PROTEIN COMPOSITION OF RAT UTERINE FLUSHINGS

A Thesis Submitted for the Degree of

MASTER OF SCIENCE

by

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CONTENTS

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DECLARATION

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any other university, and to the best of my knowledge contains no material previously published by any other persons, except where due reference is made in the text of the thesis.

The results of this thesis have also been presented to the meeting of the Australian Society for Reproductive Biology in Brisbane, August 1976.

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SUMMARY

A major protein component of uterine fluid has been described for the rabbit in early pregnancy and pseduo--pregnancy (Beier et al, 1971 and Daniel, 1971). Its function may be related to uptake of steroids and/or nutrients by the unimplanted embryo (Fowler et al, 1976). This component has not been described for rats, although secretory activity by uterine epithelial and glandular cells early in pregnancy has been claimed (Nilsson, 1972) but this has been challenged (Enders and Nelson, 1973 and Parr and Parr, 1974).

Using SDS-gel electrophoresis, originally developed by Maizel (1966) and modified by Schnaitman (1974), uterine flushings were obtained at either pro-oestrus, oestrus, dioestrus or day 5 pseudopregnancy and analysed after the protein level had been measured by Lowry assay (Lowry et al, 1951). Electrophoretic profiles of the gels were subsequently recorded both visually and with an optical densitometer.

At pro-oestrus (n=4), 7-16 bands occurred of which large peaks had Rf values of 0.5, 0.6, 0.9 (Post-albumin) and 1.0 (albumin). At oestrus (n=5), 11-14 bands occurred, Rf values of large peaks being the same as pro-oestrus. At dioestrus (n=5) and on day 5 pseduopregnancy (n=5) uterine flushings had lower total protein so these were pooled and concentrated. Although 11-17 and 12-14 bands occurred respectively, there were only two large peaks with Rf values of 0.9 and 1.0.

Preliminary study involving ovariectomized animals given replacement therapy of 1.0μg of oestradiol - 17β/day for 10 days either with (n=3) or without (n=3) 1 mg of progesterone/day for the last 5 days resulted in 11-15 and 10-12 protein bands respectively. In three out of six samples, large peaks had Rf values of 0.5, 0.6, 0.9 and 1.0. Thus these profiles are similar to those of pro-oestrus and oestrus. However, when 5 mg of progesterone/day was given for the last 5 days, the prominent protein peaks 5 and 6 were not apparent, whereas ovariectomy alone only resulted in albumin being visible.

Finally, this project was extended to include the study of the effect of IUCD on the protein composition of the uterus on day 5 pseudopregnancy (n=4). Protein levels in uterine flushings demonstrate that the IUCD significantly increased the protein levels ($P<0.05$) confirming previous reports (Kar et al, 1964 and Breed et al, 1972). However, the electrophoretic profiles of uterine flushings obtained from control and IUCD horns demonstrated 7-15 and 11-14 bands respectively with large peaks only having Rf values of 0.9 and 1.0 in both horns. Thus no qualitative differences in protein components between two horns were apparent even though quantitative differences were found.

In conclusion, therefore, when all protein peaks are considered, comparing uterine proteins with plasma, Rf values of 0.1, 0.2, 0.3, 0.4, 0.5, 0.9, 1.0, 1.2, and 3.0 were found in both plasma and uterine flusings irrespective of the endocrine states, but peaks with Rf values of 0.6, 0.7, 0.8 and 1.4 were present only in uterine flushings.

When different endocrine states are compared, uterine flushings obtained at pro-oestrus and oestrus had two components (Rf values 0.5 and 0.6) in greater amounts than in flushings taken at dioestrus and day 5 pseudopregnancy. By comparison with protein standards the molecular weights of these components are about 103,000 and 94,000 respectively. No extra protein bands were found in day 5 pseudopregnancy flushings. Thus it may be that oestrogen induces the increase of two proteins in uterine fluid, whereas it appears that progesterone does not induce any extra protein components on day 5 pseudopregnancy.

1. INTRODUCTION

1.1 GENERAL

William Harvey was the first person to describe the presence of a uterine secretion in mammalian uterus in 1665 (cited by Heap and Lamming, 1961). At that time he commented that "it might be transported by the uterine arteries and distilled into the lumen". The comparative lack of information on the chemical composition of uterine fluid is presumably attributable to the small quantity of material available and subsequent recovery and analytical problems. Recent revival of interest in this subject is based on the work done by Ringler (1956, 1961); Junge and Blandau (1958); Albers and Neverse Castro (1961); Kunitake et al (1965); Breed et al (1972), and Peplow et al (1973). These investigators have worked on protein composition of rat uterine fluid obtained at pro-oestrus and oestrus when a measurable amount of uterine fluid is obtainable and the various workers have used a wide range of analytical and electrophoretic techniques. Electrolyte composition of the uterus has been studied in various species including the rat by Heap and Lamming (1961).

Besides the different analytical procedures used in the study of uterine secretion, a wide range of methods have been used in the collection of the fluid. These methods include direct aspiration, flushing the uterus, ligation of the cervix or double ligation at distant points followed by aspiration of accumulated fluid and collection by cannulation of the uterus. As a result, a comparative study of the results presented by various workers is difficult due to possible alteration induced in the composition of uterine

fluid by the methods used in its collection.

In spite of differences in technique, some understanding of the various processes involved in the formation of the uterine fluid has been achieved as a result of its analysis. Indeed, the formation of uterine fluid is not a simple process as described by William Harvey in 1665 as proteins not present in blood are found in uterine fluid. The composition of the uterine fluid, hence the intrauterine environment, is determined by complex interaction of factors some of which are under hormonal influence. Factors which could determine the composition of the uterine fluid in various hormonal states are (i) leakage from uterine capillaries; (ii) specific and non-specific reabsorption by the uterine epithelium; (iii) glandular secretions by the uterine glands and epithelial cells; (iv) inflow of oviductal fluid and (v) in the case of early pregnancy, the presence and secretion by developing the blastocyst (Hamner, 1971).

Various functional roles have been attributed to the uterine fluid: (i) the fluid may act as a source of nutrients for the spermatozoa and the developing embryo in the case of early pregnancy; (ii) endometrial secretory proteins and uterine enzymes may be involved in the metabolic activities of the spermatozoa and the developing embryo (Heap and Lamming, 1961); (iii) the uterine fluid may have anti-bacterial activity, thus preventing uterine infection (Broome and Lamming, 1959).

The present study involves three experiments in an attempt to gain insight into the protein composition of the rat uterine fluid. Firstly, protein profiles of uterine fluid obtained at different physiological states of the rat oestrous cycle and on day 5 pseudopregnancy have been performed.

The second experiment was to determine whether the protein profiles of the uterine flushings studied were obtained from ovariectomized female rats to which oestrogen and/or progesterone replacement therapy had been given. Finally, the effect of an intrauterine foreign body on the protein electrophoretic profiles of the uterus was studied. SDS-polyacrylamide gel electrophoresis was used in all the above investigations.

1.2 THE PROTEIN COMPOSITION OF THE UTERUS IN THE VARIOUS HORMONAL STATES OF THE OESTROUS CYCLE AND ON DAY 5 PSEUDOPREGNANCY

In sexually mature females, ovarian and pituitary hormones regulate the reproductive cycle. Despite the fact that the same hormones are involved, it appears that the hormonal patterns differ from one species to another resulting in the occurrence of different reproductive cycles. Thus it is not possible to describe a cycle which represents a typical female mammal as some have short cycles, some long and some have no cycles at all.

For instance, the rabbit is an induced ovulator without a reproductive cycle. By contrast, most other species are spontaneous ovulators with a characteristic reproductive cycle which may be either an oestrous or a menstrual cycle. Further, the reproductive cycle can be classified into two catagories on the basis of its length. In the first case, (e.g. sheep and woman) the life of the functional corpus luteum regulates the reproductive cycle, by determining the length of the luteal phase. By contrast, the second type of reproductive cycle, e.g. rat, is characterized by having a short oestrous cycle of four days, reflecting the absence

of a functional corpus luteum. The timing of events in a rat oestrous cycle is regulated photoperiodically.

Since this project involves the study of protein composition during the rat oestrous cycle, it is of relevance to consider the peripheral plasma pituitary and ovarian hormonal levels during the cycle. Oestradiol levels reach a maximum concentration of 40-50 pg/ml of plasma in the morning of pro-oestrus from a constant basal level of 7 pg/ml. The pituitary hormonal levels, namely LH, FSH and Prolactin, remain low during the cycle except for a characteristic pro-oestrous surge in the afternoon of pro-oestrus. Finally, the progesterone level is characterized by the occurrence of two peaks during the cycle. The first and smaller one occurs in the morning of dioestrus-2 while the second and more pronounced peak reaches a maximum concentration of 45-50 ng/ml and occurs in the afternoon of pro-oestrus, coinciding with that of the LH surge (Smith et al, 1975.)

Some information is available on the total protein concentration of the uterus during the reproductive cycle. At pro-oestrus uterine protein concentration is about 0.5 mg per ml or about 4% of that found in rat serum (Junge and Blandau, 1958; Breed et al, 1972), whereas at dioestrus it is reduced but this could be largely due to a less absolute amount of uterine fluid at this time. During pro-oestrus and early oestrus cervical constriction occurs under the influence of oestrogen resulting in the retention of uterine fluid. This in turn causes the distention of the uterus. However, during late oestrus, the cervix relaxes resulting in drainage of most of the uterine fluid from the uterus.

On considering the different proteins present, Junge and Blandau (1958) using paper electrophoresis, demonstrated the presence of only four major components in rat oestrous fluid and they failed to show a component similar to serum albumin. Albers and Neves e Castro (1961) using immuno--electrophoresis and Ouchterlony gel-diffusion techniques found five proteins in pro-oestrous and oestrous fluid. One of the protein components was considered to be uterine specific with migration similar to that of β-globulin while the rest of the proteins were common to both rat serum and uterine fluid.

Ringler (1961) also demonstrated the presence of five proteins in pro-oestrous uterine fluid by moving boundary electrophoresis, four of which were also found in serum and had mobilities similar to γ, β, α_2 and α_1 - globulin and albumin. Kunitake et al (1965) using Ouchterlony gel diffusion analysis with rabbit anti-rat serum showed that at least four proteins in rat uterine fluid were also found in the serum, while five of the nine protein components were uterine specific. However, it is worth noting that these authors carried out a surgical ligation of the uterine tubes at the distal and proximal ends. Further, 25μg of 17β-oestradiol was administered sub-cutaneously for three days to intact animals before obtaining the uterine fluid, and cycle state was not given.

Breed et al (1972) demonstrated by disc electro--phoresis the presence of four to nine protein bands in pro-oestrous and oestrous uterine fluid. However, they stated that unfortunately during the process of disc-

electrophoresis, losses and chemical alterations of the protein samples may have occurred. These problems may however be overcome by a quantitative immunoelectrophoretic technique. Accordingly, Peplow et al (1974) by using this technique demonstrated seven to nine precipitate bands using anti-(rat plasma) serum. This implies that seven to nine uterine proteins are of plasma origin.

When analysis for specific enzymes was performed Albers et al (1961) reported higher uterine peptidase activity in rat during pro-oestrous and oestrous stages of the cycle. Joshi et al (1970), isolated a proteolytic enzyme from uterine fluid obtained from pro-oestrous rat uterus. Further, the protein fraction containing this enzyme was associated with endopeptidase and sperm decapacitating activities. Joshi and Murry (1974) by immunofluorescent protein tracing demonstrated the presence of a rat uterine endopeptidase in the luminal epithelium and the epithelium of the endometrial glands of the uteri obtained from pro-oestrous and oestrous rats. Further, they also showed that it was uterine specific and oestrogen dependent. Finally, lysozyme activity in the rat uteri was measured by Parr et al (1967); other enzymatic activities in rat uteri by Breed et al (1972); and in women by Kar et al (1968).

Interuterine environment plays an important role in the development and the implantation of blastocyst during early pregnancy. In the rabbit the presence of a uterine specific protein during early pregnancy and pseudopregnancy has been well documented, (Beier and Beier-Hellwig, 1973);

Beier et al, 1971 and Daniel, 1971). This uterine specific protein in the rabbit is called the uteroglobulin or blastokinin and it may be a source of nutrition or act as a substrate for the metabolic processes that take place during the growth of the blastocyst.

Beier (1968) showed that uteroglobulin is the predominant fraction in the rabbit endometrial secretion from day 3 to day 8 of pregnancy or pseudopregnancy. Further investigations by Daniel (1969, 1971 and 1972), Urzua et al (1970) and Beier (1971) have confirmed the above findings. Krishnan and Daniel (1967 and 1968) independently isolated a glycoprotein from the uterus of the rabbit at early pregnancy and also demonstrated that this fraction might control blastulation of rabbit morula and stimulates blastocyst development in vitro. This finding was also confirmed by El-Banna and Daniel (1972a). Hence this glycoprotein fraction was named blastokinin and is apparently similar to uteroglobulin. The molecular weight of blastokinin was estimated to be approximately 27,000 daltons by amino acid analysis and by polyacrylamide gel electrophoresis (Krishnan and Daniel, 1968). Murray et al (1972) showed that blastokinin is composed of two sub-units. Various estimations of the molecular weight of the sub-unit ranging from 10-15,000 daltons have been reported, (McGaughey and Murray, 1972; Bullock and Connell, 1973).

It has been demonstrated that blastokinin is progesterone dependent (Arthur et al, 1972; Arthur and Daniel, 1972 and El-Banna and Daniel, 1972b). Further, the fact that the earliest

detection of blastokinin occurs on day 3 of pregnancy or pseudopregnancy which coincides with the beginning of progesterone secretion by the ovary, confirms the progesterone regulation of blastokinin synthesis (Hilliard et al, 1968). Hence, the secretion of blastokinin by the rabbit endometrium in early pregnancy may be considered as one of several ways through which the maternal or uterine environment regulates the early embryonic development. However, the occurrence of blastokinin appears not to be widespread and has so far only been reported in rabbit and northern fur seal (Daniel, 1972), but not in rat, man, baboon or mouse.

Another endometrial protein during early pregnancy was reported by Mintz (1971) in mice. She demonstrated the occurrence of a uterine lytic factor (IIF) in uterine fluid on day 3 of pregnancy. She suggests that its dependence on oestrogen may explain the appearance of this factor on day 3 of pregnancy, at a time when one might expect increasing oestrogen secretion. In this regard it differs from blastokinin in rabbit which is progesterone dependent. It is postulated that IIF may be involved in the triggering of implantation possibly by affecting molecules on the surfaces of blastocyst cells.

Finally, in the rat, Nilsson (1972), presented ultra--structural evidence in support of secretory activity in the uterine epithelium at pre-implantation stage. The morphological evidence was based on the number of vesicles present, enlargement of golgi apparatus and rough endoplasmic reticulum and the presence of both microvilli and cellular protrusions on the uterine epithelial surface. He suggested

that the protrusions secrete protein(s) which may be involved in the transmission of information to the blastocyst. Investigations by Enders and Nelson (1973) confirmed the above morphological findings. They demonstrated the occurrence of abundant large ectoplasmic projections into the uterine lumen prior to both implantation and during delayed implantation. But using tracer materials demonstrated pinocytosis or endocytosis rather than secretion of these projections. Parr and Parr (1974) came to similar conclusions to Enders and Nelson in their study since reabsorption of ferritin from the uterine lumen on day 5 of pregnancy occurs.

Since there is controversy about whether there is secretion of proteins in the rat, one of the aims of this study was to ascertain whether changes in uterine protein profiles occur during the various hormonal stages of the rat oestrous cycle. Such information is not available as previous investigators have restricted their study to biochemical composition of the uterine fluid obtained at pro-oestrus or oestrus. However, changes in the protein profiles during various stages of menstrual cycle have been reported in women by Shirai, Iizuka and Notake (1972) and Wolf and Mastroianni (1975) and in baboon by Peplow et al (1973).

1.3 EFFECT OF UNILATERAL IUCD ON UTERINE PROTEIN COMPOSITION DURING THE OESTROUS CYCLE AND ON DAY 5 PSEUDOPREGNANCY IN RATS.

Previous workers in this field have shown that the presence of the IUCD (intrauterine contraceptive device) causes changes in the protein and enzymic compcsition of uterine fluid in rat (Parr et al, 1967; Breed et al, 1972),

in baboons (Peplow et al, 1973) and in women (Kar et al, 1968 and Joshi and Sujan-Tejuja, 1969). This in turn may make the uterine environment hostile to fertilized ovum and so result in the prevention of implantation.

It appears that increase in total protein level in uterine fluid on the IUCD side in different species is an established fact. Kar et al (1968) reported that the occurrence of the elevation of uterine protein level by the IUCD is independent of the stage of the cycle. By contrast, although IUCD increased protein level in uterine fluid, it occurred in a cyclic fashion and independent of the stage of the menstrual cycle. Thus the above findings imply that IUCD may alter the response of endometrium to endogenous ovarian hormones (Joshi and Sujan-Tejuja, 1969; and Peplow et al, 1973). It is possible that the above contradiction may be attributed to different techniques used in the measurement of protein levels. The former authors measured protein levels by measuring nitrogen concentration while the latter authors used Lowry assay to measure protein levels.

The following factors may be responsible individually or collectively for the increase in protein levels in uterine fluid in the presence of IUCD. Firstly, it has been observed that in the rat, the volume of uterine fluid is reduced and its consistency altered in the presence of an IUCD. It is postulated that the reduction of uterine fluid volume may be due to dehydration effect resulting in the concentration of uterine protein (Kar et al, 1964).

Secondly, the presence of IUCD induces a mild inflammatory response in the endometrium, resulting in the alteration in vascular permeability of the endometrium (Greenwald, 1965). Such vascular changes may be responsible for the 'leakage' of plasma proteins into the uterine cavity. This in turn may cause an increase in protein levels in the uterus. Another consequence of inflammatory response is the leucocytic infiltration of the endometrium and the luminal fluid which occurs in the presence of an IUCD (Greenwald, 1965; Parr et al, 1967; Havranek, Dykova and Tichy, 1967; Sahwi and Moyer, 1971; and Breed et al, 1972). Thus the increase in total protein level in the presence of an IUCD could be due to the release and disintegration of cells such as polymorphonuclear leucocytes.

Finally, increase in total protein level in the presence of IUCD may be due to the device promoting intracellular anabolic or protein synthetic activity in the endometrium (Joshi and Sujan-Tejuja, 1969). The increased protein synthetic activity in the IUCD endometrium is attributed to a hypersensitivity of the endometrium to oestrogen or to the mild, transient local inflammation (Kleinman, 1968). Further, it has also been shown that the inhibitory influence of progesterone in the oestrogen induced stimulation of the synthetic activity in rat uterus is abolished in the presence of an IUCD (Joshi, 1967).

It has been postulated that the hostile uterine environment and the prevention of implantation in the presence of an IUCD may involve the synthesis of factor(s) which exert(s) toxic influence on ova and embryos or prevents

implantation by suppressing decidual cell response. The factor(s) may or may not be of protein origin. The fact that IUCD generally causes an increase in protein level strongly suggests that the anti-fertility factor(s) may be protein. Accordingly, some attempts have been made to study the qualitative differences in the uterine fluid in the absence or presence of IUCD.

Breed et al (1972) analysing pro-oestrous and oestrous rat uterine fluid failed to demonstrate any qualitative differences in the absence or presence of IUCD by disc-electrophoresis. However, possibly an extra protein band was detected in baboon uterine flushings fitted with IUCD by disc-electrophoresis (Peplow et al 1973). By contrast, immuno-chemical analysis with anti-serum raised against rat plasma, demonstrated a larger number of immuno-precipitate bands in pro-oestrous flushings obtained from IUCD horns than in the non-IUCD horn flushings (Peplow et al, 1974). This implies that a 'leakage' of plasma proteins into the uterine lumen or breakdown of polymorphs has taken place in the presence of an IUCD. Analysis of rabbit uterine fluid obtained during early pregnancy and pseudopregnancy by electrophoresis suggests that the presence of IUCD elevated the albumin and globulin proteins but slightly reduced the level of blastokinin (Johnson, 1972).

Since it has been shown quite clearly that IUCD increases the total protein levels in the uterus, one of the aims of this study was to determine whether correspond-ing qualitative changes in the protein composition of the uterus occur in the presence of an IUCD.

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL ANIMALS

Sexually mature, virgin female albino barrier strain rats, weighing between 200-250 grams were obtained from the central animal house at the Waite Agriculture Research Institute. They were then kept at the medical school animal house until use. The animals were housed in groups of three or four per cage and given food pellets (Charlick Feeds) and water *ad lib*. The animal house was maintained at a temperature of $24 \pm 5^{o}C$ and the lighting regime was 14 hours light, 10 hours dark. The animals were conditioned for at least a week in the medical school before use.

2.2 VAGINAL SMEARS

Vaginal smears, using a Pasteur pipette, were taken in the morning of the day of investigation. The pipette was introduced into the animal's vagina and the lumen was flushed with the saline. The saline, now containing suspended cells, was recovered by suction back into the pipette and a few drops were then placed in a microscope slide which was air-dried. The smear was stained with a 1% aqueous solution of methylene blue, and viewed under the microscope with a 10x objective for assessment of abundance of different cell types.

Three types of cells occurred in varying numbers. They were (i) cornified epithelial cells with irregular outlines and no nucleus and whose cytoplasm stained only slightly; (ii) nucleated epithelial cells which were oval shaped with a round nucleus and stained more deeply and (iii) leukocytes which were small, round, densely-staining cells with lobulated nucleus.

The cell types were scored quantitatively on a zero to four '+' scale as illustrated below.

0	none
+	very few
++	some
+++	many
++++	abundant

The following cell characteristics occurred for the various stages of the cycle (i) dioestrus smears had many leukocytes and few nucleated or cornified cells; (ii) pro-oestrous smears had mainly nucleated epithelial cells and (iii) oestrous smears had mainly cornified epithelial cells with few nucleated cells and no leukocytes.

2.3 SURGICAL PROCEDURES

The surgical procedures carried out in this project were ovariectomy and insertion of the intrauterine contraceptive device (IUCD).

These procedures were carried out under semi-sterile conditions. Surgical instruments used were sterilized in boiling water for twenty minutes. Then needles, surgical silk threads, michell clips, and the sterilized instruments were immersed in aqueous solution of zephiron (1:50) for at least twenty minutes before use. Surgical gloves were also used during surgical procedures.

The animals were anaesthetised with diluted Avertin (Tribromoethanol, Bayer, Surrey). 0.5 mls of concentrated Avertin was diluted in 20 mls of sterile physiological saline (1.40 parts). The dose administered was 1 ml per 100 gram body weight. The Avertin solution was first

warmed in a boiling water bath to dissolve the Avertin before being injected intra-peritoneally.

After the animal had been anaesthetised, the fur around the mid-ventral area of the abdomen was shaved and the exposed skin swabbed with diluted solution (1.10) of Hibitane (ICI Australia).

(i) Ovariectomy

A sterile drape was placed over the animal and an incision, approximately 3-4 cm long, was made along the mid-ventral region of the abdomen. The ovary was located and the ovarian artery and vein were then ligated. Both the ovary and the oviduct were removed. Similarly, the other ovary and oviduct were also removed. The incision of the peritoneal wall was subsequently closed with a 2.0 grade surgical thread and then the skin with michell clips.

(ii) IUCD insertion

The IUCD was inserted into one of the uterine horns as follows. After the incision was made at the lower mid-ventral portion of the abdomen, the uterine horns were located. The IUCD, a 3.0 grade surgical silk suture approximately 2-3 cm in length, was gently threaded through the uterine lumen. This was achieved by gently holding the uterine horn with a pair of forceps while the mesometrial wall of the horn was pierced by the needle. The needle was removed and both ends of the silk suture were knotted closely to the uterine wall to ensure that the IUCD remained in place. The other uterine horn was subjected to a sham operation which served as a control.

Post-operatively, the animals were kept warm for at least

the first 24 hours and then rested for about thirty days before being used for experiments.

2.4 OESTROGEN-PROGESTERONE REPLACEMENT THERAPY

After a period of thirty days, hormone replacement therapy was commenced by which time endogenous ovarian steroids would probably have been depleted in the bilaterally ovariectomized animals. Hormones used were Oestradiol (17β) Benzoate, (Evans Medical, England) and progesterone (Sigma). They were dissolved in absolute alcohol before adding peanut oil. The solution was then heated on a mechanical stirrer-hot plate until hormones were completely dissolved in peanut oil and alcohol had evaporated. Hormones were injected sub-cutaneously in the back of the neck of the rats. Dosages and schedules of hormone replacement therapy employed in this study are given in Table 2.1.

2.5 INDUCTION OF PSEUDOPREGNANCY

This procedure was based on the method developed by De Feo (1966). Pseudopregnancy was induced by stimulation of the cervix of those animals in either pro-oestrus or oestrus. This was accomplished using a modified electric tooth brush. The day of stimulation was designated as day 1 of pseudopregnancy, and vaginal smears were taken daily until day 5 of pseudopregnancy. Those animals demonstrating a constant dioestrous smear during days 2-5 of pseudopregnancy were killed and uterine flushings obtained.

2.6 FLUSHING TECHNIQUE

Animals were killed by an overdose of chloroform and an incision was made in the mid-ventral region of the abdomen so that the uterine horns were exposed. A 1.0 ml syringe and a 21 gauge needle were used in the flushing procedure. 0.5 ml of sterile physiological saline was introduced into the uterine lumen at the end nearest the ovary and the syringe was held in position with a haemostat. Am empty 1.0 ml syringe, with a similar 21 gauge needle was introduced into the uterine lumen at the cervical end and was similarly held in position. As the saline was being gently flushed from the ovarian end, the contents of the lumen were drawn up into the syringe at the cervical end. Uterine flushings so obtained were centrifuged at $4^{o}C$ for 10 minutes at 2,500 rpm. The supernatant was frozen and kept ready for analysis.

2.7 THE CONCENTRATION PROCEDURE

During the early stages of this study, it became apparent that uterine flushings collected at dioestrus and on day 5 of pseudopregnancy did not have sufficient total protein content for electrophoretic analysis. Therefore pooling of uterine flushings from 6 to 8 animals in the same hormonal state was performed and these were then run through a concentration procedure. The concentrated sample was then ready for analysis.

2.8 EFFECT OF CONCENTRATION ON PROTEIN PROFILE

Since there was enough protein for electrophoresis in pro-oestrous and oestrous flushings without concentration, it was necessary to determine whether concentrating of flushings of dioestrus and day 5 pseudopregnancy affected the electrophoretic profiles. Uterine flushings were obtained from

animals in oestrus. Flushings were pooled to make a sample of total volume of 6 mls. The sample was then divided into two portions of 1 ml and 5 mls respectively. The latter was concentrated fivefold in the Amnion B-15 concentrating cell. Total protein content of both unconcentrated and concentrated portions was determined by the modified Lowry assay. A sample of 50µg of SDS protein mixture was then loaded on to the gel for the electrophoretic analysis.

Optical densitometer tracings of the unconcentrated and the concentrated samples are illustrated in Fig. 2.1 and in Table 2.2. The results obtained indicate that the concentration procedure has no significant influence on the electrophoretic profiles although slight differences in Rf values and in peak heights were observed. However, the levels of the total protein measured by the modified Lowry assay indicate that the accuracy of the concentration procedure utilized in this study was questionable. Since the protein level of the unconcentrated portion was 0.8 mg/ml and after concentrating fivefold, the protein level was 2.6 mg/ml instead of the expected 4.0 mg/ml. Therefore in all subsequent procedures where concentrated dioestrus or day 5 pseudopregnant samples were used, some material was lost.

2.9 ANALYTICAL PROCEDURES

Analytical techniques carried out in this study were (i) quantitative measurement of total protein in uterine flushings by modified Lowry assay and qualitative measurement of protein in uterine flushings by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis.

(i) Modified Lowry Assay

The following steps were involved in the assay. 2 mls of Lowry Reagent C (see appendix) was added to 0.2 ml of protein sample. The mixture was then shaken well and allowed to stand for 10 minutes at room temperature. 0.2 ml of 1:1 diluted Folin and Ciocalteus Reagent E was added and mixed within a second or two. The final mixture was allowed to stand for 30 minutes or longer for colour to develop. The absorbance values of the samples were read in a Coleman Junior 11 model 6/20 spectrophotometer at wavelength 750 mμ. The total volume in this system was 2.4 mls. Normally the protein samples were diluted five or ten fold before analysis so that the protein levels of the samples would lie within the middle of the range of the standard curve. Dilution of the samples before analysis enabled one to economize on the uterine flushings, particularly when only minute amounts were available for analysis. The samples were analysed in duplicate.

Bovine serum albumin (BSA, Sigma) was used as a standard over a range of 0 to 100 μg/0.2 ml when each series of samples was analysed. A standard curve was plotted with the absorbance reading at 750 mμ along the Y-axis and the BSA concentrations ranging from 0 to 100 μg/ 0.2 ml was plotted along the X-axis. The total protein levels of the samples were calculated from this standard curve. The mean standard curve for BSA is illustrated in Table 2.3 and graph 2.1 respectively.

(ii) SDS-Polyacrylamide Gel Electrophoresis

This technique was originally developed by Maizel (1966) and modified by Schnaitman (1973, and 1974).

Firstly, the SDS-protein complex was formed by reacting one volume of protein sample with two volumes of solubilizing solution 'A' (see appendix). The reaction mixture was thoroughly mixed and sealed. It was then incubated at 37^{o}C for two hours and was then ready for use. It could be frozen and stored if necessary.

7.5% maize gels were used in the electrophoretic analysis. Such a gel preparation has a molecular weight range of 14,000 to 120,000 daltons. The gel preparation contained the following components: 7.5% acrylamide (Sigma), 3.0% Urea (May and Baker, Dagenham England) and 0.2% N, N-methylene bisacrylamide (Eastman Kodak, N.Y.). They were dissolved in 100 mls of gel buffer solution C (see appendix) at room temperature. The preparation was stored in an amber glass container which protects the gel solution from direct light.

Pyrex glass tubes with internal diameter of 5 mm and lengths of 12 cm were used for electrophoresis. They were placed in the electrophoretic apparatus with one end of the tube being sealed gently with a piece of clear plastic cling wrap, which was held in place by a rubber band.

Normally, 0.09 ml of one-tenth diluted TEMED or N-N-N-N Tetramethyl 1-2 Diaminoethane (BDH Chemicals, Poole, England) and 0.12 ml of 50 mg/ml of ammonium per sulphate (Ajax analytical reagent) were added to a volume of 18 mls of the gel preparation. The mixture was then

transferred to the glass tubes to a height of 10 cm and then overlaid with a few drops of butan-1-01 (BDH Chemicals, Poole, England). The tubes were then left to stand at room temperature for about an hour to allow polymerization to occur. The amount of persulphate was adjusted to give polymerization in not less than 30 minutes and not more than 60 minutes, at ambient room temperature. After polymeriza--tion, the pieces of clearwrap were removed, and the tops of the tubes were rinsed a few times with buffer C.

The gels were either used immediately or within a day or two of polymerization. For electrophoresis, gel tubes were placed in the electrophoretic apparatus and both chambers of the apparatus were filled with buffer C. The SDS-protein samples were mixed with 0.2% bromophenol blue indicator in 50% sucrose, and were then injected on top of the gels with a Hamilton syringe, the dye serving as an indicator of the front of the moving zone. The 50% sucrose provided the density for the protein sample which enabled it to form a uniform zone above the analytical gel. Wherever possible, 50µg of SDS-protein samples were loaded on to the gels. The gels were then run at 5 mA/gel for about 6-7 hours or until the bromophenol blue dye had travelled to the end of the tube. The whole electrophoretic apparatus during the run was maintained at constant temperature of 29^{o}-30^{o}C by immersing it in a water bath.

Once the bromophenol blue indicator had reached the end of the tubes, the electrophoresis was stopped. The gels were then removed from the glass tubes by spraying a jet of water between the inner walls of the tubes and the gels. The gels were stained overnight with 0.25% Coomassie blue (DIFCO, Surrey UK) dissolved in water containing

45% methanol and 10% glacial acetic acid which also performed the function of fixing the SDS-protein bands. The following morning the gels were destained for 24 hours by using several changes of water containing 25% methanol and 7.5% glacial acetic acid (destaining solution). The gels were then stored in water containing 7.5% acetic acid. Electrophoretic profiles of the gels were recorded diagramatically under a viewer and an optical densitometer tracing of the gels made at wavelength 525mμ using a Quickscan (Helena Lab. Corp. Beaumont Texas). Those SDS-protein peaks observed in the densitometer tracing corresponding to the bands observed under the viewer were considered as authentic ones and marked accordingly on the tracing, while the rest of the peaks were classified as artefact (see Fig. 2.2).

During development of this method, the following preliminary investigations were carried out. Firstly, the effect of loading varying concentrations of protein samples on the electrophoretic profiles was studied. The sample used in this study was a pro-oestrous uterine flushing with a protein concentration of 1.2 mg/ml. The concentration of the protein samples loaded on the gel ranged from 25μg to 160μg per gel and the diagram of the electrophoretic profiles illustrated in Fig. 2.3.

The results in Fig. 2.3 suggested that the minimum concentration of protein sample required was 50μg. Above this concentration, the electrophoretic profiles were similar, but with the increase of protein concentration, the widths of the existing bands increased proportionally.

However, this may also be due to the increase in volume of sample loaded per gel. But the most likely effect of increasing volume loaded was to make the zone front more diffuse resulting in poorer resolution. Below 50µg of SDS-protein sample, the number of bands of the electrophoretic profiles were markedly reduced. Thus taking into account the average protein concentrations in various uterine flushings and the effect of protein concentration on the electrophoretic profiles, it was decided that 50µg of protein sample would be loaded each time.

A comparative study of the staining properties of Coomasie Blue and Amido Black (Merek, West Germany) was undertaken. Two samples, namely, unconcentrated and fivefold concentrated uterine flushing obtained at oestrus were used. The results obtained are illustrated in Fig. 2.4 and 2.5 respectively. In the case of unconcentrated samples, two very small extra peaks were detected when Coomasic Blue stain was used. But in the case of concentrated sample, no differences between the two stains were detected.

2.10 EXPERIMENTAL DESIGN

(i) Study (a) - Uterine protein composition in the various stages of the oestrous cycle and on day 5 pseudo-pregnancy.

On the basis of the vaginal smears, the animals were divided into three groups comprising animals at pro--oestrus, oestrus and dioestrus respectively, while the fourth group comprised of animals at day 5 pseudopregnancy. The animals were killed and the uterine flushings obtained as previously described. Since the protein levels in

dioestrous and day 5 pseudopregnant uterine flushings were not sufficient for electrophoretic analysis, such samples were pooled and concentrated five or ten fold.

(ii) Study (b) - The effect of oestrogen and/or progesterone replacement therapy on uterine protein composition of overiectomized rats.

The details of doses and the schedule of the hormone replacement therapy have been described previously.

(iii) Study (c) - The effect of IUCD on the protein composition of the uterine fluid in the various endocrine states of oestrous cycle and on day 5 pseudopregnancy.

Rats fitted with unilateral IUCD were divided into three groups. Groups 1 and 11 were made up of animals on oestrus and on dioestrus. Group 111 consisted of animals on day 5 pseudopregnancy.

Total protein levels of all the samples were calculated by modified Lowry assay. A constant amount of 50µg of protein sample was loaded per gel. However, in some cases less than 50µg of protein were loaded per gel because of insufficient protein levels. Such samples are indicated in the result section.

Rat plasma and a mixture of five standard proteins were included as references with each series of samples analysed electrophoretically. The five standard proteins used were transferrin, BSA, carbonic anhydrase, ovalbumin and haemoglobin. Table 2.4 illustrates the mean Rf values of the respective protein standards. By plotting a graph with molecular weight, on log scale, against mean Rf values of the protein, the molecular weight of the Rf values of the

electrophoretic profiles of uterine flushings and plasma could be ascertained. Such a representative graph is illustrated in graph 2.2 and a typical electrophoretic tracing in Fig. 2.6.

Table 2.1 - Dosages and schedule of oestrogen and progesterone replacement therapy.

DAYS	GROUP I (Control)	GROUP II	GROUP III	GROUP IV
1	0.1ml peanut oil	1µg 17β oestradiol/ 24hr	1µg 17β oestradiol/ 24hr	1 g 17 oestradiol/ 24hr
2	"	"	"	"
3	"	"	"	"
4	"	"	"	"
5	"	"	"	"
6	"	"	1µg 17β oestradiol + 1mg progesterone/24hr	1µg 17β-oestradiol + 5mg progesterone/ 24hr
7	"	"	"	"
8	"	"	"	"
9	"	"	"	"
10	"	"	"	"
11	Animals killed and the uterine flushings obtained.			

Table 2.2 - Unconcentrated Vs 5x concentrated uterine flushings.

PEAK NO.	Rf VALUE unconcentrated	concentrated	HEIGHT OF PEAK IN MM unconcentrated	concentrated
1	0.12	0.13	1	1
3	0.33	0.28	5	3
4	0.41	0.37	1	1
5^1	0.49	0.47	16	13
5^2	0.53	0.52	14	8
6	-	0.63	-	9
7^1	0.65	-	8	-
7^2	0.73	0.73	1	1
8	0.82	0.78	4	4
9	0.92	0.88	23	22
10	1.00	1.00	15	9
11	1.22	1.22	5	2

Fig. 2-1 The Effect of Concentration Procedure on Electrophoretic Profile of Uterine Flushings.

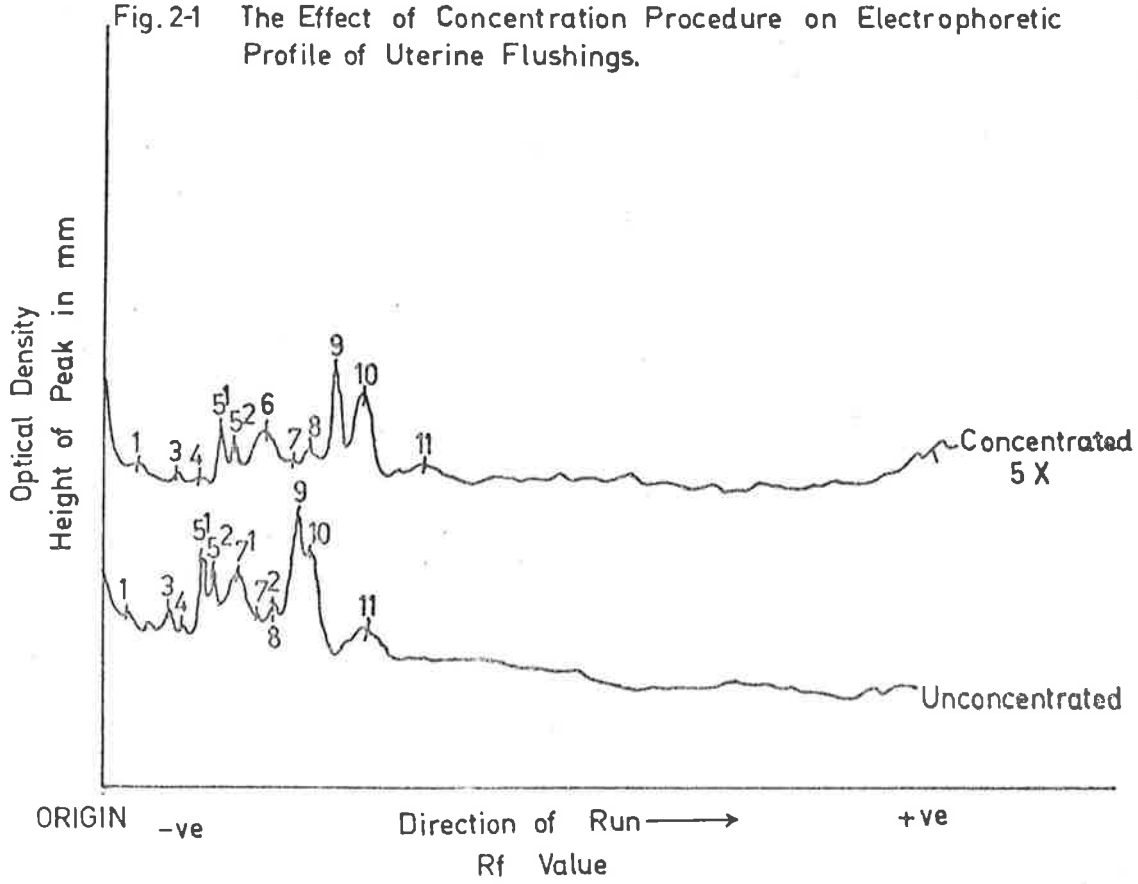


Table 2.3 - Mean BSA standard curve for Lowry Assay

(BSA)/0.2ml	Number of Samples	Mean absorption reading at 750mμ
20μg	37	0.12 ± 0
40μg	37	0.22 ± 0
60μg	37	0.32 ± 0
80μg	37	0.41 ± 0
100μg	37	0.49 ± 0

Graph 2-1 Standard Curve for BSA

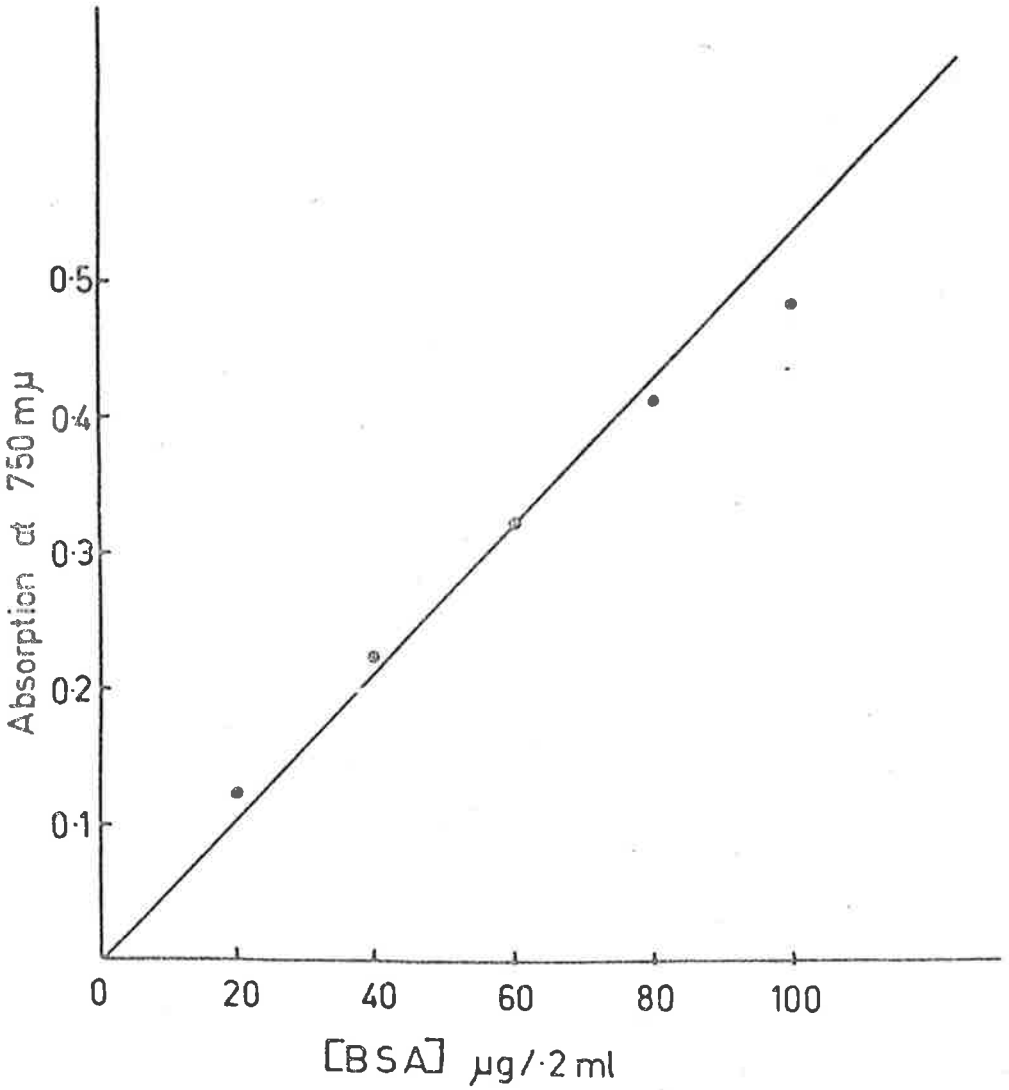


Table 2.4 - Electrophoretic profile of standard protein mixture.

Protein Standard	Mol. Weight	Number of Runs	Rf value
Transferrin	86,000	10	0.75± 0.00
BSA	68,000	10	1.00± 0.00
Ovalbumin	43,000	10	1.35± 0.02
Carbonic anhydrase	29,000	10	1.91± 0.02
Haemoglobin	15,500	10	2.74± 0.05

Graph 2-2 Standard Curve Mol. wt. of Proteins Vs Rf Values.

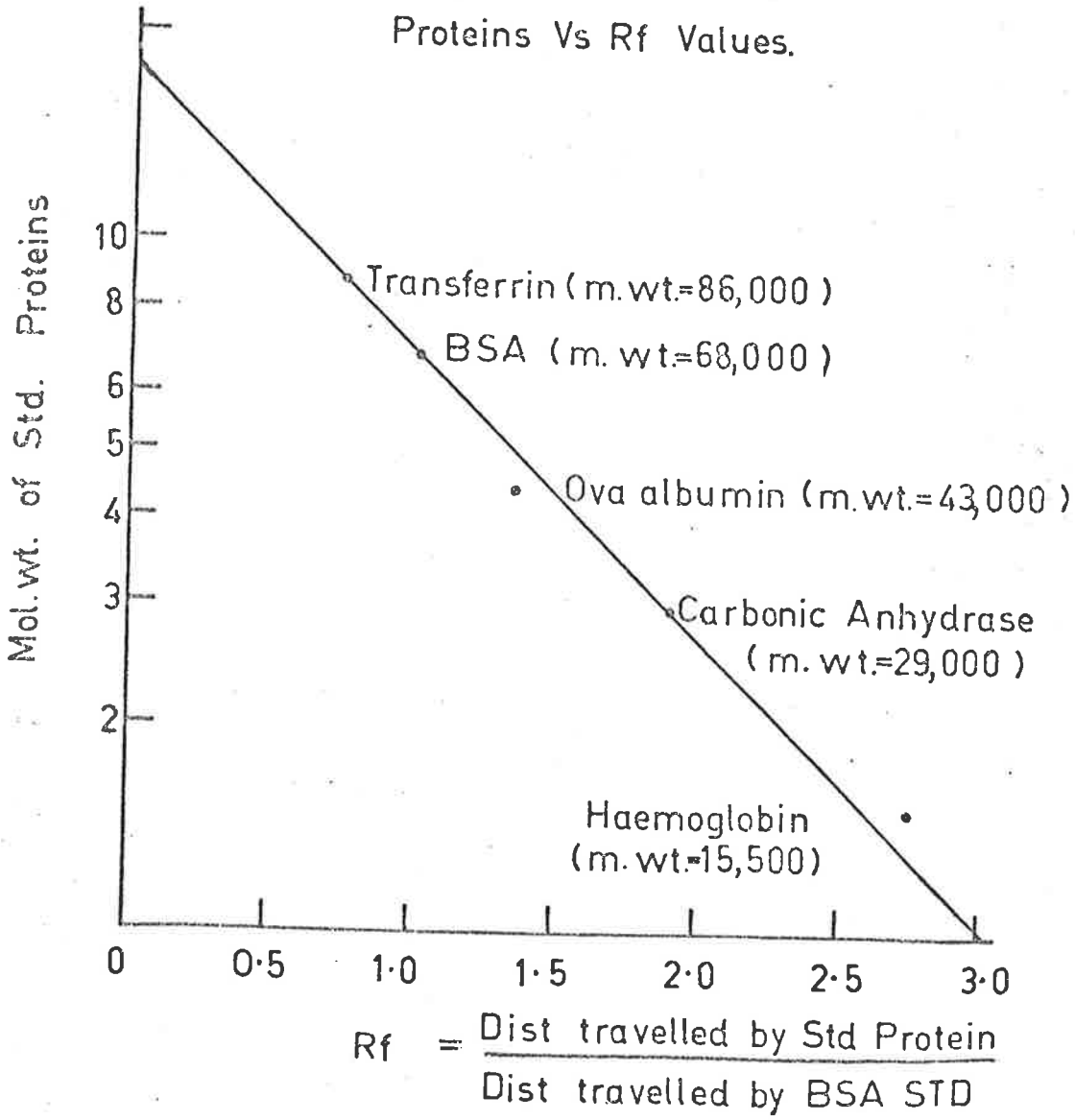


Fig. 2-2 The comparison of Densitometer tracing with that observed under a viewer.

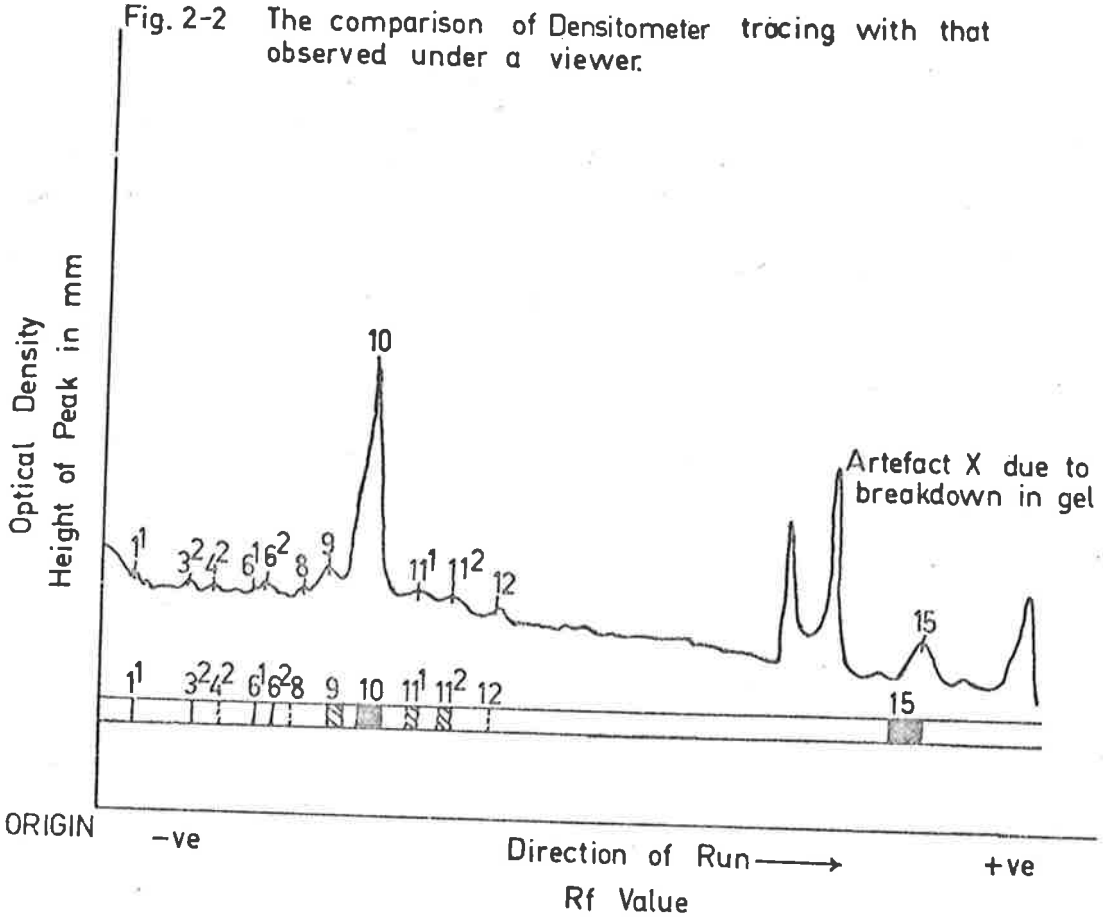
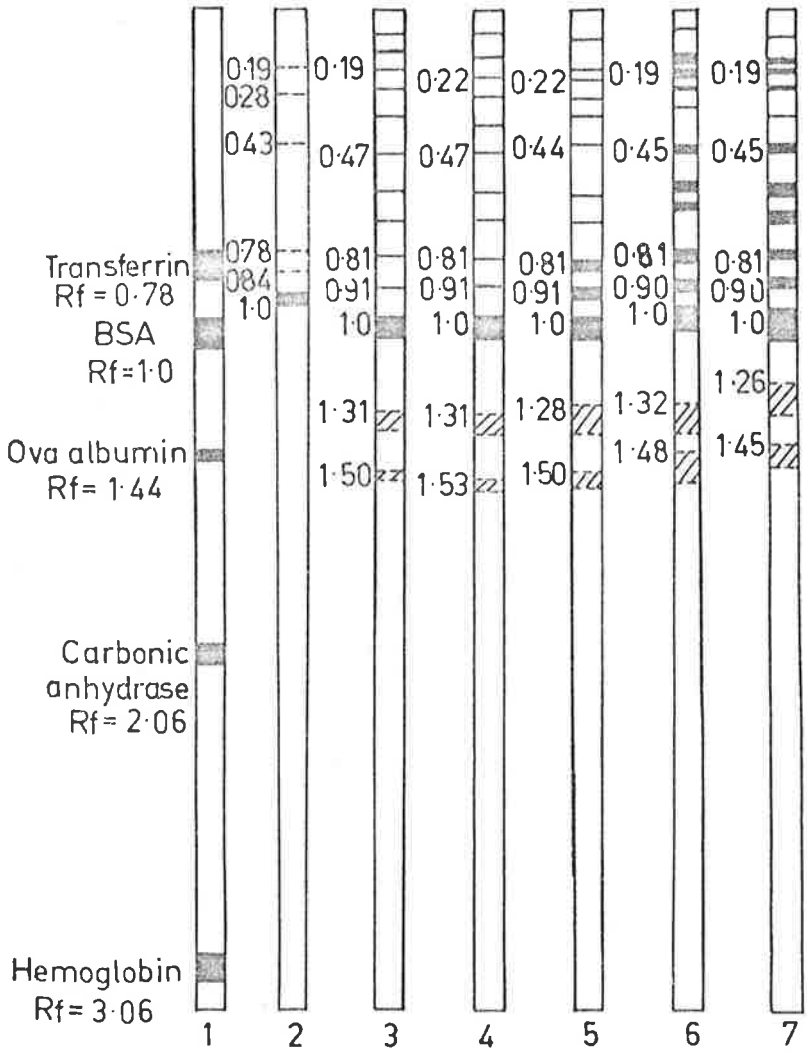


Fig. 2-3 Electrophoretic profiles of protein samples of concentration ranging from 25 µg to 161 µg.



PROTEIN loaded on gel	25µg	50µg	75µg	100µg	125µg	161µg
Vol. Loaded	21µL	42µL	63µL	84µL	105µL	136µL

Sample used was Proestrus uterine flushing of Protein = 1·2 mg/ml.

KEY: --- small or weak band
—— medium intensity band
▬ dominant band (large)
▨ small broad band

Fig. 2-4 A comparative study of staining properties of Amido Black and Coomassic Blue Dyes using unconcentrated Uterine Flushings.

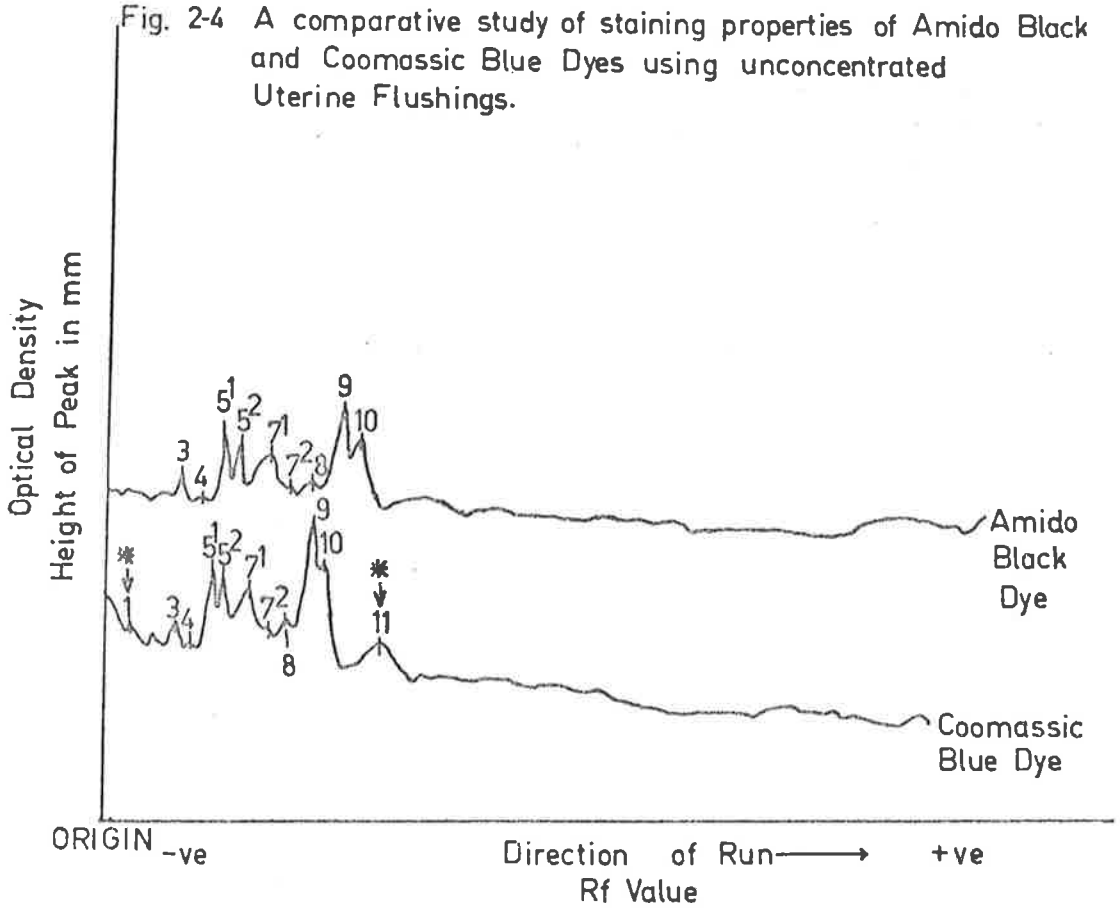


Fig. 2-5 A Comparative Study of Staining Properties of Amido Black and Coomassic Blue Dyes using Concentrated Uterine Flushings.

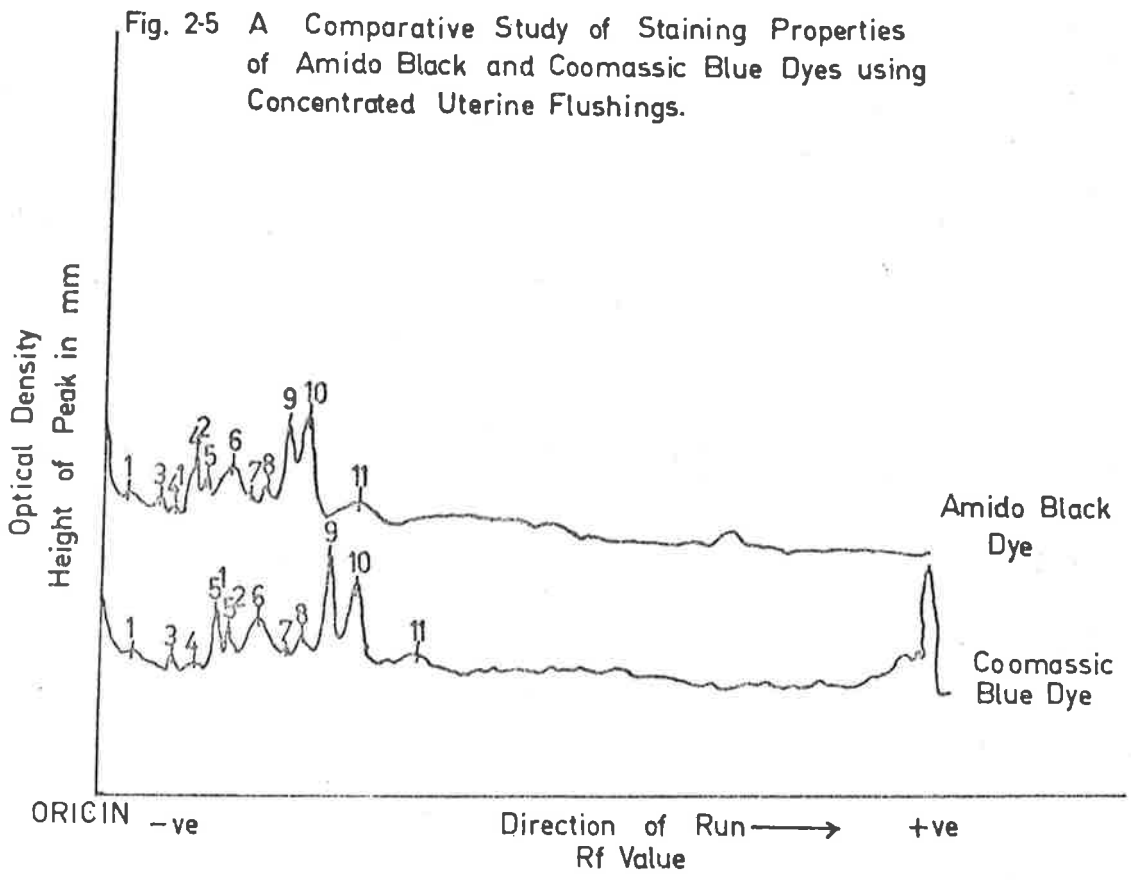
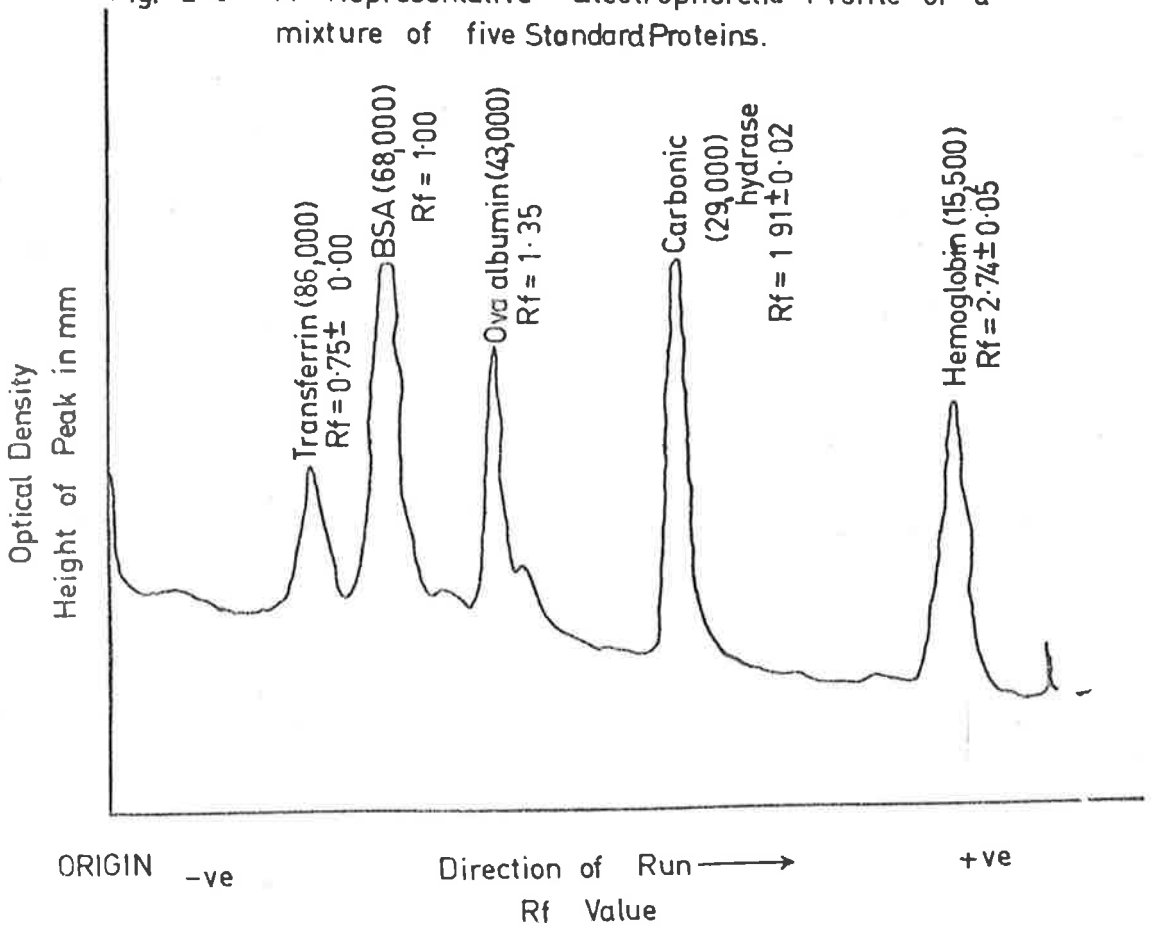


Fig. 2-6 A Representative Electrophoretic Profile of a mixture of five Standard Proteins.



3. RESULTS

3.1 AN ARBITARY CLASSIFICATION OF GEL ELECTROPHORETIC PROFILES.

An arbitary classification system of the Rf values was developed to describe the protein peaks which constitute the polyacrylamide gel profiles. This sytem is illustrated in Key 3.1

An extensive study of the profiles of the samples analysed demonstrated the invariable presence of a peak which had an electrophoretic mobility the same as that of BSA standard. Hence this peak was assumed to be albumin. With the exception of the profiles of three oestrous samples, the albumin peak was the dominant peak of uterine flushings and blood plasma. The albumin peak was assigned an Rf value of 1.0 and the rest of the protein peaks were assigned Rf values in relation to this.

With this arbitary classification system, the length of the gels were divided into fifteen zones. Each zone was represented by Rf value with standard error of $\pm$ 0.05. These zones were assigned Rf values in an ascending order from the origin of the gel. There were some instances where more than one band was present in a particular zone. Thus in theory it implies that more than one protein peak may have the same Rf value.

3.2 THE PROTEIN COMPOSITION OF THE UTERUS IN THE VARIOUS HORMONAL STATES OF THE OESTROUS CYCLE AND ON DAY 5 PSEUDOPREGNANCY

Table 3.1 shows the protein concentrations and the number of SDS-protein components present in the plasma and uterine flushings obtained at different endocrine states.

Representative optical densitometer tracing of uterine flushings are illustrated in Figs 3.1, 3.2, 3.3 and 3.4 respectively. Rf values, corresponding peak heights and frequency of occurrence are illustrated in Tables 3.2. 3.3, 3.4, and 3.5. In the analysis of the electrophoretic profiles, the peak height of the protein component may be used to indicate the percentage composition of that protein fraction to the total as determined by the Lowry assay.

The following characteristics emerge from the electro--phoretic profiles:

(i) In zones 1, 2, 3 and 4 a variable number of small peaks occurred, except in one case involving an oestrous sample.

(ii) In zones 5 and 6 peaks were usually significantly elevated at pro-oestrus and oestrus with one exception. Usually the greatest peaks occurred at oestrus, whereas only trace amounts at dioestrus and on day 5 pseudopregnancy occurred.

(iii) In zones 7 or 8 (or both) a peak corresponding to that of transferrin standard (Rf=0.75) was detected in two out of the four pro-oestrous samples (Rf=0.70 and 0.78) and three out of the five oestrous samples (Rf=0.74, 0.75 and 0.76). By contrast in only one out of the five dioestrous samples (Rf=0.75) and in two out of the five days pseudopregnant samples (Rf=0.70 and 0.71) were detected.

(iv) Component 9 was elevated in three out of the five oestrous flushings compared to other endocrine states. Further, in the above oestrous samples, peak 9 was the dominant peak, while the albumin peak had the dominant peak in plasma and the rest of the uterine flushings regardless of the endocrine states.

(v) In zone 11 two very small peaks were usually present.

(v) In zone 12 another small component occurred with the exception of two oestrous samples.

(vii) In zone 15 a protein component was detected in some uterine flushings regardless of hormonal states. The Rf value of this peak corresponds approximately to that of haemoglobin standard.

3.3 PROTEIN COMPOSITION OF THE UTERUS OF OVARIECTOMIZED RATS WITH AND WITHOUT OESTROGEN AND PROGESTERONE REPLACEMENT THERAPY

Table 3.6 shows the protein levels and range of protein components in uterine flushings obtained from ovariectomized control and hormone treated groups. Representative optical densitometer tracings of the uterine flushings are illustrated in Figs. 3.5, 3.6, 3.7 and 3.8 and a detailed analysis is presented in Tables 3.6, 3.7, 3.8 and 3.9.

In the ovariectomized group with no replacement therapy, atrophy of the uterus was observed. This may be related to the very low protein levels in the uterine

flushings (0.03± 0.01 mg/ml). Unfortunately, due to the very low protein levels, less than the normal 50µg SDS-protein mixture were loaded on to the gel for analysis, thus comparisons of valid protein profiles are difficult to make. However, the presence of a faintly staining peak corresponding to that of albumin was seen.

In the group given only 17β-oestradiol, significantly higher protein levels (0.75 ± 0.20 mg/ml) occurred in the uterine flushings than the ovariectomized group ($p<0.05$). Similarly, significantly higher protein levels (0.33 ±0.04 mg/ml) were measured in uterine flushings obtained from both the combined 17β-oestradiol and progesterone treated groups than the ovariectomized group ($p<0.01$). However, no statistically significant differences in protein levels were observed among the treated groups although progesterone tended to have a depressing effect.

Apart from protein with Rf value of 1.0 (presumed albumin) peaks were not very high. In female rats given only 17β-oestradiol or 17β-oestradiol and 1mg of progesterone, the small peaks in zones 1 to 4 usually occurred. Likewise in zones 5, 6, 8 and 9, peaks were usually visible when 17β-oestradiol alone or 17β-oestradiol and 1 mg of progesterone were given. However, 5 mg of progesterone appeared to reduce or eliminate most of the peaks.

3.4 EFFECT OF UNILATERAL IUCD ON PROTEIN COMPOSITION DURING THE OESTROUS CYCLE AND ON DAY 5 PSEUDOPREGNANCY

Table 3.11 shows the protein levels and the range of the protein bands present in the uterine flushings obtained from the control and experimental IUCD horns on day 5 pseudopregnancy. Representative optical densitometer

tracings of the uterine flushings are illustrated in Figs. 3.9 and 3.10 and detailed analysis is presented in Tables 3.12 and 3.13.

The following characteristics were observed.

(i) The presence of an IUCD significantly elevated the protein levels in uterine flushings ($p<0.05$).

(ii) Generally, no obvious differences in the profiles can be detected between the control and experimental IUCD horn uterine flushings.

(iii) Protein peak 14 was detected in 4 out of 12 uterine flushings obtained from IUCD horns. By contrast, this peak was not detected in any of the uterine flushings obtained from the control horns of the same animal.

3.5 ELECTROPHORETIC PROFILE OF RAT PLASMA

Representative optical densitometer tracings of plasma is illustrated in Fig. 3.11 and detailed analysis is represented in Table 3.14.

The number of protein bands ranged from 10 - 12 bands. The first two peaks located in zones 1 and 2 were faintly stained. These two faint peaks were followed by two prominent peaks located in zones 2 and 3. This was followed by the presence of three faintly staining bands located in zones 4 and 5. No bands were observed in zones 6, 7 and 8. Protein bands were present in zones 9 and 10. The latter was the most dominant peak with Rf value corresponding to that of BSA standard. As observed in the uterine flushings, two bands with the same Rf values were usually detected in zone 11. In 2 out of the 8 plasma profiles, a faintly staining band was detected in Zone 13, while a faint

band in zone 14 was detected in all the plasma profiles. Finally, in three plasma profiles, a protein component corresponding to that of haemoglobin standard was detected in zone 15.

3.6 COMPARISON OF THE ELECTROPHORETIC PROFILES OF THAT OF RAT PLASMA AND UTERINE FLUSHINGS

(i) Protein components 1, 2, 3, 4, 5, 9, 10, 11, and 15 appear to be common on both rat plasma and uterine flushings irrespective of the physiological state. However, protein components 2 and 3 were elevated in plasma profiles compared to uterine flushings.

(ii) Proteins in zones 6, 7, 8, and 12 were only observed in the uterine flushings but not in the plasma indicating these proteins are of uterine origin.

(iii) Conversely, proteins in zones 13 and 14 were observed only in the plasma but not in the uterine flushings. Further the protein in zone 13 was only detected in two out of the eight plasma samples.

Key 3.1 - Arbitary classification of Rf values of the electrophoretic profiles.

Peak Number	Range of Rf Values
1	0.10± 0.05
2	0.20± 0.05
3	0.30± 0.05
4	0.40± 0.05
5	0.50± 0.05
6	0.60± 0.05
7	0.70± 0.05
8	0.80± 0.05
9	0.90± 0.05
10	1.00± 0.00
11	1.20± 0.05
12	1.40± 0.05
13	1.60± 0.05
14	2.00± 0.05
15	3.00± 0.05

Table 3.1 - The protein levels and the range of protein bands in uterine flushings obtained at different endocrine states.

Physiological State	No. of samples	[Protein] in flushes mg/ml	Number of bands	Comments
(Rat plasma)	8	1.75	10-12	40 dilution
Pro-oestrus	4	0.65± 0.18	7-16	One sample pooled from 2 animals
Oestrus	5	0.86± 0.17	11-14	One sample pooled from 6 animals and concentrated 5 fold. In a further 2 samples, the oestrous fluid aspirated from lumen.
Dioestrus	5	*0.24 0.60± 0.07	11-17	All samples pooled and concentrated 10 fold.
D_5 Pseudo-pregnancy	5	*0.38 0.93± 0.07	12-14	All samples pooled; 1 sample concentrated 5 fold and 3 samples concentrated 10 fold.

* Unconcentrated uterine flushings.

Table 3.2 - Electrophoretic profiles of pro-oestrous uterine flushings (n=4).

Peak No.	Range of Rf Values				Frequency of occurrence	Height of Peak in mm			
	1	2	3	4		1	2	3	4
1	0.10	-	-	0.14	2/4	1	-	-	1
2	0.17	0.15	-	-	2/4	4	1	-	-
2^2	0.22	0.23	-	0.24	3/4	3	2	-	1
3	0.28	0.26	-	0.29	3/4	4	3	-	1
4^1	0.37	0.35	-	0.35	3/4	3	6	-	3
4^2	-	0.42	-	0.41	2/4	-	3	-	2
5	0.45	0.51	0.53	0.49	4/4	10	16	2	10
6	0.58	0.63	0.64	0.61	4/4	19	20	5	8
7	0.67	-	0.70	0.69	3/4	12	-	1	6
8^1	0.78	0.81	-	0.84	3/4	10	6	-	9
8^2	0.83	-	-	-	1/4	10	-	-	-
9	0.88	0.90	0.85	-	3/4	11	6	8	-
10	1.00	1.00	1.00	1.00	4/4	45	38	41	30
11^1	1.23	1.16	1.15	-	3/4	2	1	3	-
11^2	1.32	1.26	-	1.29	3/4	4	4	-	2
12	1.52	1.45	1.38	1.47	4/4	4	4	2	2
15	3.18	-	-	3.00	2/4	17	-	-	8

Where 1, 2, 3, and 4 refer to the electrophoretic runs of the four individual samples

Fig. 3-1 An Optical Densitometer Tracing of a Representative Electrophoretic Profile of Uterine Flushing in Pro-oestrus.

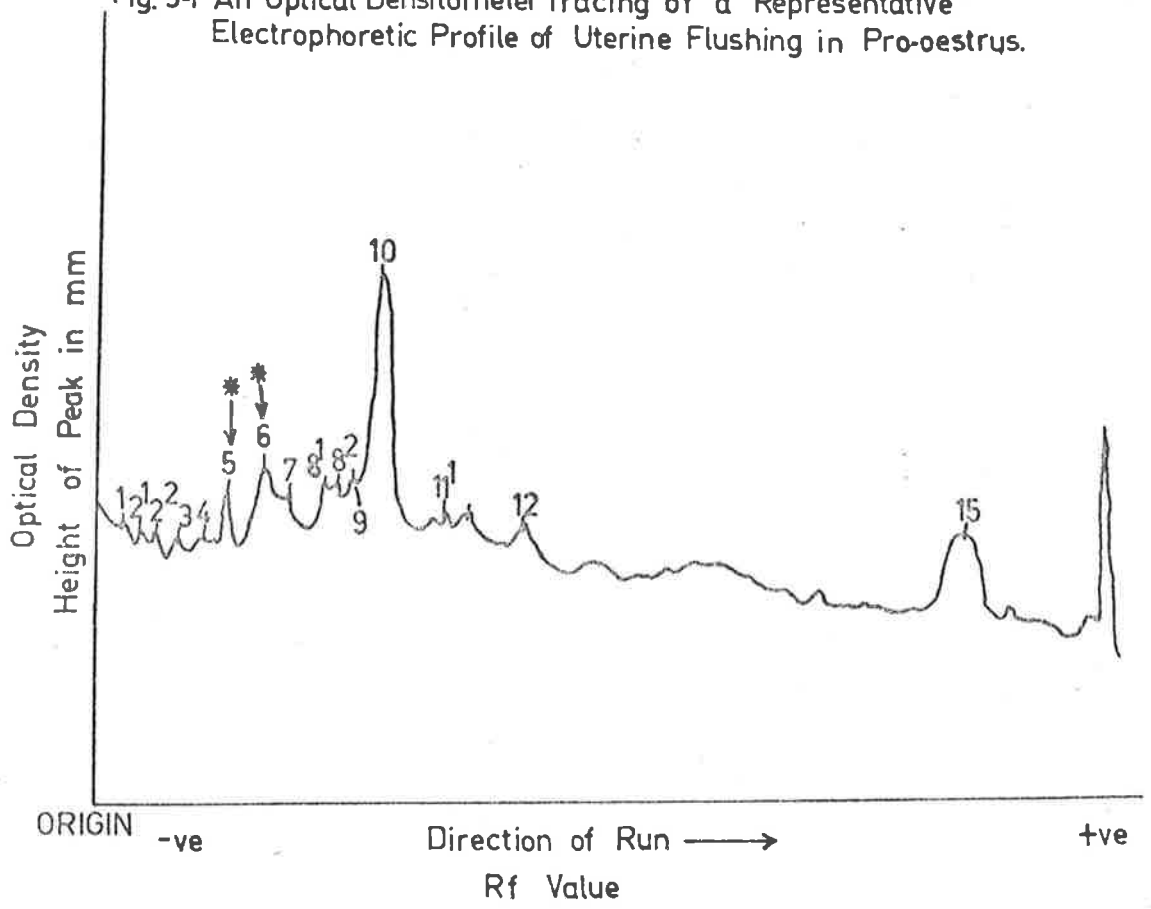


Table 3.3 - Electrophoretic profiles of oestrous uterine flushings (n = 5).

Peak No.	Range of Rf Values 1	2	3	4	5	Frequency of occurrence	Ht. of peak in mm. 1	2	3	4	5
1	0.13	0.12	0.10	-	-	3/5	1	1	1	-	-
2	-	0.22	-	0.18	0.23	3/5	-	6	-	4	1
3	0.32	0.27	0.28	0.33	0.31	5/5	5	4	2	16	3
4^1	0.38	0.39	0.36	0.39	0.38	5/5	3	47	10	2	2
4^2	0.43	-	0.43	-	-	2/5	5	-	3	-	-
5^1	0.49	-	0.49	0.49	0.46	4/5	2	-	24	19	8
5^2	-	-	0.54	0.54	0.54	3/5	-	-	25	26	3
6	0.56	0.57	-	0.64	0.63	4/5	21	37	-	18	16
7^1	0.67	-	0.66	-	0.73	3/5	13	-	23	-	1
7^2	-	-	0.74	-	-	1/5	-	-	3	-	-
8^1	-	0.76	-	0.75	-	2/5	-	7	-	2	-
8^2	0.84	-	0.80	0.82	0.81	4/5	9	-	2	7	9
9	-	0.88	0.92	0.92	-	3/5	-	58	41	53	-
10	1.00	1.00	1.00	1.00	1.00	5/5	44	45	26	16	53
11^1	1.14	1.18	1.11	-	-	3/5	2	4	3	-	-

Table 3.3 (cont.)

Peak No.	Range of Rf Values 1	2	3	4	5	Frequency of occurrence	Ht. of Peak in mm. 1	2	3	4	5
11^2	1.30	1.27	1.16	1.20	1.25	5/5	2	2	1	3	4
12	1.49	1.49	-	-	1.48	3/5	6	8	-	-	4
15	2.84	-	-	-	3.04	2/5	10	-	-	-	16

Fig. 3-2 An Optical Densitometer tracing of a Representative Electrophoretic Profile of Uterine Flushing in Oestrus.

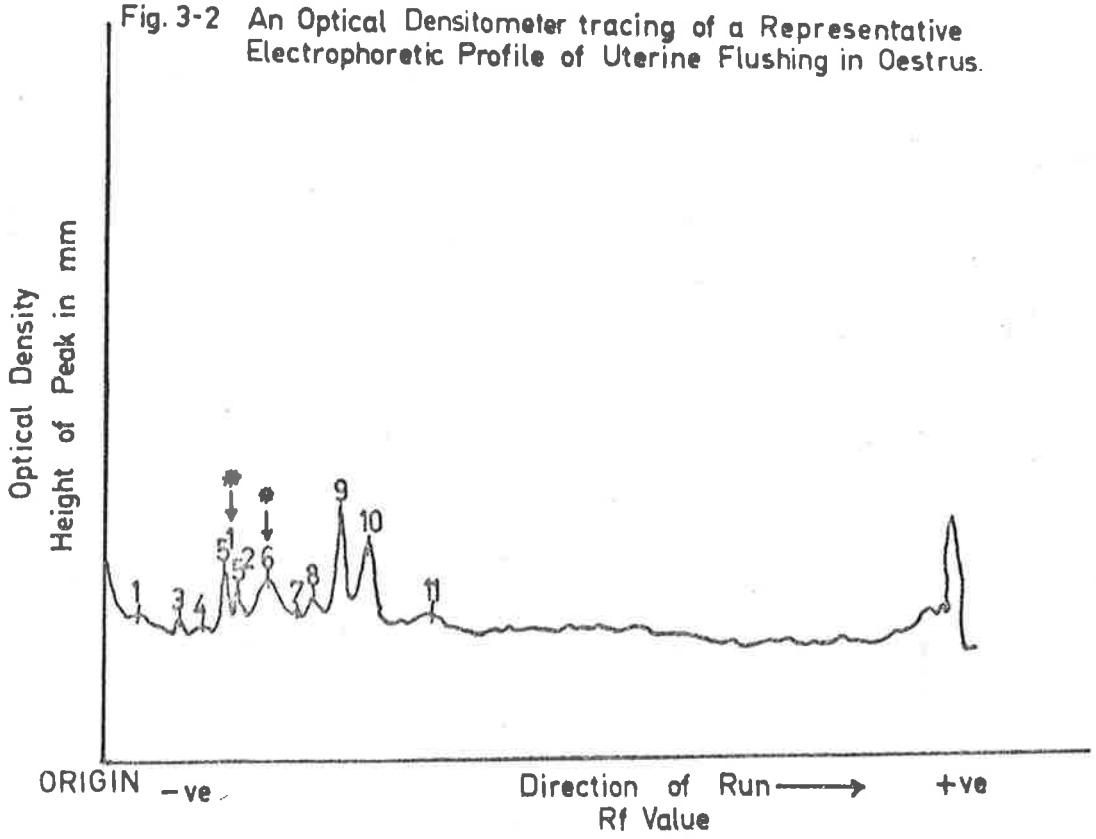


Table 3.4 - Electrophoretic profiles of dioestrous uterine flushings (n = 5).

Peak No.	Range of Rf Values 1	2	3	4	5	Frequency of occurrence	Ht. of peak in mm. 1	2	3	4	5
1^1	0.11	0.13	-	0.10	0.13	4/5	1	1	-	1	1
2^2	-	-	-	-	0.14	1/5	-	-	-	-	1
2^1	-	0.17	0.15	0.18	-	3/5	-	1	1	3	
2^2	-	-	0.24	-	0.23	2/5	-	-	2	-	4
3^1	-	0.25	-	-	-	1/5	-	9	-	-	-
3^2	0.33	0.30	-	0.32	0.30	4/5	1	1	-	1	1
3^3	-	0.33	-	-	-	1/5	-	1	-	-	-
4^1	-	0.37	0.37	0.38	0.38	4/5	-	1	2	1	2
4^2	0.43	0.41	0.42	-	0.43	3/5	1	3	1	-	2
4^3	-	0.44	-	-	-	1/5	-	1	-	-	-
5^1	-	0.48	-	0.45	0.46	3/5	-	1	-	1	1
5^2	-	-	0.51	-	0.52	2/5	-	-	1	-	2
6^1	0.56	0.56	0.58	0.58	0.57	5/5	2	1	1	2	1
6^2	0.62	0.60	0.61	-	0.63	4/5	2	4	2	-	2
7	-	0.67	0.68	0.67	0.68	4/5	-	5	3	2	3

Table 3.4 (cont.)

Peak No.	1	2	3	4	5	Frequency of occurrence	Height of peak in mm. 1	2	3	4	5
8	0.75	-	-	-	-	1/5	3	-	-	-	-
9	0.85	0.86	0.85	0.85	0.86	5/5	4	24	13	13	11
10	1.00	1.00	1.00	1.00	1.00	5/5	49	75	54	48	36
11^1	1.18	1.21	1.15	1.17	1.07	5/5	1	8	2	2	3
11^2	1.31	1.25	1.24	1.25	1.25	5/5	1	10	5	2	4
12	1.48	1.48	1.42	1.45	1.46	5/5	3	7	4	3	4
15	3.05	2.94	-	-	2.93	3/5	8	15	-	-	8

Fig. 3-3 An Optical Densitometer Tracing of a Representative Electrophoretic Profile of Uterine Flushing in Dioestrus.

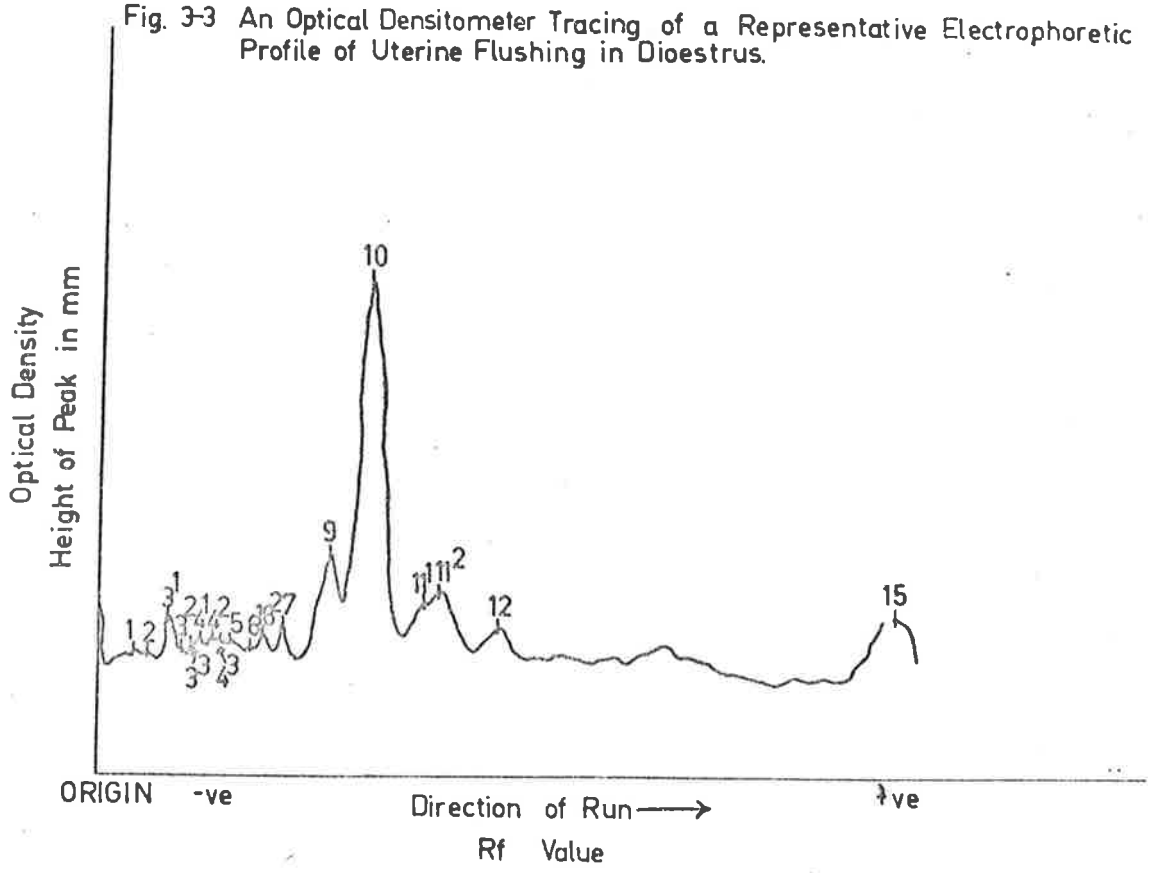


Table 3.5 - Electrophoretic profiles of day 5 pseudopregnant uterine flushings (n = 5).

Peak No.	Range of Rf Values 1	2	3	4	5	Frequency of occurrence	Ht. of Peak in mm. 1	2	3	4	5
1	-	-	0.13	-	0.10	2/5	-	-	1	-	1
2^1	-	0.19	0.17	0.19	0.15	4/5	-	1	4	6	1
2^2	0.20	-	0.21	-	0.23	3/5	4	-	1	-	4
3^1	0.26	0.26	0.27	-	-	3/5	5	2	1	-	-
3^2	0.31	-	-	0.31	-	2/5	1	-	-	2	-
4^1	0.37	0.35	0.35	0.38	-	4/5	3	1	2	1	-
4^2	0.44	0.41	-	-	0.42	3/5	2	1	-	-	1
5^1	-	-	0.46	0.46	-	2/5	-	-	1	2	-
5^2	0.52	-	0.52	0.54	0.50	4/5	1	-	1	1	3
6^1	-	0.55	0.56	0.58	-	3/5	-	1	2	2	-
6^2	0.61	0.59	-	-	0.63	3/5	4	1	-	-	2
7	0.70	0.67	0.65	0.67	0.71	5/5	4	3	4	5	3
8	-	0.84	0.83	-	-	2/5	-	12	16	-	-
9	0.87	-	-	0.85	0.88	3/5	14	-	-	14	14
10	1.00	1.00	1.00	1.00	1.00	5/5	53	54	53	62	52

Table 3.5 (cont.)

Peak No.	Range of Rf values					Frequency of occurrence	Ht. of peak in mm.				
	1	2	3	4	5		1	2	3	4	5
11^1	1.22	1.20	1.19	1.12	-	4/5	6	5	4	6	
11^2	-	1.28	1.29	1.19	1.21	4/5	-	3	4	4	4
12	1.39	1.45	1.48	1.38	1.44	5/5	4	3	5	7	5
14^1	-	2.00	-	-	-	1/5	-	2	-	-	-
14^2	-	2.10	-	-	-	1/5	-	1	-	-	-
15	3.01	-	-	2.87	3.03	3/5	5	-	-	14	9

Fig. 3-4 An Optical Densitometer Tracing of a Representative Electrophoretic Profile of uterine flushing on D_5 Pseudopregnancy.

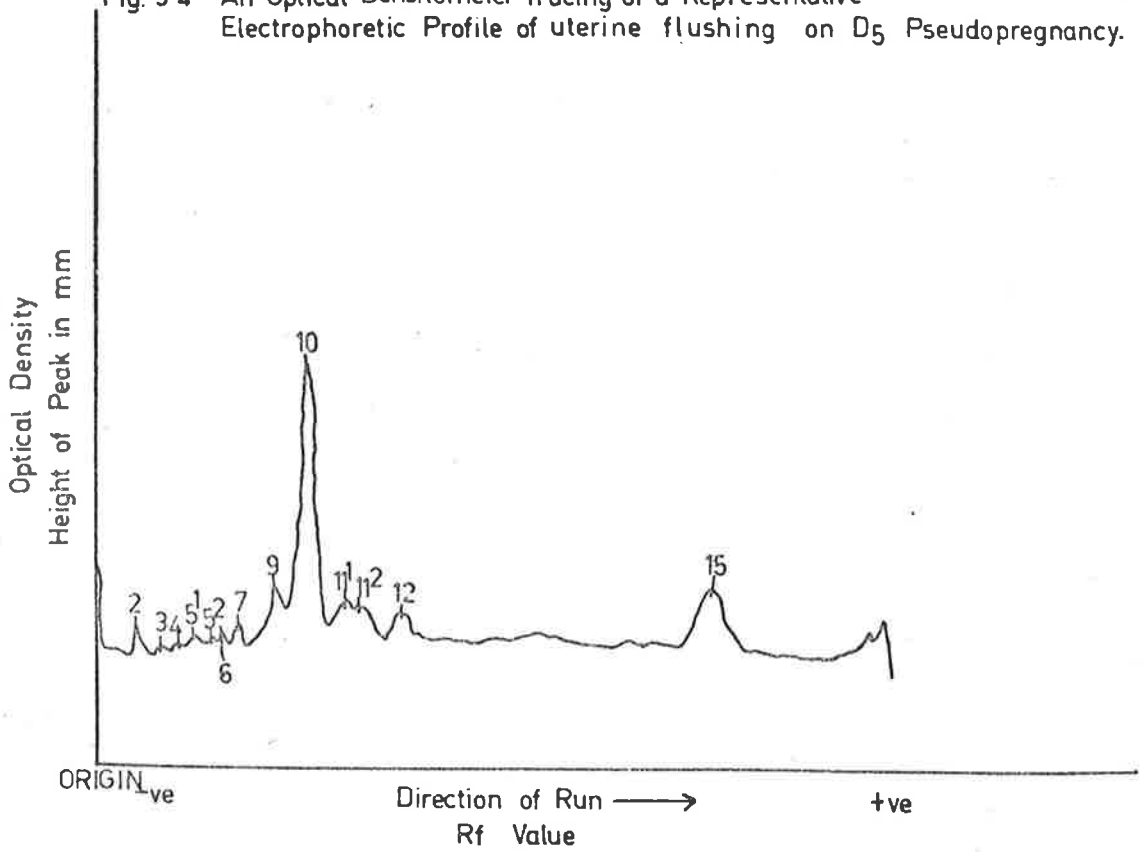


Table 3.6 - The protein levels and the range of protein bands in uterine flushings obtained from ovariectomized rats with and without hormone replacement therapy.

Physiological State	No. of samples	(Protein) in flushes mg/ml	Range of bands	Comments
Ovariectomy control	3	0.03±0.01	1	Samples obtained from individual animals.
Ovariectomy + 1 µg 17 oestradiol	3	0.75± 0.20	11-15	"
Ovariectomy + 1µg 17 oestradiol + 1 mg progest--erone	3	0.33± 0.04	10-12	"
Ovariectomy + 1 µg 17 oestradiol + 5 mg progest--erone	3	0.33 ± 0.04	8-10	"

Table 3.7 - Electrophoretic profiles of uterine flushings from ovariectomized rats with no hormone replacement therapy (n = 3).

Peak No.	Range of Values 1*	2*	3*	Frequency of occurrence	Ht. of Peaks in mm. 1	2	3
10	1.00	1.00	1.00	3/3	4	3	6

* Samples loaded on gel were less than the normal amount of 50µg protein sample.

Fig. 3-5 An Optical Densitometer tracing of a Representative Electrophoretic Profile of Uterine Flushing from Ovariectomized Rats.

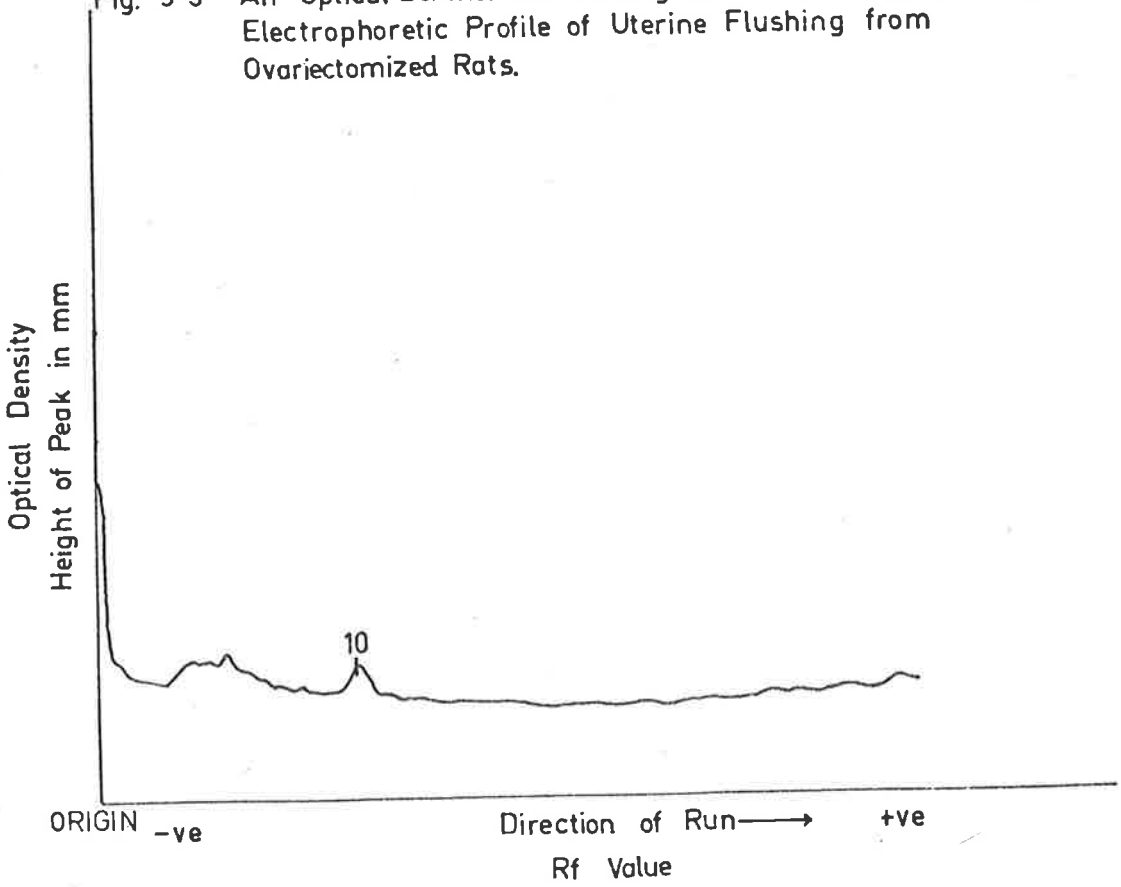


Table 3.8 - Electrophoretic profiles of uterine flushings from ovariectomized rats, treated with 1µg 17β-oestradiol.

Peak No.	Range of Rf Values			Frequency of occurrence	Ht. of Peak in mm.		
	1	2	3		1	2	3
1	0.14	0.13	-	2/3	1	3	-
2^1	-	-	0.15	1/3	-	-	1
2^2	0.20	0.21	0.22	3/3	1	5	5
3^1	0.27	0.27	-	2/3	3	1	-
3^2	-	0.34	-	1/3	-	5	-
4^1	0.38	0.39	0.36	3/3	2	2	3
4^2	0.43	-	0.41	2/3	1	-	1
5	0.50	0.48	0.51	3/3	4	12	2
6^1	-	0.55	-	1/3		2	-
6^2	0.63	0.61	0.61	3/3	4	8	3
7	-	0.68	0.69	2/3	-	8	4
8	0.84	0.80	0.81	3/3	9	11	8
9^1	-	0.86	0.86	2/3	-	13	11
9^2	-	0.91	-	1/3	-	7	-
10	1.00	1.00	1.00	3/3	48	66	50
11	1.23	1.27	1.29	3/3	4	5	8
12	-	1.48	1.49	2/3	-	8	8

Fig. 3-6 An Optical Densitometer tracing of a Representative Electrophoretic Profile of Uterine Flushing from ovariectomized rats treated with 1 µg Oestradiol.

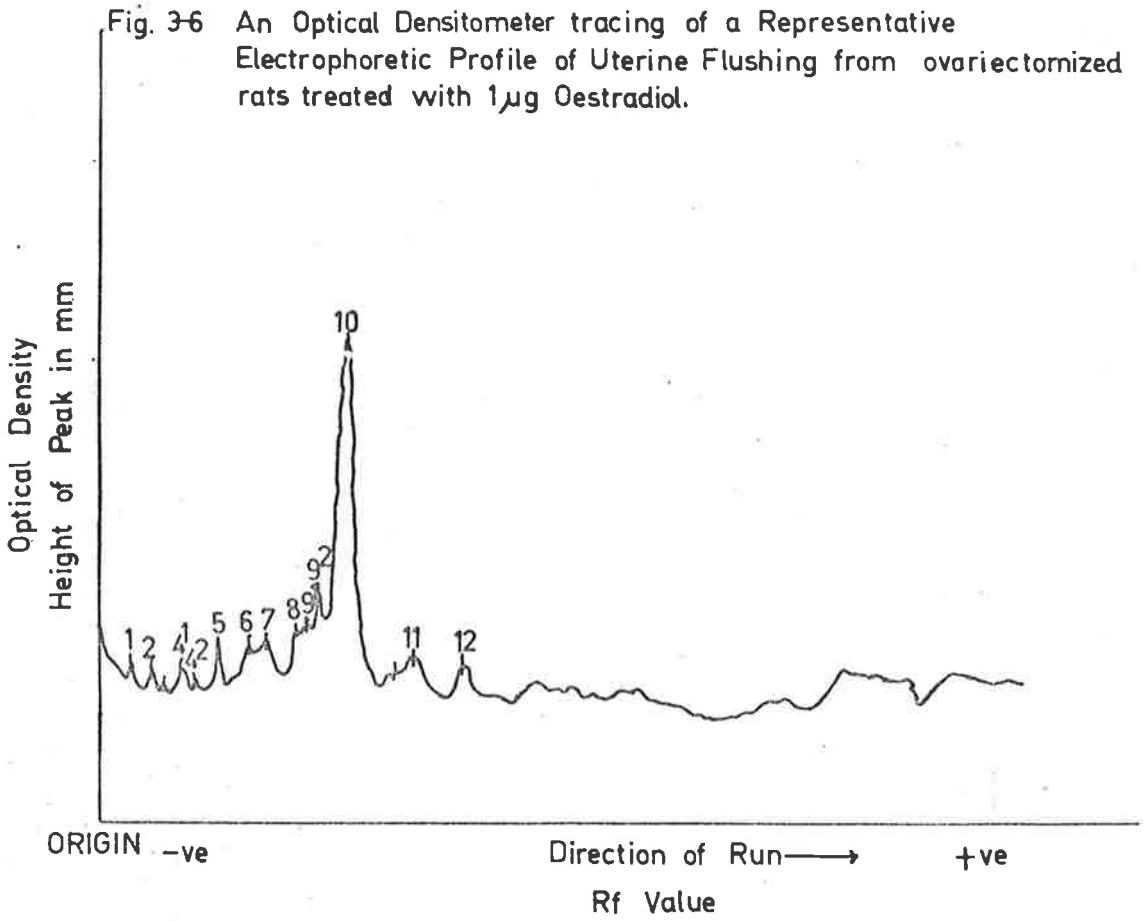


Table 3.9 - Electrophoretic profiles of uterine flushings from ovariectomized rats treated with 1µg 17β-oestradiol and 1 mg progesterone (n = 3).

Peak No.	Range of Rf Values			Frequency of occurrence	Ht. of Peaks in mm.		
	1	2	3		1	2	3
2	-	-	0.17	1/3	-	-	6
3^1	-	0.26	0.25	2/3	-	1	6
3^2	0.32	0.30	0.28	3/3	6	3	7
4^1	0.35	0.36	0.36	3/3	1	2	5
4^2	0.40	-	-	1/3	3	-	-
4^3	0.44	0.42	0.42	3/3	1	1	2
5	0.52	0.50	0.49	3/3	2	8	22
6^1	0.59	-	-	1/3	1	-	-
6^2	0.63	0.62	0.64	3/3	2	2	19
7	0.70	-	-	1/3	1	-	-
8	-	0.82	0.81	2/3	-	5	10
9	0.87	-	0.92	2/3	10	-	28
10	1.00	1.00	1.00	3/3	57	17	29
11	1.25	1.29	-	2/3	5	2	-
12	1.48	1.48	-	2/3	4	1	-

Fig. 3-7 An Optical Densitometer tracing of a Representative Electrophoretic Profile of Uterine Flushing obtained from ovariectomized rats treated with 1 µg. Oestradiol and 1 mg. Progesterone.

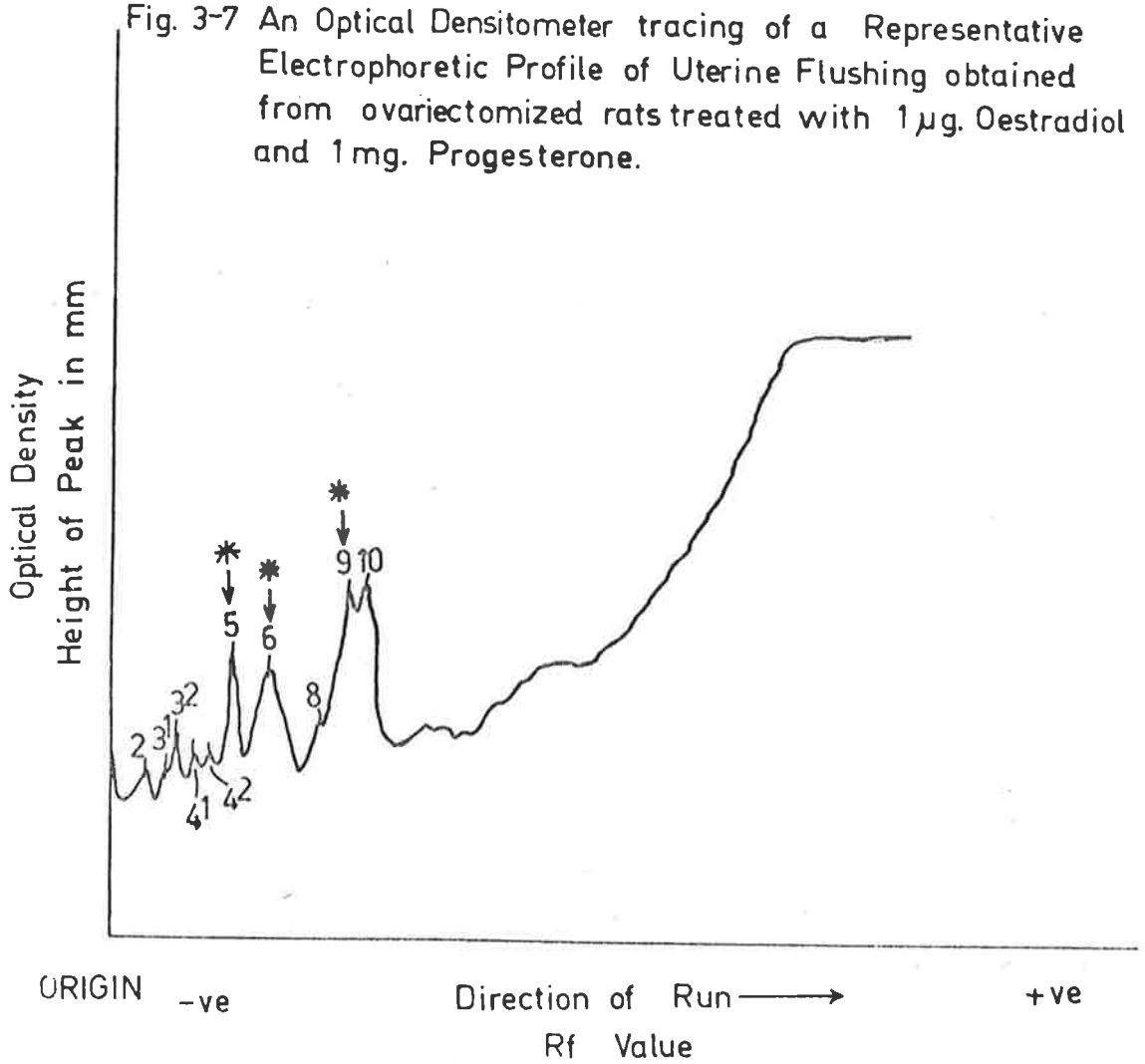


Table 3.10 - Electrophoretic profiles of uterine flushings from ovariectomized rats treated with 1µg 17β-oestradiol and 5 mg progesterone (n = 3).

Peak No.	Range of Rf Values			Frequency of occurrence	Ht. of peaks in mm.		
	1	2	3		1	2	3
2^1	-	0.16	-	1/3	-	1	-
2^2	-	0.24	-	1/3	-	3	-
3^1	-	0.29	0.27	2/3	-	2	2
3^2	-	-	0.33	1/3	-	-	1
4^1	-	0.39	0.38	2/3	-	1	1
4^2	-	0.43	-	1/3	-	2	-
5^1	-	-	0.45	1/3	-	-	3
5^2	-	0.51	0.49	2/3	-	1	3
7^1	-	0.67	0.65	2/3	-	6	3
7^2	-	-	0.73	1/3	-	-	2
9	-	-	0.85	1/3	-	-	7
10	1.00	1.00	1.00	3/3	7	30	25
11	-	-	1.29	1/3	-	-	2
12	-	-	1.47	1/3	-	-	2

Fig. 3-8 An Optical Densitometer tracing of a Representative Electrophoretic Profile of Uterine Flushing obtained from Ovariectomized Rat treated with 1 µg Oestradiol and 5 mg Progesterone.

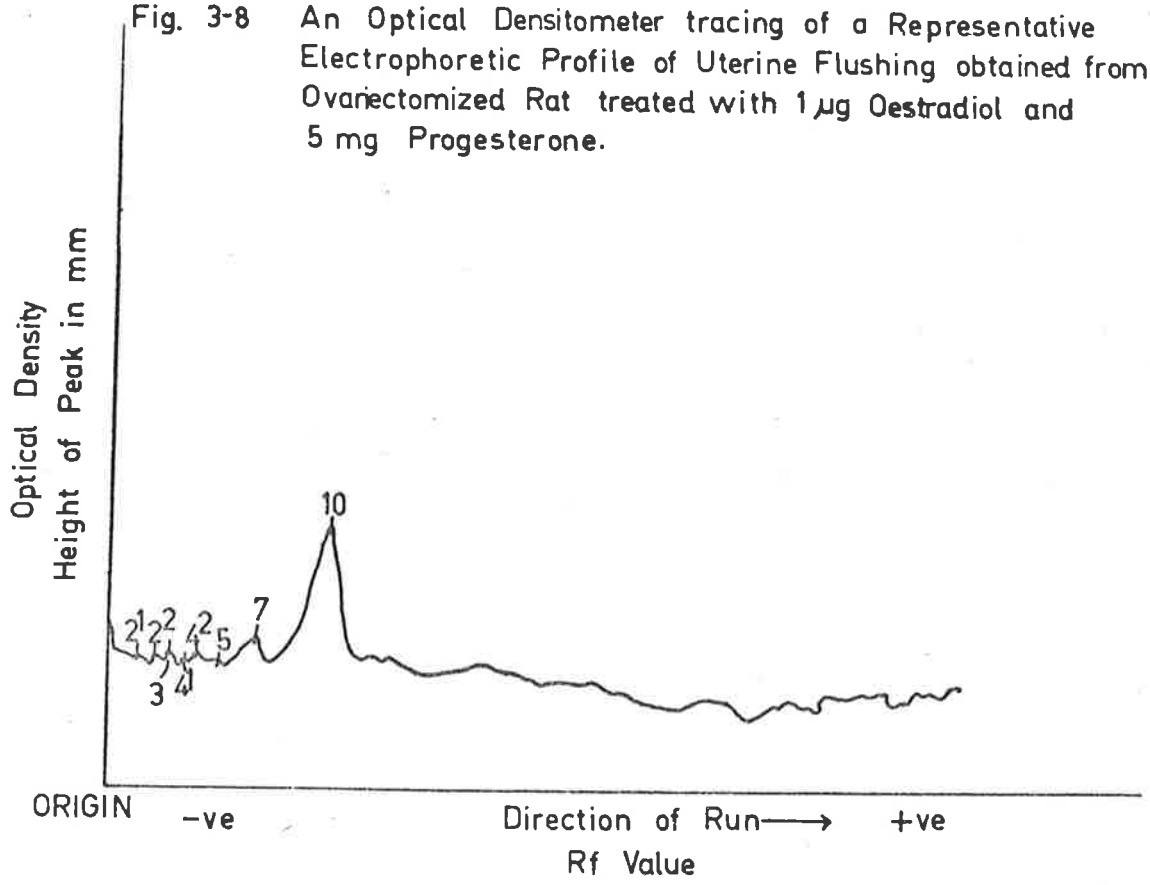


Table 3.11 - The protein levels and the range of protein bands in uterine flushings obtained from control horns and experimental horns of rats fitted with unilateral IUCD on day 5 pseduopregnancy.

Physiological State	Number of Samples	(Protein) in flushings (mg/ml)	Range of Protein Bands
Control horn	4	0.95±0.19	7-15
Experimental horn	4	0.79±0.20	11-14

Table 3.12 - Electrophoretic profiles of day 5 pseudopregnant uterine flushings obtained from control horns in rats with unilateral IUCD (n=4).

Peak No.	Range of Rf values				Frequency of occurrence	Ht. of Peaks in mm.			
	*1	2	3	*4		*1	2	3	*4
2^1	0.18	0.17	0.16	-	3/4	3	1	1	-
2^2	-	0.22	0.19	0.23	3/4	-	1	1	1
3^1	0.29	0.29	0.26	0.26	4/4	1	8	7	1
3^2	0.31	0.33	0.31	0.32	4/4	2	1	1	3
3^3	0.34	-	-	-	1/4	3	-	-	-
4^1	0.39	0.38	0.37	-	3/4	1	3	2	-
4^2	0.43	0.44	0.43	-	3/4	2	3	1	-
5^1	0.48	-	0.46	0.45	3/4	1	-	1	2
5^2	-	0.51	0.50	-	2/4	-	3	2	-
6	0.56	0.62	0.62	0.55	4/4	1	1	2	2
7^1	0.66	-	0.69	-	2/4	1	-	3	-
7^2	0.71	0.70	-	-	2/4	2	2	-	-
9	0.87	0.86	0.85	0.85	4/4	6	14	10	10
10	1.00	1.00	1.00	1.00	4/4	21	68	47	29
11^1	-	1.16	1.16	-	2/4	-	2	2	-
11^2	1.21	1.22	1.25	-	3/4	2	2	3	-
12	1.44	1.41	1.44	-	3/4	1	2	2	-

* Sample loaded on gel less than 50µg

Fig. 3- 9 An Optical Densitometer tracing of a Representative Electrophoretic Profile of Uterine Flushing, obtained from Control Horn of Rat fitted with Unilateral IUCD, on D_5 Pseudopregnancy.

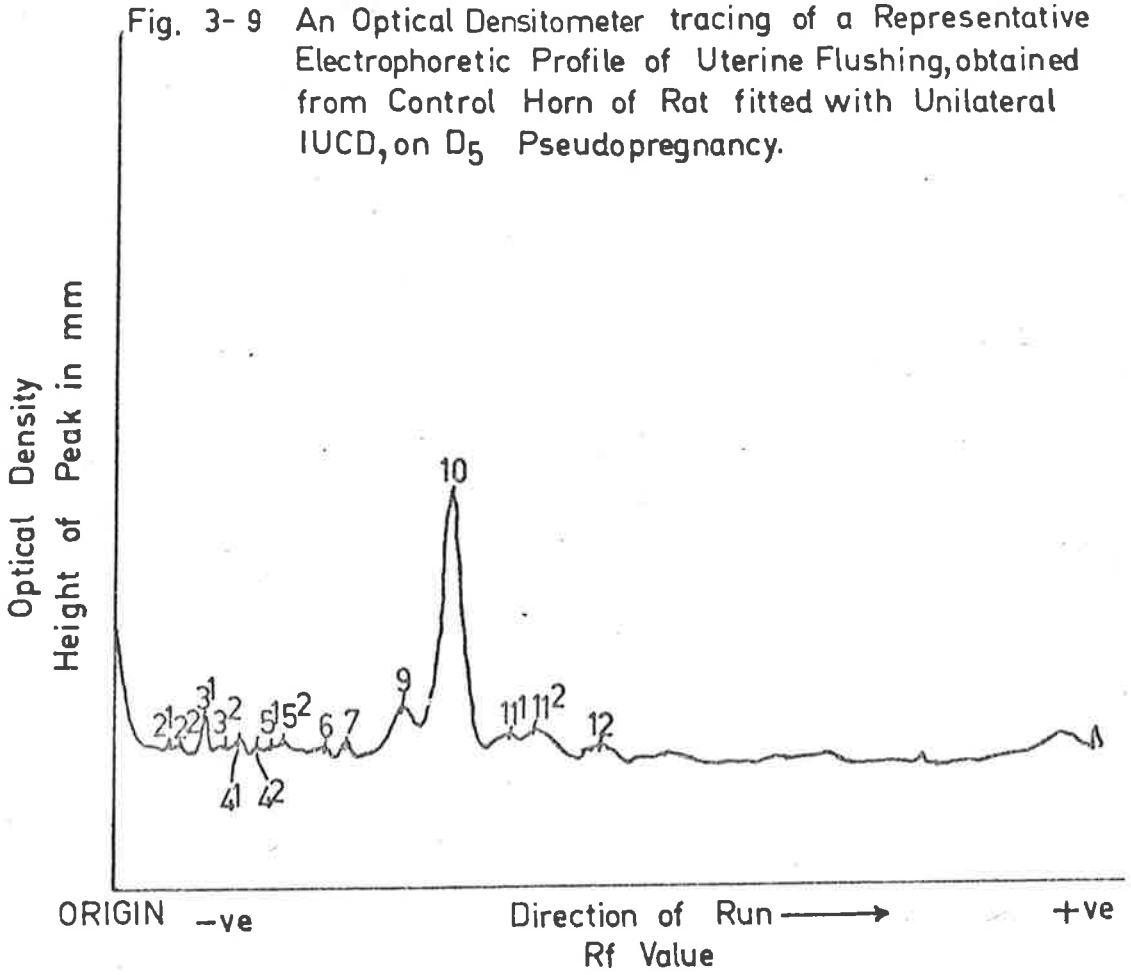


Table 3.13 - Electrophoretic profile of day 5 pseudopregnant uterine flushings obtained from uterine horn with I U C D (n = 4).

Peak No.	Range of Rf Values				Frequency of occurrence	Ht. of peak in mm.			
	1	2	3	4		1	2	3	4
1	-	-	0.10	-	1/4	-	-	1	-
2^1	-	0.17	0.15	-	2/4	-	1	2	-
2^2	-	0.23	0.21	0.21	3/4	-	1	2	2
3^1	0.29	0.29	-	0.28	3/4	1	5	-	2
3^2	0.33	-	0.31	-	2/4	3	-	1	-
4^1	-	0.38	0.36	0.36	3/4	-	1	1	2
4^2	0.43	0.43	0.39	0.43	4/4	2	3	2	1
5^1	0.48	-	-	0.48	2/4	2	-	-	1
5^2	0.54	0.51	-	0.53	3/4	1	1	-	1
6	-	0.64	0.58	0.62	3/4	-	1	3	2
7^1	0.67	-	0.67	0.69	3/4	2	-	1	1
7^2	0.71	0.70	-	-	2/4	2	1	-	-
8	-	0.84	-	-	1/4	-	11	-	-
9	0.87	-	0.85	0.86	3/4	13	-	12	12
10	1.00	1.00	1.00	1.00	4/4	45	46	52	57
11^1	-	-	1.15	-	1/4	-	-	1	-
11^2	1.24	1.23	1.22	1.22	4/4	2	2	4	4
12	1.43	1.42	1.42	1.41	4/4	2	1	4	3
14	-	-	-	2.12	1/4	-	-	-	2
15	-	-	-	2.81	1/4	-	-	-	10

Fig. 3-10 An Optical Densitometer tracing of a Representative Electrophoretic Profile of Uterine Flushings, obtained from Experimental Horn fitted with Unilateral IUCD, on D_5 Pseudopregnancy.

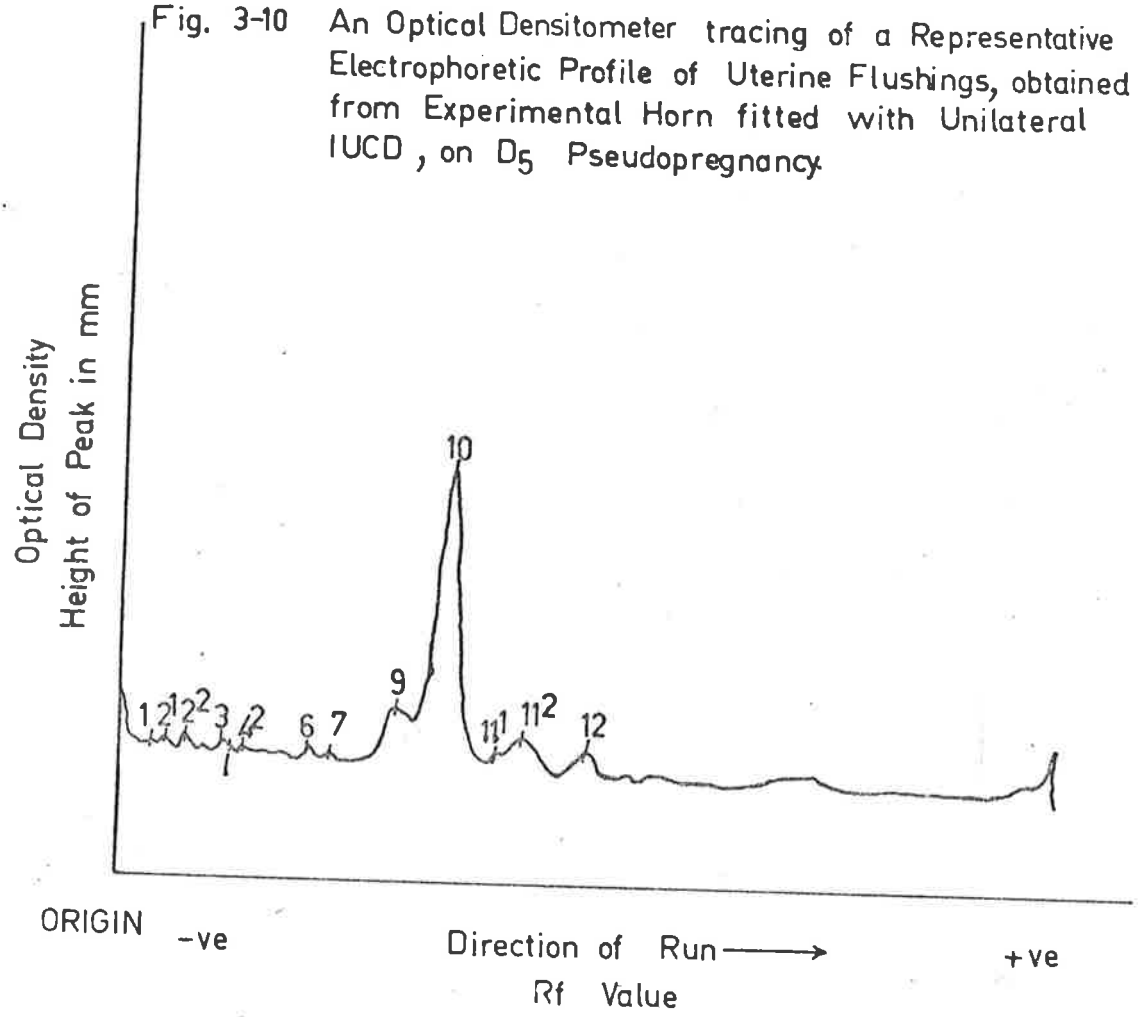


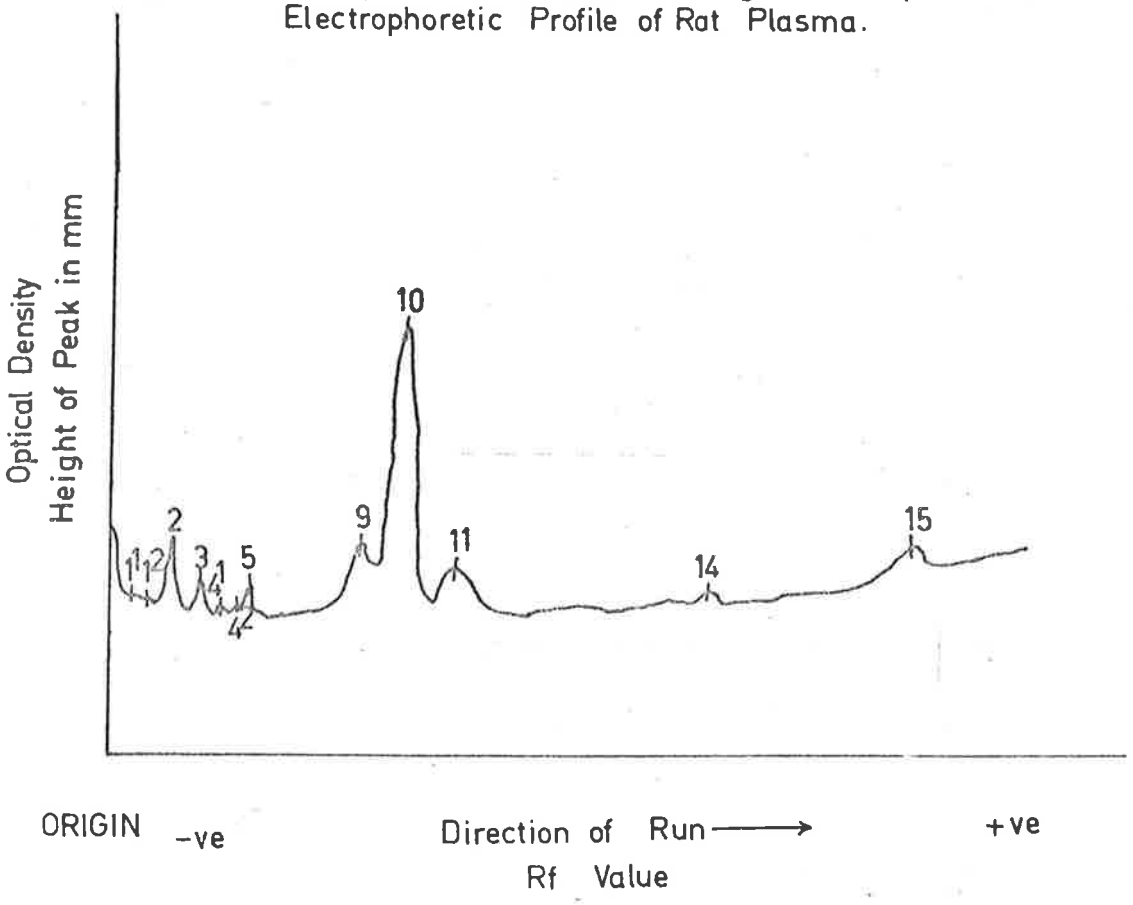
Table 3.14 - Electrophoretic profiles of rat plasma (n = 8).

Peak No.	Range of Rf Values								Frequency of occurrence	Height of Protein peaks in mm.							
	1	2	3	4	5	6	7	8		1	2	3	4	5	6	7	8
1^1	-	-	0.08	0.12	0.08	-	0.08	-	4/8	-	-	1	1	1	-	1	-
1^2	-	-	0.14	-	0.11	-	0.13	-	3/8	-	-	1	-	1	-	1	-
2^1	0.15	0.16	-	-	-	-	-	-	3/8	1	1	-	1	-	-	-	-
2^2	0.20	0.20	0.24	0.22	0.21	0.22	0.21	-	7/8	1	1	21	21	13	10	14	-
3	0.26	0.26	0.33	0.31	0.30	0.31	0.30	0.26	8/8	20	21	10	11	9	9	5	17
4^1	0.36	0.36	0.39	0.37	0.38	0.39	0.38	0.36	8/8	8	11	2	2	3	2	2	9
4^2	0.42	0.42	0.44	0.42	0.42	0.43	0.44	-	7/8	2	2	2	1	3	1	2	-
5^1	0.50	0.47	0.47	0.46	0.45	0.48	0.48	0.48	8/8	2	1	1	1	4	1	3	2
5^2	-	0.51	-	-	-	-	-	0.51	2/8	-	1	-	-	-	-	-	5
9	0.85	0.85	0.85	0.84	0.85	0.85	0.84	0.85	8/8	18	20	17	17	17	19	14	18
10	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	8/8	66	68	77	68	71	40	51	58
11^1	1.14	1.11	1.13	1.13	-	1.15	1.16	1.15	7/8	7	6	5	5	-	7	6	8
11^2	1.21	1.18	1.19	1.19	1.17	1.21	-	-	6/8	3	4	4	4	10	5	-	-

Table 3.14 (cont.)

Peak No.	Range of Rf Values 1	2	3	4	5	6	7	8	Frequency of occurrence	Height of protein peaks in mm. 1	2	3	4	5	6	7	8
13	-	-	-	1.60	-	1.63	-	-	2/8	-	-	-	1	-	2	-	-
14	1.98	1.97	2.01	2.06	2.20	1.90	2.03	2.05	8/8	6	3	3	4	3	3	2	5
15	-	-	-	-	3.02	-	2.72	2.70	3/8	-	-	-	-	4	-	9	9

Fig. 3-11 An Optical Densitometer tracing of a Representative Electrophoretic Profile of Rat Plasma.



4 DISCUSSION

In this project sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis has been used in the study of protein composition of uterine flushings under different physiological conditions. This technique was originally developed by Maizel (1966) and later modified by Schnaitman (1973 and 1974).

A review on the applications of polyacrylamide gel electrophoresis is given by Maizel (1971). The principle of this method is as follows. There is a dissociating system which brings about the disruption of hydrogen, hydrophobic and ionic bonds. The anionic detergent SDS is the most commonly used dissociating agent. According to this technique, samples are prepared by incubating one volume of sample with two volumes of solubilizing solution containing SDS at 37^{o}C for two hours. This produces SDS-protein complexes with an overall negative charge. The basis of mobility of these SDS-protein complexes is on the molecular weight of proteins and the direction is from the cationic to the anionic chamber of the electrophoretic apparatus.

Initially, an experiment was conducted to determine the effect of loading from 25 to 160µg of SDS-protein mixture on the electrophoretic profiles. Results indicated that when 50µg or more of SDS-protein mixtures were loaded on to the gels, the profiles were similar. However, with increasing amounts of SDS-protein mixture, the width of certain bands was increased and also stained more intensely.

Below 50µg, a significant reduction in the number of protein bands could be observed. Thus, it may be concluded that below 50µg of SDS-protein mixture, there was not enough protein in individual bands for them to be detected, but increasing SDS-protein mixture above 50µg did not appear to increase the number of resolvable protein components by this method. Thus taking this result together with the average protein concentration in various uterine flushings, it was decided to load 50µg of SDS-protein mixture in all cases regardless of whether or not pooling and concentrating of flushings had to be initially performed.

Total protein in uterine flushings was determined by a modified Lowry method (Lowry, et al, 1951) which involved the replacement of Lowry reagent B, 0.5% cupric sulphate in 1% sodium citrate solution (Gibbs, personal communication). The former solution proved to be unstable resulting in cupric sulphate precipitating out of the solution while the latter solution was more stable and could be stored for a few months at room temperature. Alternatively, the problem of instability of the original reagent B could be overcome by making up two separate solutions of 1% cupric sulphate solution and 2% potassium sodium tartate solution and then mixing them in the ratio of 1:1 just before use (Drew, personal communication). In this study, the former modification to reagent B was used.

Measurement of total protein content of the uterine flushings demonstrated considerable variation between samples at different physiological states and also within the same physiological state. However, since lower protein

levels were invariably present in uterine flushings obtained in dioestrus and on day 5 pseudopregnancy compared to pro-oestrus and oestrus, it was necessary to pool and concentrate them. This was achieved by using Amicon B-15 clinical sample concentrators. No concentrating of pro-oestrous and oestrous flushings was necessary. Since some uterine flushings were concentrated while others were not, it was necessary to investigate the possible effect of concentration on the electrophoretic profiles. The results demonstrated that the concentration procedure had no effect on the electrophoretic profiles. However, some loss of protein occurred possibly due to sticking to the wall of the chamber during the concentrating process.

Although the pattern of the optical densitometer tracings of different samples at the same physiological state was similar, some variation in the total number of bands visible in different samples was apparent, e.g. 7-16, 11-14, 10-17 and 12-14 numbers of bands occurred in uterine flushings obtained at pro-oestrus, oestrus, dioestrus and on day 5 pseudopregnancy respectively. This was mainly due to presence or absence of some barely detectable weak protein components in the higher molecular weight region, e.g. zones 1 and 2 close to the origin. Such variation may be a reflection of either individual variation of the protein components of the samples or variation of the resolving power of the SDS-gel electrophoresis. The latter possibility is influenced by such factors as the variation in the volume of sample loaded on to the gels and also by the staining and destaining procedures.

The presence of variation in the total number of bands may be related to the fact that in 26 out of 47 samples, a double peak or band in zone 11 occurred. Further, in 20 out of 47 samples, a band was detected in zone 15 of the electrophoretic profile where the haemoglobin sub-unit standard was detected. This may imply that either some samples are slightly contaminated with haemolysed blood although there was no visible evidence of this or that a protein sometimes occurs with similar molecular weight to haemoglobin.

As well as variation in the number of bands, some minor variation occurred in the mobility of individual bands which made identification and comparative study difficult. This factor proved to be a disadvantage of the disc electrophoretic system and may be attributed to the following factors. Differences may be present among the individual gels used which may result in an uneven starting zone or origin. Further, the unevenness may not be restricted to gel surfaces only but may also include the whole gel itself. Such unevenness may be due to gel polymerization occurring either too fast or too slowly. In this study, the polymerizing agents TEMED and ammonium sulphate were adjusted to obtain polymerization in not less than thirty minutes and not more than sixty minutes at ambient room temperature.

Further, a range of 20 to 100 µl volume of sample was loaded on to the gels in order to maintain the constant amount of 50µg of SDS-protein mixture. By loading a wide range of volume, there is a risk of overloading the gel

which produces electrical and heating effects which in turn are detrimental to resolution. Hence, the consequence of excessive sample loading is to broaden protein bands and also to increase the migration rates of protein (Maizel, 1971). This in turn may also affect neighbouring bands. Further, excessive loading of protein samples can result in the protein precipitating at the origin. However, as far as is known, this phenomenon did not occur in the present study. This was probably because the volume of the sample loaded was always small, i.e. generally less that 100 μl.

The problem of variation in the mobility of the bands could be partially resolved by expressing the mobility of the protein components as a fraction of the distance travelled by an internal reference protein or a dye. In this project, two or three drops of 0.2% bromo-phenol blue dye in 50% sucrose was added to the samples. The dye served as an indicator of the front of the moving zone as the sample descended down the gel. Unfortunately, by the time the gels have been subjected to the staining and destaining procedures, the bromo-phenol blue dye had been effectively removed. Thus the use of the dye is limited to following the electrophoretic run only. However, a peak with a mobility similar to that of bovine serum albumin was always observed. So this assumed albumin peak was assiged the Rf value of 1.0 and the rest of the protein compounds related to it and fifteen arbitary zones in descending order from the origin of the gel were assigned.

Finally, variation in the height of the peaks or staining intensity of the bands of the electrophoretic profiles was also observed. This is likely to be due to quantitative differences of the individual proteins within the different samples obtained at the same physiological state.

During the analysis of results, comparison was made between the optical densitometer scan of the gels and the protein bands present in the gels as observed under a viewer. Since the gels were stained with Coomassie blue dye, the peaks present in the optical dens--itometer tracing should correspond to the bands observed under the viewer. Such double checking eliminated any artefacts occurring as a result of the scanning procedure.

The present study demonstrates that changes occur in the electrophoretic profiles of uterine flushings obtained at different hormonal states of the oestrous cycle and on day 5 pseudopregnancy. No direct comparison can be made with the results obtained by previous workers because of the different techniques employed and a wide range of uterine protein concentrations used in the analysis of uterine flushings, except that of Surani (1975) who studied ovariectomized rats given oestrogen and progesterone and then flushed the uterus at different times after the last oestrogen injection. Further, the earlier workers also restricted their study only to uterine fluids or flushings obtained at either pro-oestrus or oestrus.

The protein concentration of the uterine flushing at pro-oestrus and oestrus was approximately 0.5 mg/ml.

This is in agreement with the values obtained by previous workers (Junge and Blandau, 1958, and Breed et al, 1972). Neat uterine fluid aspirated at oestrus had twice the amount of total protein, e.g. about 1.0 mg/ml. This value differs from that obtained by Junge and Blandau (1958) who found the average protein concentration of uterine fluid to be about 0.5 mg/ml or about 4% of that found in rat serum. This discrepancy may be attributed partly to the different techniques used in measuring protein concentration. The protein concentration of dioestrous uterine flushingswas approximately 0.25 mg/ml.

Despite the variability in the electrophoretic profiles of uterine flushings, more bands were detected by SDS-gel electrophoresis than by previous workers using disc electrophoresis, immunoelectrophoresis or ouchterlony gel diffusion analysis. Hence, this may suggest that the SDS-gel electrophoresis is more sensitive than the other techniques. Further, the overall electrophoretic profile of uterine flushings in this study indicates that a number of the protein components have molecular weight greater than 70,000.

The results of this study indicate greater protein peaks 5 and 6 of uterine flushings obtained at pro-oestrus and oestrus than at dioestrus and day 5 pseudopregnancy. These two proteins have mobilities slower than that of Transferrin standard which has a molecular weight of 86,000,so these molecular weights are approximately 103,000 and 94,000 respectively. Surani (1975) demonstrated that if progesterone alone was administered to ovariectomized rats,

there was a lack of high molecular weight proteins. However, when oestradiol was also given along with progesterone, a protein with molecular weight of 70,000 was detected within 1 hour; 13 to 20 hours after oestrogen administration, there was a shift towards the occurrence of higher molecular weight proteins which he considers coincides with the protein profiles of day 5 pseudopregnancy. Thus the higher molecular weight proteins mentioned by Surani as occurring 13-20 hours after giving oestrogen could be the same as those described in the present study as increasing at pro-oestrus and oestrus. Uterine flushings from pro-oestrus and oestrus animals were usually taken early in the afternoon and since the oestrogen surge occurs early in the morning of pro-oestrus (Yoshinaga, et al, 1969), the time scale between oestrogen inducing high molecular weight proteins mentioned by Surani and flushings obtained during the cycle in the present study may not be too different.

A small peak with Rf value of 0.75 corresponding to that of transferrin standard was detected in two out of the pro-oestrous samples (Rf=0.70 and 0.78) and three out of the five oestrous samples (Rf=0.74, 0.75 and 0.76). By contrast it was detected in only one out of the five dioestrous samples (Rf=0.75) and in two out of the five day 5 pseudopregnant samples (Rf=0.70 and 0.71). In the rabbit, however, Transferrin is one of the dominant peaks in the pre-ovulatory or oestrous uterine flushings (Beier et al, 1971; Beier and Beier Hellwig (1973). Thus indicating species differences with regard to this protein. Further protein component 9 showed an increase in concentration in 3 out of the 5 oestrous

uterine flushings compared to the other uterine flushings and that of plasma.

Uterine flushings obtained on day 5 pseduopregnancy had protein concentrations of about 0.3 mg/ml which is similar to that of dioestrus. In common with other uterine flushing profiles, a number of protein components had molecular weights greater than 70,000 daltons. These results are in agreement with Surani's findings on day 5 pseudopregnancy (1975). However, they differ from those in the rabbit where the majority of uterine proteins are relatively small macromolecules with molecular weights in the range of 25,000-70,000, in this species a specific protein called uteroglobin is particularly prominent (Beier et al, 1971). The increase in concentration of proteins 5 and 6 observed at pro-oestrus and oestrus did not occur in day 5 pseudopregnant samples. The overall electrophoretic profiles of day 5 pseudopregnant samples appear therefore to resemble closely those of dioestrus.

The results of this study also appear not to support the occurrence of uteroglobin or blastokinin in rat uterine flushings obtained on day 5 pseudopregnancy as extensively reported in rabbit. However, there appears to be a discrepancy in the estimation of the molecular weight of blastokinin sub-unit ranging from 10-15,000 (Murray, et al, 1972; McGaughey and Murray, 1972; and Bullock and Connel, 1973). Thus if the higher estimation of the molecular weight of the sub-unit is true and if such a substance did occur in the rat uterine flushings at early pregnancy or pseudopregnancy, then it would be located in zone 15 of the SDS-gel electro--phoretic profile. A faint peak was detected in this zone in some uterine flushings regardless of the physiological

states from which they were obtained and in plasma. However, the haemoglobin sub-unit standard has a molecular weight of 15,500 daltons and its peak is also located in zone 15 of the SDS-gel electrophoretic profile so it is likely that this peak is haemoglobin.

Finally, rat plasma samples were analysed with uterine flushings. The results suggest that nine proteins namely bands 1, 2, 3, 4, 5, 9, 10, 11, and 15 were common to both plasma and uterine flushings, irrespective of endocrine state but protein bands 6, 7, 8, and 12 were present only in uterine flushings while protein band 14 was only present in plasma. Thus, uterine fluid proteins appear to contain a mixture of those passing into the uterine lumen from blood plasma and those synthesized de Novo in the uterine endometrium and released into the lumen.

As a result of changes in protein profiles in intact female rats at different endocrine states, it was decided to ascertain whether the changes observed could be induced in ovariectomized rats given replacement hormonal therapy and especially whether exogenous oestrogen is able to induce the two prominent protein bands observed at pro-oestrus and oestrus. Thus ovariectomy and replacement therapy was performed with oestrogen and/or progesterone. The schedule and dosage of the hormone replacement therapy employed in this study has been presented in Materials and Methods.

The levels of protein measured in uterine flushings from ovariectomized animals without hormone therapy was significantly less than when oestrogen was given alone or

in combination with progesterone ($P<0.05$ and $P<0.01$ respectively). Thus it seems that oestrogen induces secretion of protein into the uterine lumen in some way or another. In the case of ovariectomized animals without hormone replacement therapy, atrophy of the uterus was observed which may be related to the very low protein levels in the uterine flushings. Electrophoretic profiles in all the three samples analysed demonstrated the presence of one faintly staining band corresponding in position to that of BSA standard.

In the oestradiol treated group, elevation of proteins 5 and 6 were observed in two out of three samples. In this respect, the pattern was similar to the protein profile of pro-oestrous uterine fluid. In the case of combined oestradiol and 1 mg progesterone treated group, the protein profile was also characterized by the elevation of proteins 5, 6 and 9 in one out of three samples while protein 5 was elevated in two out of three samples. This again was similar to the protein profiles of both pro-oestrous and oestrous uterine flushings with regard to proteins 5 and 6. The elevation of two protein components 5 and 6 at pro-oestrus and oestrus may be due to ovaries secreting oestradiol-17β in the morning and progesterone in the afternoon of pro-oestrus (Shaikh and Shaikh, 1975; and Smith *et al*, 1975).

The induction of intracellular *de Novo* protein synthesis by oestrogen involving the incorporation of labelled amino acids into proteins under *in vivo* and *in vitro* conditions have been reported by numerous workers (Notides and Gorski, 1966; Barnea and Gorski, 1970; Katzenellenbogen and Gorski, 1970; Mayol and Thayer, 1970, and Iacobelli *et al*, 1973). The fact that oestrogen induced

protein synthesis after *in vivo* and *in vitro* hormone administration, clearly indicates that oestrogen can stimulate the uterus without the requirements of intact vascular or nervous systems. Such proteins are known to be taken into the nucleus but extra-cellular secretion as indicated by this study also appears to occur.

Finally, in the protein profiles of uterine flushings obtained from oestradiol and 5 mg progesterone treated ovariectomzied animals, the prominent protein peaks 5 and 6 were not apparent. Thus the protein profile is similar to that obtained at dioestrus and on day 5 pseudopregnancy. Hence these results suggest that higher progesterone levels may inhibit secretion of bands 5 and 6. The protein profile of this treated group does however appear to differ from that of day 5 pseudopregnancy in that there appears to be a reduction in the number of bands occurring at the higher molecular weight range (particularly in zones 1, 2, and 3) resulting in a reduction in the number of overall protein bands observed.

This project was extended to include the study of the effect of I.U.C.D. on the protein composition of the uterus on day 5 pseudopregnancy. The measurement of protein levels in uterine flushings demonstrates that the presence of an I.U.C.D. significantly increases the protein levels ($P<0.05$). Thus the findings confirm results obtained previously (Kar *et al*, 1964 and Breed *et al*, 1972). Although the SDS-protein profiles suggest a slight reduction in the higher molecular weight proteins, the profile appears not to be consistently altered by the presence of an I.U.C.D.

Hence these results are in agreement with the results obtained by Breed et al (1972), and Peplow et al (1973) who reported that the disc electrophoretic protein patterns of uterine fluid were not consistently altered by the presence of an I.U.C.D.

The inhibition of both decidual response and implantation in rat and mice in the presence of I.U.C.D. or I.U.F.B. are well documented (Margolis and Doyle, 1964; Bartke, 1968; Batta and Chandhury, 1968; Dizzia and Bo, 1969; Kar et al, 1964 and Krueger and Bo, 1972). Hence the failure of decidual cell response to occur in the rat during pseudo--pregnancy in the presence of I.U.C.D. may be associated with the absence of protein(s) some way involved in the mechanism of DCR and subsequent implantation.

5. APPENDICES

MODIFIED LOWRY ASSAY FOR PROTEIN MEASUREMENT

Reagents

1. Reagent A = 2% $Na_2 CO_3$ in 0.10N Na OH.
2. Reagent B = 0.5% $Cu50_4 5H_2 0)$ in 1% sodium citrate.
3. Reagent C = is an alkaline copper solution which is made up fresh about 30 minutes before use by mixing 50 ml of Reagent A with 1 ml of Reagent B.
4. Reagent E = is 1:1 diluted Folin and Ciocalteu's phenol Reagent 2 normal (Sigma Lot 64c-5033) is diluted on the day of use and then discarded.
5. A stock solution of BSA - (Bovine serum Albumin) 1 mg/ml is made up and stored in the freezer in 2 ml aliquots.
6. Standard Curve for 0.2 ml System

Dilutions

BSA	Stock Solution of (BSA) = 1mg/ml or 1000µg/ml		Dilution with $H_2 0$	Total vol.
100µg/ml	0.1 ml	+	0.9ml	1.0
200µg/ml	0.2 ml	+	0.8ml	1.0
300µg/ml	0.3 ml	+	0.7ml	1.0
400µg/ml	0.4 ml	+	0.6ml	1.0
500µg/ml	0.5 ml	+	0.5ml	1.0

(BSA)/0.2 ml Standard Curve

20µg = 0.2 ml of 100 g/ml BSA solution
40 g = 0.2 ml of 200 g/ml BSA solution
60µg = 0.2 ml of 300 g/ml BSA solution
80µg = 0.2 ml of 400 g/ml BSA solution
100µg - 0.2 ml of 500 g/ml BSA solution

SCHNAITMAN SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS PROCEDURES.

Solution A (Solublizing solution)

Stock solution of 100 mls of 0.1 M sodium phosphate buffer containing 3% or 3 grams of SDS, and 7.5 mm EDTA disodium salt (Ethylen-ediamine tetra acetic acid - Sigma Lot 51c-91001).

M.wt of EDTA = 372.24

1000 mM (1M) = 372.24 gm/1000mls

10 mM = 2.7224 gm/1000mls

$$7.5 \text{ mM} = \frac{3.72}{10} \times 7.5 = 2.79 \text{gm/1000mls}$$

Since the stock solution = 1000 mls, the amount of EDTA added was 0.279 gm/100 mls.

Before the solution A was added to reacting samples, to form SDS-protein complex, 0.15%. 2-mercaptoethanol Type I (Sigma Lot 34c-1810) was added to solution A.

Calculation of the amount of gel preparation required for 10 tube.

The area of tube $= \pi r^2$

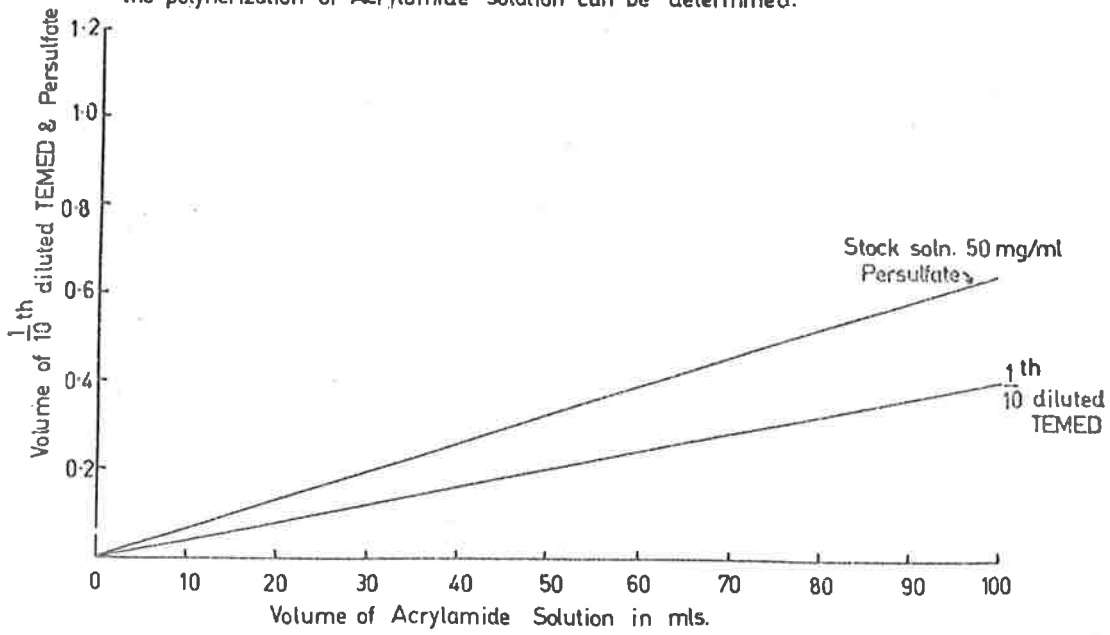
Internal diameter of tube = 5 mm ∴ radius 2.5 mm or 0.25 mm.

∴ amount of gel prep/tube = 0.196 x 10 cm = 1.96mls

Coomassie Blue Stain

0.25% Coomassie blue dye was dissolved in water containing 45% methanol and 10% acetic acid. The dye solution was filtered before use. Destaining solution consists of water containing 25% methanol and 7.5% acetic acid.

From this graph the amount of $\frac{1}{10}^{th}$ diluted TEMED and persulfate required for the polynerization of Acrylamide solution can be determined.



Calculation of the amount of protein loaded in gel. Generally a sample with 1mg;ml of protein, 50µl volume was loaded on to the gel. This volume was equivalent to 50µgs of SDS-protein mixture.

Protein with xmg/ml or xµg/1000µl, the vol. equivalent to 50µg of SDS-protein mixture = $\frac{1000}{x}$ x 50µl

PEAK HEIGHT OF MAIN BANDS UNDER DIFFERENT ENDOCRINE STATES

Endocrine States	No. of samples	Peak Height (mean ± SE)		
		5	6	10
Pro-oestrus	4	*9.5 ± 2.7	*13.0 ± 3.8	** 38 ± 3
Oestrus	5	10.2 ± 4.9	18.4 ± 5.9	37 ± 7
Dioestrus	5	*0.6 ± 0.2	*1.4 ± 0.2	52 ± 6
Day 5 Pseudopregnancy	5	0.8 ± 0.4	1.0 ± 0.4	**55 ± 1
(Plasma)	8	1.9 ± 0.4	0	62 ± 4

* Pro-oestrus Vs Dioestrus $p < 0.05$

** Pro-oestrus Vs Day 5 Pseudopregnancy $p < 0.01$

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