both were prepared according to the manufacturers instructions, and sterilised by autoclaving at 121°C for 15 minutes.

### Chemicals

Phosphate buffered saline (PBS) tablets were obtained from Oxoid Ltd., Basingstoke Hampshire. They were dissolved in distilled water according to the manufacturers instructions and the resultant buffer sterilised by autoclaving at 121°C for 15 minutes.

Hypnorm injection (a mixture of fentanyl citrate and fluanisone) was obtained from Janssen Pharmaceuticals Limited, Wantage, Oxfordshire, and used according to the manufacturer's instructions.

Valium injection (a solution of diazepam in arachis oil) was obtained from Roche Products Limited, Welwyn Garden City, Hertfordshire and was used according to the manufacturer's instructions.

Nylon monofilaments were obtained from Nymofil Limited, Poulton-le-Fylde, Lancashire.

### Sutures

Nylon (Ethilon) and silk (Mersilk) sutures were obtained from Ethicon UK, Edinburgh, Scotland.

### Tubing

Polyethylene tubing used in the manufacture of the monofilament inserting device, was obtained from Portex UK, Basingstoke, Hampshire.

Glass capillary tubes were obtained from Philip Harris Medical Supplies Limited, Birmingham, West Midlands, and sterilised packed inside stoppered test tubes at 180°C for 1 hour.

### 2.3 Apparatus

A Kilian nasal speculum was obtained from Downs Surgical Limited, Mitcham, Surrey and was attached to a fibre-optic light source obtained from Schott of Germany. Prior to insertion into the guinea-pig vagina, the speculum was lubricated with sterile K-Y Jelly obtained from Johnson and Johnson Limited, Slough, Berkshire.

Optical density/absorbance measurements were performed using a Lambda 2 spectrophotometer obtained from Perkin-Elmer Limited, Beaconsfield, Buckinghamshire.

Centrifugation was performed using a Sorvall RC-5B refrigerated superspeed centrifuge, Du Pont (U. K.) Ltd., Sorvall products, Stevenage, Hertfordshire, U. K.

## 2.4 Methods

### 2.4.1 Construction of a calibration plot of *Serratia marcescens* against absorbance

Determination of the cell density of *Serratia marcescens* was made using a nutrient broth culture of the organism obtained by inoculating 100 mL of broth with a single colony and incubating at 37°C, without shaking, for 24 hours. The culture was centrifuged for 20 minutes at 4300g and 4°C, the supernatant liquid decanted, the pellet resuspended in PBS, (pH 7.4), and centrifuged again using the same parameters. The pellet was then resuspended in PBS and diluted to give an optical density of 0.5 at 430 nm against a blank of sterile PBS, determined on a Perkin-Elmer lambda 2 spectrophotometer. This suspension was then diluted to give suspensions with absorbances of approximately 0.4, 0.3, 0.2 and 0.1. Each of these dilutions was further diluted to give a cell concentration of approximately 200 to 1000 colony forming units (cfu) mL<sup>-1</sup>. 0.2 mL of these final dilutions were then plated out onto over-dried nutrient agar plates in triplicate. These plates were then incubated at 37°C for 24 hours. After incubation the plates were removed and the colonies counted. From these data a plot of absorbance against cfu mL<sup>-1</sup> was constructed (Figure 2.1). This plot was used to determine approximate numbers of bacteria which were inoculated into the guinea-pig vagina (see later).

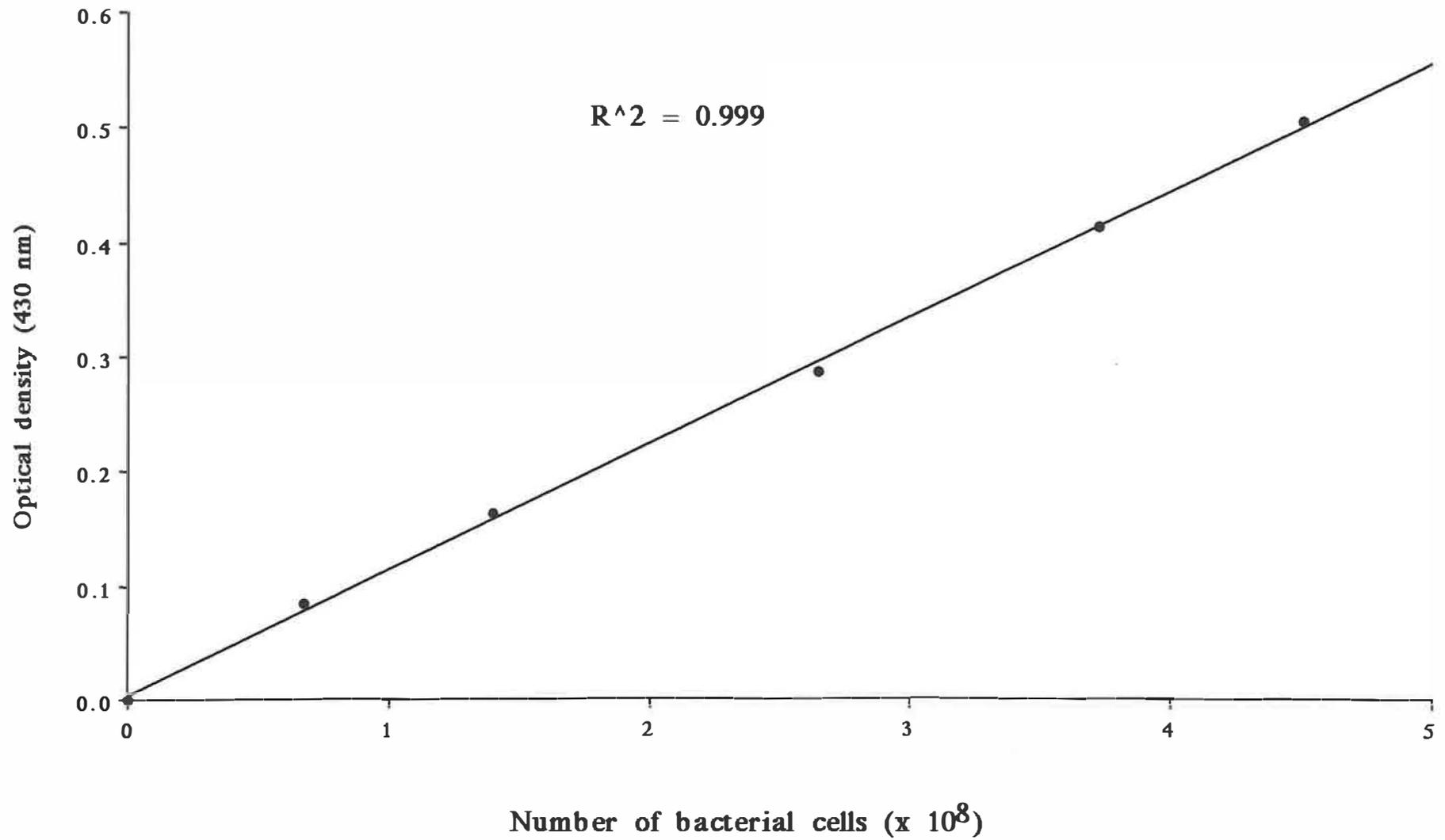


Figure 2.1. Calibration plot of *Serratia marcescens* concentration against optical density

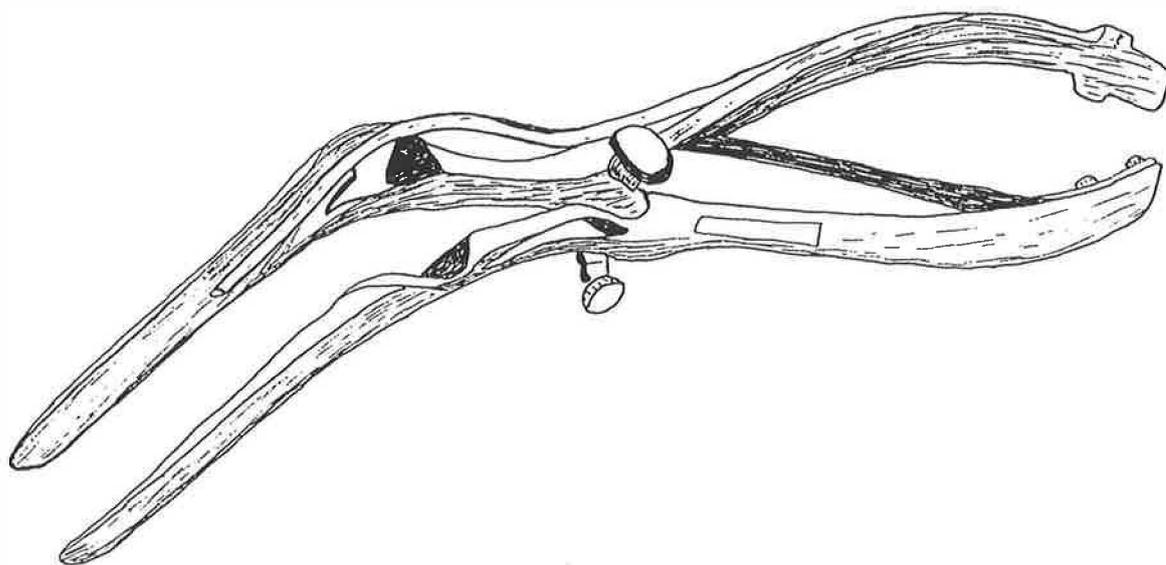
#### 2.4.2 Animal husbandry

Female Dunkin-Hartley guinea-pigs, (*Cavies porcellus*), were either bred at the University of Brighton, or obtained from commercial breeders (Harlan Porcellus, Sussex).

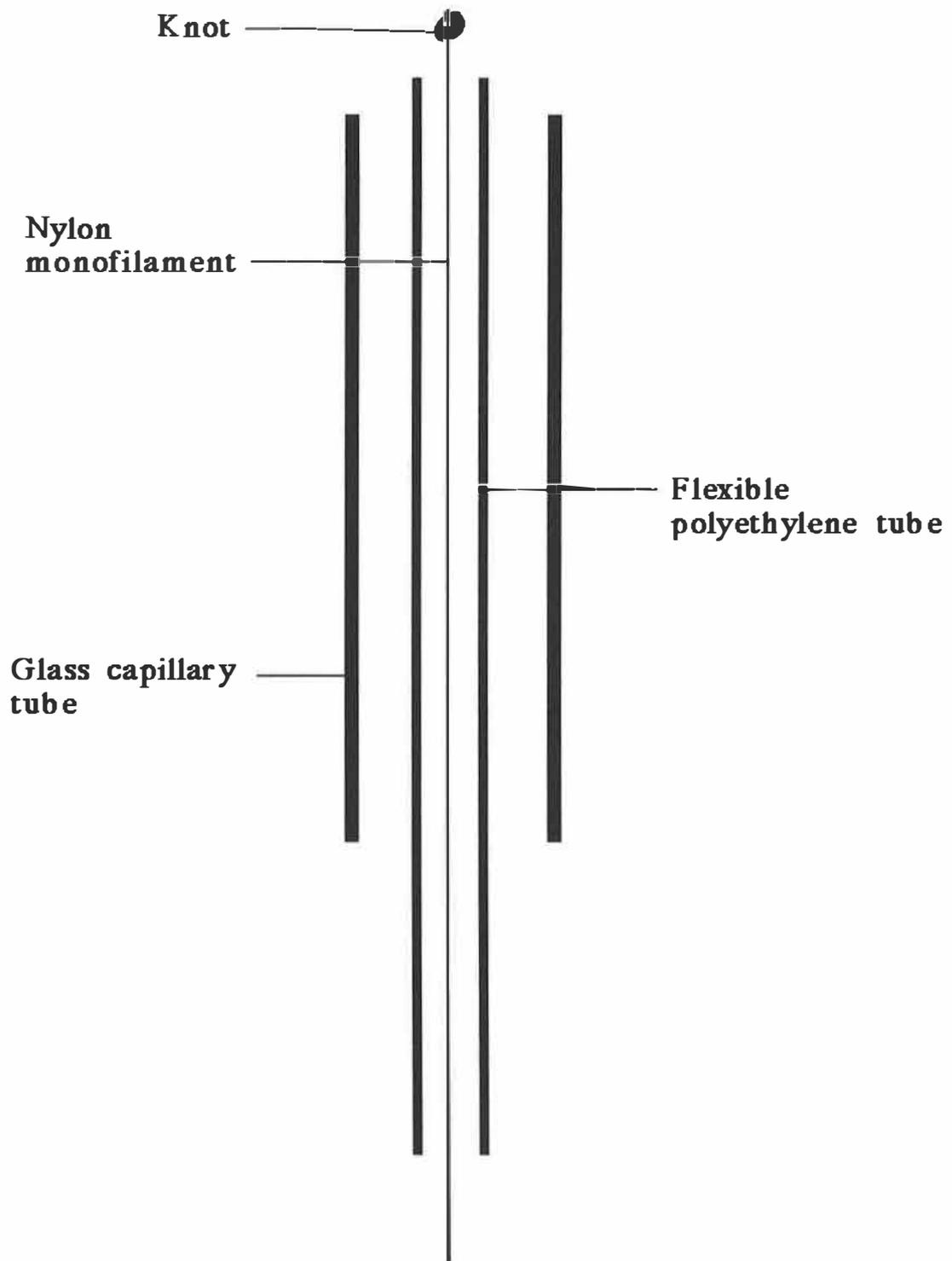
The animals were of body weight 350 - 550 grams and were housed in wire bottomed cages (1000 mm x 1000 mm x 300 mm) from North Kent Plastic Cages, Dartford, Kent. Subsequent to weaning, the guinea-pigs were housed in groups of eight for at least 14 days in order to ensure that the animals were familiar with one another thus reducing the risk of fighting. The animal holding room was maintained at a temperature of 21.5°C with a 12 hour light - 12 hour dark cycle. Animals received diet pellets (FD1, Southern Dietary Services Limited) and tap water *ad libitum*

#### 2.4.3 Animal surgery

Prior to surgery, anaesthesia was induced in the guinea-pigs by administration of an intraperitoneal injection, (1 mg Kg<sup>-1</sup>), of diazepam, followed several minutes later by an intramuscular injection, (1 mg Kg<sup>-1</sup>), of Hypnorm. Once the guinea-pig was anaesthetised, the abdomen of the animal was shaved. The guinea-pig was then placed onto a heated table, (to maintain the animals normal body temperature), and the area surrounding the orifice of the vagina was gently washed using cotton wool soaked in a 1% Hibitane solution. K-Y jelly was placed onto the vaginal opening, and a 1 mL syringe liberally covered with K-Y jelly was



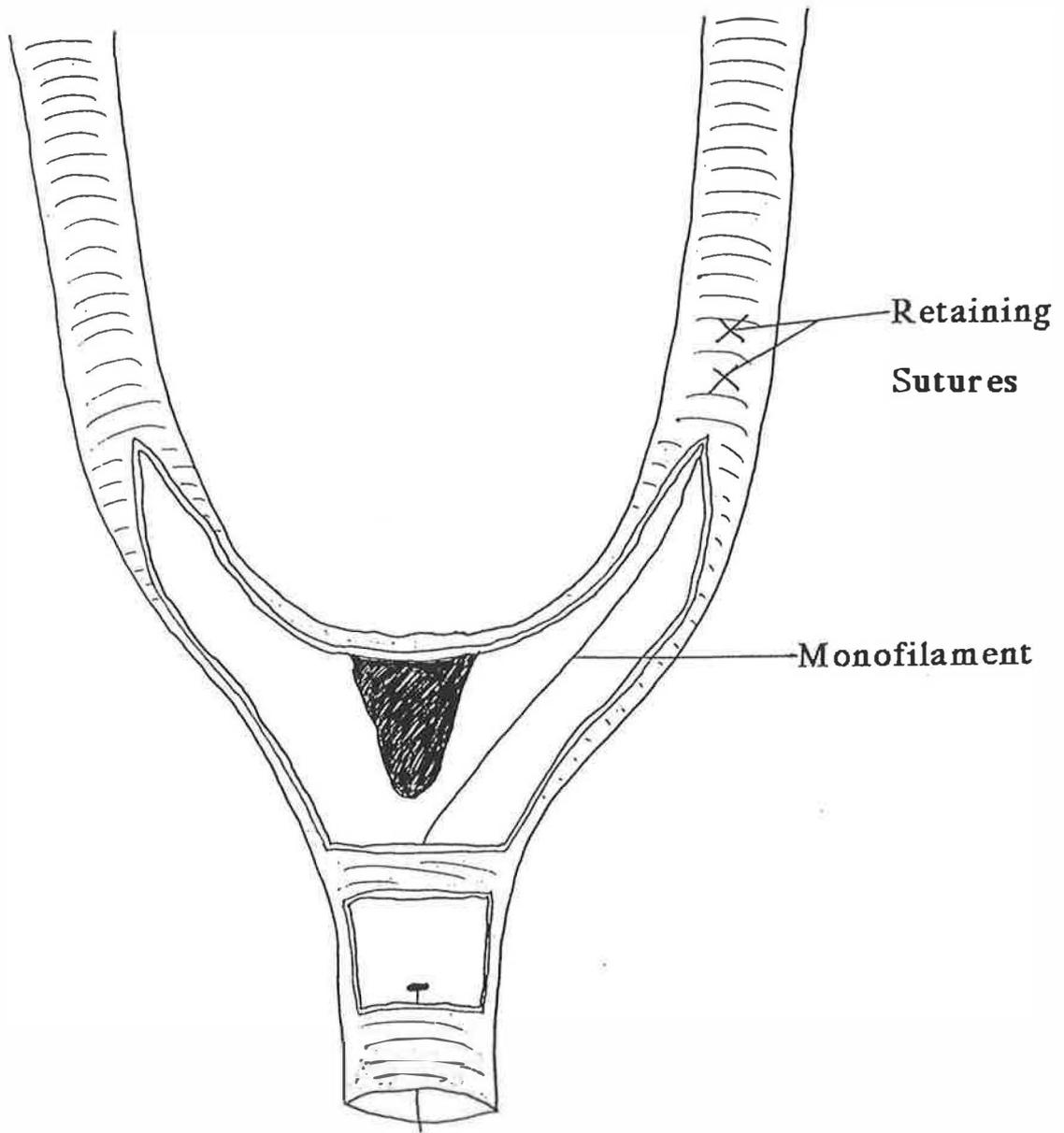
**Figure 2.2. Kilian nasal speculum with fibre optic light facility used to insert devices into the guinea-pig uterus**



**Figure 2.3 Representation of device used for insertion of nylon monofilaments into the guinea-pig uterus**

inserted carefully into the vagina until just below the cervix. The syringe was then gently withdrawn, whilst applying mild suction to remove excess cervical mucus which may prevent viewing of the cervix. A modified Kilian nasal speculum (Figure 2.2), also liberally coated with K-Y jelly, was then inserted carefully into the vagina so that the cervix could be viewed. Once the cervix was located, the device, (figure 2.3), was introduced into the vagina. The glass capillary tube was then pushed through the cervix to a depth of no more than 5 mm and the plastic tube was pushed further in, until resistance prevented it from going deeper. The glass capillary tube was then withdrawn. The abdomen was washed with cotton wool soaked in a 1% Hibitane solution, and a 2 cm incision was made through the skin. Once through the skin, the abdominal muscle was revealed and a further 2 cm cut was made along the *linea alba*, revealing the intestines. The uterine horn containing the device was then brought to the surface and prevented from re-establishing its position within the peritoneal cavity by use of forceps.

The nylon monofilament was secured in the uterine horn by a silk suture, which was threaded through the uterine wall, trapping the knot of the monofilament against the wall. A second stitch was put in just below the first, as a backup (figure 2.4). (In the case where controls were performed and no nylon monofilament was to be secured within the uterus, the stitches were still made). The flexible polyethylene tube, (figure 2.3), was then removed from the guinea-pig cervix and vagina, and the uterine horn allowed to re-establish its position within the peritoneal cavity. The abdominal muscular layer was then drawn



**Figure 2.4. Anchoring of monofilament to myometrium with sutures**

together, and sutured using silk suture thread, after which, the whole area was gently dabbed with cotton wool soaked in a 1% Hibitane solution. The skin layer was then drawn together and sutured with a nylon suture thread. Again, after the wound edges had been drawn together, the whole area was dabbed with cotton wool soaked in a 1% Hibitane solution. The animal was then kept in a wire bottomed cage until it was needed for determination of the microbial status of the uterine horns, after which it was killed by cervical dislocation.

#### **2.4.4 Collection and determination of uterine microflora**

The abdominal cavity of the killed animals was cut open aseptically to reveal the intestines, which were moved aside to reveal the vagina, the cervix and the uterine horns.

The unthreaded horn was removed aseptically and placed into 9 mL of lecithin-tween broth.

The remaining uterine horn was removed aseptically with the monofilament intact. The part of the monofilament which had extended into the vagina was cut away from that attached to the uterine horn, to prevent any contamination by vaginal microorganisms. The uterine horn was washed through repeatedly with a 2 mL volume of lecithin-tween broth, using a sterile Pasteur pipette, (10-20 times). After washing through with the broth, 0.2 ml aliquots of the broth were plated out onto overdried nutrient agar plates in replicates of 5. All samples were then

incubated for 24 - 48 hours after which they were examined for bacterial growth. One group of guinea-pigs (n = 6) which was untreated had their uterine horns removed and treated as described above, as a control to determine the normal microbial status of the guinea-pig uterus.

#### 2.4.5 Inoculation of guinea-pig vaginae with *Serratia marcescens*

Female guinea-pigs which had been obtained and housed as described in section 2.4.2 were inoculated, (intravaginally), with approximately 1 mL of an overnight culture of *Serratia marcescens* treated as described in section 2.4.1, containing  $1 - 5 \times 10^8$  colony forming units mL<sup>-1</sup>. After inoculation of the vaginae of the animals, vaginal swabs were taken at time 2, 4, 6 and 24 hours, and then daily for the following 5 days. Nutrient agar plates were then inoculated with the swabs and incubated at 37°C for 24 to 36 hours. After incubation, the plates were examined for red colonies which would indicate the presence of *Serratia marcescens*

At times 2, 4 and 6 hours swabs taken and inoculated onto nutrient agar plates produced an abundance of red colonies, indicative of vaginae containing a dense population of *Serratia marcescens*. After 24 hours, numbers of the organism had greatly reduced, and by 5 days after inoculation, no red colonies were observed.

#### 2.4.6 Inoculation of guinea-pig vaginae with *Serratia marcescens* in the presence of a transcervical monofilament

Female guinea-pigs which had been obtained and housed as described in section 2.4.2, had knotted nylon monofilaments inserted transcervically and secured as described in section 2.4.3. 24 hours after the animals had recovered, an overnight culture of *Serratia marcescens* prepared as described in section 2.4.1 was inoculated into the vaginae of the animals by use of a 1 mL graduated pipette. Re-inoculation of the vaginae of these animals was performed every 3 days for a total of 21 days, to ensure that the organism remained present in these animals for the duration of the experiment, after which the animals were killed by cervical dislocation, and the uteri and the nylon monofilament removed and treated as described in section 2.4.4. A second group of animals were treated similarly without insertion of the nylon monofilament. In both groups of animals, the presence of red colonies was indicative of *Serratia marcescens*. In both groups of animals the last vaginal inoculation with *S. marcescens* was performed 2 days before the animals were killed.

#### 2.4.7 Statistical analysis

The data obtained were statistically analysed using the Fischer exact test (Fischer, 1948).

## 2.5 Results

The results of determining the normal microbial status of the guinea-pig uterus are presented in table 2.1.

cfu mL <sup>-1</sup>	No. of animals
< 10	3
10 - 99	2
99 - 1000	1
> 1000	0

**Table 2.1 Colony forming units per mL obtained from the untreated uterine horns of guinea-pigs**

The presence and absence of *Serratia marcescens* for those animals which had a monofilament inserted is given for the uterine horn containing the monofilament, the contralateral uterine horn and the vaginae in table 2.2. In those animals where no monofilament was inserted, one uterine horn was washed through and one was incubated in nutrient broth. The presence and absence of *Serratia marcescens* for these two horns and the vaginae for this group of animals is given in table 2.3.

Animal Number	Monofilament present	Monofilament absent	Vaginal Swab
1	- (5)	- (0)	+
2	+ (1)	- (0)	+
3	+ (7)	- (0)	-
4	+ (4)	- (0)	-
5	- (170)	- (0)	+
6	- (1)	- (0)	-
7	- (1)	- (0)	-
8	- (2)	- (0)	+

Table 2.2 The presence of *Serratia marcescens* in guinea-pig uterine horns and vaginae after repeated vaginal inoculation with the organism in animals with a transcervical monofilament inserted. (Numbers of commensal bacteria isolated from the uterus are given in brackets).

Animal Number	Uterine horn washed through	Uterine horn incubated whole in broth	Vaginal Swab
1	- (9)	- (0)	-
2	- (1)	- (0)	+
3	- (1)	- (0)	-
4	- (1)	- (0)	+
5	- (1)	- (0)	-
6	- (2)	- (0)	-
7	- (1)	- (0)	+
8	- (1)	- (0)	-

Table 2.3. The presence of *Serratia marcescens* in guinea-pig uterine horns and vaginae after repeated vaginal inoculation with the organism in animals without a transcervical monofilament inserted. (Numbers of commensal bacteria isolated from the uterus are given in brackets).

When comparing those results obtained from the guinea-pig uteri with a monofilament inserted to those uteri of guinea-pigs which were washed through but had no monofilament inserted, differences are not statistically significant at a 5 % level, when considering the presence or absence of *S. marcescens*

## 2.6 Discussion

The involvement of the locating monofilament attached to IUCDs in pelvic infections is surrounded with controversy stemming from the high incidence of infection associated with the thread which was attached to the Dalkon Shield (Tatum *et al* 1975). This particular thread was composed of many fibres enclosed in a plastic sheath, enabling the thread to act as wick, which could draw microorganisms up within it (Tatum *et al* 1975). If this wick effect occurred with devices which had been inserted into women, microorganisms may be drawn from the vagina into the normally sterile uterus, causing infection. Withdrawal of this device from the market lead to a reduction in the number of cases of pelvic infection associated with IUCDs, since users of the Dalkon Shield were at least five times more likely to be hospitalised for a first episode of pelvic infection compared with users of other types of IUCD (Population Report, 1982). Despite removal of this device from the market, the infection promoting ability of the IUCD locating monofilament was still debated. Sparks *et al* (1981), showed microorganisms to be present in the uteri of women who used IUCDs with threads, but failed to show bacteria in the uteri of those women who used IUCDs that lacked the thread. This suggested that those microorganisms that were found in the uteri did not represent survival of bacteria introduced at insertion, as they were absent from the uteri containing tailless devices, but arose by the continuous ascent of vaginal bacteria along the IUCD locating monofilament (Sparks *et al* 1981). However, Galvez *et al* (1985), found that the rate of PID between groups of women using IUCDs with or without strings were not significantly

different. Wilkins *et al* (1989), using an *in vitro* system to model the IUCD locating monofilament which traverses the cervix, were able to show that microorganisms could migrate through S-carboxymethyl-L-cysteine (SCMC) gels provided a monofilament was present where the gel provided a model for the cervical mucus plug. The speed of this migration was dependent on the motility of the microorganism and the viscoelasticity of the gel. In the absence of a monofilament, microorganisms were unable to pass through the gel no matter how motile they were.

Malhi *et al*(1987) examined the effects of mucolytics on the guinea-pig uterine microflora. They found that the use of mucolytics was associated with a 50 % uterine contamination rate compared with animals in which mucolytics were not used which showed a 0 % contamination rate. This difference must be primarily due to the reduction in the viscoelasticity of cervical mucus allowing access of vaginal microorganisms into the uterus. This suggests that any factor which compromises the cervical mucus plug may facilitate access of bacteria from the vagina into the uterus.

In untreated animals, (animals where no hollow fibre had been inserted, and no sham control had been performed), bacterial counts were very low (Table 2.1). These results concur with the findings of Bilbruck (1991), who observed that in 50 % of the uteri of guinea-pigs that had not had a nylon monofilament transcervically inserted, the uteri were sterile, and in the other 50 %, numbers of bacteria were very low. Results from the work of Sparks *et al*(1981), who determined the bacterial status of uteri

from hysterectomy patients and Malhi *et al* (1987) who examined the uterine horns of guinea-pigs for bacteria, are also in agreement with these findings showing that the uterus (human and guinea-pig) is normally sterile.

In the case of inoculation of *S. marcescens* into the guinea-pig vagina, the presence of *S. marcescens* was observed in guinea-pig uteri when the monofilament was present, but was not isolated when the monofilament was absent. This suggests that the monofilament was assisting with the ascension of the microorganism from the lower genital tract to the upper genital tract, perhaps by the microorganism adhering to the monofilament in the vagina and growing along it to reach the uterus. Although statistically this effect is not significant, ( $p > 0.05$ ), the presence of the microorganism in the uterus is indicative of some compromise of the cervical mucus plug being made. It should be noted that had the population of animals in both groups been larger, with the same proportion of uteri in the test group being contaminated with *S. marcescens* then differences between the 2 groups would have been significant. The actual value for  $p$  with the number animals used in this study is 0.1, but if all values are doubled,  $p$  is calculated to be 0.0088. The fact that only three of the animals were observed to have *S. marcescens* in the uterine cavity may reflect the findings found in women, where compromise of the cervical mucus plug, and hence colonisation of the uterus is only shown in a small proportion of women using IUCDs with a transcervical monofilament. Rank and Saunders, (1990), who inoculated the guinea-pig vagina with low doses of

Chlamydia, were able to show the presence of the microorganism in the upper genital tract of these guinea-pigs several days after inoculation. However, Rank and Saunders, (1990), did not insert a transcervical monofilament into the animals, and they inoculated the guinea-pig vagina with Chlamydia once only. This study has shown that *S. marcescens* could not be isolated from the guinea-pig vagina more than five days after inoculation. The absence of *S. marcescens* several days after inoculation may be explained by the putative protective role of avidly tissue adherent lactobacilli which resist colonisation by non-commensal microorganisms (Dominigue *et al* 1991). The mechanism of competitive exclusion has been suggested to be steric hindrance rather than to a specific blockade of receptor sites (Chan *et al* 1985). If this exclusion was due to steric hindrance, then the size of the Chlamydia cells may allow them to reach the mucus membrane of the vagina, enter the epithelial cells and replicate within these cells. This would give the Chlamydia cells a 'foothold' in the lower genital tract from where they could ascend to the upper genital tract. This may explain the delay that was seen by Rank and Saunders (1990) between inoculation of the microorganism and location of Chlamydia within the uterine cavity.

*S. marcescens* was only isolated from the vaginae of 3 out of 8 guinea-pigs when the monofilament was absent. In those animals where the monofilament was present *S. marcescens* was isolated from the same proportion of vaginae as when the monofilament was absent. This similarity in the proportion of vaginae colonised by *S. marcescens* was unexpected as it was believed that the presence of a clean substrate onto

which the microorganism could adhere when inoculated into the vagina in great numbers would convey advantages not available in the case where the monofilament was absent. It was expected that *S. marcescens* would adhere to the monofilament, develop a biofilm and modify its growth cycle (Anwar et al, 1992), preventing competitive exclusion from the vagina. However, from the results tables (tables 2.2 and 2.3), this is not the case and may be due to the monofilament becoming immediately encased within vaginal mucus laden with commensal microorganisms excluding colonisation by *S. marcescens*

Since the finding that *S. marcescens* is able to cross the cervical mucus plug is not statistically significant, then the period of greatest concern, at least for most women, is that immediately after insertion of the IUCD when bacteria are introduced into the uterus from the vagina. It would therefore appear that this period immediately post-insertion is most likely to cause pelvic infection, and use of a device that could kill microorganisms at the time of insertion may prove of benefit to IUCD users.

**3 CHLORHEXIDINE RELEASE  
FROM HOLLOW POLYMER  
FIBRES *IN VITRO***

### 3.1 Introduction

The transcervical insertion of a device into the uterus will inevitably introduce microorganisms from the vagina. This has been shown by Bilbruck (1991) and by Malhi (1991) when inserting monofilaments transcervically into guinea-pigs.

In the case of transcervical insertion of IUCDs, there appears to be little risk of development of infection after 3 months, although the risk of pelvic inflammatory disease (PID) does appear to increase for the first 1 to 3 months post-insertion (Lee *et al* 1988). This transient increase in risk of development of PID is likely to be due to the introduction of vaginal bacteria into the uterus. Any incidence of PID which may occur several months after IUCD insertion is believed to be mainly due to sexually transmitted pathogens. As well as this increase in the risk of developing PID, 20 % of women who use an IUCD produce a malodourous, homogenous grey and thin non-purulent discharge which resembles non-specific vaginosis 4 times more common than in non-users (Kivijarvi, 1984).

The previous chapter was able to show that a non-commensal microorganism when introduced into the vagina of a guinea-pig was able to traverse the cervix in 3 out of 8 animals in the presence of a monofilament, compared to 0 out of 8 animals when the monofilament was absent. This finding is not statistically significant ( $p > 0.1$ ), and is therefore in agreement with the findings of Galvez *et al* (1975) who

showed no difference in the rates of PID in women using IUCDs with a monofilament and women using IUCDs with no monofilament. These data suggests that the greatest risk associated with intrauterine devices relates to the carriage of vaginal bacteria into the uterus during the insertion procedure. Modification of the device to reduce this risk therefore needs to be focussed on the rapid release of antibacterial agent to eliminate the bacteria adhered in this mucus layer. A more sustained release designed to contend with microorganisms ascending at a later stage seems to be of lesser importance.

One way in which the device could be altered is by modification of the monofilament itself. Bilbruck *et al* (1990), have shown that by coating the locating monofilament of an IUCD with poly (2-hydroxyethyl methacrylate) (poly-HEMA), they were able to reduce the numbers of bacteria which adhered to the thread. The use of nylon monofilaments coated with poly-HEMA impregnated with chlorhexidine have been shown to release sufficient amounts of chlorhexidine to be bactericidal (Bilbruck *et al* 1991).

This study is based upon the work of Dunn, Lewis and Laufe (1986), who looked at a povidone-iodine dispensing fibre to be used in conjunction with IUCDs. The fibre consisted of a hollow core filled with povidone-iodine and coated with a shell of polypropylene or polycaprolactone. The usual monofilament fibre that is attached to an IUCD is solid whereas the fibres to be investigated in this study are hollow, and loaded with the antibacterial agent chlorhexidine acetate, which when released would act

directly on the bacteria attached to the monofilament.

The aims of this study were to determine the release kinetics of chlorhexidine acetate from different hollow polymer fibres and to establish if modification of the kinetics of release was possible from these hollow fibres by using different vehicles, different concentrations of antibacterial agent and varying geometries.

### **3.2 Materials**

Parafilm was obtained from Fisons Scientific Equipment, Bishop Meadow Road, Loughborough, Leicestershire, England.

#### Chemicals

Chlorhexidine acetate powder was obtained from The Sigma Chemical Company, Poole, Dorset.

Ether, chloroform and Decon (for cleaning of glassware) were supplied by BDH Chemicals Ltd., Poole, Dorset, U. K.

Absolute ethanol was supplied by J. Burroughs Ltd., London, U. K.

Sesame oil was obtained from Thornton and Ross Ltd., Linthwaite Laboratories, Huddersfield, England.

#### Tubing

The polyethylene and nylon hollow fibres to be used for release studies were obtained from Portex UK, Hythe, Kent. For details of the fibres see section 3.4.2.

### **3.3 Apparatus**

Absorbance measurements - As section 2.3.

### **3.4 Methods**

#### **3.4.1 Construction of a calibration plot for chlorhexidine acetate**

Determination of chlorhexidine concentration was made using a Perkin-Elmer Lambda 2 spectrophotometer. To determine the wavelength of maximum absorbance a wavelength scan of a dilute solution of chlorhexidine acetate in absolute ethanol, ( $500 \mu\text{g mL}^{-1}$ ), was performed from 190 nm to 500 nm. Three peaks were shown, at 200 nm, 230 nm and 253 nm, of which the greatest absorbance was given at 253 nm. This was therefore used in all subsequent experiments.

A calibration plot for chlorhexidine was constructed by measuring the absorbances of standard solutions of chlorhexidine acetate in absolute ethanol against an absolute ethanol blank at 253 nm. Use of this plot, (figure 3.1), was made to establish the concentration of chlorhexidine in the bathing fluid after release from the hollow fibres. ( $n = 4, \pm \text{SEM}$ ).

#### **3.4.2 Optimisation of vehicle, fibre material and fibre geometry on chlorhexidine acetate release**

30 mL glass bottles were treated in Decon overnight, and thoroughly rinsed in tap water followed by freshly distilled water prior to use. They were then filled with 30 mL of distilled water. Nylon and polyethylene hollow fibres were used as drug containing devices, the dimensions of

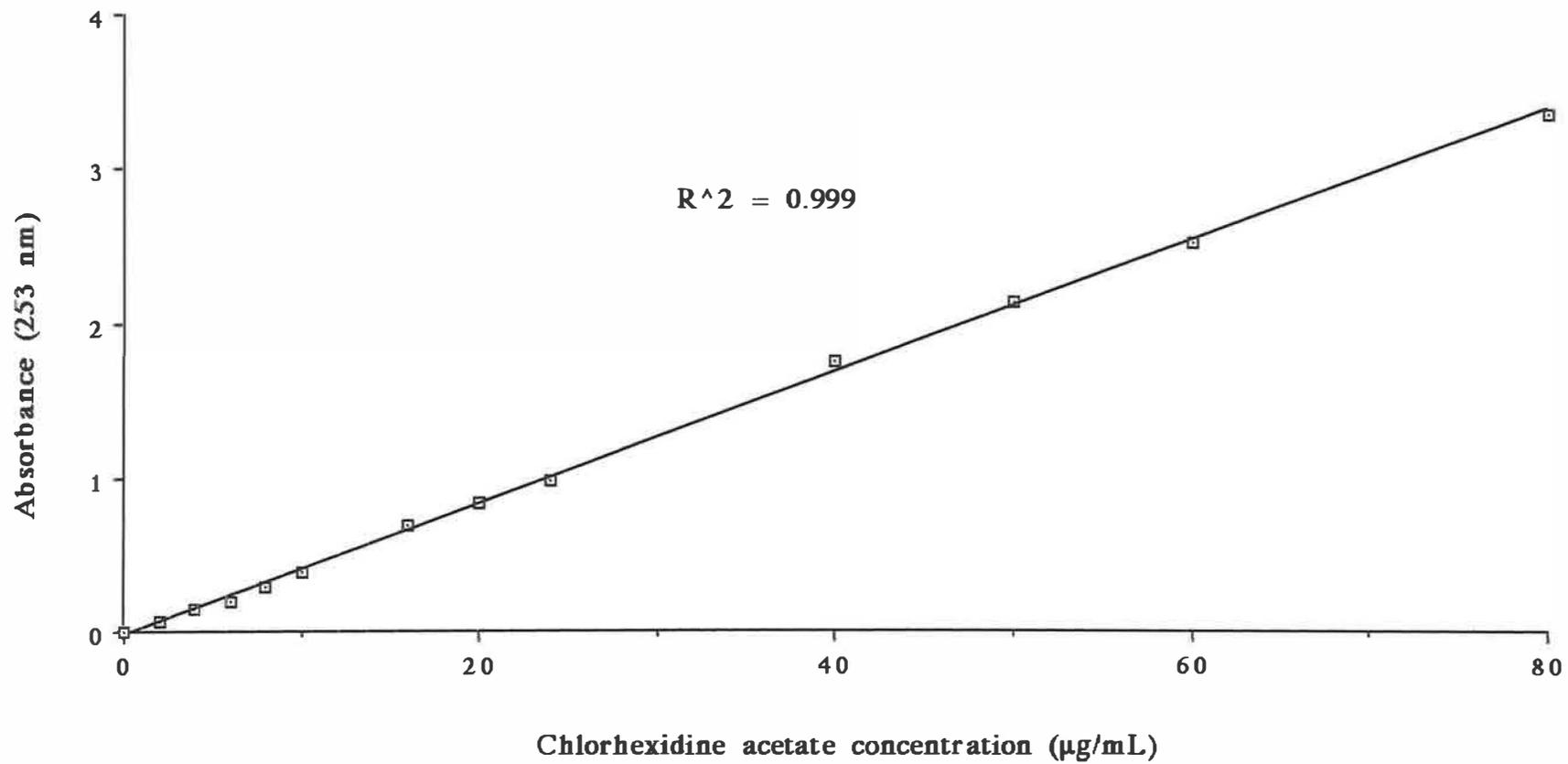


Figure 3.1. Calibration plot of chlorhexidine concentration against absorbance ( $\mu\text{g/mL}$ )

which are given below.

<b>Material</b>	<b>internal diameter (mm)</b>	<b>outer diameter (mm)</b>
standard grade nylon	0.50	0.63
standard grade nylon	0.25	0.75
flexible grade nylon	0.58	1.02
flexible grade nylon	0.75	0.96
polyethylene	0.28	0.61
polyethylene	0.40	0.80
polyethylene	0.50	1.00
polyethylene	0.58	0.96

When referred to in the text, a shorthand version of the dimensions of the hollow fibres is used, such that a nylon hollow fibre with an internal diameter of 0.25 mm and an outer diameter of 0.75 mm would be abbreviated to N,0.25/0.75. The hollow fibres were cut to a length of 9 cm, placed within glass capillary tubes, (to prevent coiling of the fibres when being boiled), and boiled for 15 minutes. This was repeated twice more, each time in a fresh volume of distilled water, in order to remove any plasticisers and excipients added during manufacture which may have affected subsequent absorbance readings. This was checked by performing a wavelength scan of the final volume of water in which the hollow fibres

had been boiled, compared with a distilled water blank. In every case when this was checked no deviation from the distilled water blank was observed. The hollow fibres were then filled with a solution of 140 mg mL<sup>-1</sup> chlorhexidine acetate in absolute ethanol, (the highest concentration of chlorhexidine that would easily dissolve in absolute ethanol), or a suspension of 140 mg mL<sup>-1</sup> chlorhexidine acetate in sesame oil, to a length of 6 cm and then heat sealed. Control hollow fibres were treated identically and loaded to a length of 6 cm with either absolute ethanol or sesame oil. To ensure no leakage from the ends of the hollow fibres, the ends were brought together and wrapped in Parafilm. The Parafilm was then drawn into a thread, the hollow fibre lowered into the bottle, and the thread attached to the side of the bottle, so that the ends constantly remained above the surface of the water. A second piece of Parafilm was used to seal the top of the bottle, in order to prevent evaporation when the bottles were placed into a thermostatically controlled environment at 37 ± 2°C. 2 mL samples were removed from the bottles every 2 to 3 days, and measurements made by UV spectrophotometry using distilled water as the reference, at 253 nm. The absorbances obtained were then converted to concentrations by use of a calibration plot (figure 3.1). After measurements were taken, samples were returned to the bottles, and the tops of the bottles resealed with Parafilm. In each case n = 6, unless otherwise stated.

The results are presented in figures 3.2 and 3.3.

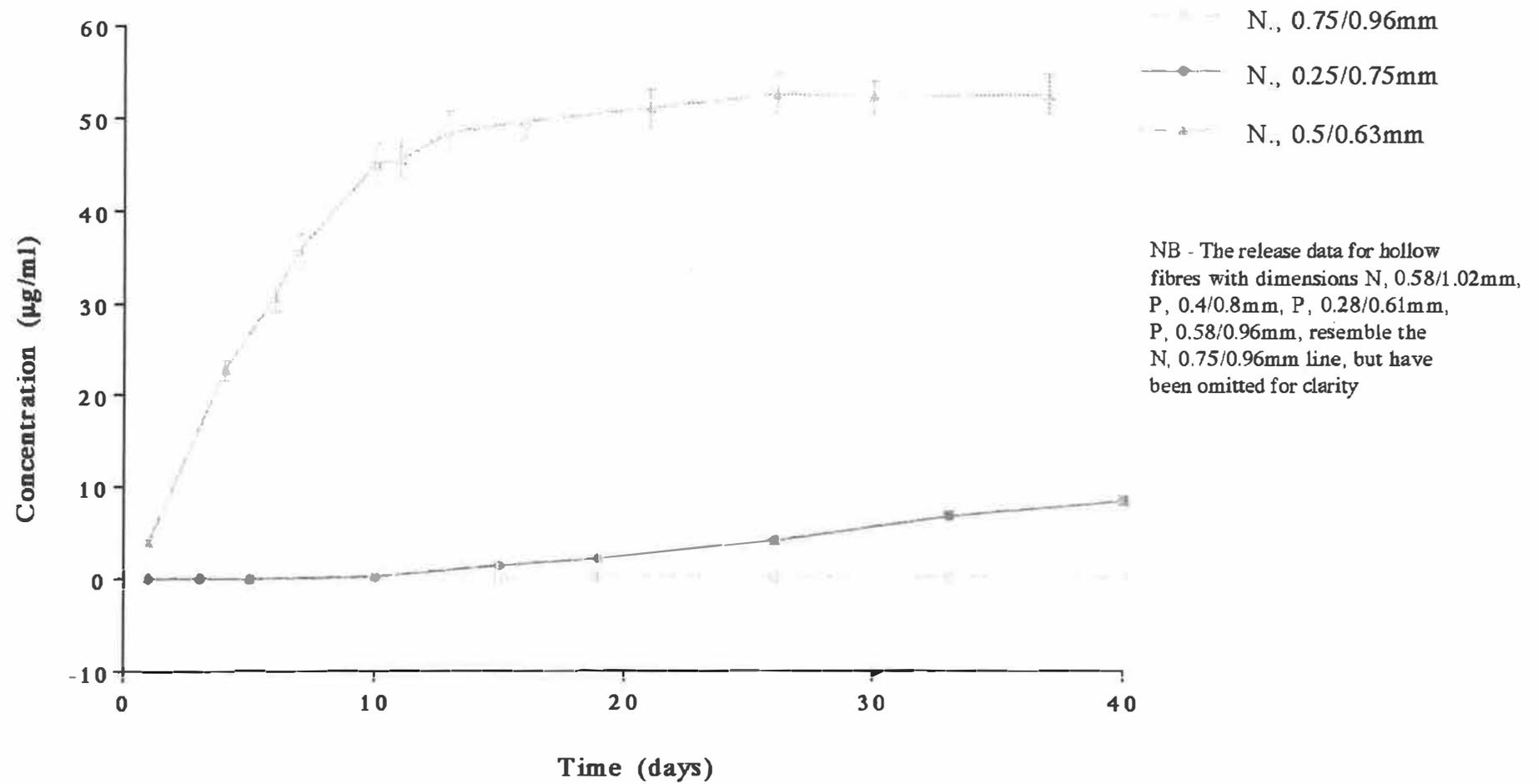


Figure 3.2 Release of 140 mg/ml chlorhexidine acetate in absolute ethanol from hollow polymer fibres (mean  $\pm$  sem, n=6)

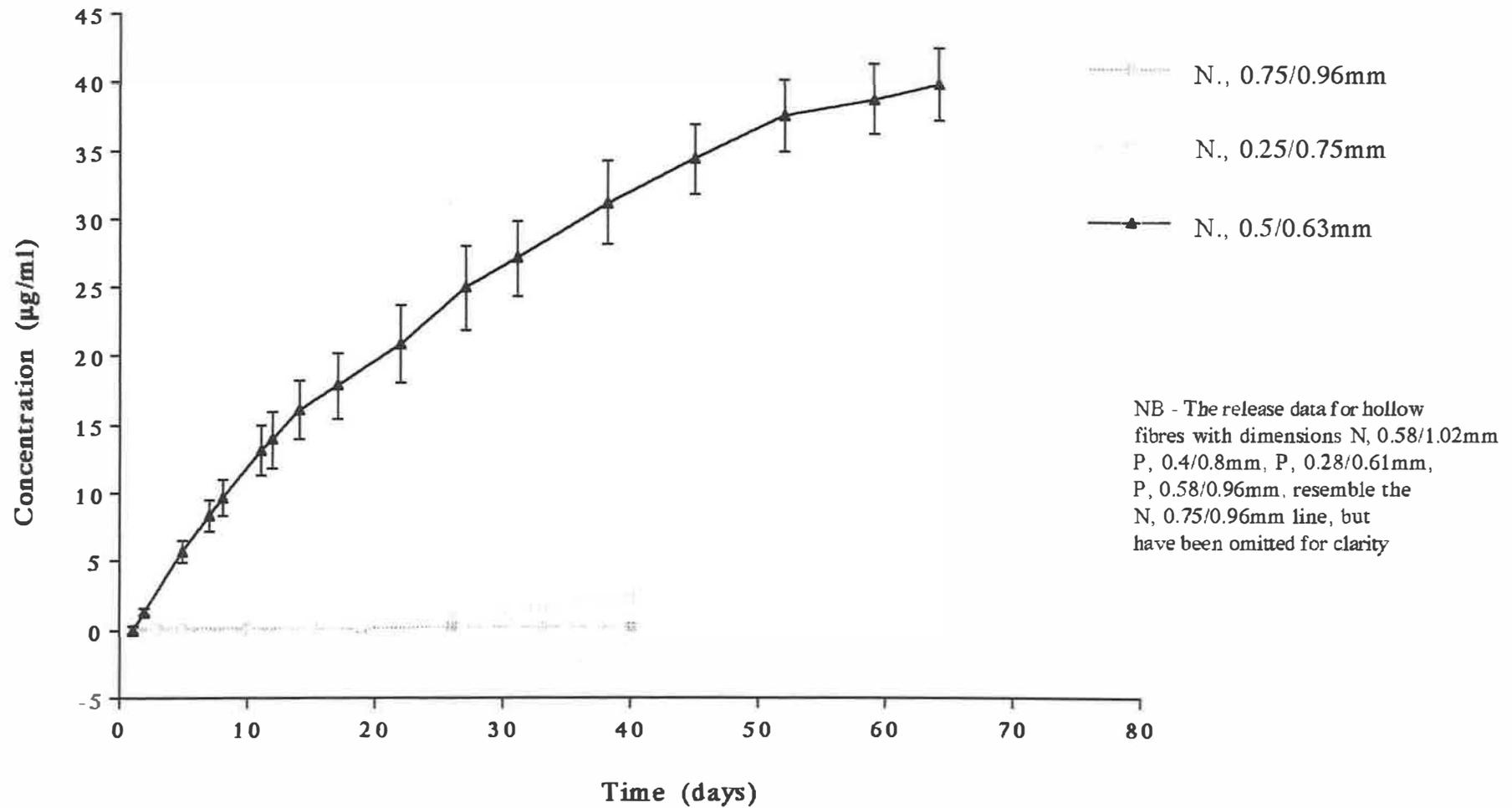


Figure 3.3 Release of 140 mg/ml chlorhexidine acetate in sesame oil from hollow polymer fibres (mean  $\pm$  sem, n=6)

These preliminary studies were performed to determine which fibre gave the quickest release of chlorhexidine acetate. The release of chlorhexidine acetate from 4 nylon and 4 polyethylene hollow fibres possessing different geometries was monitored for up to 64 days. Of the fibres with chlorhexidine acetate in absolute ethanol, the hollow fibre N,0.50/0.63 mm produced the quickest initial rate of release ( $6.49 \mu\text{g day}^{-1}$ ), where release after day 13 was minimal. The fibre that produced the next quickest rate of release, was the nylon hollow fibre with an i.d. of 0.25 mm and an o.d. of 0.75 mm. However, with this fibre it was noted that there was a 10 day lag before any substantial release of chlorhexidine acetate was achieved. The other nylon hollow fibres and the polyethylene hollow fibres gave negligible release, with no apparent increase from the beginning to the end of the study (figure 3.2).

Of the fibres with chlorhexidine acetate in sesame oil, it was again the hollow fibre N,0.50/0.63 mm that gave the quickest release. However, the initial rate of release over the first 15 days was much slower than when chlorhexidine acetate was in the absolute ethanol vehicle, (approximately  $1.3 \mu\text{g day}^{-1}$ ), but release was sustained for much longer, such that chlorhexidine was still being released from the fibre after 50 days. Again, the second quickest release was produced by the nylon hollow fibre with an i.d. of 0.25 mm and an o.d. of 0.75 mm. In this case, the lag before significant amounts of chlorhexidine acetate were released was approximately 20 days.

Negligible release of chlorhexidine acetate from the other hollow nylon

and polyethylene hollow fibres was observed over the course of the experiment (figure 3.3)

### **3.4.3 Influence of concentration and vehicle on the release of chlorhexidine acetate**

Once it had been established which hollow fibre gave the fastest rate of chlorhexidine release, this was then used for the remaining studies.

30 mL glass bottles were treated as described previously in section 3.4.2, and filled with 30 mL of distilled water. Nylon hollow fibres, (N,0.5/0.63) were cut to a length of 9 cm, and treated as described in section 3.4.2. The fibres were then loaded with a solution of 30, 50 or 140 mg mL<sup>-1</sup> chlorhexidine acetate in absolute ethanol, or a suspension of 30, 50 or 140 mg mL<sup>-1</sup> chlorhexidine acetate in sesame oil, to a length of 6 cm. Control hollow fibres, which had been treated in an identical manner to the test fibres, were filled with only sesame oil or absolute ethanol, again to a length of 6 cm. The tubes were placed in the bottles and incubated as described previously (section 3.4.2). Samples were taken daily for the first 2 days, after which measurements were taken at progressively longer intervals. Readings were made at 253 nm as described previously (section 3.4.2).

The results are presented in figure 3.4 and 3.5.

When the nylon hollow fibre, (N,0.50/0.63), was filled with a solution or

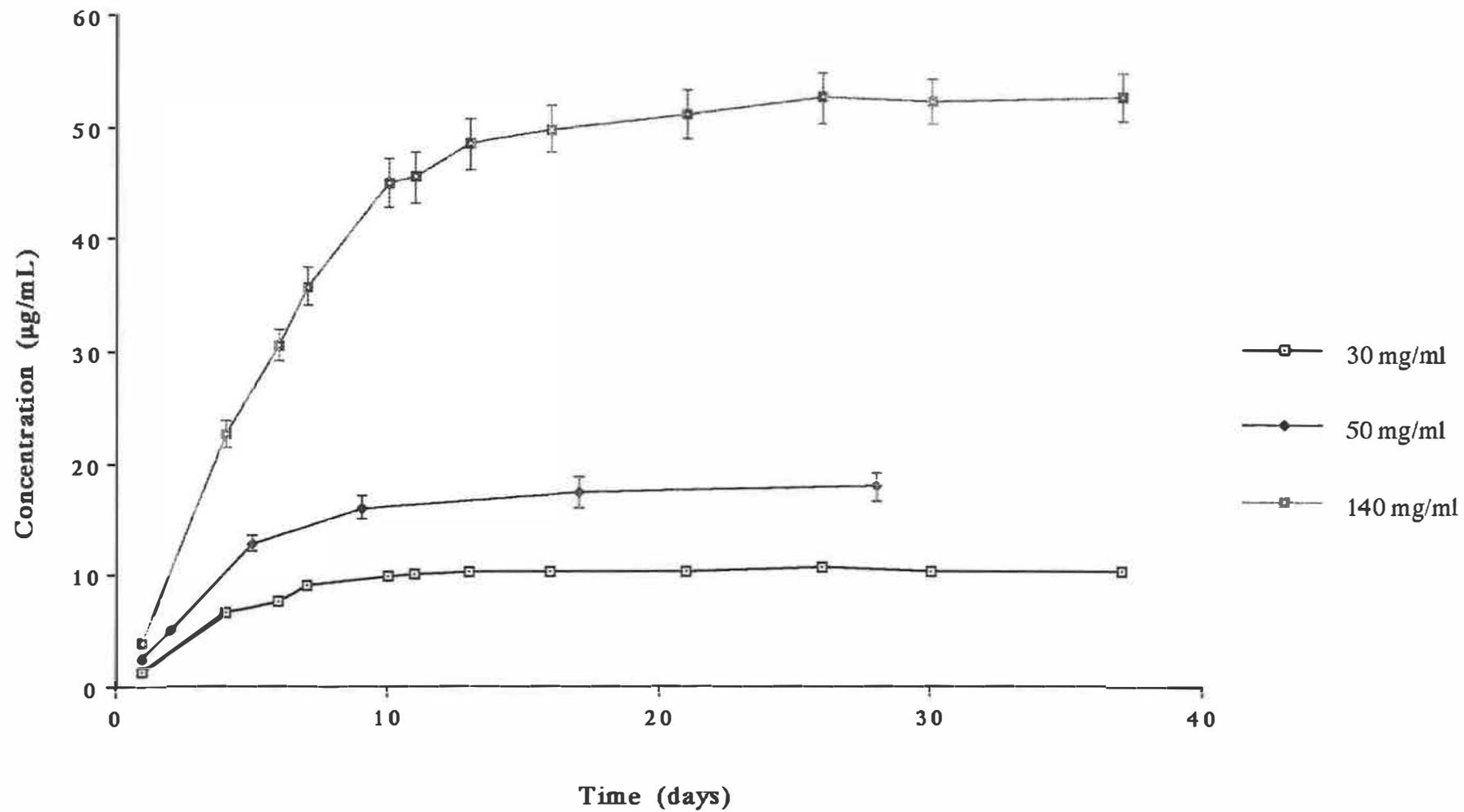


Figure 3.4 Release of various concentrations of CA in absolute ethanol from a nylon hollow fibre (N,0.5/0.63mm) (mean  $\pm$  sem, n=6)

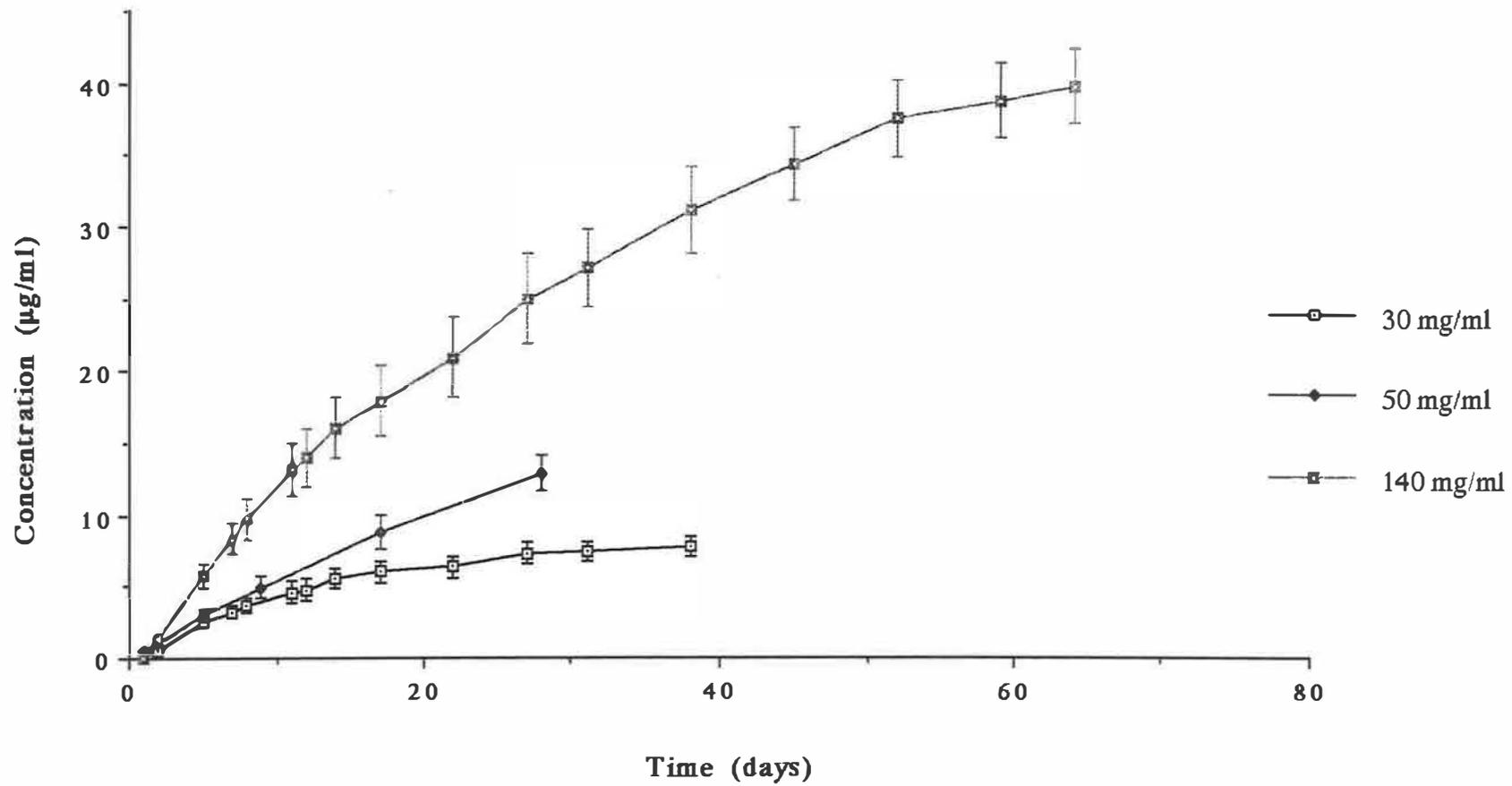


Figure 3.5 Release of various concentrations of CA in sesame oil from hollow fibre (N, 0.5/0.63mm) (mean  $\pm$  sem, n=6)

suspension of 30 or 50 mg mL<sup>-1</sup> chlorhexidine acetate in either absolute ethanol or sesame oil, and the release of these 2 concentrations was compared against the release rate from the hollow fibres loaded with 140 mg mL<sup>-1</sup>, it can be seen from figure 3.4 and figure 3.5 that the release of chlorhexidine acetate from the hollow fibre is concentration dependent. Comparing the figures, it can be seen that for each concentration, the initial release rate is quickest from the fibre with absolute ethanol as the vehicle. Where the initial release rates are 1.86, 2.59 and 6.49 µg mL<sup>-1</sup> day<sup>-1</sup> from the hollow fibres filled with solutions of 30, 50 and 140 mg mL<sup>-1</sup> of chlorhexidine in absolute ethanol and 0.72, 0.74 and 1.30 µg mL<sup>-1</sup> day<sup>-1</sup> from the hollow fibres filled with suspensions of 30, 50 and 140 mg mL<sup>-1</sup> of chlorhexidine in sesame oil respectively. A slower more sustained release is produced with the chlorhexidine acetate in the sesame oil.

#### **3.4.4 Influence of ethanol concentration on chlorhexidine release**

30 mL glass bottles were treated as described in section 3.4.2, and filled with 30 mL of distilled water. A hollow fibre, (N,0.50/0.63), was cut to lengths of 9 cm, and treated as described in section 3.4.2. The hollow fibre was then loaded with a solution of 140 mg mL<sup>-1</sup> of chlorhexidine acetate in 100%, 75% or 50% ethanol, or a suspension of chlorhexidine acetate in distilled water or 25% ethanol, to a length of 6 cm. Control hollow fibres which had been treated identically to the test fibres

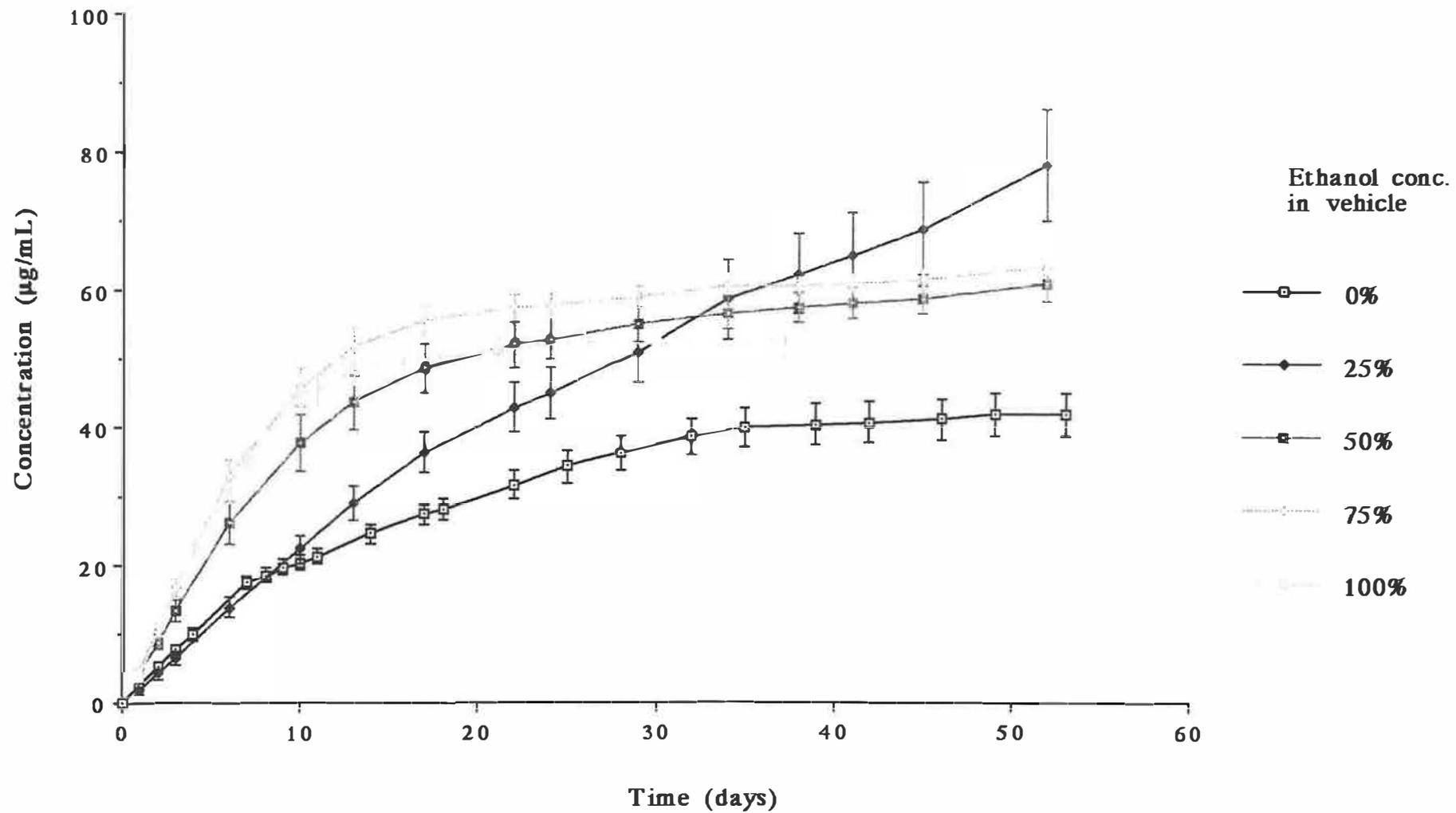


Figure 3.6 Release of CA in various concentrations of ethanol from a nylon hollow fibre (0.5/0.63 mm) (mean  $\pm$  sem, n=6)

were loaded with either distilled water, 25, 50, 75% or absolute ethanol and placed into the glass bottles containing the distilled water bathing fluid. Measurements were taken daily for the first 3 days, after which they were taken after longer intervals. Readings were made at 253 nm as described in section 3.4.2. (n = 6 in each case).

Results are presented in figure 3.6.

The rate of release of chlorhexidine acetate was dependent upon the concentration of ethanol in the vehicle. However, it was noted that with an ethanol concentration of 75% and above, the initial rate of release of the chlorhexidine acetate did not increase, with an initial release rate of  $6.49 \mu\text{g mL}^{-1} \text{day}^{-1}$ . Likewise, below a concentration of 25%, the initial rate of release of the chlorhexidine acetate did not differ appreciably from the rate of release when it was in distilled water, where the initial release rate was  $2.35 \mu\text{g mL}^{-1} \text{day}^{-1}$ . The initial rate of release of chlorhexidine acetate was slower from the hollow fibre when in sesame oil than when in any of the ethanol vehicles or distilled water with an initial release rate of  $1.3 \mu\text{g mL}^{-1} \text{day}^{-1}$  of chlorhexidine when released from the nylon hollow fibre in the sesame oil vehicle.

### **3.4.5 Effect of solvent pretreatment of hollow nylon fibres on the release kinetics of chlorhexidine acetate**

To determine whether or not the rate of release was influenced by the vehicle altering the nature of the fibre wall, hollow nylon fibres were incubated in various solvents prior to being loaded with a suspension of chlorhexidine acetate in distilled water, and the release was then monitored. 30 mL glass bottles were treated as described previously in section 3.4.2, and filled with 30 mL of distilled water. Hollow fibres, (N,0.5/0.63 mm), were then cut to lengths of 9 cm, and incubated in distilled water, 25, 50, and 75% ethanol, absolute ethanol, ether and chloroform, for 1 hour and 5 days after which they were treated as described in section 3.4.2. The fibres were then loaded with a suspension of 140 mg mL<sup>-1</sup> chlorhexidine acetate in distilled water, to a length of 6 cm. Control hollow fibres which had been treated identically to the test fibres were loaded with vehicle alone. Readings were taken daily for the first 3 days, after which, they were made after longer intervals. All readings were made at 253 nm as described previously.

Results are presented in figures 3.7 and 3.8.

From figure 3.7 it can be seen that incubation of the fibres in the various solvents for only 1 hour produces a spread of points after 30 days, where the greatest release of chlorhexidine after this time is given by the fibres which were pre-incubated in distilled water. Using one way analysis of variance the computed F value (variance ratio) was 4.6. This suggests that

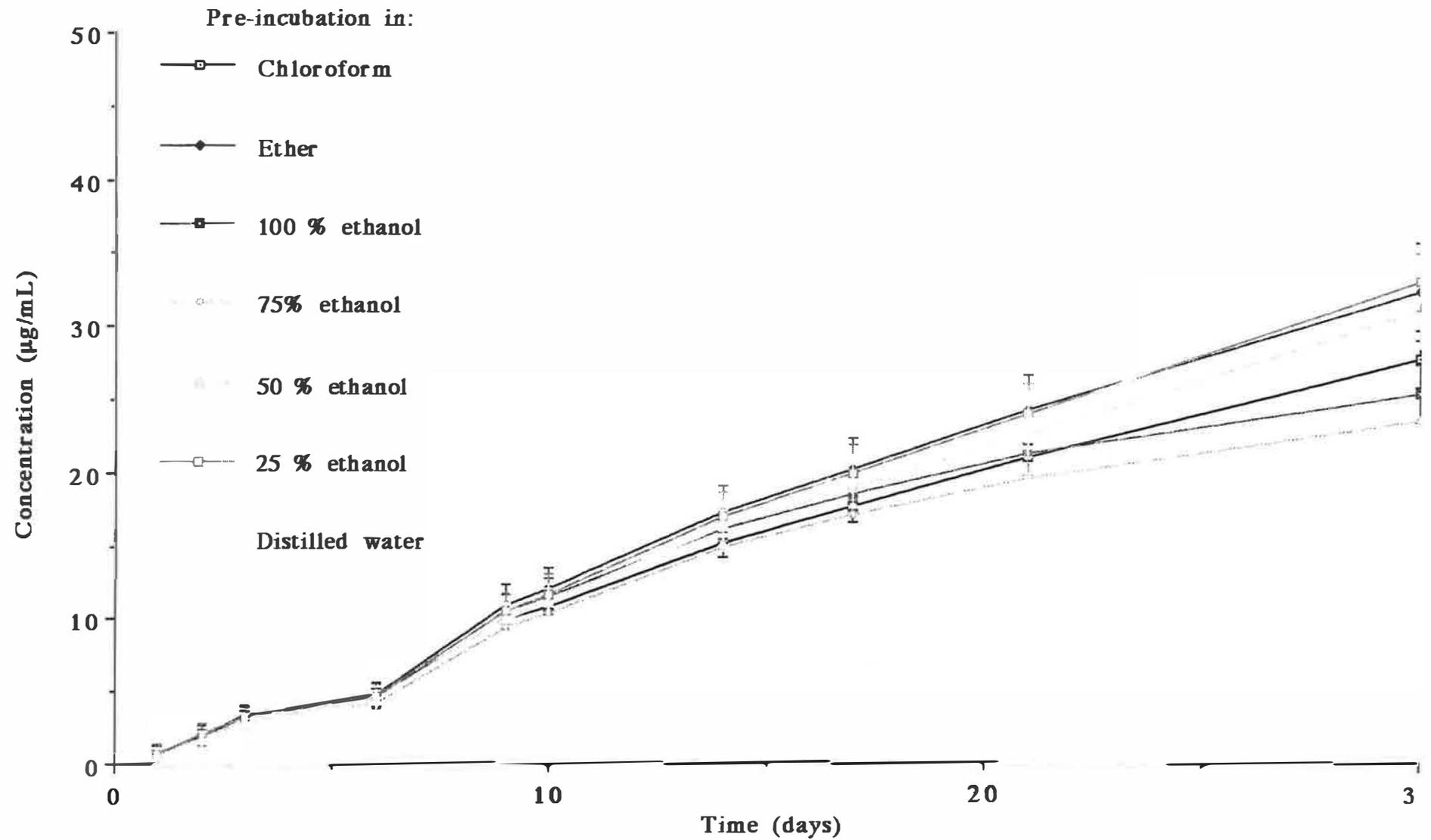


Figure 3.7 Release of chlorhexidine from a nylon hollow fibre after incubation in various solvents for 1 hour (mean  $\pm$  sem,  $n=6$ )

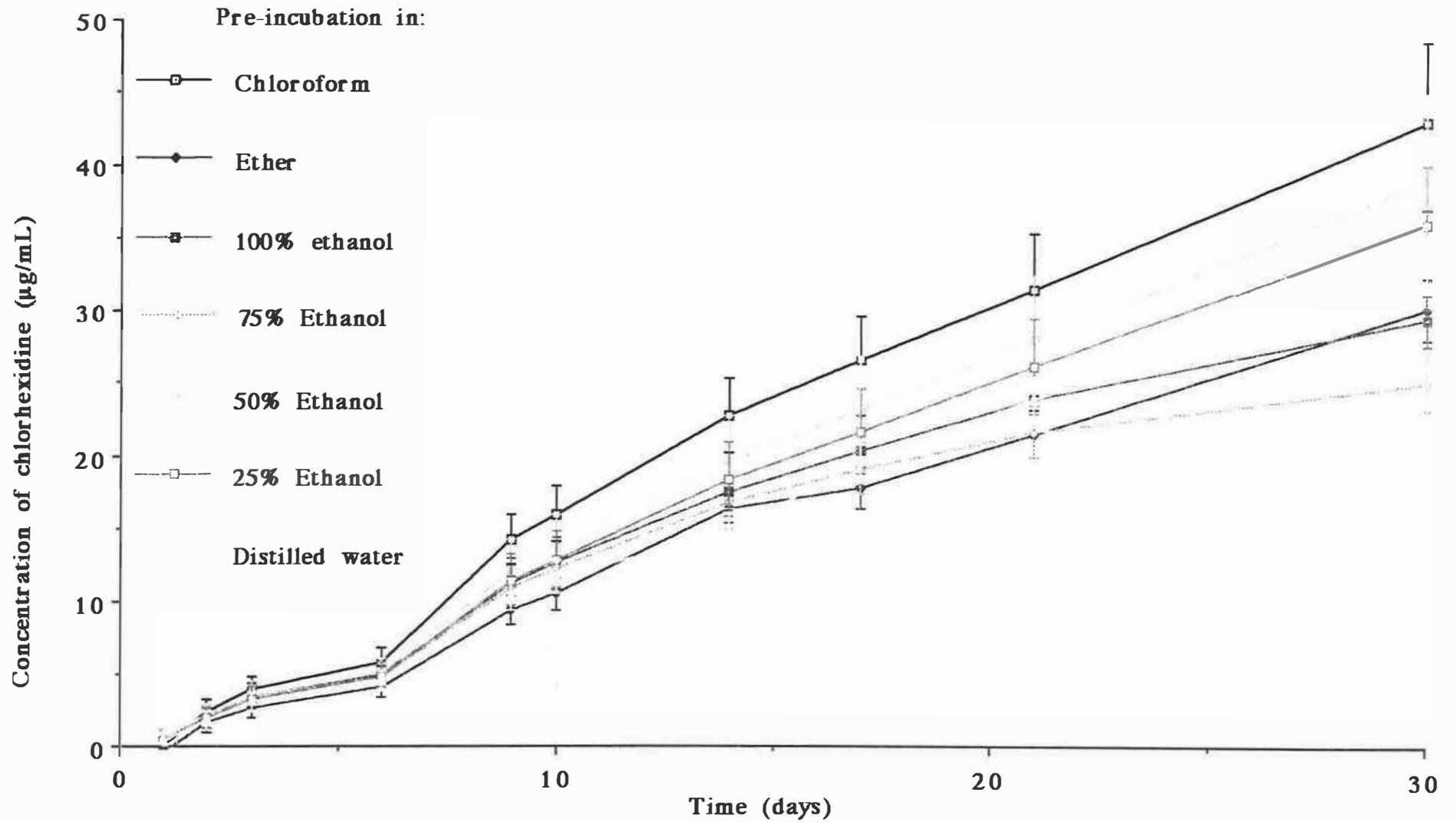


Figure 3.8. Release of chlorhexidine acetate from a hollow fibre (N, 0.5/0.63 mm) after pre-incubation in various solvents for 5 days (mean  $\pm$  sem, n=6)

the within group variation and between group variation was significant ( $p > 0.05$ ). Similarly, after 5 days incubation in the solvents, and 30 days after release was monitored an even larger spread can be seen (figure 3.8). In this case the fibre pre-incubated in chloroform produced the highest concentration of chlorhexidine in the bathing fluid. Again, using one way analysis of variance the computed F value, was 3.4. This suggests that the within group variation and between group variation was significant ( $p > 0.05$ ).

#### **3.4.6 Release of chlorhexidine from nylon hollow fibres after prior evaporation of the vehicle**

30 ml glass bottles were treated as described previously in section 3.4.2, and filled with 30 mL of distilled water. Nylon hollow fibres, (N,0.50/0.63 mm), were cut to lengths of 9 cm and treated as described in section 3.4.2. The fibres were loaded with a solution of 140 mg mL<sup>-1</sup> chlorhexidine acetate in absolute ethanol to a length of 6 cm. The fibres were sealed as described in section 3.4.2, and were placed in an incubator at 37°C for 2 days to allow the ethanol to evaporate, leaving the chlorhexidine powder within the hollow nylon fibre. Control hollow fibres which had been treated identically to the test fibres were loaded with vehicle alone, which was also allowed to evaporate. The hollow fibres were then placed into the glass bottles and Parafilm was employed as described in section 3.4.2. Readings were taken daily for the first 3 days, after which, they were made after longer intervals. All readings were made at 253 nm as described previously.

Results are presented in figure 3.9.

After the ethanol had been allowed to evaporate the appearance of chlorhexidine within the hollow fibre was as a white powder. Within 24 hours of the hollow fibres being placed into the distilled water bathing fluid, liquid was seen within these hollow fibres, and chlorhexidine was detected in the bathing fluid itself. The rate of release of chlorhexidine from these hollow fibres is shown in figure 3.9.

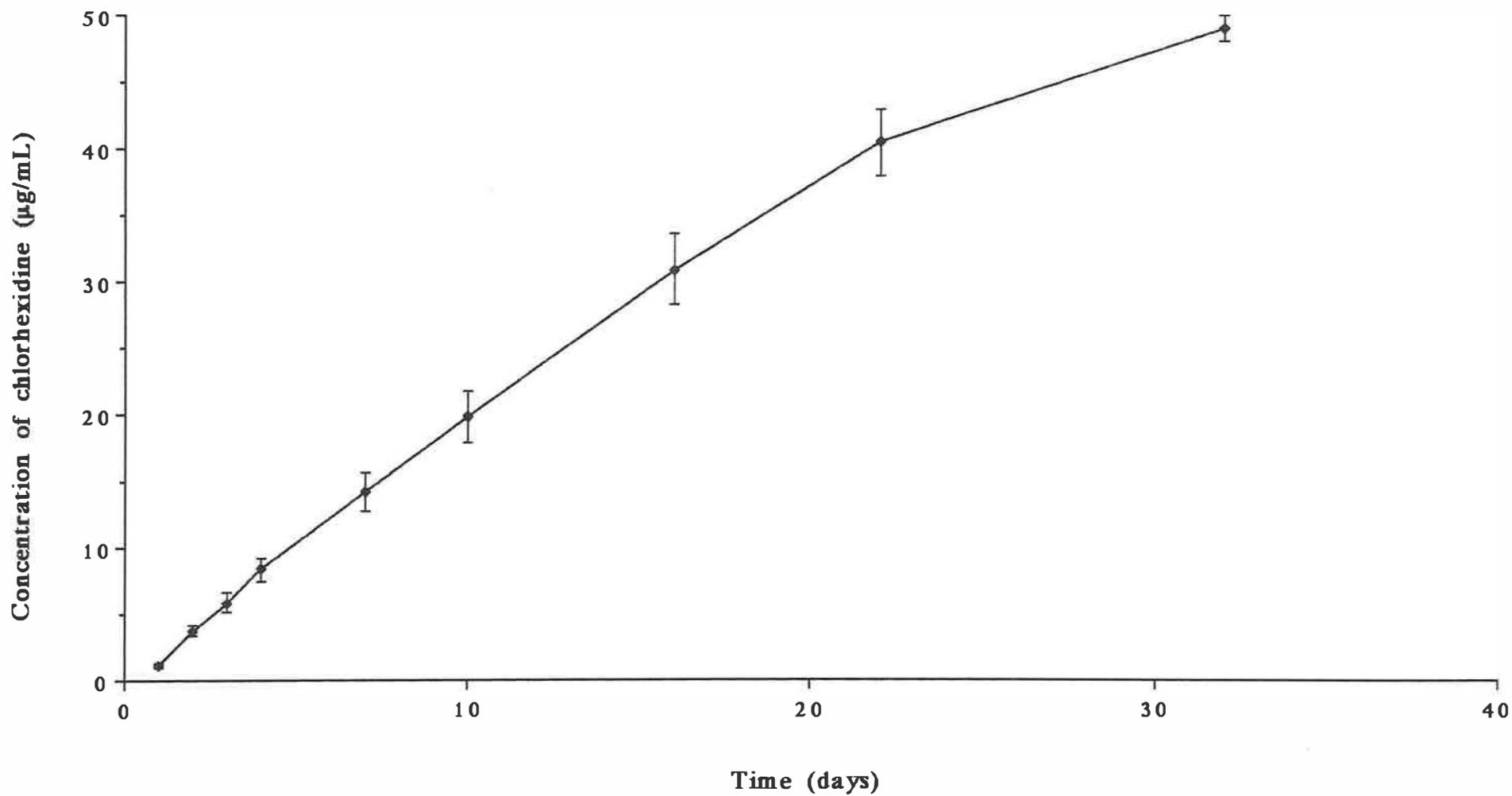


Figure 3.9 Release of chlorhexidine acetate after evaporation of the absolute ethanol solvent (mean  $\pm$  sem, n=6)

### 3.5 Discussion

No release of chlorhexidine was detected from the hollow polyethylene fibres. Release from the nylon hollow fibres was limited to 2 of the 4 studied regardless of the vehicle used. Within a given polymer, whether it is nylon or polyethylene, permeability is a function of the degree of crystallinity, which is itself a function of the molecular weight. The crystalline regions of the solid polymer present an impenetrable barrier to the movement of most molecules. Diffusing molecules must therefore circumnavigate these ordered or crystalline regions which act as obstructions, passing through the disordered or amorphous regions of the polymer (Florence and Attwood, 1989). Another factor that will influence the permeability of the polymer is its density, since the more dense the polymer, the greater the number of obstacles in a given volume, decreasing the speed of passage of the diffusing molecule. The 2 fibres that were able to release chlorhexidine were N,0.25/0.75 mm and N,0.50/0.63 mm, having a wall thickness of 0.25mm and 0.065mm respectively. As can be seen in both figures 3.2 and 3.3, release is clearly shown from these 2 hollow fibres, although that with the thicker wall, (N,0.25/0.75mm), shows a time lag between immersion of the fibres and detection of chlorhexidine in the bathing fluid. This delay would appear to be related to the thickness of the wall of these hollow fibres and as such appears to act as a diffusional barrier to release of chlorhexidine into the bathing fluid. With the other 2 nylon hollow fibres, no release of chlorhexidine was observed throughout the course of any of the experiments performed. In the case of both of these nylon fibres,

(N,0.58/1.02mm and N,0.75/0.96mm), the thickness of the wall was less than that of one of the nylon hollow fibres which was able to release chlorhexidine (N,0.25/0.75mm which had a wall thickness of 0.25 mm), having wall thicknesses of 0.22 mm and 0.105 mm. The reason for the inability of these fibres to release chlorhexidine must be due to the type of nylon from which they were constructed. Those fibres that were able to release the chlorhexidine were fabricated from what was called by the manufacturers standard grade nylon, which may be a less dense polymer with a larger area of amorphous material than those fibres that were unable to release chlorhexidine, which were fabricated from what was described as flexible grade nylon. The manufacturers were unable to supply any further information on the nature of these materials.

The release of chlorhexidine from the hollow fibres is a first order process. This may be shown by plotting the logarithm of the amount of chlorhexidine remaining within the hollow fibre against time. This produces a straight line graph, which is characteristic of first order processes. This graph is shown in figure 3.10, the values of which were derived from figure 3.9. A straight line graph will also be produced when plotting similar graphs using values derived from the release of chlorhexidine, when chlorhexidine is in any vehicle, at any concentration used in this study, provided that chlorhexidine is able to be released from the hollow fibre. The chlorhexidine was released into the bathing fluid from the hollow fibres by passive diffusion. This is the process where matter moves from one region of a system to another following random molecular motions. The basic hypothesis underlying the mathematical

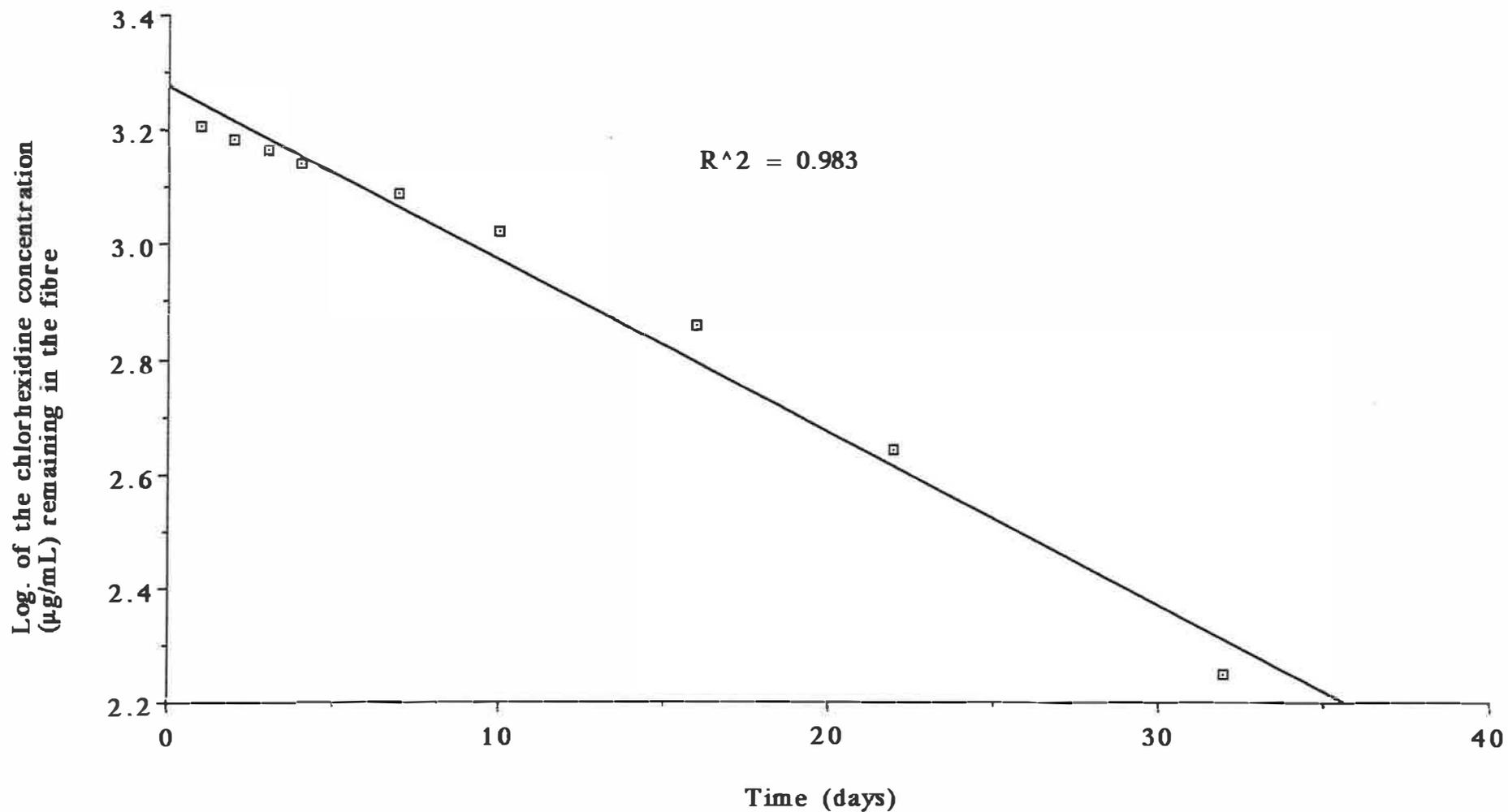


Figure 3.10. Plot of chlorhexidine remaining within a hollow fibre against time in days to demonstrate first order release

theory for isotropic materials (which have identical structural and diffusional properties in all directions) is that the rate of transfer of diffusing substance per unit area of a section is proportional to the concentration gradient. This is expressed as Fick's first law of diffusion.

$$J = -D \frac{\partial c}{\partial x}$$

where J is the rate of transfer per unit area of surface (flux), C is the concentration of diffusing substance, x is the space coordinate measured normal to the section, and D is the diffusion coefficient. The negative sign indicates that the flux is in the direction of decreasing concentration.

The initial rates of release of chlorhexidine from the hollow fibre (N, 0.50/0.63 mm), filled with solutions of 30, 50 and 140 mg of chlorhexidine per mL of absolute ethanol were 1.86, 2.59 and 6.49  $\mu\text{g mL}^{-1} \text{ day}^{-1}$  respectively. Since the volume of the bathing fluid was 30 mL, the total initial rate of release for the 30, 50 and 140 mg  $\text{mL}^{-1}$  solutions were 55.81, 77.61 and 194.70  $\mu\text{g day}^{-1}$  respectively. If the hollow fibres are considered as being cylinders 60 mm long, with an external diameter of 0.63 mm, then the total surface area of the hollow fibre is 119.38 mm. Therefore, the initial rates of release of chlorhexidine from the hollow fibres filled with 30, 50 and 140 mg  $\text{mL}^{-1}$  of chlorhexidine solution per  $\text{mm}^2$  of hollow fibre are 0.468, 0.650 and 1.631  $\mu\text{g day}^{-1} \text{ mm}^2$  respectively, which is equal to the initial flux. Since

these values are derived from the initial rates of release, it may be assumed that at this point the concentration gradient is at its maximum, which is equal to the initial concentration of chlorhexidine within the hollow fibres, which is 30, 50 and 140 mg mL<sup>-1</sup>. Therefore, by plotting a graph of flux against concentration gradient it is possible to obtain D from the slope of the curve produced. Figure 3.11 shows this plot, and D was calculated as being  $4.52 \times 10^{-2} \text{ mm}^2 \text{ day}^{-1}$ .

From the results obtained in this chapter, it may be seen from the plots of chlorhexidine release against time, that in all cases where substantial release occurred, initially release is linear, after which it slows down and reaches a plateau. The release of 140, 50 and 30 µg mL<sup>-1</sup> of chlorhexidine acetate is monitored over 37, 28 and 37 days respectively and actually contain 1.649 mg, 0.589 mg and 0.353 mg of chlorhexidine respectively. After monitoring release until each of the fibres containing chlorhexidine had reached a plateau it was possible to calculate the actual amount of chlorhexidine that was released. The amount of chlorhexidine released was 1.583 mg, 0.540 mg and 0.315 mg for the fibres loaded with 140, 50 and 30 µg mL<sup>-1</sup> of chlorhexidine respectively. This is in the region of 90 % exhaustion of the chlorhexidine containing reservoir over the period release was monitored. Fick's law states that flux (J), which is the net amount of solute that diffuses through unit area per unit time, is proportional to the concentration gradient. As such, it would appear that release from the hollow fibres in this study obeys Fick's law, since as the concentration gradient decreases, so does the flux.

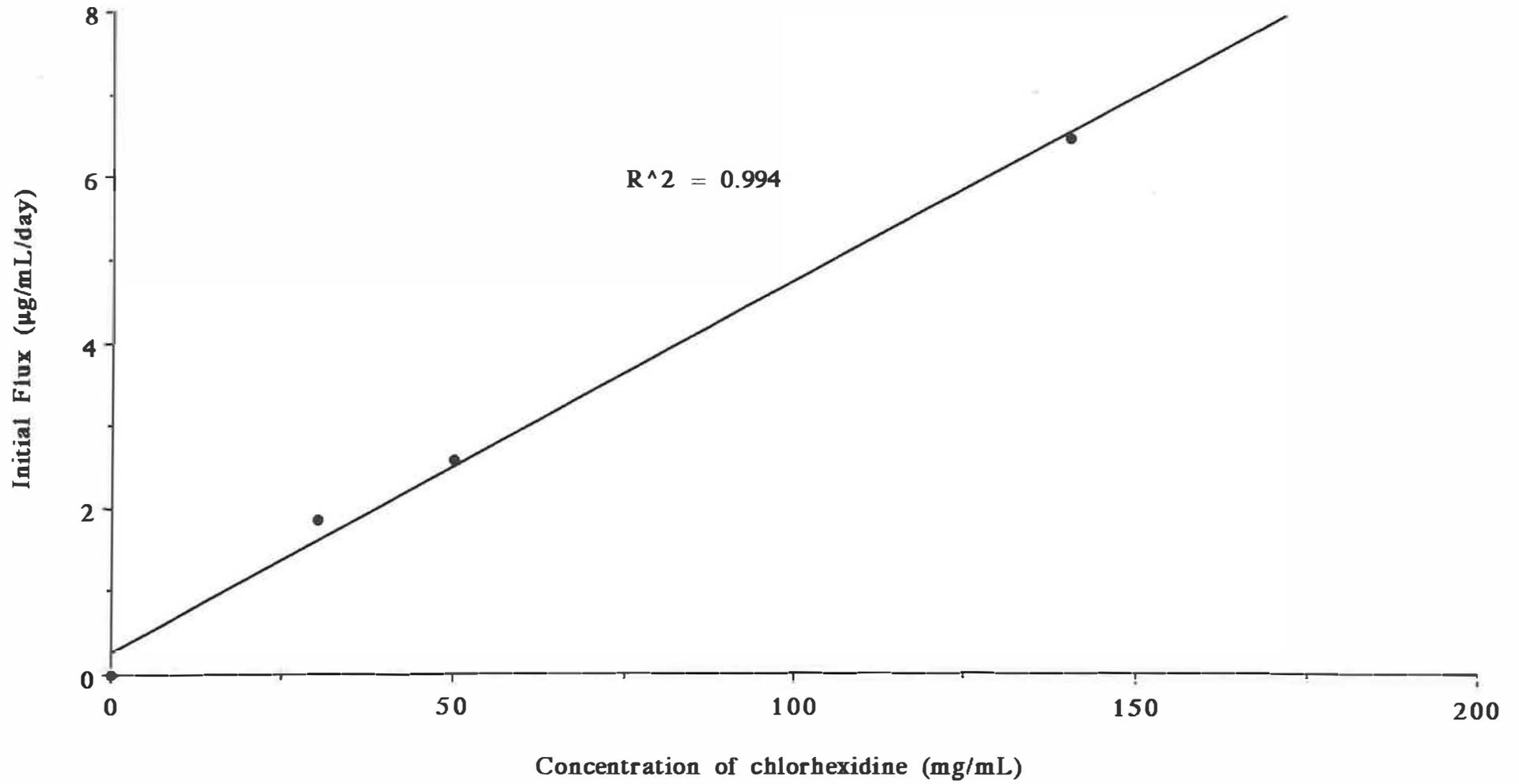


Figure 3.11. Plot of initial flux of chlorhexidine against the initial concentration gradient from hollow fibres (N, 0.5/0.63 mm)

This has also been demonstrated in figures 3.4 and 3.5 where the initial rate of release in each case is proportional to the amount of chlorhexidine within the hollow nylon fibres at the beginning of the experiment. Such that the initial rates of release differ with the greatest initial rate of release being given by those fibres with the greatest chlorhexidine concentration.

The rate of release of chlorhexidine, as well as being dependent on the dimensions of the fibre itself, could also be controlled by using different vehicles. This has been demonstrated by the use of several different vehicles, namely sesame oil and various concentrations of ethanol in distilled water. The greatest rate of release was obtained from the fibre (N,0.5/0.63) when absolute ethanol was used as the vehicle, where the initial rate of release was  $6.49 \mu\text{g day}^{-1}$ . This was also the initial rate of release when a vehicle of 75 % ethanol was used. When the concentration of ethanol was reduced to 50 %, the rate of release had decreased to  $4.32 \mu\text{g day}^{-1}$ . With the lowest concentration of ethanol used, which was 25 %, the rate of release did not differ from that when chlorhexidine was in distilled water. The rate of release for these two vehicles was  $2.42 \mu\text{g day}^{-1}$ . By far the slowest release was produced when sesame oil was used as the vehicle. When this was used, the initial rate of release was only  $1.3 \mu\text{g day}^{-1}$ .

The difference in initial rates of chlorhexidine release appeared to be related to the solubility of the chlorhexidine within each vehicle. In the absolute ethanol and the 75 % ethanol vehicles, the chlorhexidine was

freely soluble. In 50 % ethanol chlorhexidine was in a saturated solution at a concentration of 140 mg mL<sup>-1</sup>. In both 25 % ethanol and in distilled water, the chlorhexidine was only sparingly soluble, with most of the chlorhexidine remaining in suspension. In the sesame oil, chlorhexidine was practically insoluble which may explain why the release rate from this particular vehicle was the lowest of all the vehicles used.

Release of chlorhexidine was also monitored from a fibre loaded with 140 mg mL<sup>-1</sup> of chlorhexidine in absolute ethanol which was immersed in the bathing fluid only when the vehicle had evaporated away, leaving chlorhexidine in the form of a powder within the fibre. Within 24 hours of immersion of the fibre, liquid was observed inside the previously dry hollow fibre, and chlorhexidine was detected in the surrounding bathing fluid. This may give an indication of the mechanism of action for the release of chlorhexidine from these hollow fibres, as well as giving an insight into the reason for the differences in release rate with different vehicles. If it is necessary that prior to release of chlorhexidine the hollow fibre wall must be saturated with either vehicle or bathing fluid, then the driving force of chlorhexidine movement from the fibre will be the concentration gradient. Those fibres which contain the greatest amount of chlorhexidine in solution will give the greatest concentration gradient and therefore the highest initial rate of release. Thus, chlorhexidine in absolute ethanol and 75 % ethanol vehicles, where all the chlorhexidine is in solution will give the fastest initial rates of release. In the case of the 50 % and 25 % ethanol vehicles, and the distilled water vehicle where the chlorhexidine is also in suspension in varying degrees,

the effective concentration gradient will be less when compared with those fibres containing chlorhexidine that is completely dissolved. This is clearly demonstrated by the fibre that had the vehicle evaporated off prior to immersion, where release could only be expected with the chlorhexidine in solution which could only occur once the bathing fluid had crossed the wall of the fibre dissolving the chlorhexidine allowing it to cross the fibre wall and enter the bathing fluid. With this particular experiment, release from the nylon hollow fibre was slower than from all the other vehicles, ( $2.35 \mu\text{g day}^{-1}$ ), (except that from the sesame oil vehicle), because in all the other vehicles there is at least some chlorhexidine in solution that could be released immediately once the wall of the fibre is saturated with bathing fluid and/or vehicle. In the case of the sesame oil vehicle in which chlorhexidine is virtually insoluble, practically all of the chlorhexidine would have been in suspension within the hollow fibre. Since the bathing fluid, which is distilled water, and the sesame oil are immiscible, they will not freely diffuse into one another. This suggests that the only places where the chlorhexidine could therefore dissolve into the bathing fluid would be either at the external surface of the hollow fibre, presuming that the sesame oil crosses the nylon wall, at the inner surface of the nylon wall should the bathing fluid cross the fibre wall, or at a point within the wall itself. It is most likely however, that the way in which the chlorhexidine will cross the wall of the nylon hollow fibre will be by the bathing fluid crossing to the inner surface of the nylon hollow fibre, from where the chlorhexidine will be able to dissolve and diffuse into the bathing fluid since chlorhexidine in the form of a suspension would be unable to cross the wall.

Figures 3.7 and 3.8 show the results of chlorhexidine release from a hollow fibre (N,0.5/0.63) after pre-incubation in various solvents for 1 hour and 5 days respectively. The purpose of this experiment was to determine whether there was an effect of solvent pre-treatment on the integrity of the wall of these hollow fibres. After 1 hours incubation in the various solvents, and after thirty days suspended in the bathing fluid, the greatest effect was produced by pre-incubation in the distilled water (figure 3.7). Using one way analysis of variance the computed F value (variance ratio) was 4.6. This suggests that the within group variation and between group variation was significantly different ( $p < 0.01$ ). After 5 days incubation in each of the solvents, the effect of chloroform on the release of chlorhexidine is most significant and by using one way analysis of variance the computed F value, was 3.4 suggesting that the within group variation and between group variation was significantly different ( $p < 0.01$ ), but was not as great as that found with the hollow fibres that were incubated in the solvents for only 1 hour. It is probable that the different solvents used were able to penetrate the nylon hollow fibre. In doing so they may have increased the porosity of the nylon wall by causing it to swell, easing the passage of chlorhexidine molecules through it. The greatest release is likely to have been produced by those hollow fibres that were exposed to the solvents that caused the highest degree of swelling. Since the actual nature of the nylon used could not be obtained from the manufacturers, the action of the solvents is purely speculative. The change in the order of greatest release after 5 days incubation may be due to a chemical action of the solvents upon the polymer itself as well as an ability to cause a swelling of the hollow fibre wall.

**4 THE BACTERICIDAL EFFECT  
OF CHLORHEXIDINE  
ACETATE *In Vitro***

## 4.1 Introduction

From chapter 3 the release of chlorhexidine acetate was demonstrated in only 2 hollow nylon fibres. Release from the other 2 nylon fibres and all 4 of the polyethylene hollow fibres was negligible. Although release has been established, the antibacterial effect of the chlorhexidine that is released needs to be ascertained.

The aims of this study were to isolate and identify the most predominant microorganisms found in the guinea-pig vagina and determine the minimum inhibitory concentrations of chlorhexidine acetate upon those microorganisms. This involved establishment of a chlorhexidine/vehicle combination that produced the greatest antimicrobial effect, and testing the antibacterial action of this combination after release from hollow nylon fibres. In order to ascertain the action of this combination in an environment that was considered to mimic that of the guinea-pig vagina, the antibacterial action of chlorhexidine was also investigated after release from hollow nylon fibres into purified mucus inoculated with a commensal of the guinea-pig vagina. This was then compared with the antibacterial effect of chlorhexidine released into phosphate buffered saline inoculated with the same microorganism.

## 4.2 Materials

### Bacteria

*Escherichia coli* NCIB 8196 was obtained from the National Collection of Industrial Bacteria (NCIB), Aberdeen, and was maintained on Nutrient Agar at 4 °C and subcultured at approximately 3 month intervals.

### Media

Nutrient broth and nutrient agar - as section 2.2.

API 20 E and API STAPH identification systems were supplied by API Laboratory Products, Basingstoke, Hampshire, U. K.

API 20 E identifies Enterobacteriaceae and other gram negative rods using 23 tests, whilst API STAPH is an identification system for Staphylococci and Micrococci based on 19 tests.

### Chemicals

Tween 80 (polyoxyethylene sorbitan mono-oleate), egg lecithin, sodium chloride, sodium azide and ammonium chloride were supplied by BDH Chemicals Ltd., Poole, Dorset, U. K.

Absolute ethanol as section 3.2.

Chlorhexidine as section 3.2.

Phosphate buffered saline (PBS) as section 2.2.

Sepharose CL-4B was supplied by Pharmacia Ltd., Milton Keynes, Buckinghamshire, U. K.

Phenylmethylsulphonyl fluoride (PMSF) and EDTA were obtained from The Sigma Chemical Company, Poole, Dorset, U. K.

### Tubing

Nylon monofilaments as section 2.2.

Glass capillary tubes as section 2.2.

Silicone rubber tubing was obtained from Jencons (Scientific Ltd.), Leighton Buzzard, Bedfordshire, U. K.; it was sterilised by closure in aluminium foil and then heating in an oven at 180°C for 2 hours.

Purified porcine gastric mucus as section 4.4.8.1.

### **4.3 Apparatus**

Optical density as section 2.3.

Centrifugation as section 2.3.

Minitan ultracentrifugation system obtained from Millipore, Blackmore Lane, Watford, Hertfordshire, England.

Amicon model 402 ultrafiltration cell with a PM 30 membrane (with a cut off at a molecular weight of 30,000). This was obtained from Amicon Ltd., Upper Mill, Stonehouse, Gloucestershire, England.

## **4.4 Methods**

### **4.4.1 Microbiological identification using API systems**

A single colony from a nutrient agar plate was emulsified in 5 mL sterile distilled water to produce a homogenous bacterial suspension. This suspension was used to inoculate the relevant identification strip according to the manufacturers instructions. The strip was then placed in a tray, containing 5 mL of distilled water, covered with a lid and incubated at 37°C for 24 hours. At this time results were recorded and the bacteria identified using the tables provided.

### **4.4.2 Collection and determination of vaginal microflora**

Vaginal lavage with 2 mL of sterile quarter strength Ringer solution was performed by repeatedly aspirating 0.5 mL aliquots with a 1 mL graduated pipette in 6 appropriately restrained guinea-pigs. From the resultant cell suspensions 0.2 mL aliquots were plated onto overdried nutrient agar plates which were incubated either aerobically or anaerobically at 37°C for 24 - 48 hours. It was noted that the numbers of bacteria found in the different animals varied markedly, although the actual morphological types of bacteria exhibited showed little variation. Only the predominant microorganisms were isolated and identified using the systems described in section 4.4.1. These microorganisms are listed in table 4.1.

Gram-negative

Gram-positive

*Escherichia coli*

*Staphylococcus aureus*

*Proteus mirabilis*

*Klebsiella pneumoniae*

**Table 4.1 Microflora of the virgin guinea-pig vagina**

#### **4.4.3 Collection and determination of uterine microflora**

The female guinea-pigs were killed by cervical dislocation and the bicornate uterus exposed by laparotomy. Using sterilised instruments, both horns of the uterus were removed aseptically and one horn incubated whole in nutrient broth at 37°C for 24 - 48 hours. The lumen of the other horn was repeatedly aspirated with 2 mL sterile lecithin-tween peptone water and 0.2 mL aliquots plated onto nutrient agar and incubated in air at 37°C for 24 - 48 hours. If bacteria were found to be present, those that predominated were then isolated, and identified using the systems described in section 4.4.1.

Examination of the nutrient broth solutions after incubation for 36 - 48 hours showed them all to be clear. This suggests that no microorganisms were present in the normal environment of the guinea-pig uterus. These findings agree with those of Malhi (1991) and Bilbruck (1991).

#### **4.4.4 Determination of the minimum inhibitory concentration of chlorhexidine acetate against guinea-pig vaginal microorganisms**

Varying concentrations of chlorhexidine acetate were added to equal volumes of sterilised double strength molten nutrient agar, to give final concentrations of 0.0, 0.8, 1, 2, 4, 8, 10, 20, 40, 80 and 100  $\mu\text{g mL}^{-1}$  in normal strength agar. (This dilution scheme was adopted as preliminary data suggested that it would be appropriate with the selection of microorganisms isolated). Once the plates were poured, they were allowed to solidify, and overdried prior to use.

The surface of each of the plates was inoculated with the predominant microorganisms isolated from the vaginae of several guinea-pigs (see table 4.1 for a list of microorganisms). The plates were then incubated for 36-48 hours at 37°C after which time they were examined for the presence or absence of growth to determine the lowest concentration of chlorhexidine acetate in the nutrient agar which prevented growth of each of the microorganisms. These were recorded as the minimum inhibitory concentration (MIC). The MIC of those microorganisms isolated from the guinea-pig vaginae and identified and given in table 4.2.

Microorganism	Concentration of chlorhexidine acetate ( $\mu\text{g mL}^{-1}$ )
<i>Escherichia coli</i>	4
<i>Proteus mirabilis</i>	100
<i>Klebsiella pneumoniae</i>	40
<i>Staphylococcus aureus</i>	4

**Table 4.2 MIC values for those microorganisms isolated from the guinea-pig vagina against chlorhexidine**

#### **4.4.5 Construction of a calibration plot of *Escherichia coli* against absorbance**

Determination of the cell density of *Escherichia coli* was made as described in section 2.4.1, where all parameters used were identical, replacing *Serratia marcescens* with *E. coli*. A plot of optical density against  $\text{cfu mL}^{-1}$  has been constructed and is shown in figure 4.1.

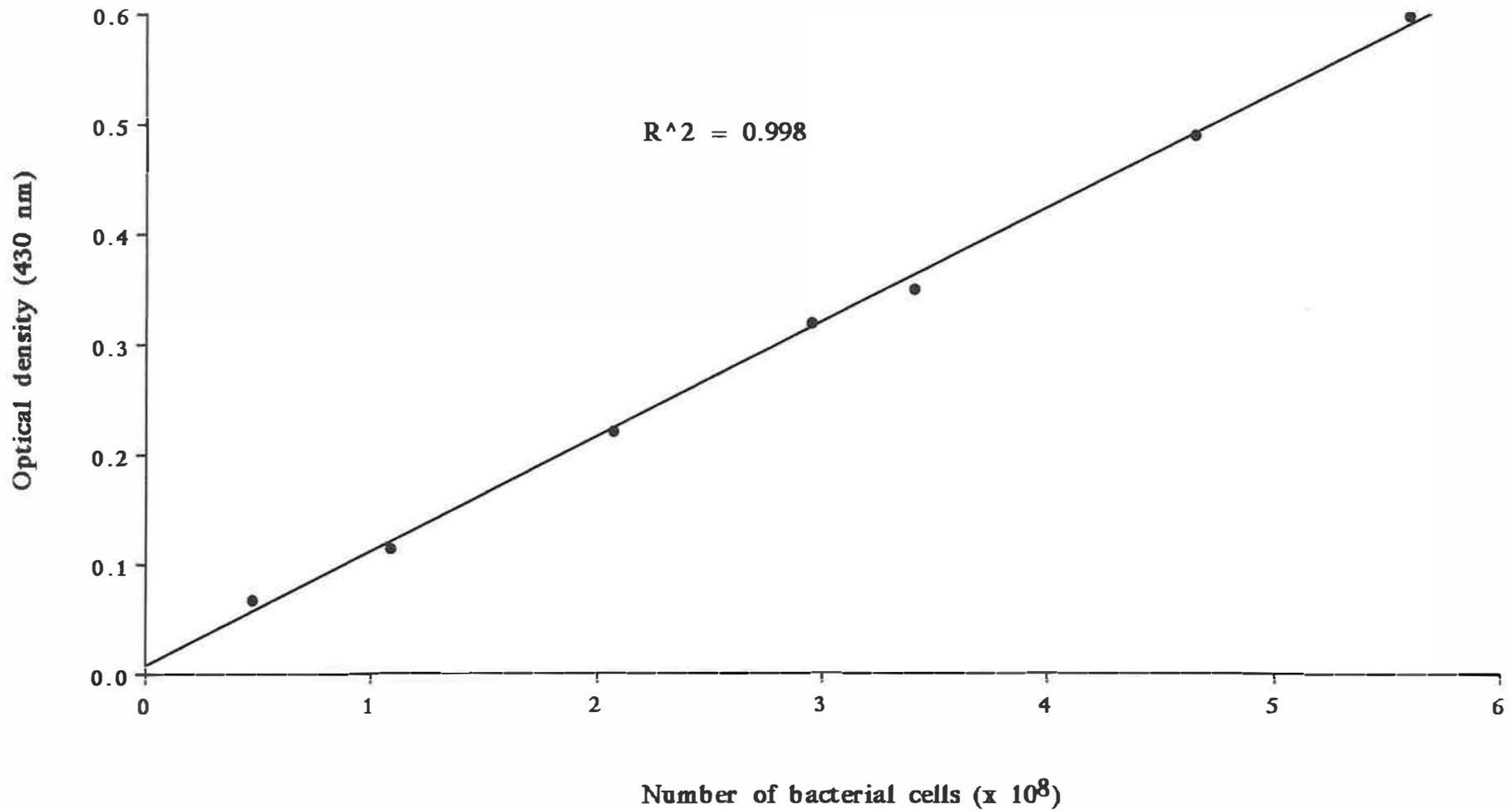


Figure 4.1 Calibration plot of *Escherichia coli* cell concentration against optical density

#### 4.4.6 Investigation of the bactericidal activity of chlorhexidine acetate alone, and in various solvents

0.2 mL of an overnight culture of *E. coli* grown in nutrient broth, was plated onto the surface of freshly poured, overdried nutrient agar. 4 wells were cut in each of the plates by use of a flamed and cooled cork borer. Into the wells, a solution of 50 mg mL<sup>-1</sup> chlorhexidine acetate in either water, 70% ethanol or absolute ethanol was introduced, as well as chlorhexidine acetate in the form of a powder. As controls, wells were filled with absolute ethanol alone or 70% ethanol. After addition of the samples to the wells, the plates were incubated at 37°C for 18-24 hours. After incubation, the plates were examined for zones of growth inhibition surrounding each well. Measurements were made using callipers, accurate to 0.1 mm, measuring from the edge of the well to the point where bacterial growth began.

The results are shown in figure 4.2.

Between 8 and 13 wells were used to determine an overall estimate of the mean zone of inhibition for each parameter investigated.

It was observed that of the 6 test and control samples investigated, chlorhexidine acetate in absolute ethanol produced the largest uniform area of inhibition, this was closely followed by chlorhexidine in 70 % ethanol, (although the differences between the two were not found to be statistically significant, ( $0.2 > p > 0.1$ )), and then chlorhexidine in distilled

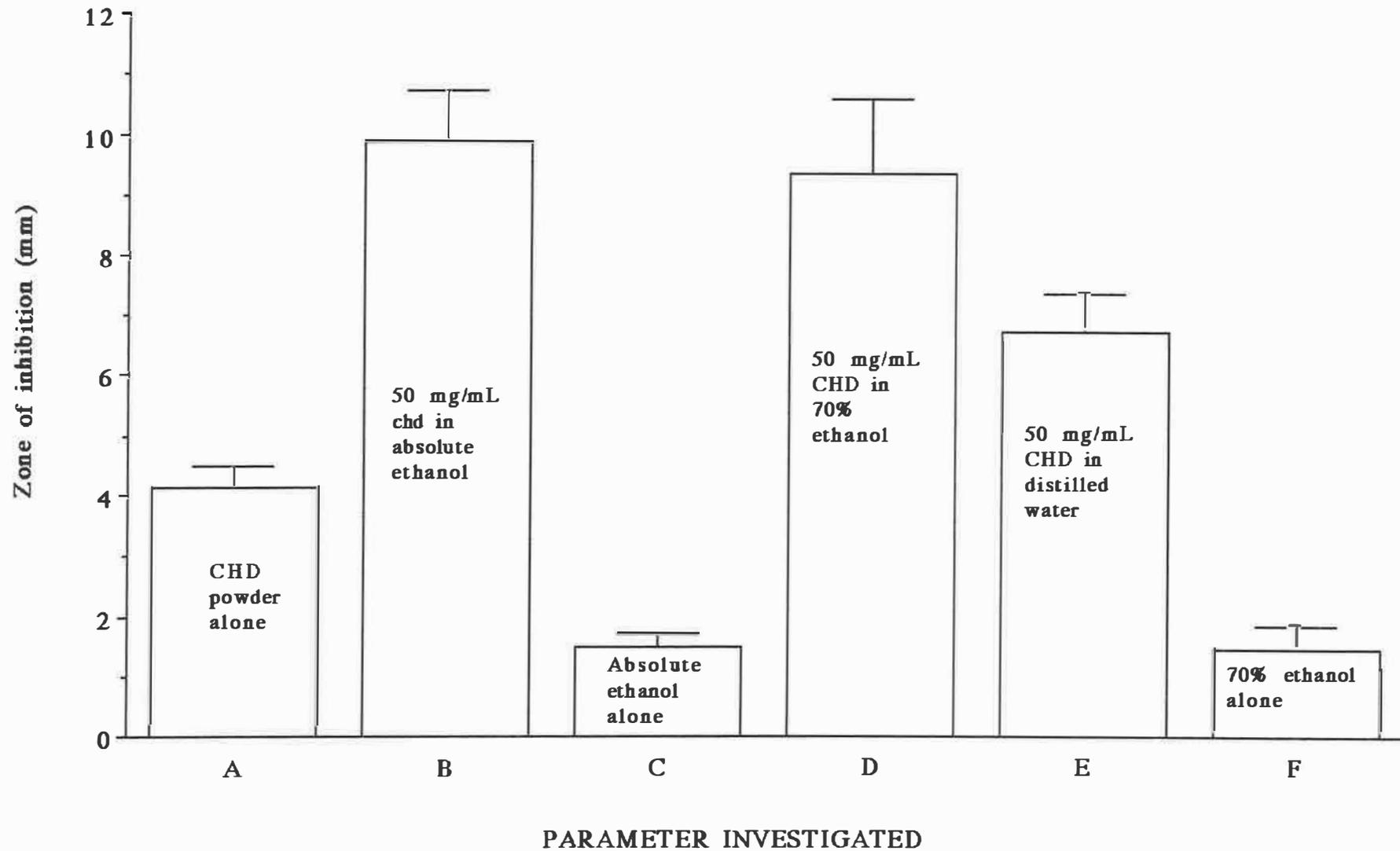


Figure 4.2. Zones of inhibition produced by various mixtures of chlorhexidine acetate and ethanol applied to wells in nutrient agar which had been inoculated with *E. coli*

water, which was significantly different from chlorhexidine in absolute and 70 % ethanol ( $0.001 > p$  in both cases). The area of growth inhibition produced by absolute ethanol alone was much less than that produced when used in conjunction with chlorhexidine. The area of inhibition produced with chlorhexidine powder alone, when looking at the most uniformly circular area of inhibition was between absolute ethanol alone and solvent plus chlorhexidine. (Figure 4.2). It appears, therefore, that chlorhexidine in the absolute ethanol has the greatest bactericidal activity, and is the logical choice of solution to be loaded into the hollow fibres for the purpose of investigating their bactericidal activity.

#### **4.4.7 Bactericidal activity of chlorhexidine acetate on *E. coli* after release from hollow nylon fibres**

A hollow nylon fibre (N, 0.5/0.63 mm) was cut into 4 cm lengths and treated as described in section 3.4.2. The fibres were then loaded with 140 mg mL<sup>-1</sup> chlorhexidine acetate in absolute ethanol and heat sealed. Appropriate vehicle controls were also set up. The hollow nylon fibres were then placed onto freshly poured nutrient agar plates. 500 mL of freshly autoclaved molten agar which had cooled to 45 - 50 °C, was inoculated with 1 mL of  $1 \times 10^9$  cfu mL<sup>-1</sup> suspension of *E. coli* and mixed to disperse the inoculum throughout the agar. Sufficient inoculated agar was then poured over the agar plates onto which the loaded hollow fibres had been placed to just completely cover the fibres, making sure that the fibres remained close to the centre.

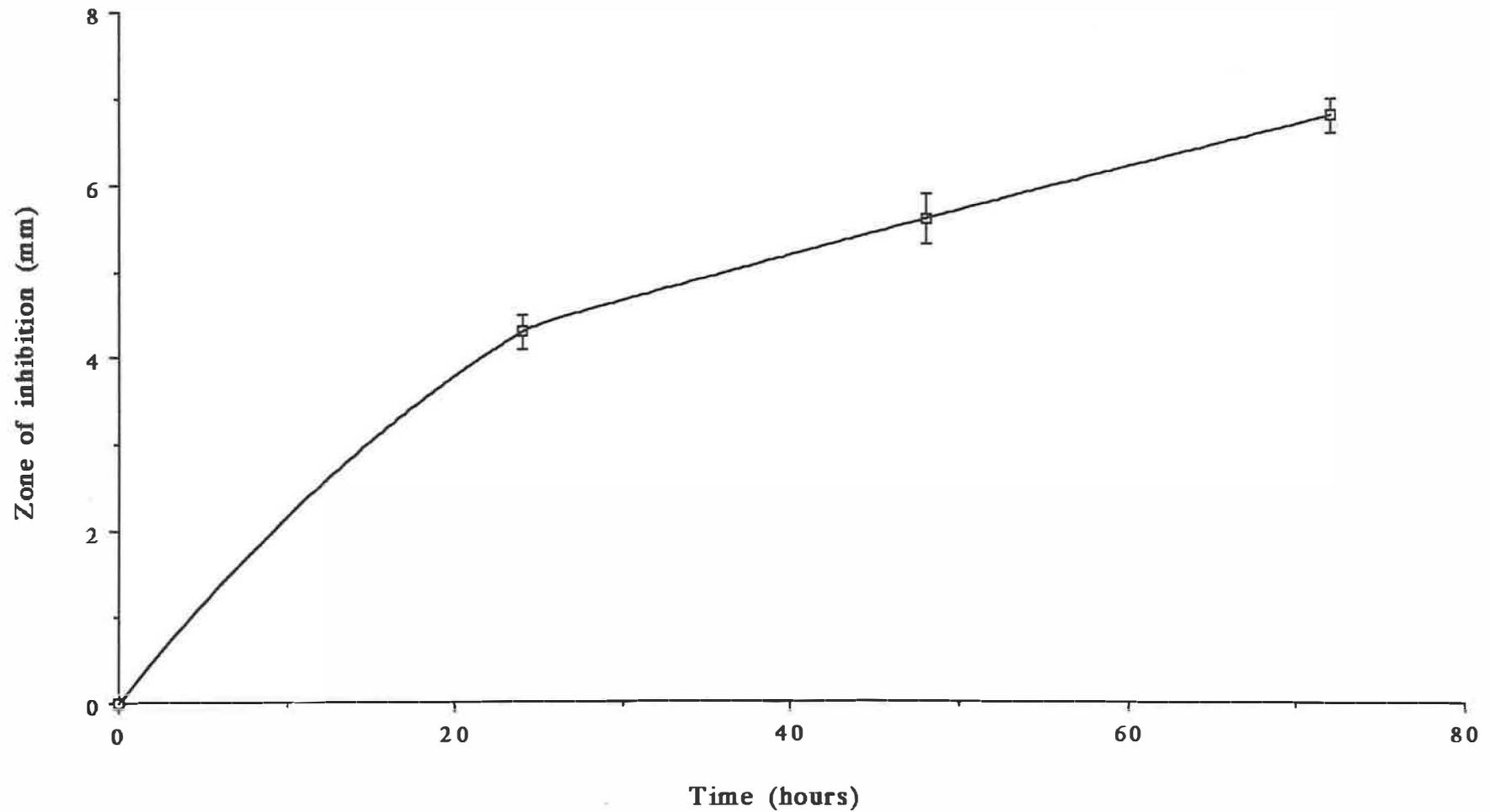


Figure 4.3 Plot showing increase in zone of inhibition with time produced by release of chlorhexidine from hollow fibres within agar inoculated with E. coli (mean  $\pm$  sem, n=3)

Immediately after the inoculated agar had solidified, the plates were placed into a refrigerator at 4°C to allow release of the chlorhexidine acetate from the fibres before allowing bacterial growth. Groups of plates were removed from the refrigerator after 0, 24, 48 and 72 hours and placed into an incubator at 37°C for 18-24 hours. After incubation the plates were removed and examined for zones of inhibition. The zone widths were measured with callipers. Figure 4.3 shows the increase of zone width with increasing pre-diffusion time. For each time point, n = 3.

#### **4.4.8 Chlorhexidine release into purified mucus**

##### **4.4.8.1 Purification of porcine gastric mucus**

Approximately 50 pig stomachs were obtained from a local abattoir on the day of slaughter, and kept on ice until being rinsed with water, to remove food debris from mucosal surfaces. Using a wooden spatula, the mucus was gently scraped from the surface of the stomachs, and put into a blender to which an equal volume of protease inhibiting saline, (NaCl - 11.7 g; sodium azide - 0.2 g; EDTA - 1.46 g; phenylmethylsulphonyl fluoride (PMSF) - 0.17 g, made up in one litre), was added. The crude mucus was homogenised in the blender for 30 seconds to 1 minute, this was then centrifuged at between 12,000 g and 17,000 g, for 1 hour at 4°C. This procedure was performed to separate the solubilised mucus from the food and cellular debris that formed the pellet. The supernatant liquid was filtered twice through glass wool, and each 50 mL of the resultant filtered solution was diluted to 180 mL with protease inhibiting saline.

Approximately 200 mL of the diluted, filtered solution was then added to an exclusion chromatography column, (30 cm in length and 9.5 cm in diameter), consisting of Sepharose CL4B. The sample was eluted with protease inhibiting saline at a rate of 300 mL day<sup>-1</sup>, at a temperature of 4°C. The eluate was monitored spectrophotometrically at 280 nm.

The excluded fraction which contained the glycoprotein was concentrated to between 50 mL and 100 mL, by use of an ultrafiltration cell equipped with 100,000 Dalton ultrafiltration plates. The resultant solution was transferred to a minitan ultrafiltration system and dialysed against water, at 4°C, with at least 3 changes of water over 36 hours, using dialysis tubing with a 12,000 Dalton cut-off.

After reduction of the volume of the purified mucus to between 50 and 100 mL, the pH was adjusted to 7.4 and the solution was further concentrated down to between 30 and 50 mL, again using the minitan ultrafiltration system, checking pH and adjusting accordingly. The resultant solution was then transferred to an Amicon model 402 ultrafiltration cell and concentrated to a gel containing approximately 8% w/v mucin, which was confirmed by dry weight determination.

#### **4.4.8.2 Use of absolute ethanol as vehicle**

3 cm lengths of silicone rubber tubing 1.5/5 mm were sterilised by wrapping in foil and placing into an oven at 180°C for 2 hours. An

overnight culture of *E. coli* was harvested as described in section 4.4.5. The pellet was then resuspended, in PBS to give a concentration of between  $1 \times 10^8$ -  $5 \times 10^8$  cfu mL<sup>-1</sup> (for *E. coli* calibration curve see section 4.4.5). A hollow nylon fibre (N, 0.5/0.63 mm) was cut into 4 cm lengths and treated as described in section 3.4.2. Before loading the fibres with chlorhexidine they were autoclaved for 15 minutes at 121°C.

The purified mucus (obtained as described in section 4.4.8.1), was inoculated with the suspension of *E. coli* giving a final concentration of mucus of 8% w/v and a final cfu mL<sup>-1</sup> of approximately  $1 \times 10^8$  cfu mL<sup>-1</sup>. The 3 cm lengths of silicone rubber tubing were then filled with inoculated mucus. Once the tubes were filled, the hollow nylon fibres were loaded with 140 mg mL<sup>-1</sup> chlorhexidine acetate in absolute ethanol, a vehicle control or left empty, were inserted into the mucus filled silicone rubber tubes. A series of control silicone rubber tubes were set up containing inoculated mucus only. The tubes were then placed into a humidity chamber (which consisted of a desiccator containing a saturated solution of ammonium chloride in water), to prevent evaporation of the mucus at 37°C. Silicone rubber tubes were removed at 1, 2, 4, 6, 12, 24, 48 and 72 hours after insertion of the hollow nylon fibres.

The tubes were removed from the humidity chamber at the appropriate times and the nylon hollow fibre and the mucus were washed out of the silicone rubber tubes with a 10 ml volume of lecithin-tween broth, which

<b>Time (hours)</b>	<b>chlorhexidine loaded fibre (cfu mL<sup>-1</sup> +/-SD)</b>	<b>absolute ethanol loaded fibre (cfu mL<sup>-1</sup> +/-SD)</b>	<b>inoculated mucus only (cfu mL<sup>-1</sup> +/-SD)</b>
<b>1</b>	5.70 +/-1.33 n=6	7.41 +/-0.591 n=6	4.32 +/-0.929 n=6
<b>2</b>	7.66 +/-2.86 n=6	7.98 +/-0.928 n=6	5.28 +/-0.485 n=6
<b>4</b>	5.95 +/-0.788 n=5	5.47 +/-0.721 n=6	6.03 +/-0.665 n=6
<b>6</b>	8.12 +/-1.95 n=6	6.13 +/-0.568 n=6	4.85 +/-0.481 n=6
<b>12</b>	3.44 +/-1.48 n=5	3.92 +/-0.595 n=5	4.03 +/-0.685 n=5
<b>24</b>	0.065 +/-0.0767 n=5	1.06 +/-0.0835 n=5	10.5 +/-4.96 n=5
<b>48</b>	0.166 +/-0.287 n=5	0.0643 +/-0.111 n=5	25.0 +/-8.19 n=5
<b>72</b>	0.163 +/-0.344 n=5	0.112 +/-0.131 n=3	11.0 +/-5.15 n=5

**Table 4.3. Colony forming units per mL (x 10<sup>7</sup>) of inoculated mucus remaining in the silicone rubber tubes.**

deactivated the bactericidal activity of chlorhexidine. This mixture was then vigorously aspirated ensuring the inoculated mucus was evenly dispersed within the 10 ml volume of lecithin-tween broth. Serial dilutions of this mixture were then made, and 0.2 mL aliquots were plated onto overdried nutrient agar plates in duplicate and incubated for 24 - 48 hours at 37°C after which the number of colonies were counted. The results are presented in table 4.3. From table 4.3, it can be seen that the number of bacteria decreased from 1 hour to 72 hours with silicone rubber tubes containing both ethanol loaded fibres and those containing chlorhexidine, the differences being statistically significant ( $0.001 > p$  in each case). However, from 1 hour to 72 hours with the silicone rubber tube containing inoculated mucus only, the number of bacteria increased and again the difference was statistically significant ( $0.02 > p > 0.01$ ). When the 72 hour time points are compared statistically between groups, it was shown that the differences between the chlorhexidine group and the inoculated mucus only group were significant ( $0.01 > p > 0.001$ ). The same was true when the inoculated mucus only group and the absolute ethanol control group were compared ( $0.01 > p > 0.001$ ). However, when the absolute ethanol control group and the chlorhexidine groups were compared, no statistically significant difference was shown ( $0.8 > p > 0.7$ ), which suggests that absolute ethanol alone appears to produce an effect upon bacterial growth which is quantitatively very similar to that produced by the chlorhexidine releasing fibre.

#### 4.4.8.3 Use of 50% ethanol vehicle

Section 4.4.8.2 has shown that chlorhexidine is unable to produce a bactericidal effect which is greater than that given by the absolute ethanol vehicle alone. Strong solutions of ethanol are known to be bactericidal and use of a more dilute solution as a vehicle may allow the bactericidal nature of chlorhexidine to be demonstrated. 50 % ethanol was chosen, since 140 mg of chlorhexidine was still soluble in only 1 mL of vehicle.

3 cm lengths of silicone rubber tubing, and an overnight culture of *E. coli* were prepared as described in section 4.4.8.2. Purified pig gastric mucus, (obtained as described in section 4.4.8.1), was inoculated with the washed culture and introduced into the silicone rubber tubing as described in section 4.4.8.2.

4 cm lengths of nylon hollow fibre were treated as described in section 3.4.2, and loaded with a solution of 140 mg mL<sup>-1</sup> chlorhexidine acetate in 50% ethanol, 50% ethanol only or left empty. The tubes were then heat sealed as described in section 3.4.2, and inserted into the silicone rubber tubes containing the inoculated mucus. The tubes were then placed into a humidity chamber and incubated as described in section 4.4.8.2.

At 0, 12, 24, 48 and 72 hours, sample and control tubes were removed from the humidity chamber and washed through with lecithin-tween broth as described in section 4.4.8.2, plated out and diluted as described in section 4.4.8.2.

The results are presented in table 4.4.

<b>Time (hrs)</b>	<b>chlorhexidine loaded fibre (cfu mL<sup>-1</sup> +/- SD)</b>	<b>50% ethanol loaded fibre (cfu mL<sup>-1</sup> +/- SD)</b>	<b>inoculated mucus only (cfu mL<sup>-1</sup> +/- SD)</b>	<b>unloaded fibre (cfu mL<sup>-1</sup> +/- SD)</b>
<b>0</b>	42.0 +/-26.3 n=11	42.9 +/-17.8 n=10	27.6 +/-9.95 n=5	47.4 +/-18.4 n=6
<b>12</b>	104 +/-32.4 n=5	86.2 +/-17.0 n=5	-	299 +/-135 n=5
<b>24</b>	513 +/-60.4 n=5	609 +/-187 n=5	-	6770 +/-4210 n=6
<b>48</b>	2120 +/-921 n=6	748 +/-3380 n=6	-	3830 +/-1580 n=6
<b>72</b>	22.0 +/-16.1 n=4	942 +/-363 n=5	450 +/-105 n=5	584 +/-84.0 n=6

**Table 4.4. Colony forming units per mL (x 10<sup>7</sup>) of inoculated mucus remaining in the silicone rubber tubes.**

At time 0 hour, when the value for chlorhexidine is compared statistically with that for the 50% ethanol control group, no significant difference is apparent ( $p > 0.9$ ). The same is also true when this value is compared against the  $\text{cfu mL}^{-1}$  for the unloaded hollow fibre and the inoculated mucus only control group ( $0.7 > p > 0.6$  and  $0.5 > p > 0.4$  respectively).

After 12 hours, there was still no statistically significant difference between the chlorhexidine group and the 50% ethanol control group ( $0.4 > p > 0.3$ ). However, when the chlorhexidine group is compared with the unloaded fibre group, the difference is statistically significant ( $0.05 > p > 0.01$ ), but the same is also true when the unloaded fibre group is compared against the 50% ethanol control group ( $0.01 > p > 0.001$ ).

After 24 hours, there is no statistically significant difference between the 50% ethanol control group and the chlorhexidine group ( $0.3 > p > 0.2$ ). Again, however, both the 50% ethanol control group and the chlorhexidine group are statistically different from the unloaded fibre ( $0.05 > p > 0.01$  and  $0.01 > p > 0.001$  respectively).

At 48 hours, when the  $\text{cfu mL}^{-1}$  for chlorhexidine is compared against the 50% ethanol control group and against the unloaded hollow fibre control group, the differences are significantly different in both cases ( $0.01 > p > 0.001$  and  $0.05 > p > 0.01$  respectively). However, when the actual values for the 50% ethanol control group and the chlorhexidine group are compared, it can be seen that that for the chlorhexidine group

is greater than that for the 50% ethanol group. When the unloaded hollow fibre is compared with the 50% ethanol control group, again the differences are significant, with the greater  $\text{cfu mL}^{-1}$  being given by the unloaded hollow fibre group.

After 72 hours, the chlorhexidine group is significantly different from the 50% ethanol control group, the unloaded hollow fibre group and the inoculated mucus only group ( $0.01 > p > 0.001$ ,  $0.0001 > p$  and  $0.0001 > p$  respectively). At this time point, the  $\text{cfu mL}^{-1}$  for the 50% ethanol group is not significantly different from that of the unloaded hollow fibre control group or the inoculated mucus only control group ( $0.6 > p > 0.5$  and  $0.5 > p > 0.4$  respectively). From table 4.4, the  $\text{cfu mL}^{-1}$  for chlorhexidine is less than those from the three control groups, suggesting that after this time the chlorhexidine is able to exert an action upon bacterial growth which is greater than any effect that may have been produced by any of the control groups.

When the  $\text{cfu mL}^{-1}$  of the control groups for time 0 hour are compared with the corresponding  $\text{cfu mL}^{-1}$  at 72 hours, in each case they are significantly different ( $0.0001 > p$  for all controls), where the values at time 0 hour are less than those at time 72 hours (see table 4.5). With the chlorhexidine groups, when the  $\text{cfu mL}^{-1}$  at time 0 hour is compared with that at time 72 hours, the values are not significantly different from one another ( $0.2 > p > 0.1$ )

#### **4.4.9 Release of chlorhexidine in 50% ethanol vehicle from a nylon hollow fibre into phosphate buffered saline**

3 cm lengths of silicone rubber tubing 1.5/5 mm were sterilised as described previously (section 4.4.8). An overnight culture of *E. coli* was harvested as described as described in section 4.4.5. The pellet was then resuspended, in PBS to give a concentration of between  $1 \times 10^8$  -  $5 \times 10^8$  cfu  $\text{mL}^{-1}$  (for *E. coli* calibration curve see section 4.4.5), this suspension was then introduced into the silicone rubber tube. A hollow nylon fibre (N, 0.5/0.63 mm) was cut into 4 cm lengths and treated as described in section 3.4.2. Before loading the fibres with chlorhexidine they were autoclaved for 15 minutes at  $121^\circ\text{C}$ , after which they were filled with  $140 \text{ mg mL}^{-1}$  chlorhexidine acetate in 50% ethanol, 50% ethanol only or left empty. The tubes were then sealed as described in section 3.4.2 and inserted into the silicone rubber tubes containing the inoculated PBS, which remained within the silicone rubber tubes due to surface tension. The tubes were then placed into a humidity chamber and incubated as described in section 4.4.8.2.

At 0, 2, 4, 6 and 24 hours, sample and control tubes were removed from the humidity chamber and the PBS and nylon hollow fibre were washed out of the silicone rubber tubes with a 10 ml volume of lecithin-tween broth, vigorously aspirated ensuring the inoculated PBS was evenly dispersed within the 10 ml volume of lecithin-tween broth as previously described (section 4.4.8.2). Serial dilutions of this mixture were then

made, and 0.2 mL aliquots were plated onto overdried nutrient agar plates in duplicate and incubated for 24 - 48 hours at 37°C after which the number of colonies were counted.

The results are presented in table 4.5.

<b>Time (hours)</b>	<b>chlorhexidine loaded fibre (cfu mL<sup>-1</sup>)</b>	<b>50 % ethanol loaded fibre (cfu mL<sup>-1</sup>)</b>	<b>inoculated PBS alone (cfu mL<sup>-1</sup>)</b>
0	9.35	9.42	10.3
2	7.73	6.77	8.68
4	4.61	6.41	7.96
6	1.74	6.59	7.18
24	<0.00005	0.0199	0.272

**Table 4.5. Colony forming units per mL (x 10<sup>7</sup>) of inoculated PBS remaining in the silicone rubber tubes. (n = 6 in each case).**

At time 0 hour, when the value for chlorhexidine is compared statistically with that for the 50% ethanol control group, no significant difference is apparent ( 0.9 > p > 0.8). The same is also true when this value is compared against the cfu mL<sup>-1</sup> for the inoculated PBS only control group (0.2 > p > 0.1).

After 2 hours, there is no statistically significant difference between the chlorhexidine group and the 50% ethanol control group (0.4 > p >0.3). or

the chlorhexidine group the inoculated PBS only group, ( $0.4 > p > 0.3$ ).

After 4 hours, there is no statistically significant difference between the 50% ethanol control group and the chlorhexidine group ( $0.3 > p > 0.2$ ). However, both the 50% ethanol control group and the chlorhexidine group are statistically different from the inoculated PBS only group ( $0.01 > p > 0.001$  and  $0.05 > p > 0.01$  respectively).

After 6 hours, when the  $\text{cfu mL}^{-1}$  for chlorhexidine is compared against the 50% ethanol control group and against the inoculated PBS only control group, the differences are significant in both cases ( $0.001 > p$  in each case). When the 50% ethanol control group and the inoculated PBS only group are compared, the differences are not statistically significant, ( $0.5 > p > 0.4$ ).

After 24 hours, no organisms were recovered from the PBS obtained from the silicone rubber tubes which had the hollow fibre loaded with chlorhexidine, whereas many organisms were recovered from the silicone rubber tubes into which inoculated PBS alone was placed, and from those silicone rubber tubes into which the 50% ethanol loaded hollow fibres were placed, ( $2.72 \times 10^6$  and  $1.99 \times 10^5$  respectively). Statistically, the differences between the vehicle control and the chlorhexidine loaded fibre and the inoculated PBS control and the chlorhexidine loaded fibre were found to be significant ( $0.0001 > p$  in both cases). When compared statistically, the differences between the two control groups were also

found to be significant ( $0.001 > p$ ).

The results indicate that the release of chlorhexidine acetate from the hollow fibres produces a significant effect on the growth rate of *E. coli* compared with the controls. This suggests that such a fibre attached to a device that is to be inserted into the uterus transcervically, may be able to reduce the numbers of bacteria introduced into the uterus associated with such a procedure. Investigation of this concept *in vivo* needs to be performed to establish the usefulness of the device.

## 4.5 Discussion

From section 4.4.4 it was established that the highest minimum inhibitory concentration (MIC) of chlorhexidine needed to inhibit completely growth of the hardest commensals found in the guinea-pig vagina was  $100 \mu\text{g mL}^{-1}$  (against *Proteus mirabilis*). In chapter 3, release of chlorhexidine was demonstrated from a nylon hollow fibre (N,0.5/0.63), that would reach a concentration of approximately  $45 \mu\text{g mL}^{-1}$  in the bathing fluid after only 7 days. This was dependent on the initial concentration of chlorhexidine loaded into the fibre, the geometry of the fibre, the vehicle which was used and the volume of bathing fluid. Although this figure is somewhat less than that needed to completely inhibit growth of *P. mirabilis* when the actual size of the guinea-pig lower genital tract and upper genital tract are considered, which together will have an approximate volume of distribution of only 5 mL, then the effective concentration of chlorhexidine released will be approximately  $270 \mu\text{g mL}^{-1}$ . This concentration would be sufficient to inhibit the growth of any commensal bacteria associated with the guinea-pig genital tract.

The most effective vehicle and chlorhexidine combination was determined as described in section 4.4.6. It was found that a combination of chlorhexidine with 70 % ethanol or absolute ethanol provided the greatest antimicrobial action (figure 4.2). It was decided to use chlorhexidine in absolute ethanol for the remainder of experiments in this section since chlorhexidine was marginally more soluble in absolute ethanol than in 70

% ethanol.

To demonstrate that the hollow fibre could release chlorhexidine in sufficient quantities to inhibit growth, some chlorhexidine loaded fibres were embedded in nutrient agar inoculated with *Escherichia coli* (NCIB 8196). This microorganism is often found associated with the guinea-pig vagina and was demonstrated to exist in the vaginae of animals used in this study. The zones of inhibition produced were directly proportional to the length of time allowed for the chlorhexidine to diffuse through the hollow fibre wall at 4°C. Once the plates were incubated at 37°C the *E. coli* was able to grow (figure 4.3). The microorganism was able to grow as close to the fibre as the concentration of chlorhexidine in the agar would allow. Once it had grown however, further increases in the chlorhexidine concentration beyond where the zone of inhibition was initially seen could not be determined since the bacteria remained, even where the chlorhexidine concentration reached levels high enough to inhibit the growth of the *E. coli*

The release of chlorhexidine from the hollow fibres into the silicone rubber tubes loaded with phosphate buffered saline (PBS) (pH 7.4), which had been inoculated with *E. coli* caused a significant reduction in the numbers of viable bacterial cells in the sample tubes compared with the control tubes in only 6 hours ( $0.001 > p$ ) (table 4.5). After 24 hours, no viable cells were found in the PBS of the silicone rubber tubes containing the chlorhexidine loaded nylon hollow fibres whereas significant numbers of viable cells were obtained from the PBS of the 2 controls ( $0.0001 > p$

in both cases) (table 4.5).

Purified porcine gastric mucus has been used previously as a model of cervical mucus (Bilbruck *et al.* 1991). It was also used in this study to produce an environment that was thought to mimic that of the guinea-pig vagina. To give a closer approximation of the events that may occur when nylon fibres loaded with chlorhexidine are inserted into the guinea-pig genital tract, silicone rubber tubes were loaded with purified porcine gastric mucus, which has been reported to have a similar structure to cervical mucus (Meyer, 1977). Using hollow nylon fibres loaded with chlorhexidine in absolute ethanol and the hollow fibres containing absolute ethanol only, the numbers of bacteria obtained were significantly lower than the number of bacterial cells obtained from the inoculated mucus only control tubes ( $0.01 > p > 0.001$ ). However, when the chlorhexidine and the absolute ethanol only bacterial counts were compared, there was no significant difference between the two ( $0.8 > p > 0.7$ ). It would appear, therefore, that the antibacterial action of absolute ethanol is at least as great as that of the chlorhexidine. However, it must be considered that absolute ethanol will remain in the hollow fibre for only 36 - 48 hours before diffusing out completely. The effect of chlorhexidine will be exerted for as much as 10 days longer than this. Ideally the release of chlorhexidine into mucus would have been performed over a longer period of time, but after only 72 hours, even when using the humidity chamber, at 37°C the mucus had become greatly dehydrated, which may explain the extremely high bacterial counts obtained in table 4.4. To determine if the chlorhexidine could exert a

substantial bactericidal effect in the presence of mucus, a more dilute concentration of ethanol was used so that the vehicle itself did not swamp any activity. 50 % ethanol was used in replacement of the absolute ethanol vehicle.

In the case of those silicone rubber tubes loaded with inoculated mucus, no significant difference between the 2 controls and the chlorhexidine containing silicone rubber tube was observed until after 72 hours (table 4.5). Even after this length of time however, the numbers of bacterial cells that were viable in the mucus was not significantly different from the number of viable cells determined at time 0 hour. With the 2 controls, however, the number of viable cells obtained after 72 hours was greater than that determined at time 0 hour. This suggests that although the chlorhexidine is not killing the bacterial cells within the mucus, it is at least exerting a bacteriostatic action when compared with the 2 controls. Where no antibacterial action was being exerted, it is obvious that *E. coli* has proliferated. This suggests that the mucus environment is conducive to replication which leads to the conclusion that *E. coli* must be using the mucus as a growth medium. The mucus gel itself is made up of very large and structurally complex glycoproteins (Carlstedt and Sheehan, 1989) which must provide sufficient nutrients for the bacteria to proliferate for the period of study.

The release of chlorhexidine from the nylon hollow fibre is clearly bactericidal when the chlorhexidine is released into the inoculated PBS, reducing the viable count of microorganisms to zero after 24 hours. This

is not the case when chlorhexidine is released into inoculated mucus. In fact, the microorganisms appear to proliferate until 72 hours after the chlorhexidine loaded fibre is inserted into the inoculated mucus, when the bacterial numbers decrease, but only to approximately the same value as was inoculated into the mucus at time 0 hours. It is possible that the surviving microorganisms were those only found at the periphery of the silicone tube. No attempt was made to determine whether the killing effect was reduced with increasing distance from the hollow fibre. If the bacteria close to the thread were killed, then there is still a case for using the system to kill biofilm bacteria. If the chlorhexidine loaded fibres placed within the silicone tubes had been placed at 4°C for 24 - 72 hours prior to allowing growth of the *E. coli* diffusion of the chlorhexidine may have occurred sufficiently to enhance the killing effect of these fibres.

Chantler *et al.* (1989), have reported on the mucospissic effect of chlorhexidine, and compared the diffusion of chlorhexidine and glucose, which are molecules of comparable size, through mucus. Under the same conditions, 74 % less chlorhexidine entered the mucus than glucose, and the distance of the diffusion front observed with chlorhexidine was only 60 % that seen with the glucose after 1 hour of surface contact. Bilbruck *et al.* (1991), have shown that the bactericidal activity of chlorhexidine impregnated monofilaments, which are very effective against bacteria in PBS, is greatly reduced when these monofilaments are inserted into samples of inoculated mucus.

Mucus is predominantly composed of water (95%), the majority of which is bound within a glycoprotein matrix. It is widely accepted that it is this mucus glycoprotein which determines the bulk properties of the mucus gel (Pain, 1980). The glycoprotein component consists of macromolecules, rich in carbohydrates, called mucins, which exist as a series of monofilaments held together by disulphide bridges. The oligosaccharides, covalently attached to the peptide core of mucin, contain ionised anions e.g. sulphate and N-acetylneuraminate. These groups render mucin polyanionic in nature, and make it likely that the greatest interaction of mucus will be with polycations (Sharman, 1987). Chlorhexidine is characterised by two pKa values of 2.2 and 10.3 (Hugo and Longworth, 1964), and so at pH 6 the chlorhexidine molecule will be positively charged by virtue of its ammonium residues. Plaut *et al* (1980), considered that at this pH chlorhexidine existed in aqueous solution as the di-cation (figure 4.4). It is therefore anticipated that there would be a strong electrostatic attraction binding the mucus and the chlorhexidine. It is clear, therefore, that the bactericidal activity of chlorhexidine is reduced in the presence of mucus and it is likely that both the mucospissic action of the chlorhexidine and the electrostatic attraction of chlorhexidine to the mucus, contribute to this reduction in the bactericidal activity of chlorhexidine. However the diffusion of chlorhexidine, although retarded by the presence of mucus would be expected to reduce effectively the numbers of bacteria adjacent to the hollow fibre itself. Indeed the chances of successfully killing those bacteria firmly adhered to the substrate by means of an extracellular glycocalyx, may be improved by the use of chlorhexidine as the antibacterial agent, as Costerton (1984)

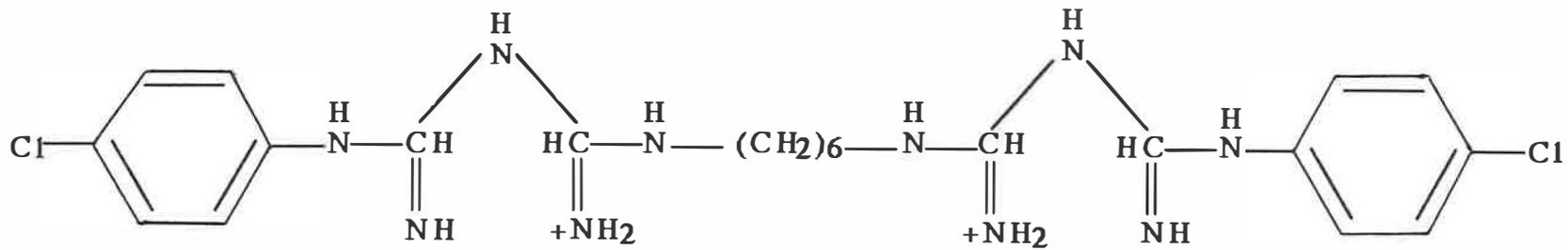


Figure 4.4. Structural formula of chlorhexidine, showing the positively charged ammonium residues which render the molecule cationic in nature

suggested that use of positively charged antimicrobial agents, which can penetrate the anionic glycocalyx, might be a promising approach to eradicate those infections associated with indwelling devices.

**5 TRANSCERVICAL INSERTION  
OF CHLORHEXIDINE  
ACETATE LOADED FIBRES  
INTO GUINEA-PIGS**

## 5.1 Introduction

As mentioned previously (1.6.3), it has been shown that the uterus is normally sterile. In contrast, the cervix and vagina support a dense polymicrobial population. Insertion of devices transcervically into the uterus will therefore introduce microorganisms which may become strongly adherent to the inserted device. These bacteria may secrete protective polysaccharide substances forming a glycocalyx, making systemic treatment of this uterine contamination very difficult. If the substrate itself were to release an antimicrobial agent directly into the biofilm, the chances of killing microorganisms growing there would be greatly increased.

The release of chlorhexidine acetate from hollow fibres has been shown to be sufficient to reach concentrations which are greater than the minimum inhibitory concentrations of any of the organisms isolated from the lower genital tract of the female guinea-pig. It has also been shown that chlorhexidine released from the nylon hollow fibre is bactericidal, when that nylon hollow fibre has been inserted into a tube containing *E. coli* inoculated into PBS. When these chlorhexidine loaded hollow nylon fibres are inserted into tubes containing purified mucus inoculated with *E. coli*, although there is substantial interaction of chlorhexidine with mucus there is a significant difference in the numbers of bacteria obtained from test and control groups of mucus containing tubes.

For the purpose of determining the effect of these nylon hollow fibres on

a bacterial population ~~in vivo~~ it was necessary to insert drug loaded devices into guinea-pig uteri. This would also provide information on the ability of the biocide released from the device to kill biofilm bacteria close to the fibre surface.

## 5.2 Materials

### Media

Nutrient Broth (C. M. 1) and Nutrient Agar (C. M. 3) as section 2.2.

### Chemicals

Hypnorm injection as section 2.2.

Valium injection as section 2.2.

Nylon hollow fibres as section 3.2.

K-Y lubricating jelly as section 2.3.

### Sutures

Nylon (Ethilon) and silk (Mersilk) sutures as section 2.2.

### Tubing

Polyethylene tubing used in the manufacture of the monofilament inserting device as section 2.2.

Glass capillary tubes as section 2.2.

### **5.3 Apparatus**

The modified Kilian nasal speculum as section 2.2.

Optical density/absorbance as section 2.3.

Centrifugation as section 2.3.

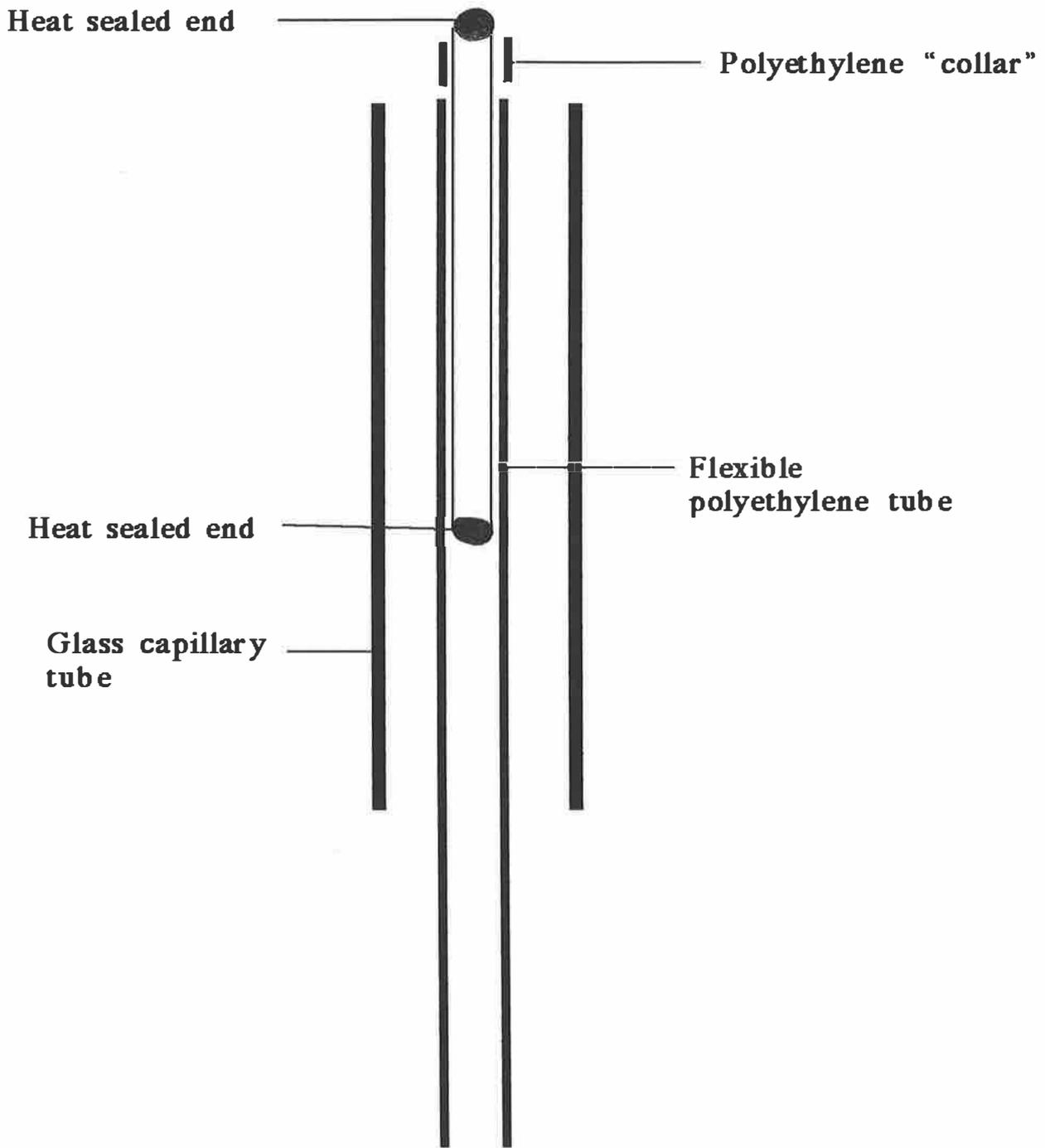
## **5.4 Methods**

### **5.4.1 Transcervical insertion of chlorhexidine loaded fibres into guinea-pigs**

Female guinea-pigs which had been obtained and housed as described in section 2.4.2, had hollow nylon fibres with collars, (to aid with removal from the device when inserted into the uterine horn), (figure 5.1), inserted transcervically and secured as described in section 2.4.3. The hollow nylon fibres were loaded with  $140 \text{ mg mL}^{-1}$  of chlorhexidine acetate in 50% ethanol. Controls which were performed were empty hollow nylon fibres, hollow nylon fibres loaded with 50% ethanol only and sham controls in which the device was inserted, and stitches made in the uterine wall without securing the hollow nylon fibre. The guinea-pigs were killed either 1 day, 5 days or 10 days after the devices were inserted in the manner described in section 2.4.3, and the uteri and the hollow nylon fibres removed and treated as described in section 2.4.4. The group size was 8 in each case unless otherwise indicated.

### **5.4.2 Statistical analysis of data**

The uterine microflora results are presented as the colony forming units per mL of original cell suspension and are compared using the Mann-Whitney U-test.



**Figure 5.1 Representation of device used for insertion of hollow fibres into the guinea-pig uterus**

## 5.5 Results

After attachment of the nylon hollow fibre to the side of the uterine wall, or after performing the sham control, the results after 1 day are given below.

cfu mL <sup>-1</sup>	Sham control	Ethanol loaded fibre	Empty fibre	Chlorhexidine loaded fibre
< 10	4	3	3	4
10 - 99	2	1	2	3
99 - 999	1	2	2	1
> 1000	1	2	1	0

**Table 5.1** Number of animals exhibiting the colony forming units per mL indicated after insertion of the test and control hollow fibres after 1 day

After 5 days, the colony forming units for each of the control and the test fibres after insertion into the guinea-pigs were as listed below.

cfu mL <sup>-1</sup>	Sham control	Ethanol loaded fibre	Empty fibre	Chlorhexidine loaded fibre
< 10	5	3	5	5
10 - 99	3	5	0	2
100 - 1000	0	0	0	1
> 1000	0	0	3	0

**Table 5.2** Number of animals exhibiting the colony forming units per mL indicated after insertion of the test and control hollow fibres after 5 days

The colony forming units 10 days after insertion of the control devices or the test device were as listed below.

cfu mL-1	Sham control	Ethanol loaded fibre	Empty fibre	Chlorhexidine loaded fibre
< 10	4	2	1	3
10 - 99	4	6	1	2
99 - 1000	0	0	1	1
>1000	0	0	5	0

**Table 5.3 Number of animals exhibiting the colony forming units per mL indicated after insertion of the test and control hollow fibres after 10 days**

When the chlorhexidine results are compared with the sham vehicle control results from the corresponding day using the Mann - Whitney U test, there are no significant differences ( $p > 0.05$  in each case), however, when compared against the group of animals in which an empty hollow fibre had been secured the results were significantly different ( $p < 0.05$ ).

The results for the contralateral uterine horns of the animals from the various time points are given in table 5.6. The results indicate that the contralateral horns are more often sterile even after insertion of the hollow fibres.

cfu mL <sup>-1</sup>	Sham control	Ethanol loaded fibre	Empty fibre	Chlorhexidine loaded fibre
1 Day	√1 x7	√0 x8	√3 x5	√2 x6
5 Days	√2 x6	√0 x8	√0 x8	√1 x7
10 Days	√0 x8	√2 x6	√0 x8	√0 x6

**Table 5.4. Numbers of guinea-pigs showing the presence (√) or absence (x) of bacterial growth in the contralateral uterine horns of the test animals.**

## 5.6 Discussion

The choice of guinea-pig for use as a model of the human female genital tract was made since the guinea-pig, unlike the rat which possesses two cervixes, has only one, and unlike rabbits which are spontaneous ovulators, the guinea-pig has a definite oestrus cycle of approximately 18 - 21 days. As such, the environments of the guinea-pig vagina, cervix and uterine horns will undergo a full range of histological and hormonal changes in one full cycle, as is observed in the human female.

24 hours after insertion of the device a range of contamination was observed (table 5.1). Since this is such a short time after insertion of the device this range of contamination must be a reflection of the numbers of microorganisms present in the vaginae of the guinea-pigs suggesting that there is much diversity with respect to the microflora found in one guinea-pig vagina compared to another (see section 4.4.2). No significant differences with respect to the degree of contamination between groups of guinea-pigs treated differently were noted after only 24 hours.

5 days after insertion the amount of contamination associated with the sham controls had reduced, as it had with the animals in which nylon hollow fibres had been secured. The degree of contamination observed with the chlorhexidine animals was marginally less than that with the other controls, although this difference was again not significant.

The influx of neutrophils and mononuclear cells into the endometrial

cavity during the first week after insertion of a foreign body into the uterus has been documented by Moyer and Mishell (1971), and may explain this temporary decrease in the number of microorganisms isolated from the uterus of the guinea-pigs after insertion of the hollow nylon fibres and the sham control.

10 days after insertion, the contamination of the sham control animals had marginally increased as it had with both the ethanol control animals and the unloaded hollow fibre animals. However, that for the chlorhexidine group had marginally decreased. Only when the 10 day results for chlorhexidine were compared with those of the empty hollow fibre were any differences shown to be significant.

Buret *et al*(1991) implanted silastic material colonised with *Pseudomonas aeruginosa* subdermally into adult rabbits and noted that from the time of implantation until 4 days after, there was a gradual decrease in the number of viable microorganisms associated with the device. From then on for the duration of the experiment when implant material was removed at 8, 28 and 42 days, a gradual increase in the number of bacteria isolated was observed. This closely parallels the findings in this study, where at 1 day post-insertion most animals showed a greater degree of contamination than at 5 days, and yet at 10 days the level of contamination had increased in most cases.

The initial decrease in bacterial contamination may be explained in terms of the leucocyte infiltration as described by Moyer and Mishell (1971),

thereafter, the protection of the biofilm on the hollow fibre in which the bacteria are embedded must have developed to a stage where the bacteria are able to multiply within it increasing the bacterial count found in the uterus. Buret *et al*(1991) observed that the majority of the biofilm mass associated with the silastic material was host generated. That is to say after the device was inserted, a foreign body reaction was initiated causing an influx of host inflammatory cells which were attracted to the foreign body and became trapped within a mesh of fibrin which would further protect against attack from other inflammatory cells and other host defence mechanisms.

This study investigated release of chlorhexidine from hollow nylon fibres over a period of 10 days. A similar device developed by Bilbruck *et al* (1991) consisted of polyHEMA coated monofilament threads which had been impregnated with chlorhexidine where most of the chlorhexidine was released in the first hour. When inserted into guinea-pigs these impregnated monofilaments showed significantly fewer microorganisms in the uterine horn compared with unimpregnated monofilaments after only 24 hours. This suggests that the initial high release of chlorhexidine with the polyHEMA coated device is more efficient at reducing bacterial numbers in the uterus than a slower more sustained release over 10 days. This difference may arise by the "dumping" effect of a high concentration of chlorhexidine exerting a greater bactericidal effect than the slower release from a nylon hollow fibre where the chlorhexidine may be washed away during the course of release before sufficient amounts of chlorhexidine accumulate to bactericidal concentrations. Also, a slower

release may cause an increase in the viscosity of the mucus, thereby preventing further release at the same rate, such that release becomes so slow that local concentrations of chlorhexidine are simply too low to be bactericidal.

Those animals on which sham control experiments were performed showed a similar reduction in bacterial numbers by day 5 and a marginal increase by day 10. These fluctuations were not significant, however. In the absence of a nylon hollow fibre it would be expected that after 10 days very few microorganisms would be found as no point of attachment for development of a protecting biofilm was available. In this case, bacteria present may have been residual from the day the cervix was breached by the device or organisms that were introduced at this time may have become attached to the suture material which was used to attach the "phantom" hollow fibre to the uterine wall. Malhi (1991) who performed similar sham control experiments with 3 different monofilaments noted that in each case 10 days after the procedure, uteri were again sterile.

Contamination of the contralateral uterine horn (with respect to the hollow fibre) was minimal. It would appear that the majority of microorganisms introduced into the guinea-pig uterus on insertion of the device remain associated with it after insertion. Any bacteria disseminating from the device during the first week after insertion may be destroyed by the influx of neutrophils and mononuclear cells, (Moyer and Mishell, 1971). Dissemination after the first week is unlikely as contamination of the contralateral horn after 10 days is minimal.

**6 STEROID RELEASE FROM  
HOLLOW NYLON FIBRES IN  
VITRO**

## 6.1 Introduction

Associated with the menopause and the climacteric there are a number of uncomfortable symptoms which are detailed in section 1.1.2.

Women in the climacteric period benefit from oestrogen treatment for subjective complaints such as vasomotor symptoms (Tacchi, 1960, 1976). This treatment also appears to protect against osteoporosis and cardiovascular disease (Lindsay *et al* 1984; Barrett-Connor and Bush, 1991). To protect the endometrium against hyperplasia and the possible development of malignancy, a progestogen supplement is believed to be effective (Persson *et al* 1989), although a cyclical regimen of progestogen leads to resumption of bleeding patterns in most women (Whitehead, 1978). Since most women prefer not to bleed during hormone replacement, discontinuation of therapy because of the bleeding disturbances often occurs (Hahn, 1989).

Several routes of administering hormone replacement therapy (HRT) are available. Oral administration is the most commonly used due to its simplicity, low cost and familiarity to the patient. However, because of the variable absorption and hepatic metabolism during the first pass enterohepatic recirculation, there is a wide variation in bioavailability and patient compliance cannot be guaranteed. The use of implants does ensure patient compliance, although reports of 'tachyphylaxis' have been made to the repeated administration of oestrogen by implant (Gangar *et al* 1989). The delivery of both oestrogen and progestogen by transdermal

administration is also available although difficulties have been noted with skin irritation, allergy to the adhesive, compliance not being guaranteed and problems with adhesion (Read and Sturdee, 1993; Siddle and Knight, 1991).

It is possible to administer progestogens directly to the uterine cavity (Nilsson and Luukkainen, 1977), and the levonorgestrel releasing IUCD, (which releases levonorgestrel from a polydimethyl siloxane reservoir), was found to cause a reduction in menstrual blood flow as well as endometrial suppression (Nilsson, 1977). This device was also effective in the treatment of menorrhagia (Andersson and Rybo, 1990). Andersson and co-workers (1992), noted that such a device when inserted into perimenopausal women prevented endometrial proliferation and reduced uterine bleeding when used in combination with orally administered oestradiol. When this treatment was compared with conventional cyclic oral administration of oestradiol for 21 days, combined with levonorgestrel for the last 10 days, the women were found to bleed regularly. In both groups subjective symptoms such as hot flushes, night sweats, irritability and sleep disturbances were equally diminished.

The use of a hollow fibre loaded with a progestogen inserted into the uterus may prove helpful in treatment of symptoms of the climacteric when used in conjunction with an orally administered oestrogen. The actual size of the hollow fibre would be conducive to easy intrauterine insertion when it is considered that the opening of perimenopausal and postmenopausal cervix is reduced in size. This study involved an

investigation into the release characteristics of the progestogens levonorgestrel and progesterone from the hollow fibre system.

## 6.2 Materials

### Chemicals

Progesterone and levonorgestrel were obtained from The Sigma Chemical Company, Poole, Dorset.

Octanol and Decon (for cleaning of glassware) were supplied by BDH Chemicals Ltd., Poole, Dorset, U. K.

Absolute ethanol as section 3.2.

Sesame oil as section 3.2.

### Tubing

As section 3.2.

### **6.3 Apparatus**

Absorbance measurements - As section 2.3.

Parafilm as section 3.3.

## **6.4 Methods**

### **6.4.1 Construction of a progesterone calibration plot**

Determination of progesterone concentration was made using a Perkin-Elmer Lambda 2 spectrophotometer. To determine the wavelength of maximum absorbance a wavelength scan of a dilute solution of progesterone in absolute ethanol, ( $2 \mu\text{g mL}^{-1}$ ), was performed from 190 nm to 350 nm. Only one maximum absorbance peak was shown, at 248 nm. This was therefore used in all subsequent experiments. (See appendix).

A calibration plot for progesterone was constructed by measuring the absorbances of standard solutions of progesterone at 248 nm with absolute ethanol as the blank. Use of this plot, (figure 6.1), was made to establish the concentration of progesterone in the bathing fluid after release from the hollow fibres.

### **6.4.2 Release of progesterone from a nylon hollow fibre**

30 mL glass bottles were treated as described in section 3.4.2, and filled with 25 mL of distilled water. Hollow fibres, (N,0.50/0.63; N,0.25/0.75), were cut to lengths of 9 cm, and treated as described previously in section 3.4.2. The hollow fibres were then loaded to a length of 6 cm with a solution of  $10 \text{ mg mL}^{-1}$  progesterone in absolute ethanol. Control hollow

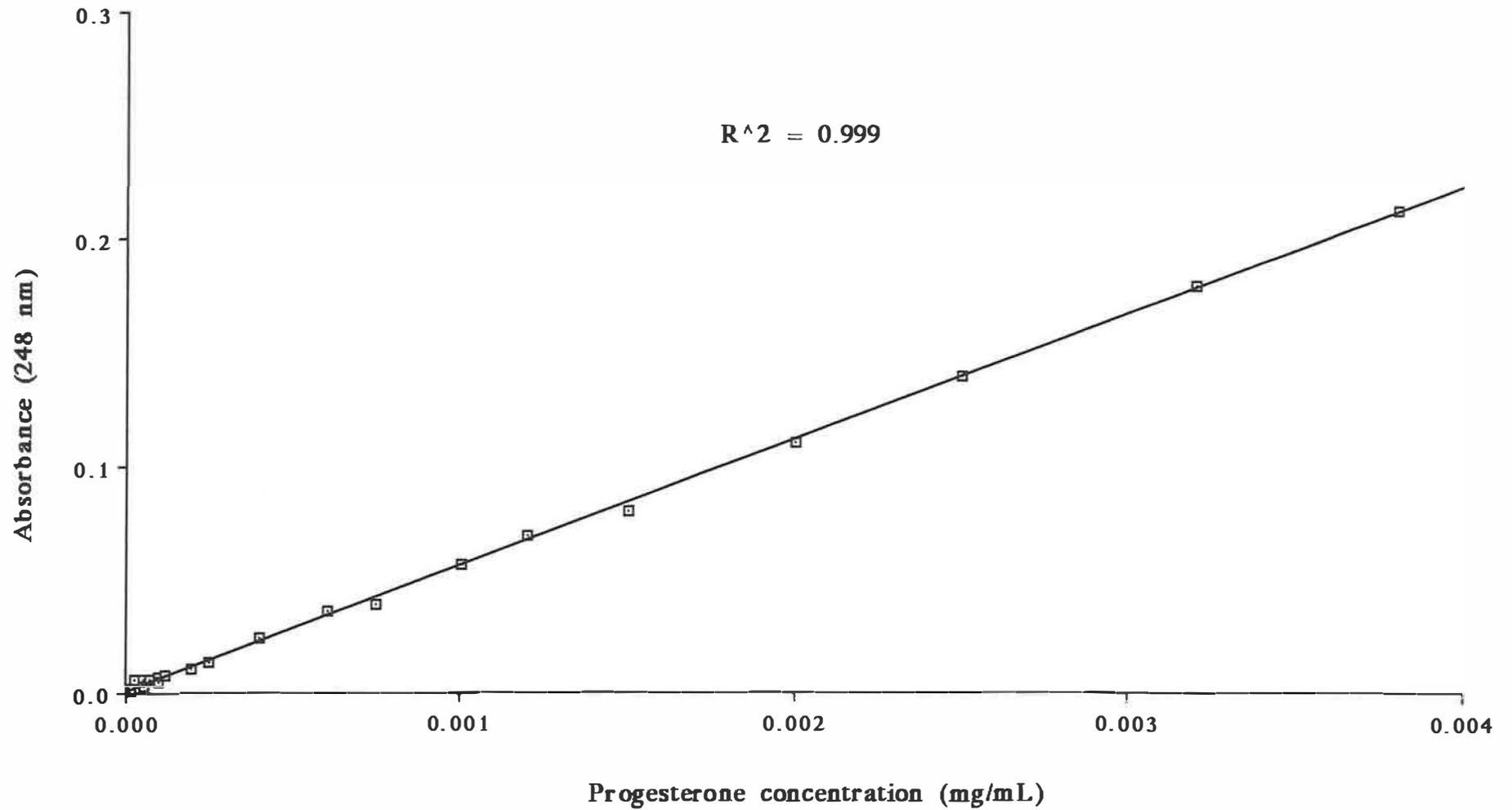


Figure 6.1. Calibration plot of concentration of progesterone against absorbance

fibres which had been treated identically to the test fibres were filled with absolute ethanol only, and sealed, or were left empty and placed into the 25 mL of distilled water. Leakage from the ends of the hollow fibres was prevented using Parafilm as described in section 3.4.2. Parafilm was also used to prevent evaporation when the bottles were placed into a thermostatically controlled environment at  $37 \pm 2^\circ\text{C}$  (see section 3.4.2). 2 mL samples were removed from the bottles daily for the first 3 days after which samples were removed at progressively longer intervals and measurements made by UV spectrophotometry using distilled water as the reference, at 248 nm. The absorbances obtained were then converted to concentrations by use of a calibration plot (figure 6.1). After measurements were taken, samples were returned to the bottles, and the tops of the bottles resealed with Parafilm. In each case  $n = 6$ , unless otherwise stated.

The results for the release of progesterone from the two hollow nylon fibres are presented in figures 6.2 and 6.3.

The amount of progesterone loaded into N,0.50/0.63 was 0.1178 mg. The initial rate of release of progesterone from this hollow fibre was  $27.75 \times 10^{-3} \text{ mg day}^{-1}$ . The maximum concentration of progesterone obtained by release from this fibre was  $3.41 \times 10^{-3} \pm 1.80 \times 10^{-4} \text{ mg mL}^{-1}$ . This concentration corresponds to a total release of 0.08513 mg, i.e. 72.3 %.

The hollow fibre with the smaller bore, (N,0.25/0.75), was loaded with

0.029 mg of progesterone. The initial rate of release of progesterone from this hollow fibre was  $12.5 \times 10^{-4} \text{ mg day}^{-1}$ . The maximum concentration of progesterone obtained by release from this fibre was  $8.30 \times 10^{-4} \pm 1.65 \times 10^{-5} \text{ mg mL}^{-1}$ . This concentration corresponds to a total release of approximately 0.021 mg, i.e. 72.4 %.

In the case of release of progesterone from N,0.50/0.63, approximately 30 % of the progesterone remained in the hollow nylon fibre reservoir, corresponding to 0.033 mg. With the narrow bore fibre, (N,0.25/0.75), again 30 % of the progesterone remains within the nylon hollow fibre reservoir.

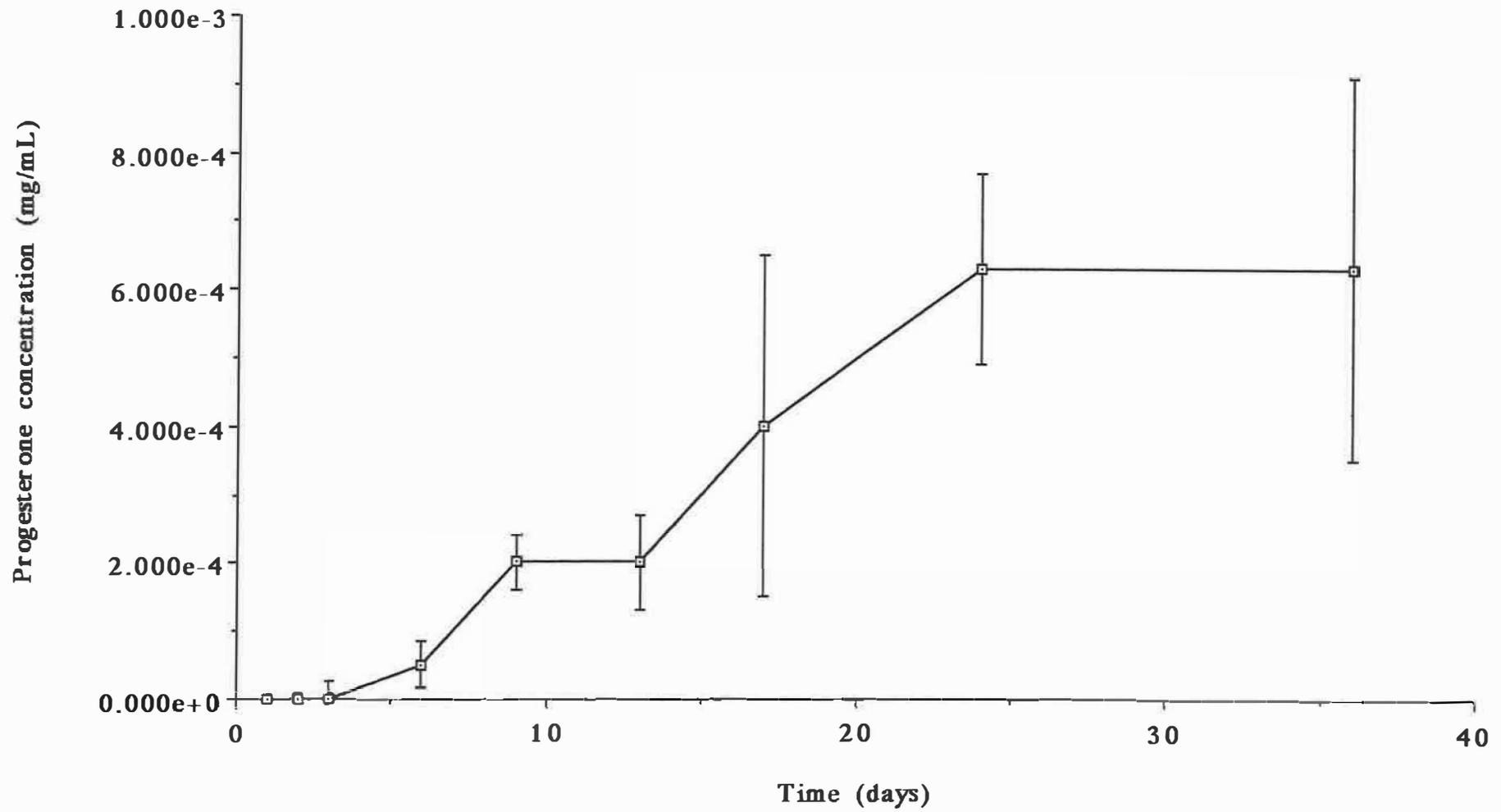


Figure 6.2. Release of progesterone from a hollow fibre (N, 0.25/0.75 mm) (mean  $\pm$  sem, n=6)

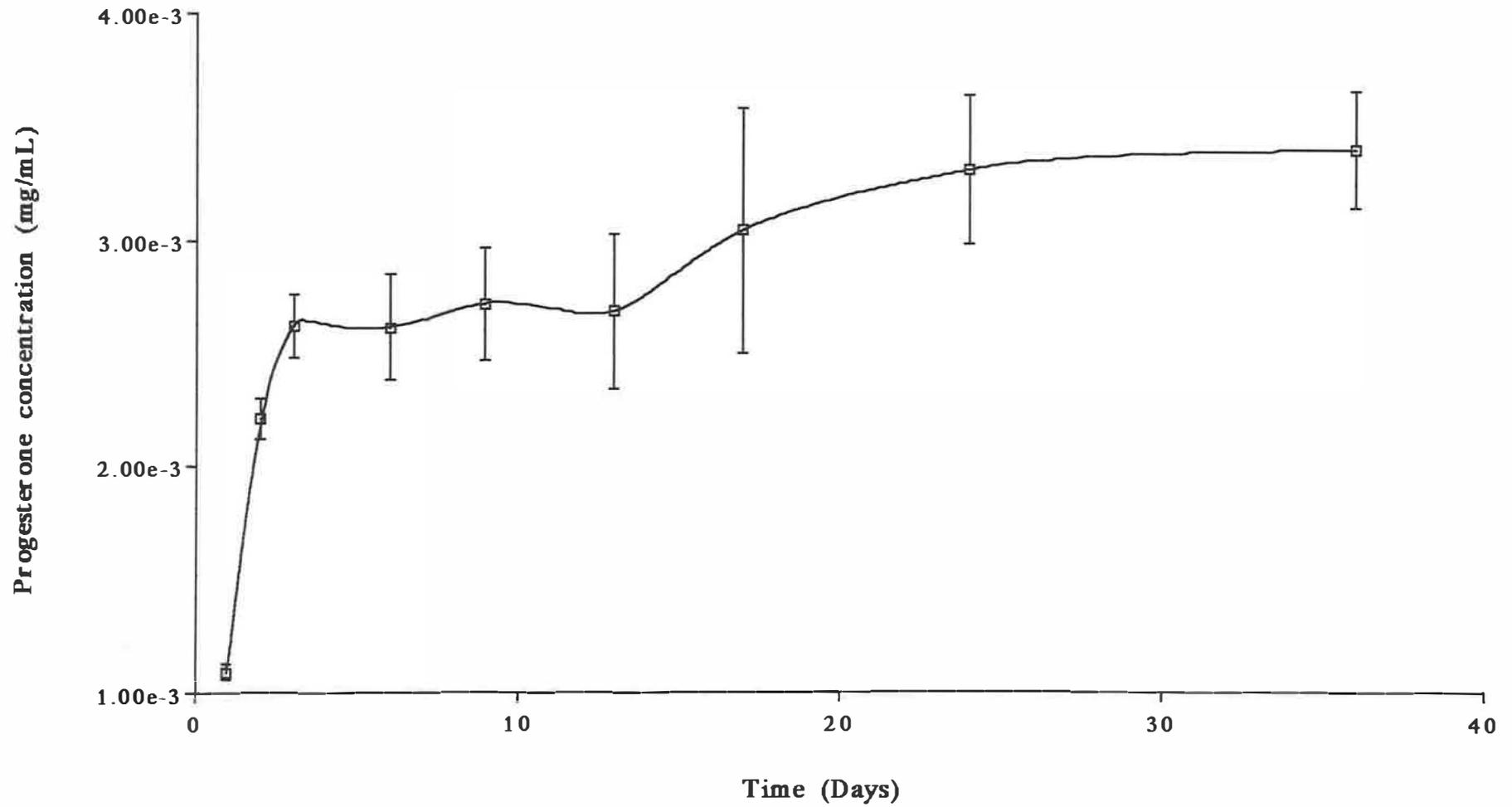


Figure 6.3 Release of progesterone from a hollow fibre (N,0.50/0.63) (mean  $\pm$  sem, n=6)

### **6.4.3 Release of levonorgestrel from hollow polymer fibres**

#### **6.4.3.1 Construction of a levonorgestrel against absorbance calibration plot**

Determination of levonorgestrel concentration was made using a Perkin-Elmer Lambda 2 spectrophotometer. To determine the wavelength of maximum absorbance a wavelength scan of a dilute solution of levonorgestrel in octanol ( $10 \mu\text{g mL}^{-1}$ ), was performed from 190 nm to 300 nm. The wavelength of maximum absorbance was at 239 nm and was therefore used in all subsequent experiments (see appendix for scan).

A calibration plot for levonorgestrel was constructed by measuring the absorbances of standard solutions of levonorgestrel in octanol at 239 nm. Use of this plot, (figure 6.4), was made to establish the concentration of levonorgestrel in the octanol 'sink' after release from the hollow fibres.

#### **6.4.3.2 UV spectroscopy for monitoring release of levonorgestrel**

30 mL glass bottles were treated as described in section 3.4.2 and filled with 30 mL of distilled water. 8 fibres, (N,0.25/0.75mm; N,0.5/0.63; N,0.58/1.02; N,0.75/0.96; P,0.28/0.61; P,0.4/0.8; P,0.5/1.0mm; P,0.58/0.96), were cut to 9 cm in length and treated as described in section 3.4.2. The hollow fibres were then filled to a length of 6 cm,

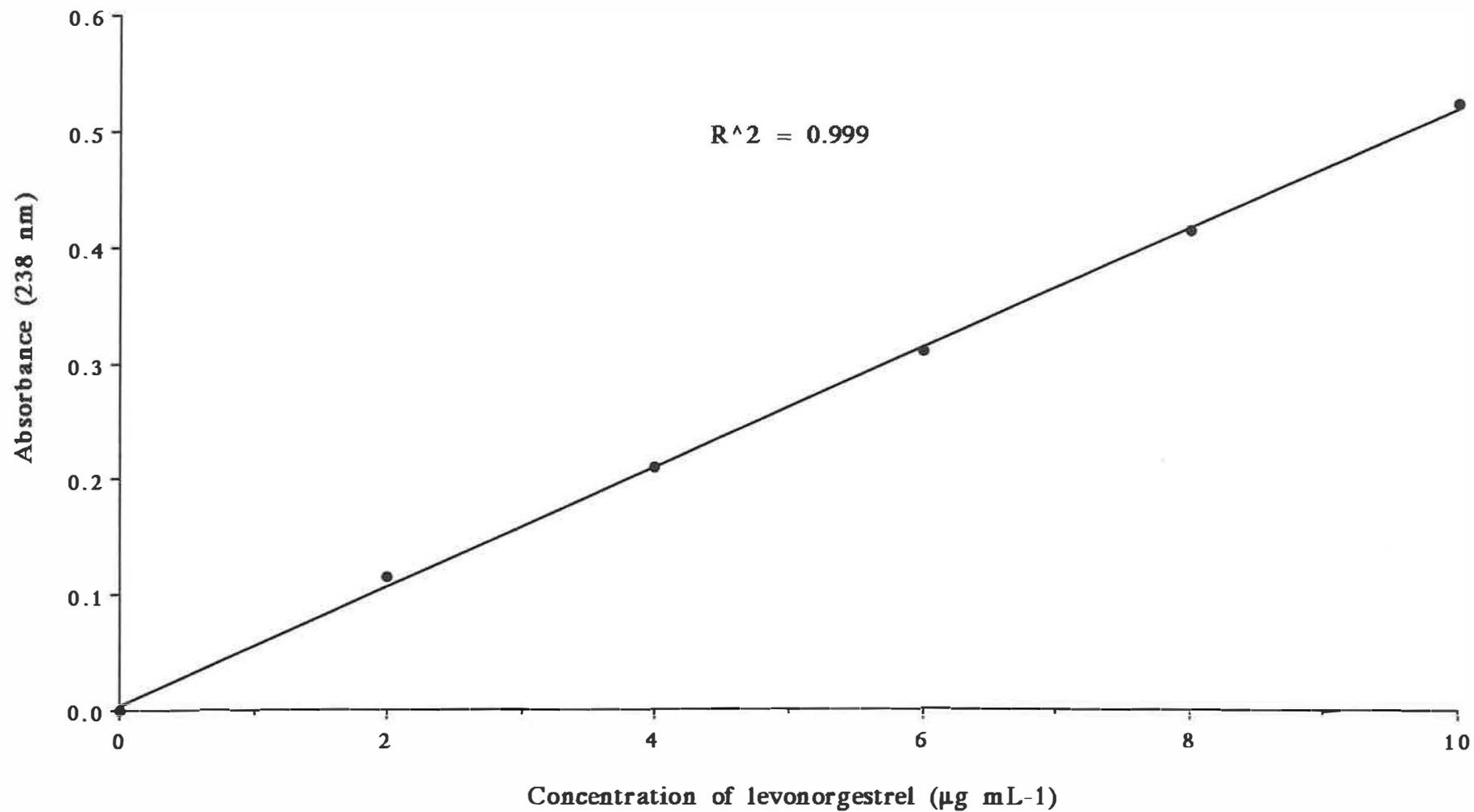


Figure 6.4. Calibration plot of levonorgestrel concentration against absorbance

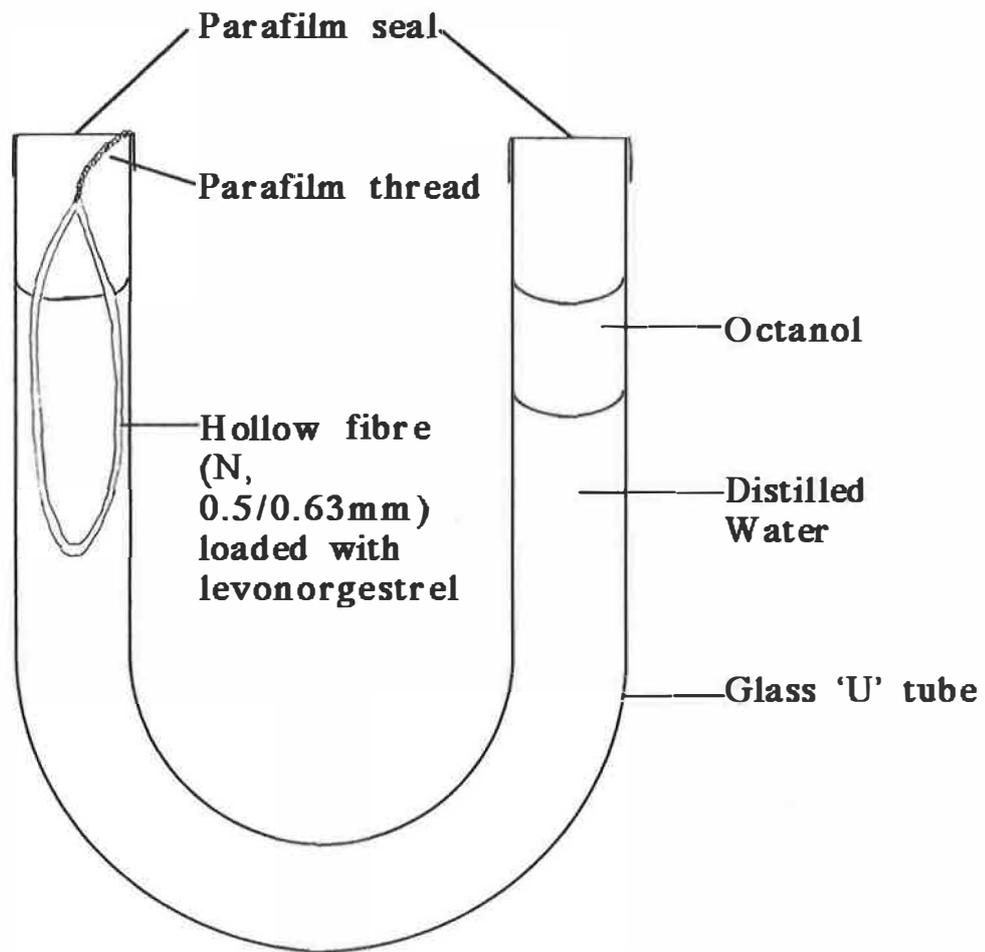
either with a solution of  $5 \text{ mg mL}^{-1}$  levonorgestrel in absolute ethanol, or with a suspension of  $15 \text{ mg mL}^{-1}$  levonorgestrel in sesame oil. Control hollow fibres were treated identically and loaded with 6 cm of the vehicle only. Leakage from the ends of the hollow fibres was prevented using Parafilm as described in section 3.4.2. Parafilm was also used to prevent evaporation when the bottles were placed into a thermostatically controlled environment at  $37 \pm 2^\circ\text{C}$  (see section 3.4.2). 2 mL samples were removed from the bottles daily for the first 3 days after which samples were removed at progressively longer intervals and measurements made by UV spectrophotometry using distilled water as the reference, at 239 nm. The absorbances obtained were then converted to concentrations by use of a calibration plot (figure 6.4). After measurements were taken, samples were returned to the bottles, and the tops of the bottles resealed with Parafilm. In each case  $n = 4$ , unless otherwise stated.

Readings were made at 239 nm.

The experiment was carried out over a 32 day time period. In this time negligible levonorgestrel was released from all of the hollow fibres in either vehicle.

#### **6.4.3.3 Release of levonorgestrel from a nylon hollow fibre into an octanol 'sink'**

To concentrate the levonorgestrel released, a different system was



**Figure 6.5. Arrangement of U tube with hollow fibre for levonorgestrel release studies**

adopted. Glass 'U' tubes were treated in Decon overnight, and rinsed thoroughly in tap water followed by distilled water prior to use. The 'U' tubes were filled with 15 mL of distilled water, and 2 mL of octanol, (purified by filtering through a calcium carbonate/activated charcoal column), introduced into one arm of the 'U' tube. The octanol was to act as a 'sink' to concentrate any levonorgestrel released. A hollow fibre, (N,0.5/0.63), was cut into 9 cm lengths, and treated as described in section 3.4.2 and filled to a length of 6 cm with a solution of 5 mg mL<sup>-1</sup> levonorgestrel in absolute ethanol and the ends heat sealed. To ensure no leakage from the ends of the fibres, the ends were brought together and wrapped in Parafilm. The Parafilm was then drawn into a thread, the hollow fibre lowered into the opposite arm of the 'U' tube to which the octanol was placed, so that the ends remained above the surface of the water. To prevent evaporation, when the 'U' tubes were placed into a thermostatically controlled environment at 37°C, the open ends of the 'U' tube were covered using more Parafilm (figure 6.5). Release was monitored every 1 to 3 days for the first 3 weeks and then every 3-4 days for the next 7-8 weeks. This was done by removing 0.3 mL of octanol from the 'U' tube using a syringe, injecting it into a semi-microcuvette, and measuring the absorbance at 239 nm. After the absorbance reading was taken, the octanol was removed from the cuvette, and returned to the 'U' tube. Control hollow fibres which had been treated identically, were filled with absolute ethanol.

The results for the release of levonorgestrel from the nylon hollow fibre into the octanol sink are given in figure 6.6.

The amount of levonorgestrel loaded into N,0.50/0.63 mm was 0.0589 mg. The initial rate of release of levonorgestrel from this hollow fibre was  $0.318 \mu\text{g day}^{-1}$ . The maximum concentration of levonorgestrel obtained by release from this fibre was  $7.798 \pm 0.543 \times 10^{-3} \text{ mg mL}^{-1}$ . This concentration corresponds to a total release of 0.015596 mg, i.e. 26.48 %.

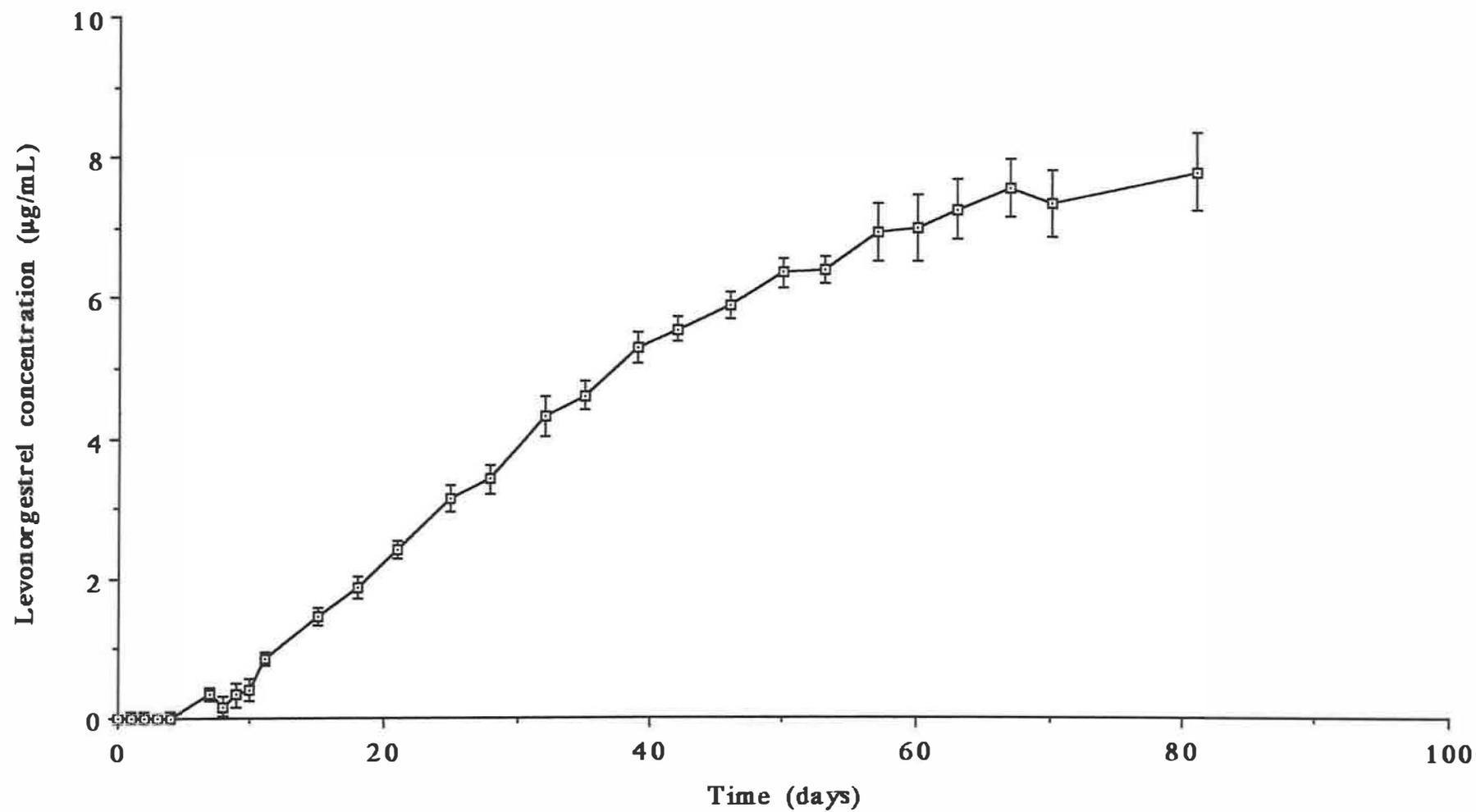


Figure 6.6. Release of levonorgestrel from a hollow fibre (N, 0.5/0.63 mm) (mean  $\pm$  sem, n=6)

## 6.4 Discussion

The release of both levonorgestrel and progesterone was determined initially using the same procedure as that for monitoring the release of chlorhexidine, by placing the hollow nylon fibres loaded with the drug into glass bottles filled with distilled water. For the release of progesterone this method proved suitable, despite release being slow due to the practical insolubility of progesterone in water. Release was observed and then confirmed by performing a wavelength scan of the bathing fluid to establish that the scan produced was the same as that when progesterone was added direct to a volume of water, and this sample was scanned. However, in the case of levonorgestrel, the use of the same system employed for monitoring chlorhexidine release was unsuccessful since levonorgestrel is even less soluble in water than progesterone and was not detected in the bathing fluid throughout the 32 day course of the experiment.

To establish that levonorgestrel could be released from the nylon hollow fibres a system was developed to demonstrate that levonorgestrel could at least be released into the distilled water bathing fluid to a minimal extent. To do this a 'U' tube system was adopted with distilled water filling the majority of the 'U' tube, the hollow nylon fibre loaded with levonorgestrel in absolute ethanol at one end and a solvent in which levonorgestrel was far more soluble in the opposite end. Octanol was chosen as it is less dense than water and is only sparingly soluble in water (0.05g of octanol will dissolve in 100g of distilled water (Morrison and

Boyd, 1983)). This system would allow the levonorgestrel to pass from the hollow nylon fibre into the distilled water, and pass from the distilled water and be taken up into the octanol, such that the octanol would act as a sink for the levonorgestrel. Using this system release of levonorgestrel from the hollow fibres was demonstrated.

From the results it was determined that 85.13  $\mu\text{g}$  of progesterone was released over a 36 day period from N,0.5/0.63, which corresponded to a concentration in the bathing fluid of 3.41  $\mu\text{g mL}^{-1}$ . This meant that of a total of 117.8  $\mu\text{g}$  of progesterone loaded into the hollow fibre, after 36 days 32.67  $\mu\text{g}$  of progesterone remained within the hollow fibre. This amount of progesterone remaining within the hollow fibre corresponds to a total percentage of 27.7 %. In the case of the other hollow fibre (N, 0.25/0.75) which was loaded with 29.45  $\mu\text{g}$  of progesterone, 20.81  $\mu\text{g}$  was released into the bathing fluid, leaving 8.64  $\mu\text{g}$  left within the hollow nylon fibre, which corresponds to a percentage of 29.3 %.

The actual concentration of progesterone in the bathing fluid at the stage when further release of progesterone is negligible, is 3.41  $\mu\text{g mL}^{-1}$  and 0.832  $\mu\text{g mL}^{-1}$  for the hollow fibres N,0.5/0.63 and N,0.25/0.75 respectively. At this stage the effective concentration of progesterone remaining in the nylon hollow fibres N,0.5/0.63 and N,0.25/0.75 is 2780  $\mu\text{g mL}^{-1}$  and 2934  $\mu\text{g mL}^{-1}$  respectively. As can be seen these concentrations far exceed those found in either of the surrounding bathing fluids and it would normally be expected that if equilibrium were to be

established internal and external concentrations would be approximately the same. However, due to the relative insolubility of progesterone in water, and the fact that each hollow fibre has ceased to release further progesterone once the internal concentration of progesterone has reached a certain point, it must be assumed that there is an internal threshold concentration below which further release of progesterone is unable to occur.

The concentration of progesterone that enters the bathing fluid with respect to the N,0.5/0.63 and N,0.25/0.75 hollow fibre is 1.22 % and 0.028 % of that which remains within the nylon hollow fibres respectively. The difference here may be a result of the much thicker wall of the latter hollow fibre since a further barrier, that of the thicker wall itself, needs to be overcome before release of progesterone. This fact is demonstrated in figures 6.2 and 6.3 where substantial release of progesterone is observed after only 2 days in the bathing fluid with the N,0.5/0.63 fibre, yet a substantial release of progesterone from the N,0.25/0.75 fibre is not shown until after 10 days. It is possible that progesterone becomes "trapped" in the walls of the hollow fibres themselves, and if this is the case, more progesterone would become trapped within the N,0.25/0.75 fibre than in the N, 0.5/0.63 fibre as the former has a greater wall thickness than the latter.

With the 'U' tube system for monitoring release of levonorgestrel, after 81 days 7.72  $\mu\text{g mL}^{-1}$  of levonorgestrel was detected in the octanol sink. The total amount of levonorgestrel taken up into the octanol was therefore

15.44  $\mu\text{g}$ . As can be seen this is a very small amount to be released over an 81 day period, and from figure 6.6 it can be calculated that the average daily release of levonorgestrel is 0.191  $\mu\text{g day}^{-1}$ .

The total amount of levonorgestrel loaded into the nylon hollow fibre was 58.9  $\mu\text{g}$  which means that after the 81 days 43.46  $\mu\text{g}$  of levonorgestrel still remained in the hollow nylon fibre. From figure 6.6, the general trend of the curve is still upwards, which suggests that levonorgestrel was still being released after 81 days. This must be related to the fact that the saturation point of octanol with levonorgestrel had not been reached, since this study has shown that 1 mg of levonorgestrel can be dissolved easily into 1 mL of octanol.

The amount of levonorgestrel and progesterone released daily from commercially available IUCDs is 20  $\mu\text{g day}^{-1}$  and 65  $\mu\text{g day}^{-1}$  respectively (Population Report, 1988). No information was available on the lowest release rate needed to produce an effect, and despite the fact that daily release of the two progestins from the devices investigated in this study was less than that from the commercially available devices, the effect of the devices investigated in this study *in vivo* needed to be ascertained to determine whether or not an effect would be seen.

**7 THE *IN VIVO* RELEASE OF  
STEROIDS FROM HOLLOW  
NYLON FIBRES**

## 7.1 Introduction

The use of progestins for peri-menopausal and post-menopausal hormone replacement therapy has been discussed in Section 6.1. The studies undertaken in Chapter 6 showed that levonorgestrel and progesterone could be released from the hollow nylon fibres at a rate which is much slower than that from levonorgestrel and progesterone releasing IUCDs, which release  $20 \mu\text{g day}^{-1}$  and  $65 \mu\text{g day}^{-1}$  respectively.

The administration of levonorgestrel locally to the uterus from an IUCD is likely to show a uniform suppression characterised by a marked atrophy of the glands, a decidualisation of the stroma, a dilatation of the thin-walled venules and an inflammatory reaction of the endometrium (Pengdi *et al* 1989). Similarly, local administration of progesterone to the uterus will show abundant stroma with decidual reaction and marked atrophy of the glands (Scommegna *et al* 1970).

The normal guinea-pig oestrus cycle lasts between 18 and 21 days and is divided into five phases, proestrus, oestrus, metoestrus, dioestrus and anoestrus. The length of each phase is given in table 1.1. Metoestrus is the phase of the cycle where progesterone secretion is dominant, causing secretion of vaginal mucus of high viscosity and development of a thin endometrium with deep tortuous glands. It would be expected that a progestin releasing device would produce changes in the uterine histology which parallel physiological changes which occur naturally.

Despite the rate of release of progestins from the devices investigated in chapter 6 falling short of those from commercial devices, the effect of these devices needed to be established *in vivo*. It was the aim of this study to determine the effect of these devices on the guinea-pig endometrium and vagina, and establish whether these devices would exert a systemic effect by white blood cell count determination.

## 7.2 Materials

### Chemicals

Levonorgestrel and progesterone as section 6.2.

Absolute ethanol as section 3.2.

The constituent chemicals of the neutral buffered formaldehyde used in histological preparation of samples consisted of 40% formaldehyde, (100 mL), distilled water, (900 mL), 4 g of sodium dihydrogen phosphate, (monohydrate) and 6.5 g of disodium hydrogen phosphate, (anhydrous) and were obtained from BDH Chemicals Ltd., Poole, Dorset.

Hypnorm injection as section 2.2.

Valium injection as section 2.2.

### Media

Nutrient Broth (C. M. 1) and Nutrient Agar (C. M. 3) as section 2.2.

### Tubing

Glass capillary tubes as section 2.2.

Polyethylene tubing used in the manufacture of the monofilament/hollow fibre inserting device as section 2.2.

Nylon hollow fibres releasing chemicals as section 3.2.

### Sutures

Nylon (Ethilon) and silk (Mersilk) sutures as section 2.2.

### **7.3 Apparatus**

The modified Kilian nasal speculum as section 2.3.

Light microscopy was performed using a Leitz Biomed Microscope, Leica U. K. Ltd., Milton Keynes, Buckinghamshire, U. K.

White blood cell counts were determined using an improved Neubauer haemocytometer obtained from Philip Harris Medical Supplies, Birmingham, West Midlands. Any dilutions were made using Gilson automatic pipettes obtained from Gilson Medical Electronics (France) S.A.

Optical density/absorbance as section 2.3

Centrifugation as section 2.3.

## **7.4 Methods**

### **7.4.1 Animal husbandry**

Dunkin-Hartley guinea-pigs were obtained and housed as described in section 2.4.2.

### **7.4.2 White blood cell counts**

Samples of fresh whole blood were collected (post-mortem) by cardiac puncture from each of the relevant animals. 50  $\mu$ L of blood was diluted appropriately using the diluting fluid (1.5% v/v glacial acetic acid, 0.5 % methylene blue and made up to 100% with distilled water). The diluted blood was placed into the counting chamber of an improved Neubauer haemocytometer, and the number of white cells determined by bright field light microscopy.

### **7.4.3 Histological preparation and examination of guinea-pig uterine specimens**

The portion of uterine horn to be used for histological examination was placed into neutral buffered formaldehyde, and left for at least 3 hours. After at least 3 hours in the neutral buffered formaldehyde, the uterine horn was transferred into a solution of 80 %  $\nabla/\nabla$  ethanol for at least 1 hour, after which, it was placed into absolute ethanol for a minimum of 4.25 hours, changing the volume of ethanol for a fresh volume after

every hour, except for the fourth volume of ethanol where it remained for at least 1.25 hours.

After leaving the horn in absolute ethanol for 4.25 hours, it was placed into a mixture of 50% ethanol and 50% clearing fluid, (Citroclear), for a minimum of 1 hour, after which it was placed into 100% clearing fluid for 3.25 hours, changing the volume of the clearing fluid for a fresh volume after each hour, except for the third hour when the uterine horn was left in the clearing fluid for at least 1.25 hours. The uterine horn was placed into pure paraffin wax, (m.p. 56°C), at a temperature of 60°C for 2.5 hours, changing the wax after 1 hour, and leaving it in the second volume for 1.5 hours. After this time, the uterine horn was placed into a suitable mould, where molten wax was carefully poured onto it, such that when it solidified, the uterine horn was completely encased within the wax ensuring no air pockets were formed. The wax was then placed into a refrigerator at 4°C to harden. When embedded the tissue was cut using a microtome to give sections of 5 µm in thickness. These sections were transferred onto glass microscope slides, where the wax was dissolved using xylene. Once dried, the section was stained with a hematoxylin and eosin stain, and examined under light microscopy.

#### **7.4.4 Transcervical insertion of hollow nylon fibres loaded with progesterone and levonorgestrel into guinea-pig uteri**

Insertion of nylon hollow fibres which had been loaded with levonorgestrel, ( $5 \text{ mg mL}^{-1}$  in absolute ethanol), or progesterone, ( $10 \text{ mg mL}^{-1}$  in absolute ethanol), was performed according to the method described in Section 2.4.3. Controls performed involved insertion of hollow nylon fibres which had been left empty, and hollow nylon fibres which had been loaded with absolute ethanol. After insertion of the hollow fibres, the animals were kept for 40 days, after which they were killed by cervical dislocation, and cardiac puncture was performed to remove sufficient blood in order to determine white blood cell numbers (section 7.4.2) for each of the animals. The uterine horns were then revealed, as described previously, (section 2.4.3), and the horn containing the hollow nylon fibre was excised, cut into 2 pieces, one piece was washed through with lecithin-tween broth for determination of the bacteriology of the uterine horn, and the other piece was placed into neutral buffered formaldehyde solution for histological examination (section 7.4.3 for histological methodology). Vaginal lavage was performed as described in section 4.4.2. The resultant suspension was viewed under a light microscope and from the cell types present it was possible to ascertain the stage of oestrus of the animals (section 1.2.2).

#### **7.4.5 Statistical analysis**

The data obtained from all white blood cell counts was statistically analysed using Students independent 2-tailed t-test.

The uterine microflora results are presented in tables 7.2 to 7.5 as colony forming units per mL ( $\text{cfu mL}^{-1}$ ) of original cell suspension, and are compared using the Mann-Whitney U-test.

## 7.5 Results

The white blood cell counts and the stage of oestrus as determined by light microscopy are given in table 7.1. (Validation of the white blood cell counting technique is given in appendix). When compared statistically, the difference in white blood cell numbers between the groups was not significant ( $p > 0.2$  in each case). Vaginal cell cytology allowed determination of the stage of oestrus and insertion of the empty hollow fibre and the control fibre filled with ethanol did not appear to influence the stage of oestrus of the animals. Similarly the oestrus cycle of the animals that were fitted with the levonorgestrel loaded hollow fibres appeared not to be under the influence of levonorgestrel release, or at least release at these low levels did not bring about a change in the vaginal cytology. However those animals fitted with hollow fibres loaded with progesterone were all found to be in metoestrus. This suggests that progesterone released from the hollow fibres is exerting an effect upon the vaginal cytology.

Tables 7.2 to 7.5 are the bacterial counts taken from the guinea-pigs in which were secured hollow nylon fibres loaded with absolute ethanol alone, progesterone in absolute ethanol, levonorgestrel in absolute ethanol or a hollow fibre which was left empty. The bacterial counts were obtained from the guinea-pig uterine horn in which the hollow fibre was secured (Ut.) and from the inserted hollow fibre itself (Tube) when the hollow fibre could be easily removed. Only the presence or absence of bacteria was established in the contralateral uterine horn (C. U. H.) and is

indicated in the tables as a tick (✓) or a cross (x) respectively.

<b>Animal no.</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>Prog.</b> (wbc/mm <sup>3</sup> ) (Stage of oestrus)	5520 Metoestrus	5040 Metoestrus	1960 Metoestrus	4800 Metoestrus
<b>Empty</b> (wbc/mm <sup>3</sup> ) (Stage of oestrus)	3400 Proestrus	5520 Dioestrus	2840 Dioestrus	- -
<b>Ethanol</b> (wbc/mm <sup>3</sup> ) (Stage of oestrus)	3560 -	4920 Proestrus	3160 Metoestrus	- -
<b>LNG</b> (wbc/mm <sup>3</sup> ) (Stage of oestrus)	2960 Proestrus	5040 Dioestrus	2360 Proestrus	1840 Metoestrus

**Table 7.1. Table showing white blood cell counts per cubic mm of fresh blood and the stage of the oestrus cycle as determined from vaginal cytology for each animal.**

<b>Animal no.</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>UT.</b>	32	68	27	19
<b>TUBE</b>	-	45	18	29
<b>C.U.H.</b>	x	x	x	x

**Table 7.2. Progesterone loaded fibre (Bacterial counts (cfu mL<sup>-1</sup>) from the animal fitted with hollow nylon fibre loaded with 10 mg/ml of Po. in absolute ethanol after approximately 40 days).**

<b>Animal no.</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>UT.</b>	3	9	13	10
<b>TUBE</b>	2	2	9	1
<b>C.U.H.</b>	x	x	x	x

**Table 7.3. Levonorgestrel loaded fibre (Bacterial counts (cfu mL<sup>-1</sup>) from animals fitted with hollow nylon fibre loaded with levonorgestrel after 40 days).**

<b>Animal no.</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>UT.</b>	315	>2500	26	0
<b>TUBE</b>	86	10	-	5
<b>C.U.H.</b>	x	x	x	x

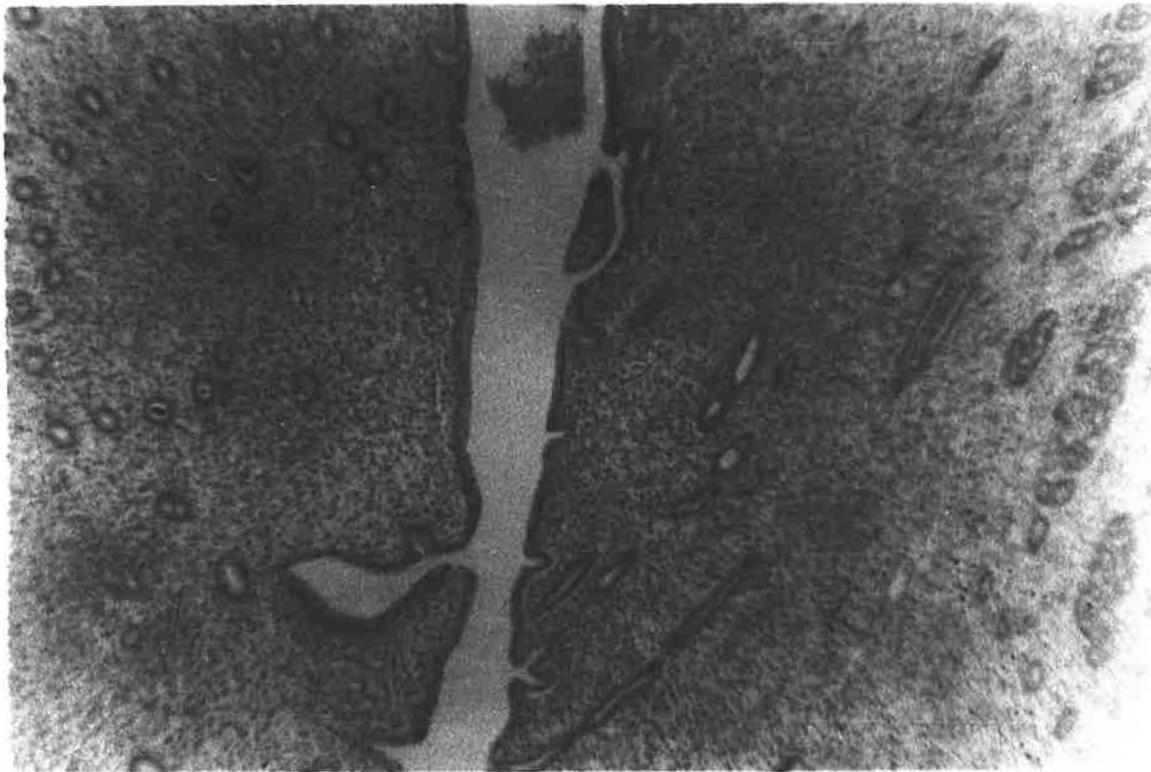
**Table 7.4. Fibre loaded with ethanol only (Bacterial counts (cfu mL<sup>-1</sup>) from animals fitted with hollow nylon fibre loaded with absolute ethanol only, after 40 days).**

<b>Animal no.</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
UT.	15	18	16	11
TUBE	4	19	47	0
C.U.H.	x	x	x	x

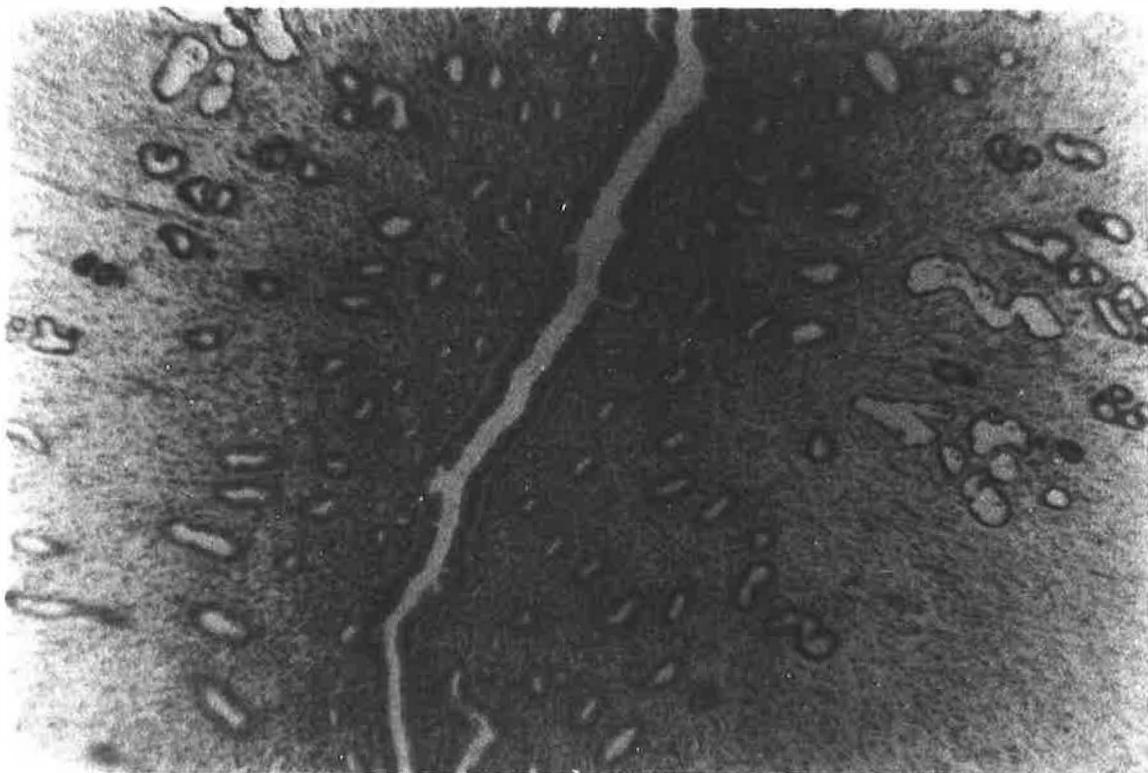
**Table 7.5. Empty hollow fibre (Bacterial counts (cfu mL<sup>-1</sup>) from the animals fitted with an empty hollow nylon fibre, after 40 days).**

In each case where a hollow nylon fibre was inserted and attached to the uterine wall, the contralateral uterine horn remained free of bacterial contamination (tables 7.2 to 7.5). Of the horns in which the hollow fibres were actually fitted, contamination was observed in each case, except in one guinea-pig from the group fitted with ethanol only loaded devices. When uterine bacterial counts obtained from the different groups of animals are compared statistically, differences are only found to be significant in two cases. (i) When counts from animals fitted with empty devices and those from animals fitted with progesterone releasing devices are compared ( $p < 0.05$ ), where counts in animals fitted with progesterone releasing devices were higher. (ii) When counts from animals fitted with levonorgestrel and progesterone releasing devices are compared ( $p < 0.05$ ), where counts in animals fitted with progesterone releasing devices were also higher.

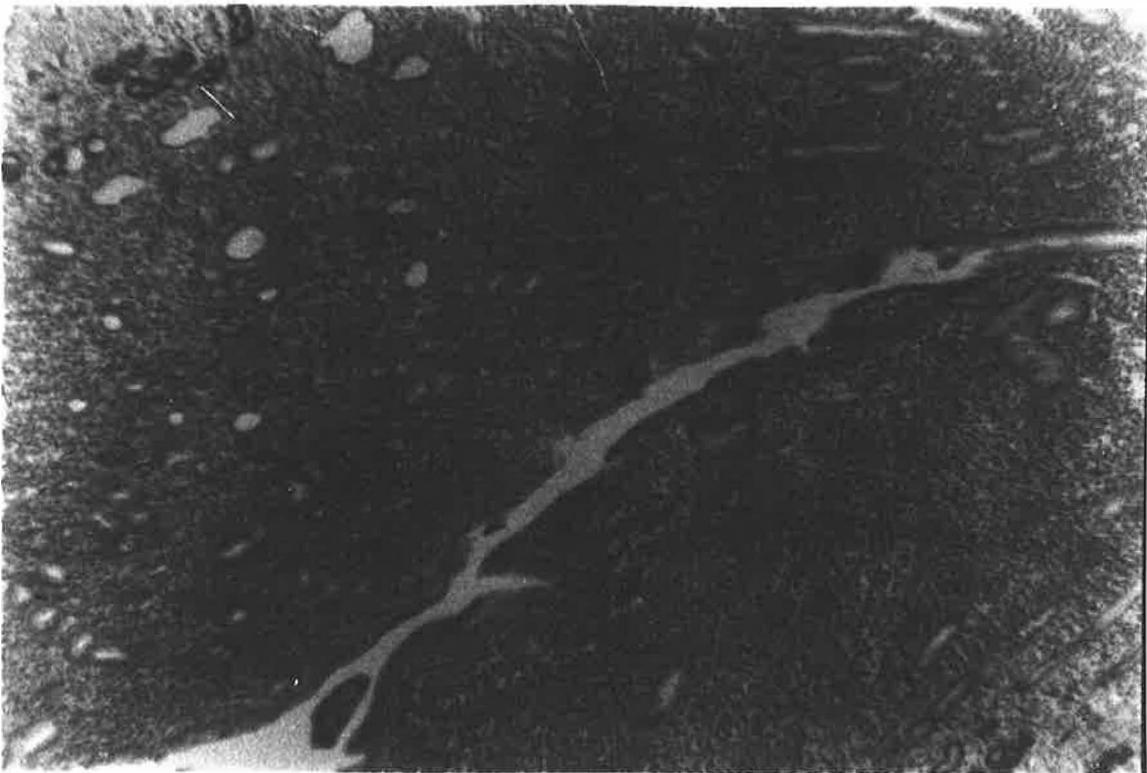
The photographs showing the histology for each of the groups are shown in figures 7.1 to 7.4, where a representative picture from each group is given.



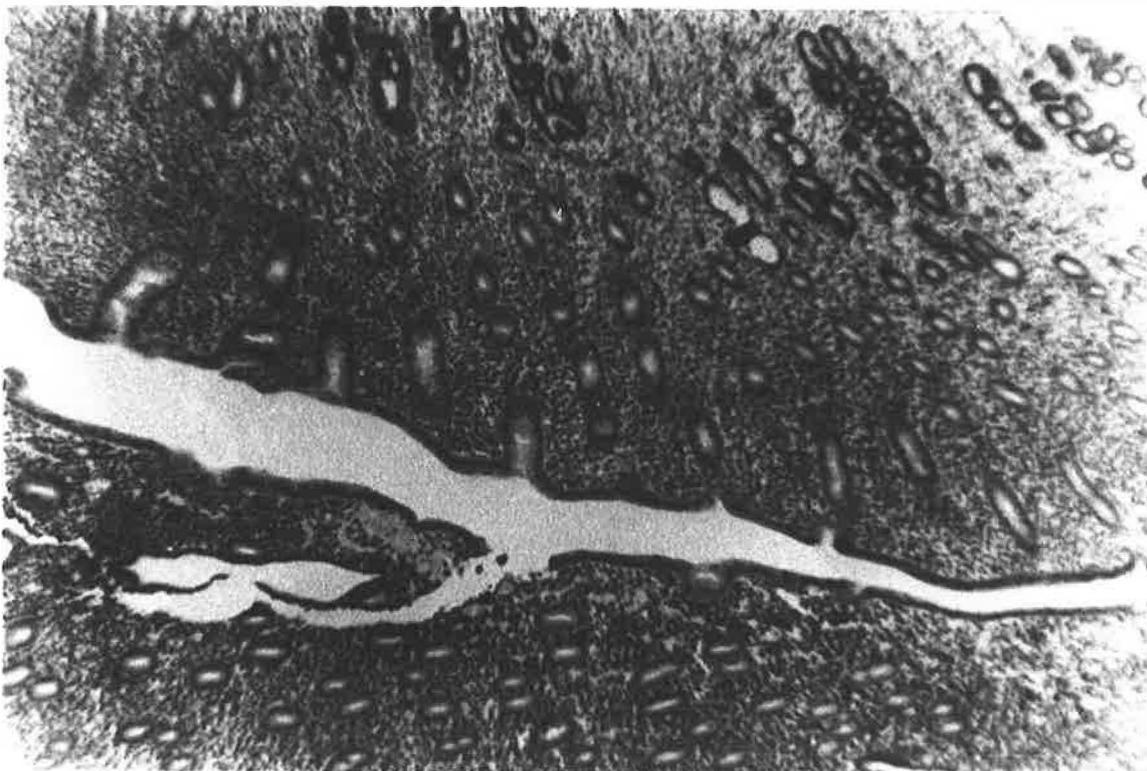
**Figure 7.1.** Transverse section of a guinea-pig uterine horn after treatment with levonorgestrel released from a nylon hollow fibre for 40 days.



**Figure 7.2.** Transverse section of a guinea-pig uterine horn after treatment with progesterone released from a nylon hollow fibre for 40 days.



**Figure 7.3.** Transverse section of a guinea-pig uterine horn 40 days after being fitted with an empty nylon hollow fibre.



**Figure 7.4.** Transverse section of a guinea-pig uterine horn after treatment with ethanol released from a nylon hollow fibre for 40 days.

## 7.6 Discussion

On average women reach the menopause when they are approximately 50 years of age (Gray, 1976), although differences between racial groups have been observed (MacMahon and Worcester, 1966; Treloar, 1974). Whatever the race, however, at some stage in their life, women will experience the menopause, and the symptoms that are associated with it. It has been estimated that 16 % of women experience mild symptoms, 33 % suffer moderately from perimenopausal symptoms and 51 % suffer severely from the symptoms associated with the menopause (Samsioe *et al* 1985). Of the effects caused by the withdrawal of oestrogen at the menopause, those which are of the greatest concern are the development of cardiovascular disease and osteoporosis. Hormone replacement therapy (HRT) has been shown to give a reduction in cardiovascular disease in up to 50 % of women who receive treatment, which suggests that the benefit conferred by this form of treatment would outweigh the other adverse oestrogen effects (Goldman and Tosteson, 1991). Osteoporosis also represents a severe challenge and 50 % of women will have sustained an osteoporotic fracture by the age of 70. It has been shown that caucasian women run a life time risk of a fractured neck of femur of 15 % and, as a result of such a fracture, have a 20 % chance of dying and a 50 % chance of losing their independence. The cost to the NHS due to osteoporosis is estimated to exceed £700 million per year (Hillard *et al* 1991).

Of those women who suffer from perimenopausal symptoms, it has been shown that HRT is successful in the treatment of 90 % of users (Hunt,

1988) and yet at present only 10 % of women are receiving HRT in the U.K. (Siddle and Knight, 1991; Read and Sturdee, 1993). One of the reasons for this small number of women who undergo HRT is that unopposed oestrogen therapy can increase the risk of endometrial cancer 3 to 25 fold (depending on the study), and this applies to all types of oestrogens (Barrett-Connor, 1992). It is current practice to add a progestogen to HRT in order to protect the uterus (provided that a uterus is still in place) and addition of progestogen for a minimum of 10 days in the cycle eliminates the excess risk (Voigt *et al*, 1991). Although addition of a progestin decreases the risk of developing endometrial cancer, it will also reduce the protecting effect of the oestrogen on cardiovascular disease (Henderson *et al*, 1988). However, there does not appear to be any counteracting action of the progestin on the oestrogen-induced increase in bone mass (Read and Sturdee, 1993).

It was the intention of this section to utilise a method of delivering a very low dose of progestin to the uterine cavity and to establish if this would exert a local and/or systemic action. Physiological changes produced by the device would indicate the intensity of the effect of the release of progestin.

During the luteal phase of the normal menstrual cycle, release of progesterone leads to the development of a secretory endometrium. Abrupt decline in the release of progesterone from the corpus luteum at the end of the cycle is the main determinant of the onset of menstruation (section 1.1.2). If the duration of the luteal phase is artificially

lengthened, either by sustaining luteal function or by treatment with a progestogen, decidual changes in the endometrial stroma similar to those seen in early pregnancy can be induced. Under normal circumstances oestrogen antecedes and accompanies progesterone in its action upon the endometrium and is essential to the development of the normal pattern.

When progestogens are administered orally from day 5 of the menstrual cycle for 20 days, the endometrial stroma will show intense luteal actions while the glands, stimulated at first will become atrophic. Under the influence of progestogens, the abundant watery secretion produced by the endocervical glands of the oestrogen stimulated structures is changed to a scant viscid material.

The oestrogen induced maturation of the human vaginal epithelium is modified toward the condition of pregnancy by the action of progestogens (section 1.3).

These physiological changes induced by progestogens may be easily determined. However in the guinea-pig very little cervical mucus is secreted and determination of the relative consistency of the mucus produced would have proved very difficult. Determination of the uterine histology and vaginal cytology was performed as an indicator of the local effects of any release of progestogen. Since all steroids cause a degree of immunosuppression a reduction in the white blood cell count was used as an indicator to determine whether the devices produced any systemic side effects.

Malhi (1991), who administered ethinyloestradiol (0,05 mg Kg<sup>-1</sup> day<sup>-1</sup>), norethisterone acetate (1 mg Kg<sup>-1</sup> day<sup>-1</sup>) or prednisolone acetate (2 mg Kg<sup>-1</sup> day<sup>-1</sup>) to guinea-pigs via the intraperitoneal route, was able to show that in each case there was a reduction in the total white blood cell count. In this study, when comparing white blood cell counts of the animals fitted with hollow fibres containing levonorgestrel and those which were empty or loaded with ethanol alone, differences were found not to be significant ( $p > 0.2$ ). The same was true when counts from progesterone treated animals were compared with those of the two control groups ( $p > 0.2$ ). This suggests that any steroid release is insufficient to exert any action upon the production of white blood cells, and therefore unlikely to have any action systemically.

Figures 7.1 to 7.4 show representative sections of the guinea-pig uterus in animals fitted with the different devices. From examination of the photographs, it is clear that it would be very difficult to determine what action the release of steroid had upon the endometrium since no section from those animals in which progestin was released is obviously different from the control fibres.

However, when examining the vaginal cell cytology of the guinea-pigs fitted with hollow fibres filled with progesterone, all animals were found to be in metoestrus. This suggests that progesterone released from the hollow fibres is exerting an effect upon the vaginal cytology. The vaginal cell cytology of the animals fitted with the empty hollow fibre and those fitted with the fibres filled with ethanol alone did not appear to influence

the stage of oestrus of the animals. Similarly the oestrus cycle of the animals that were fitted with the levonorgestrel loaded hollow fibres appeared not to be under the influence of levonorgestrel release, or at least release at these low levels did not bring about a change in the vaginal cytology.

It appears that of the two progestin releasing hollow nylon fibres, only that delivering progesterone to the guinea-pig genital tract caused any synchronisation of the oestrus cycle. However, this finding was not reinforced by uterine histology, which may suggest that higher doses may be required.

Toivonen *et al* (1991) have observed a reduction in the incidence of pelvic inflammatory disease (PID) in women using a levonorgestrel releasing IUCD compared with women using copper releasing IUCDs. The mechanisms by which these workers believe the levonorgestrel releasing IUCD may reduce the incidence of PID are by the thickening of the cervical mucus, suppression of the endometrium and by decreasing uterine bleeding. Although investigation of infection in the guinea-pigs was not performed here, the action of each of the devices upon uterine contamination was determined. When the microbial counts from the groups were compared differences found to be statistically significant in only two instances. These occurred when comparing the group of animals fitted with an empty fibre with animals fitted with a progesterone releasing fibre, where greater numbers of bacteria were associated with the progesterone releasing device, and when animals fitted with

progesterone and levonorgestrel releasing fibres were compared. When considering the values for uterine bacterial contamination for the second two groups of animals, less contamination was seen with those animals fitted with levonorgestrel releasing devices, which agrees with the findings of Toivonen *et al*(1991). Since the bacteria obtained from the uterus after insertion of the device are likely to have been within a biofilm, they are difficult to remove, and enumerate.

## **8 GENERAL DISCUSSION**

Most women will at some stage in their lives experience the climacteric and the symptoms that are associated with it. Hormone replacement therapy (HRT) may be prescribed to alleviate many of these symptoms, and generally takes the form of either an oestrogen only therapy or a combined oestrogen/progestin therapy. However, in women who are prescribed oestrogen only therapy where the uterus is intact, there is a greater risk of developing endometrial carcinoma. To reduce this risk, a progestin is generally prescribed with the oestrogen in these women, which protects the endometrium from development of this condition.

When combined therapy is prescribed some of the desired effects produced by the oestrogens are antagonised by the actions of the progestin component. For example, one of the most beneficial actions produced by oestrogens which are prescribed perimenopausally is protection against cardiovascular disease. However, when co-administration of progestin is prescribed this cardio-protective action is reported to be reduced (Ross, 1989). The most important effect of progestins in combined HRT is to produce suppression of the endometrium. If this could be produced by local administration of a progestin then it would be possible to use a much reduced dose to cause endometrial suppression whilst reducing interference with the oestrogen benefits. One method by which this could be achieved is by fitting a sustained release device into the uterus. The simplest way to fit such a device into the uterus would be transcervical insertion. However, since both the cervix and the vagina possess a dense polymicrobial population, and the uterus is sterile (Sparks *et al*, 1977), transcervical introduction of devices into the uterus is likely to cause

bacterial contamination which may result in pelvic infection. The major aims of this project were to investigate the feasibility of developing a device that could locally administer a progestin to the endometrium and at the same time keep bacterial contamination of the uterus to a minimum. As such two devices that could be inserted into the uterus transcervically were developed. One which released the progestin, and a second that delivered an antibacterial agent, capable of reducing the chance of multiplication of any microorganisms introduced into the uterus on insertion. This would allow delivery of the progestin to the endometrium and release of the antibacterial agent into the uterine cavity to kill any bacteria introduced. Since this fibre would be transcervical, it could act a locating monofilament for user assurance, and would aid with the removal of the device once release of progestin is complete. However, the role of the fibre which traverses the cervix in future contamination of the uterus, needs to be considered. With the intrauterine contraceptive device (IUCD), the involvement of the locating monofilament in pelvic infection was controversial. This stems from the thread attached to the Dalkon Shield IUCD, which, because of its structure, was able to draw bacteria up within it by capillary action from the cervix and vagina to the uterus. This resulted in an incidence of pelvic inflammatory disease (PID) five times greater than that associated with other IUCDs (Tatum *et al*, 1975). In the case of the monofilament attached to other IUCDs, some workers believed them to be associated with uterine contamination long after insertion of the devices (Sparks *et al*, 1981), whilst others found rates of PID between women using IUCDs with and without monofilament attached not to be significantly different (Galvez *et al*, 1985). Since whether or not bacteria

could traverse the cervix along the monofilament, dictates what the life of the antibacterial releasing device should be, this had to be determined. Two groups of guinea-pigs were taken, one group of animals were fitted with a transcervical monofilament, and the other group was not. The microorganism *Serratia marcescens* was then introduced into the vaginae of both groups of animals. This particular microorganism was chosen as it is a non-pathogenic microorganism which is not associated with the vagina, and whose colonies produce a pigment which is a distinctive red colour. After 20 days, reinoculating the vaginae of these animals every 3-5 days, the animals were killed, the uteri excised, and examined for *S. marcescens*. In animals where the monofilament was absent, no *S. marcescens* were found associated with the uterus. In animals with a monofilament, 3 out of 8 animals were found to have *S. marcescens* in the uterus. However, this finding is not significant, ( $p > 0.05$ ) and indicates that a non-pathogenic microorganism such as *S. marcescens* could not cross the cervix whether a monofilament was present or not. However in the case of a pathogenic microorganism, entry into the uterus would be possible with a monofilament present or not. This has been shown in guinea-pigs where no monofilament had been inserted, in which chlamydia was inoculated into the vagina. After about 7 days, chlamydia was isolated from the uterus (Rank and Saunders, 1990). Because in most cases bacteria isolated from the vagina will be non-pathogenic, release of an antibacterial agent need only be for a short period to cover the time just after insertion when numbers of bacteria introduced into the uterus are likely to be greatest.

Delivery of the antibacterial agent chlorhexidine acetate was made from hollow fibres. Several fibres were initially investigated varying in both material and geometry. Other variables were the vehicle in which the chlorhexidine was dissolved or suspended, and the concentration of chlorhexidine used. The combination of material, geometry, vehicle and concentration giving the quickest initial rate of release was investigated more fully. A hollow fibre, N, 0.50/0.63 mm was the fibre that gave the quickest initial rate of release when chlorhexidine was dissolved in absolute ethanol at a concentration of  $140 \text{ mg mL}^{-1}$ . The initial release rate when the hollow fibre was in a bottle containing 30 mL of distilled water was  $6.49 \text{ } \mu\text{g mL}^{-1} \text{ day}^{-1}$ , which means the device is actually releasing  $194.7 \text{ } \mu\text{g day}^{-1}$ . If it is considered that when *in situ* sink conditions apply, then with a device loaded with 1.649 mg of chlorhexidine, the life of the device will be approximately 8.5 days. Since the chlorhexidine released is only to act upon those bacteria introduced into the uterus, it is believed that this length of time would be sufficient to kill any bacteria introduced. Had any bacteria been able to enter the uterus after release of chlorhexidine was complete (which would have been shown with the *S marcescens*) a more prolonged release of chlorhexidine may have been more appropriate.

The commensal microorganism from the guinea-pig vagina having the greatest minimum inhibitory concentration (MIC) when exposed to chlorhexidine was *Proteus mirabilis* with an MIC of  $100 \text{ } \mu\text{g mL}^{-1}$ . Since sink conditions apply, the rate of release of chlorhexidine has been

established at  $194.7 \mu\text{g day}^{-1}$ . Provided that the chlorhexidine released is not immediately washed away, which is a reasonable assumption, it is likely that all the bacteria introduced into the vagina would be killed.

To test that release of chlorhexidine from the hollow fibres was bactericidal, fibres were placed onto nutrient agar plates inoculated with *E. coli* (a commensal of the guinea-pig vagina), and left to pre-diffuse at  $4^{\circ}\text{C}$  prior to incubation. Zones of inhibition were obtained which were proportional to the length of time of pre-diffusion. A further test of the bactericidal activity of chlorhexidine was made where hollow fibres were placed into silicone rubber tubes containing phosphate buffered saline (PBS) inoculated with *E. coli*. After only 6 hours, release of chlorhexidine from the hollow fibres had produced a significant effect ( $p < 0.001$ ), and after only 24 hours no viable cells were obtained.

Since the hollow nylon fibres were to be used in an environment where they would come into contact with substantial quantities of mucus, one further *in vitro* test of the bactericidal capability of the device was performed. Hollow fibres were placed into silicone rubber tubes loaded with purified gastric mucus, (as it was reported to have similar structure to cervical mucus (Meyer, 1977)), inoculated with *E. coli*. In this experiment it was found that a significant difference between the chlorhexidine releasing fibre and the vehicular controls was only seen after 72 hours when 50 % ethanol was used as the vehicle, such that the ethanol content did not swamp chlorhexidine activity. In this case, although chlorhexidine shows reduced numbers of bacteria after 72 hours

compared with the controls, bacterial counts were not significantly different when chlorhexidine counts at 72 hours were compared with counts at time 0 hours. This suggests that whilst *E. coli* was able to proliferate within the mucus, in those tubes where chlorhexidine was present, a bacteriostatic effect was exerted. No attempt was made to establish whether the killing effect of the fibre diminished with distance from the fibre. If bacteria very close to the fibre were killed then this system may still be useful, since on insertion into the uterus, the fibre would become covered with mucus laden with bacteria, and it is these bacteria which are a potential nidus of infection.

The mechanisms by which mucus reduces the bactericidal activity of chlorhexidine involves the mucospissic action of chlorhexidine, which has been reported by Chantler *et al* (1989), and a strong electrostatic binding between mucus and chlorhexidine. Since chlorhexidine increases the viscoelasticity of mucus, it decreases the ability of chlorhexidine to diffuse through it, effectively producing a diffusional barrier to the chlorhexidine. Mucus itself is polyanionic in nature, so it is likely that its greatest interactions will be with polycations (Sharman, 1987). Plaut *et al* (1980), have reported that in aqueous solution chlorhexidine will exist as a dication, suggesting a strong electrostatic attraction between mucus and chlorhexidine. It is clear that the decreased bactericidal activity of chlorhexidine in the presence of mucus must be due to both the mucospissic action of chlorhexidine and the electrostatic binding between the mucus and the chlorhexidine.

The guinea-pig was the chosen animal to test the bactericidal activity of the chlorhexidine releasing hollow nylon fibre *in vivo*. Under normal circumstances, the guinea-pig uterus was sterile. This was shown in this study, and had previously been shown by Malhi *et al* (1987), and Bilbruck (1991). After transcervical insertion of devices into the guinea-pig uterus, the uterus became contaminated with bacteria introduced from the cervix and vagina. When devices loaded with chlorhexidine were transcervically inserted into the guinea-pig uterus, no significant difference in the level of bacterial contamination was shown when compared with the vehicular controls. Several explanations for the inability of the chlorhexidine device to kill sufficient bacteria to produce a significant effect exist.

(i) Since no significant effect is seen, the mucospissic activity of chlorhexidine may have been greater than initially believed, increasing viscoelasticity producing a diffusional barrier to the chlorhexidine and preventing access of chlorhexidine to the bacteria.

(ii) Again, since no significant effect was seen, electrostatic interaction may also be greater than expected, such that the mucus binds with the chlorhexidine, preventing chlorhexidine reaching the bacteria.

(iii) Most bacteria that come into contact with surfaces can secrete a polysaccharide substance which will aid in adhesion to the surface, and may also protect the microorganism from environmental factors as well as antibacterial agents (Costerton, 1987). However it is unlikely that bacteria would be able to get close enough to the

fibre and remain viable to adhere to it and secrete the polysaccharide.

(iv) It is possible that because of fluid movement within the uterus, the chlorhexidine is removed before bactericidal concentrations are reached. Bilbruck (1991) was able to show reduced bacterial contamination within the uterus after transcervical intrauterine insertion of nylon monofilaments coated in hydrogel loaded with chlorhexidine. In this instance, initial chlorhexidine release was so rapid, that it was effectively dumped. This may have overcome the diffusional barrier produced by the mucus, and also may have overcome the mucus/chlorhexidine electrostatic interactions.

For the device in this study to overcome the mucus interactions, a much higher release rate is needed. This would not only produce a much greater concentration gradient and therefore increase the flux of diffusion, but would also provide many more chlorhexidine dications to compensate for those which bind with the mucus. Alternatively, co-administration of a mucolytic agent may counteract the mucospissic effect produced by chlorhexidine, although use of mucolytics on guinea-pigs have been observed to compromise the integrity of the cervical mucus plug, allowing bacterial contamination of the uterus (Malhi *et al*, 1987). It is possible that a hollow fibre loaded with chlorhexidine and coated with hydrogel impregnated with chlorhexidine could bring about the desired effects, with an initial high rate of release followed by a slower more sustained delivery of the antimicrobial agent. It should be noted that although the actions of chlorhexidine in mucus were investigated *in vitro*

the hollow fibres were inserted into the uterine cavity, where uterine fluid would predominate. It is possible that the uterine fluid would modify/neutralize the actions chlorhexidine to a greater extent than mucus alone.

The release of the 2 progestins was clearly demonstrated in chapter 6. In the case of progesterone, where the same system as used for monitoring chlorhexidine release was employed, 2 nylon hollow fibres were able to demonstrate release of progesterone into the distilled water bathing fluid. The rate of release and the amount of progesterone released was then determined. It was noted that with these hollow fibres, a time lag was noted between placing the fibre into the bottle and detection of progesterone in the bathing fluid. This time lag was dependent upon the thickness of the hollow fibre wall. With levonorgestrel, initially 8 hollow polymer fibres were investigated, 4 nylon and 4 polyethylene, monitoring release using the same system as that for monitoring chlorhexidine release. However, throughout the 32 day course of the experiment, no levonorgestrel was detected in the bathing fluid with any of the hollow fibres. This led to the development of an alternative system for monitoring levonorgestrel release, involving the use of a glass 'U' tube filled with distilled water with the hollow fibre suspended below the level of the water at one side and a small volume of octanol at the other. This allowed minute quantities of levonorgestrel to diffuse from the hollow fibre, pass through the water and enter the octanol, such that the octanol acted as a 'sink' for the levonorgestrel since the levonorgestrel was more soluble in the octanol than in the water. This system showed that

levonorgestrel was able to diffuse out of the hollow fibre, and as with release of progesterone, a time lag was observed prior to detection of the steroid in the octanol.

The intraperitoneal injection of steroids in guinea-pigs has been shown to decrease total white blood cell counts (Malhi, 1991). This study determined white blood cell counts 40 days after insertion of steroid-releasing devices into the uterus to establish whether progestins released from the devices exerted a systemic effect. In each case, no significant effect was seen. Therefore, the small release of steroids from the hollow fibres did not appear to lead to an increase in uterine contamination by causing immunosuppression, although microorganisms were introduced during the insertion procedure. Examination of vaginal cytology was performed to determine the effect of the release of the progestins on the immediate environment. In the case of levonorgestrel no effect was seen, that is, no synchronisation of the oestrus cycle was apparent. This was also the case with the animals in which control devices had been inserted. However, in those guinea-pigs in which progesterone releasing devices had been inserted, all animals were in metoestrus. Since metoestrus only lasts for between 4 to 6 hours, findings suggest that the synchronisation of the oestrus cycle produced by progesterone release was a real effect. This was not confirmed by uterine histology, which may suggest higher doses are required.

The studies performed in this thesis have demonstrated that both progestins and chlorhexidine can be released from the hollow nylon fibres

in a controlled manner over a long period of time. This control of release rate and the length of time release occurs is related to the geometry of the device, the vehicle, the concentration of drug compound within the vehicle and the nature of the polymer itself. Because of the actual size of the device, it was able to be inserted through the guinea-pig cervix, and hence should not cause problems if used in women, especially postmenopausal women where the cervix is smaller.

The potential of this device as a means of drug delivery to the uterus has been demonstrated in these studies. Further investigation to determine the appropriate doses of progestin to produce the desired physiological effects are required, as is an investigation of a device loaded with a combination of a bacterial agent and a progestin.

## **9 APPENDICES**

## A.1 Validation of viable counting technique

To demonstrate the reproducibility of the viable counting technique, five dilutions of a cell suspension of *Bacillus subtilis* were plated onto nutrient agar plates and five replicate plates prepared for each sample and the data subjected to statistical analysis (Table A.1).

**Table A.1. Statistical analysis of five replicate viable counts. The numbers indicate the numbers of colonies per plate.**

Samples	Replicates					Total	Mean
	A	B	C	D	E		
1	42	42	61	47	40	232	46.4
2	42	32	34	62	57	227	45.4
3	47	55	33	41	46	222	44.4
4	55	55	43	45	58	256	51.2
5	43	64	44	70	52	273	54.6
<b>Total</b>	<b>229</b>	<b>248</b>	<b>215</b>	<b>265</b>	<b>253</b>		
<b>Mean</b>	<b>45.8</b>	<b>49.6</b>	<b>43.0</b>	<b>53.0</b>	<b>50.6</b>		

**Equation B1** =  $\sum x^2 = 60988$  - (i)

(Sum of squares of each individual count)

**Equation B2** =  $T^2/n = 58880.8$  - (ii)

(Mean of squared totals for within replicates).

**Equation B3** =  $\sum^2x/N = 58564$  - (iii)

(Grand total squared divided by the total number of counts).

Where, n = number of replicate plates per dilution = 5

m = number of dilutions = 5

N = total number of observations =25

**Table A.2. Analysis of variance of viable counts**

Source of variation	Sums of squares	Degree of freedom	Mean squares
Between counts	B2-B3 = 316.8	m-1 = 4	79.2
Within counts	B1-B2 = 2107.2	mn-m = 20	105.36

F = variance ratio =  $105.36/79.2 = 1.33$

Tabulated value for p at 0.05, with 4/20 degrees of freedom = 2.87

Therefore there is no significant variation between count and within count values obtained.

From the values obtained in table A.1, the coefficient of variation of the mean values within counts was 8.96 % and the coefficient of variation between counts was 8.22 %.

## A.2 Validation of white blood cell counting technique

Five dilutions of the same sample of blood were counted on an improved Neubauer haemocytometer, (section 2.8), and replicated 5 times. The data was then analysed statistically using the analysis of variance test.

**Table A.3. Statistical analysis of five replicate white blood cell counts. The numbers indicate the numbers of cells  $\text{mm}^{-3}$ .**

Samples	Replicates					Total	Mean
	A	B	C	D	E		
1	6800	6120	5560	7040	6920	<b>32440</b>	<b>6488</b>
2	7160	6960	6640	7400	4800	<b>32960</b>	<b>6592</b>
3	8600	6800	6000	6680	6760	<b>34840</b>	<b>6968</b>
4	4720	7040	8160	7400	7640	<b>34960</b>	<b>6992</b>
5	5320	6880	6960	7880	7600	<b>34640</b>	<b>6928</b>
<b>Total</b>	<b>32600</b>	<b>33800</b>	<b>33320</b>	<b>36400</b>	<b>33720</b>		
<b>Mean</b>	<b>6520</b>	<b>6760</b>	<b>6664</b>	<b>7280</b>	<b>6744</b>		

**Equation B1** =  $\sum x^2 = 1175660800$  - (i)

(Sum of squares of each individual count)

**Equation B2** =  $T^2/n = 1155484160$  - (ii)

(Mean of squared totals for within replicates).

**Equation B3** =  $\sum^2_{x/N} = 1153825024$  - (iii)

(Grand total squared divided by the total number of counts).

Where,  $n$  = number of replicates per dilution = 5

$m$  = number of dilutions = 5

$N$  = total number of observations = 25

**Table A.4. Analysis of variance of white blood cell counts**

Source of variation	Sums of squares	Degree of freedom	Mean squares
Between counts	B2-B3 = 1659136	$m-1 = 4$	414784
Within counts	B1-B2 = 20176640	$mn-m = 20$	1008832

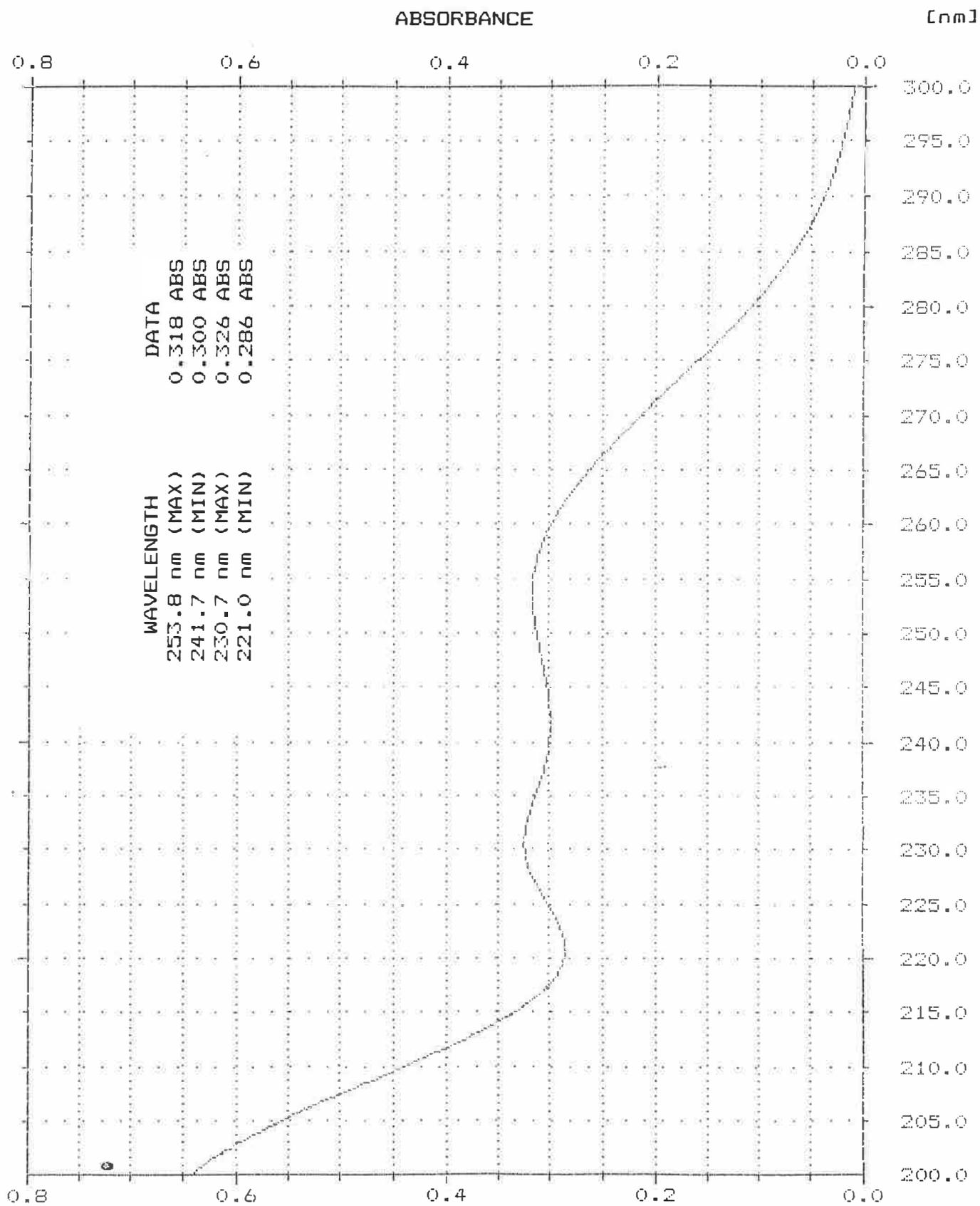
$F = \text{variance ratio} = 1008832/414784 = 2.43$

Tabulated value for  $p$  at 0.05, with 4/20 degrees of freedom = 2.87

Therefore there is no significant variation between count and within count values obtained.

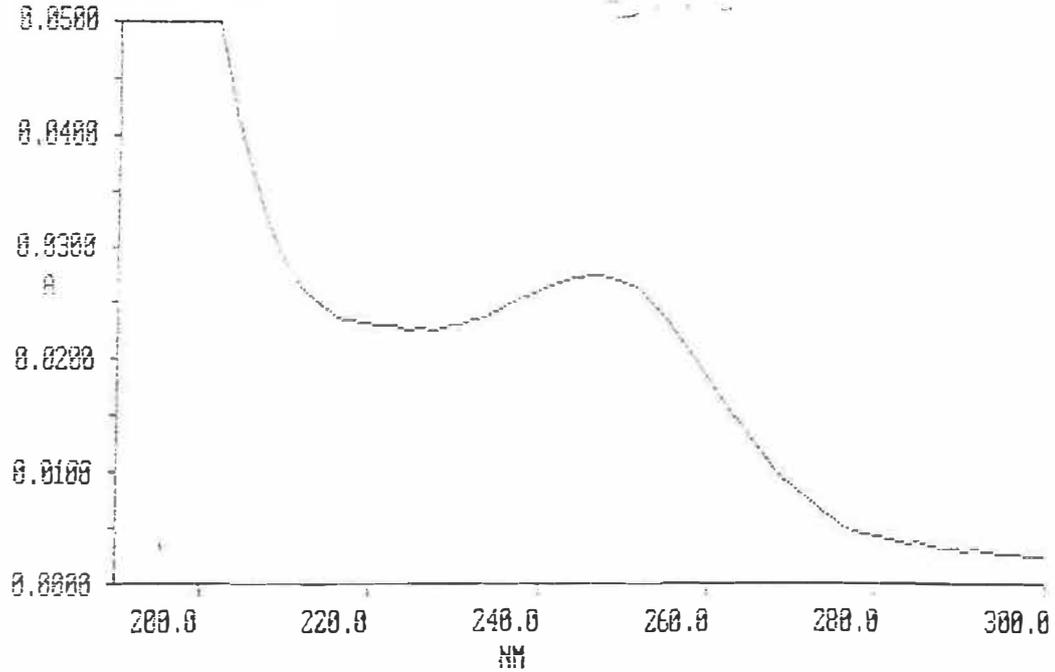
From the values obtained in table A.3, the coefficient of variation of the mean values within counts was 3.47 % and the coefficient of variation between counts was 4.24 %.

### A.3 Scan to show maximum absorbance wavelength for chlorhexidine



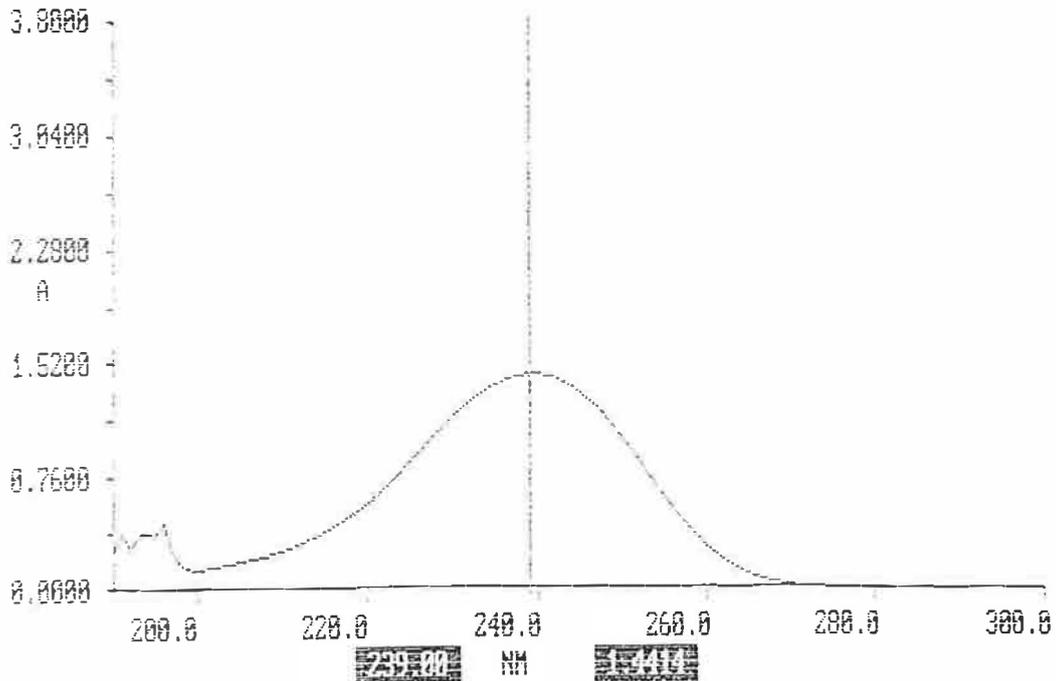
**A.4 Scan to show maximum absorbance wavelength for progesterone**

X: USER000; absc 300.0- 190.0; pts 111; int 1.00; ord 0.0024-0.1852; A  
inf: 12:39:04 98/03/09



**A.5 Scan to show maximum absorbance wavelength for levonorgestrel**

X: USER071; absc 300.0- 190.0; pts 111; int 1.00; ord -0.003-1.4414; A  
inf: 12:16:50 98/03/09



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