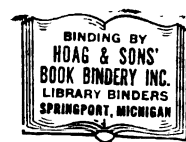


OVIDUCT FLUID AND ITS EFFECT ON
THE FERTILIZING ABILITY OF
CAPACITATED RABBIT SPERM AND
STUDIES ON IN VITRO FERTILIZATION

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ABSTRACT

OVIDUCT FLUID AND ITS EFFECT ON THE FERTILIZING ABILITY OF CAPACITATED RABBIT SPERM AND STUDIES ON *IN VITRO* FERTILIZATION

By

Michael Peter Johnson

In vitro fertilization experiments were conducted to determine the effect of oviduct fluid on the fertilizing ability of capacitated rabbit sperm. Sperm were recovered from uteri at various times after mating and were incubated in culture dishes containing 50% oviduct fluid-50% medium or 100% medium for one hour. Ova were added to the incubation dishes after the hour of sperm incubation. Ova were examined 24 hours later for cleavage. Sperm incubated in 50% oviduct fluid-50% medium fertilized ova at a rate of 24%. Sperm incubated in 100% medium fertilized at a rate of 59%.

Rabbit oviduct fluid was analyzed for protein using gel chromatography, spectrophotometry and the ninhydrin test. Sephadex G-75 chromatography and u.v. spectrophotometry at 280 m μ revealed oviduct fluid to contain two protein peaks. As oviduct fluid daily yield varied, protein did not change quantitatively, but did change qualitatively.

Experiments were conducted in an attempt to *in vitro* fertilize *Saimiri sciureus* ova. Sperm were recovered by electroejaculation and incubated in a medium for one hour. Ova were aspirated from ovarian follicles following ovulation induction. Ova were added to the sperm-containing medium and after 24 to 30 hours incubation, were examined for signs of fertilization. Sperm penetration of the zona pellucida was achieved in one ovum. Sperm entry into the cumulus mass occurred in two ova. No fertilization was achieved.

Macaca fascicularis blood plasma samples were collected and analyzed for estrone, estradiol, progesterone and 17 α -hydroxyprogesterone by radioimmunoassay. Estradiol and progesterone concentrations were similar to those reported for *Macaca mulatta*. Estrone and 17 α -hydroxyprogesterone concentrations were inconsistent among the samples analyzed.

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Michael Peter Johnson

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INTRODUCTION

Little is known of the site at which fertilization occurs *in vivo*, the oviduct. An understanding of the process of fertilization as it occurs naturally cannot be complete until the function of the oviduct and its secretions is known. Paradoxically, the best way to study the factors influencing *in vivo* fertilization is to observe fertilization *in vitro*. *In vitro* fertilization systems and techniques for quantitative and qualitative measurement of substances have been developed which allow for examination of the factors and occurrences of fertilization.

In vitro fertilization systems and analysis techniques were used in these experiments to learn more about the process of fertilization as it occurs in the rabbit and the monkey. The objectives of these investigations were:

1. To determine the effect of oviduct fluid upon the fertilizing ability of capacitated rabbit sperm,
2. To study the nature of rabbit oviduct fluid,
3. To develop an *in vitro* fertilization system for the squirrel monkey (*Saimiri sciureus*),
4. To obtain values for the blood plasma concentrations of sex steroids in the crab-eating macaque (*Macaca fascicularis*).

RABBIT *IN VITRO* FERTILIZATION EXPERIMENTS

Literature Review

Studies of *In Vitro* Fertilization

Since the discovery of the need for capacitation (Austin, 1951; Chang, 1951), a number of investigators have successfully attempted *in vitro* fertilization of mammalian ova. *In vitro* fertilization has been achieved in several animals, including the rabbit, hamster, mouse and human.

Thibault, Dautier and Wintenberger (1954) reported a fertilization rate of 87% using ova flushed from oviducts of rabbits and sperm recovered from the uterus, as opposed to 4% fertilization using ejaculated sperm. The 4% was believed to be parthenogenic cleavage due to working with gametes at lower than physiological temperatures. Dautier and Thibault (1956) later reported 25% fertilization using sperm recovered from the uterus 12 hours after mating, 3.3% using sperm recovered 8 hours after mating, and no fertilization using sperm recovered 6 hours after mating. Gamete manipulations in these experiments were done at 37 to 38°C.

Chang (1959) provided the first indisputable evidence of *in vitro* fertilization. *In vitro* incubations of 12 hour uterine sperm with oviductal ova resulted in

cleavage to the 4 cell stage in 21% of the ova. The cleaved ova were then transferred to host does, with 42% of those transferred resulting in living young. The initial incubation medium was a Krebs-Ringer bicarbonate solution with 0.25% glucose. After 4 hours the ova were transferred to a 50% heat inactivated serum in saline medium. Bedford and Chang (1962a) reported that uterine sperm motility was prolonged if 5% serum was added to an acidic saline medium composed of NaCl, KCl, CaCl_2 , NaH_2PO_4 , and MgCl_2 in water. The pH of this medium was 4.8.

Williams, Hamner, Weinmann and Brackett (1964) using the system described by Chang (1959) reported a fertilization rate of 15% when relative humidity was held at 75% during the manipulation of gametes. When the humidity was maintained at 90% they achieved 35% fertilization. They suspected that cooling of the incubation medium by excessive evaporation was the reason for the lower fertility.

Brackett and Williams (1965) achieved 73% fertilization when 96-97% relative humidity and a temperature of 37-39°C were maintained during gamete handling. Also, the gametes were covered with parafin oil and 20-40% fresh uterine fluid was added to the medium. The medium used was an acidic saline solution with 5% serum. After 4 hours incubation, ova were transferred to a medium with 10% serum.

Suzuki and Mastroianni (1965) incubated tubal ova and 12 hour uterine sperm in oviduct fluid. When mineral oil was used to cover the gametes, they reported 31% fertilization. When the mineral oil was equilibrated with 5% CO₂ the rate was 64%. In these experiments there was no incubation of sperm alone in oviduct fluid before the addition of ova, and after 4 hours incubation the ova were transferred to a prepared medium with 10% serum. The effect of the oviduct fluid was not clear, however, since no control medium was used. Suzuki and Mastroianni (1968) later achieved 29% fertilization of ovarian follicular ova recovered 12 hours after human chorionic gonadatropin administration, using 12 hour uterine sperm which were incubated in oviduct fluid for 15 minutes prior to the addition of ova. A control using a Krebs-Ringer bicarbonate medium, with and without serum, produced no fertilization.

Brackett and Williams (1968) developed a defined media system. To acidic saline they added glucose, NaHCO₃, and bovine serum albumin. The NaHCO₃ was to maintain the pH of the system, which was 7.2. The B.S.A. was to replace serum. This system apparently eliminated the need to add uterine fluid since the medium without uterine fluid allowed a fertilization rate of 65%, while adding uterine fluid resulted in 67% fertilization.

Brackett, Rocha and Seitz (1969) studied the fertilizing ability of sperm recovered from the uterus at

varying times after mating and reported that 18 hour sperm fertilized a greater proportion of ova than did 13 hour sperm.

Brackett (1970) used time-lapse microcinematography to record the events of *in vitro* fertilization. He reported sperm penetration through the zona pellucida was completed 3 hours after exposure of ova to sperm. Pronuclei were developed 9 hours after insemination. Cleavage to 2 cells occurred as early as 15.5 hours after insemination, to 4 cells by 18.5 hours, and 8 cells as early as 26 hours. By 48 hours some ova had developed to the morula stage. Penetration of the vitellus was observed in one ovum; the sperm was free in the perivitelline space for 20 minutes before attaching to the vitelline membrane, and entry of the entire sperm into the vitellus required 23 minutes at room temperature. The process of cleavage required 5 to 8 minutes. There was no observable difference in cleavage between ova fertilized *in vitro* and those fertilized *in vivo*.

Seitz, Brackett and Mastroianni (1970) reported 81% fertilization of ova recovered from the ovarian surface using 12 hour uterine sperm. This showed that physical contact of the ova with the oviduct is not necessary for a high rate of fertilization to occur. The medium used was that previously described by Brackett and Williams (1968) with 20% serum added. They reported that the addition of this amount of serum eliminated the need to transfer ova

after the initial few hours of incubation. They did report that although the percentage of fertilization was not affected, the time of early cleavage was retarded when there was no transfer.

Brackett, Killen and Peace (1971) studied the influence of the layers about the ova by experimentally removing them and then exposing these ova to 12 hour uterine sperm. Cumulus masses were removed using hyaluronidase. Coronae radiatae were then removed by placing ova in a bicarbonate solution and mechanically removing this layer with probes. Zonae pellucidae were then removed by placing the ova in a trypsin solution and mechanically removing this layer. The remaining portion of the ova were then transferred to a sperm suspension medium or a nonsperm containing medium. They reported normal appearing cleavage when ova were incubated with sperm and no cleavage when incubation was in the absence of sperm. Also, when incubated with sperm concentrations greater than 5×10^4 /ml, ova with their cumulus, their cumulus and corona, and their cumulus, corona and zona removed, cleaved at the same rate as intact ova. When the sperm concentration was less than 5×10^4 /ml, ova with layers removed had a higher percentage of fertilization than did intact ova. Ejaculated sperm were able to induce cleavage in ova with layers removed but not in intact ova.

Studies of Sperm Capacitation

Austin (1951) and Chang (1951) independently discovered the dependence rabbit sperm have upon the female reproductive tract in order to achieve fertilizing ability. They reported sperm must reside in the female reproductive tract for a matter of hours before becoming able to penetrate the zona pellucida. Many experiments in several species since then have made it apparent that functional changes occur in sperm as they are in the presence of the female tract. These changes have collectively been termed capacitation (Austin, 1952).

Chang (1951) and Austin (1951) reported that capacitation took place in either the oviduct or uterus. Their experiments involved inseminating oviducts with ejaculated or uterine sperm at varying times before and after sheep pituitary extract induced ovulation. Ejaculated sperm placed in the oviducts less than 4 hours before ovulation fertilized no ova. Insemination of ejaculated sperm 4 to 8 hours before ovulation resulted in fertilization. It was inferred that the fertilizable life of the ova expired before the later deposited sperm were capacitated. Chang (1951) reported that sperm which had resided in the uterus for 5 hours were able to fertilize ova when the sperm were deposited in oviducts as late as 2 hours after ovulation, so apparently they had been capacitated at least in part while in the uterus.

The time required for capacitation of rabbit sperm has been the subject of some debate. Adams and Chang (1962) showed that 4 to 6 hours is sufficient in the uterus, while 10 hours are required in the oviduct. Most experimenters working with *in vitro* fertilization with the rabbit today use sperm which have resided in the female tract from 12 to 18 hours. Fertilization has been obtained many times using 12 hour sperm, but some authors (Brackett, Rocha and Seitz, 1969; Bedford, 1969) have reported higher fertilization rates using 16 to 18 hour sperm. Dziuk (1965) suggested that sperm are capacitated at different rates, the first within 10 to 11 hours, the last by 16 to 18 hours.

The hormonal state of the female tract is of importance in capacitation. A number of authors (Chang, 1958; Soupart, 1967; Bedford, 1967a; Hamner et al., 1968) have reported that capacitation is depressed when the rabbit uterus is under progesterone dominance. Bedford (1970) found that when no ovarian estrogen is present, the rabbit uterus requires 17 to 20 hours to capacitate sperm. The oviduct, however, may not be sensitive to hormonal changes. Chang (1958) reported that progesterone dominance did not affect capacitation in the oviduct.

The changes brought about by capacitation are not precisely known, although experimenters have provided some explanation. Chang (1957) reported that seminal plasma from rabbits caused an inhibition of the fertilizing ability of

capacitated sperm, and that this inhibition could be reversed by returning the sperm to the female tract. This substance which reverses capacitation has been termed decapacitation factor (D.F.). A number of workers have studied the nature of D.F. Dukelow, Chernoff and Williams (1967) reported that recapacitation of decapacitated sperm in the oviduct requires the same amount of time as did the original capacitation. Zaneveld and Williams (1970) reported the presence of an enzyme in sperm acrosomal extracts which was able to disperse the corona radiata from about the ovum. They also found that D.F. had the ability to inhibit the action of this enzyme. Robertson, Bhalla and Williams (1971) reported purification procedures revealed D.F. to be a low molecular weight peptide containing arginine and histidine.

Zaneveld, Srivastava and Williams (1969) reported the presence of a trypsin-like enzyme in rabbit acrosomes which readily removed the zona of rabbit ova. An inhibitor of this enzyme was found in seminal plasma. They suggested this enzyme-enzyme inhibitor relationship was similar to the corona penetrating enzyme-decapacitation factor relationship.

Zaneveld, Srivastava and Williams (1970) found epididymal sperm to have both high corona penetrating enzyme and trypsin-like enzyme activity, ejaculated sperm to have low activities of these enzymes, and capacitated sperm to have high activities of them. Incubation of epididymal or

capacitated sperm in seminal plasma was reported to reduce corona penetrating enzyme and trypsin-like enzyme activity. Apparently, as epididymal sperm are ejaculated, the inhibitors to these enzymes are transferred from the seminal plasma to the sperm. Then while the sperm are residing in the female tract these inhibitors are removed, this likely being a part of the capacitation process.

Several workers have suggested the oviduct may have an adverse affect on sperm once they have been capacitated. Bedford (1967b) reported that rabbit sperm inseminated into rat uteri retained their fertility longer than they did in the rabbit oviduct. He suggested that in the rat uterus the sperm never became fully capacitated. But, when they were transferred to the rabbit oviduct to complete capacitation, they rapidly lose their fertility.

Dukelow and Williams (1967) reported that 8 or 12 hour uterine capacitated rabbit sperm deposited in the oviducts of rabbits which had received human chorionic gonadatropin 6 hours earlier, which would place deposition approximately 4 hours before ovulation, fertilized at a lower rate than capacitated sperm deposited in oviducts of rabbits which had received human chorionic gonadatropin 10 hours earlier, which would make deposition at about the time of ovulation. They also reported that capacitated sperm which had been ejaculated 12 or 16 hours earlier which had spent 4 of those hours residing in the oviduct had a

greatly reduced fertility when compared to sperm which spent the entire 12 or 16 hours in the uterus. Seitz, Rocha, Brackett and Mastroianni (1970) reported that sperm confined to the uterus maintained their fertility for as long as 34 to 36 hours. Dukelow and Williams (1967) suggested that the oviductal environment in which the sperm had to reside while waiting for ovulation had a detrimental effect on the fertilizing ability of capacitated sperm.

Wettemann, Ingalls and Hafs (1971) found that ejaculated sperm inseminated at the time of an ovulatory injection into oviducts fertilized at a lower rate than when inseminated into the uterus. When using superovulated sperm recipients, when 10 hour uterine capacitated sperm were inseminated at 13 hours after an ovulatory injection into oviducts the fertilization rate was lower than when inseminated into the uterus. It would seem the oviductal environment interfered with the fertilizing ability of sperm here also. When capacitated sperm were inseminated into oviducts and uteri of nonsuperovulated rabbits, however, there was no difference in fertilization between the two sites.

Materials and Methods

Experiments were conducted to determine the effect of rabbit oviduct fluid on the fertilizing ability of capacitated rabbit sperm. Some procedures were a modification of those used by Harrison (1971). Some procedures were adapted from other workers (Hamner and Williams, 1965). Some procedures were designed specifically to meet the experimental requirements.

Animals

All rabbits used were adults of the New Zealand White or mixed breeds, and were obtained from local dealers. The rabbits were housed individually in wire mesh cages and females were isolated for a minimum of 21 days before being used in order to insure that none would be in progesterone dominated pseudopregnancy, which lasts 17 to 18 days in the rabbit. All males used were of proven fertility. The building housing the rabbits was heated during the cold months. Rabbits were fed 5 to 6 ounces of commercial rabbit pellets (Triumph Feed Co.) daily and water was supplied *ad libitum*.

Collection of Oviduct Fluid

Rabbit oviduct fluid was collected using the glass flask described by Hamner and Williams (1965). Flask installation involved surgery on a sodium pentobarbital

(Halatal Solution, Jensen-Salsbery Labs) anesthetized rabbit. The rabbit was opened with a midline incision. Plastic tubing (P.E. 100) was inserted approximately one cm into the infundibular end of each oviduct and was held in place by tying with silk suture. Initially, the tubing was attached to the flask using an adapter made from plastic disposable 21 gauge needles reduced to approximately one cm in length. The first flask installations were not successful, however, resulting in either infection and no oviduct fluid collection or oviduct fluid collection lasting for only a few days. Upon opening the rabbit in an attempt to determine the cause of no collection, infection was often noted in the flask and tubing. In the final flask installation the needle adapter was replaced with a stainless steel adapter. Oviduct fluid collection continued for 16 weeks following this installation. After attaching the adapters to the flask, the oviducts were ligated near the tubal-uterine junction to prevent any mixing of uterine fluid and to prevent loss of oviduct fluid into the uterus. The flask was then placed into the abdominal cavity. An incision was made through the lateral body wall just posterior to the ribs and the withdrawal portion of the flask was pushed through this incision. The flask was positioned such that the reservoir pointed ventrally and the incisions were sutured. Cotton soaked in a streptomycin solution was inserted into the exposed withdrawal portion of the flask to

prevent infection in the flask. This cotton was changed daily. Antibiotic (Combiotic, Pfizer Labs) was then administered to the rabbit and continued daily for 3 or 4 days to reduce the possibility of infection. The rabbit was returned to its cage after recovery from the anesthesia. The first oviduct fluid collection was made either 24 or 48 hours after flask installation. Oviduct fluid was collected from the flask using a 5 ml syringe with a plastic tubing adapter which fit onto the withdrawal tube of the flask. Upon collection, the oviduct fluid was centrifuged at 862x gravity for 10 minutes and the supernatant was frozen at -15°C until use. Collection of oviduct fluid was done daily.

Superovulation Procedure

Rabbits serving as ova donors were superovulated. These donors received 0.25-0.75 mg of follicle stimulating hormone (F.S.H.) (F.S.H., Armour-Baldwin Labs) subcutaneously both a.m. and p.m. for 3 days to promote follicle development. The final injection of F.S.H. was given approximately 12 hours before an ovulatory injection of human chorionic gonadatropin (H.C.G.) (A.P.L., Ayerst Labs) was administered. The dose of H.C.G. was 100 i.u. and was administered intravenously into an ear vein. This procedure was first described by Kennelly and Foote (1965).

Recovery and Incubation of Sperm

Rabbits serving as sperm capacitors were mated at least twice to fertile males. Twelve to 18 hours after mating, the capacitor was killed either by cervical dislocation or sodium pentobarbital overdose. A midline abdominal incision was made to expose the reproductive tract. Each uterine horn was clamped at the cervical end with a hemostat. Using a 5 ml syringe with a 20 gauge needle, sperm were recovered from each horn by injecting 2 to 3 ml of culture medium into the horn and aspirating the contents. The fluid was reinjected and reaspirated several times. The resulting sperm suspensions were placed into culture dishes, under silicone oil (Dow Corning 200 Fluid, Dow Corning), with equal volumes from each horn going into each dish. To one of these dishes was added a volume of culture medium equal to the volume of sperm suspension. To the other dish an equal volume of oviduct fluid was added. The culture dishes were then placed for one hour in an incubation chamber at a temperature of 36 to 39°C, high humidity, and a 5% CO₂ in air atmosphere. A drop of sperm suspension from the aspirating syringes was placed on a hemacytometer and the number of sperm counted. Once the rabbits were killed and their uterine horns aspirated or reproductive tracts removed, all manipulations of gametes and tissues were done under a hood at a temperature of 37 to 38°C. All handling of the gametes after the aspiration of sperm was done with the

gametes under 5% CO₂ in air-equilibrated silicone oil. The culture medium and silicone oil were at 37 to 38°C at all times throughout the experiment. All equipment used in working with tissues and gametes was sterilized by autoclaving. The culture medium was sterilized by use of millipore filters (H.A. 0.45μ, 25 mm diameter, Millipore Corp.).

Recovery of Ova

Twelve and one-half to 13 hours after H.C.G. administration the ova donors were killed and their reproductive tracts exposed. The ovary, oviduct and proximal 1/3 of the uterine horn were removed in one piece, from each side. Ova were recovered by passing a 24 gauge needle with 5 ml syringe attached through the tubal-uterine junction into the oviduct and flushing 1.5 ml of culture medium through the oviduct and out the fimbria, which was submerged in silicone oil in a watch glass. By this procedure the ova are flushed out with the first few drops. Most often, the ova were recovered in their cumulus masses. In order to facilitate sperm penetration, as much cumulus as possible was removed after transferring the ova to a culture dish. This was done mechanically originally, under a stereoscopic dissecting microscope using microforceps and microprobes to pull cumulus cells away from the ovum. Later, hyaluronidase (General Biochemicals, 450 i.u./mg) was used for cumulus

removal. Flushed masses were placed in 0.01 or 0.005% hyaluronidase in culture medium solutions. At the end of 10 minutes ova were freed of their cumulus masses. After an hour the sperm suspension culture dishes were removed from the incubator and the ova added randomly to the dishes. The dishes were returned to the incubator for 24 hours, after which time the ova were examined for fertilization. Transfer of ova, either in their cumulus masses or free, was accomplished using Pasteur pipettes with an attached suction device.

The Culture Medium

The contents of the culture medium per liter were as follows: 6.550 gm NaCl; 0.300 gm KCl; 0.250 gm CaCl_2 ; 0.113 gm $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; 0.106 gm $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$; 3.104 gm NaHCO_3 ; 2.500 gm glucose; 3.000 gm bovine serum albumin; 50,000 units K-penicillin G; distilled water to 1000 ml. This, with a few minor changes, is the defined media first used by Brackett and Williams (1968). Fifty ml of this media was prepared. Ten ml were withdrawn and replaced with 10 ml of heated rabbit serum. Serum was prepared as follows: blood was obtained from rabbits by heart puncture and allowed to coagulate for one hour. The serum was drawn off and centrifuged at 862x gravity for 10 minutes. The supernatant was heated in a water bath at 55°C for 20 minutes, and frozen until use.

Criteria for Fertilization

A determination of fertilization was made as follows: (1) 24 hours after the addition of ova to the sperm suspensions, ova were examined for cleavage. Ova in the 2 to 4 cell stage with cells of equal size and normal appearance (nearly spherical, not irregular in shape) were considered fertilized (Austin, 1963). (2) If 2, 3 or 4 cells were present and there was slight irregularity in their shape, the zona pellucida and perivitelline space were examined for sperm. If sperm were found the ova were considered fertilized. (3) Ova which were still a single cell or which were multiple celled but irregular in shape were not considered fertilized.

Experimental Design

The design of the experiments involved in testing the effect of oviduct fluid on the fertilizing ability of capacitated sperm is shown in Figure 1.

The variations which occurred among experiments were these: (1) three dose levels (0.25, 0.50 and 0.75 mg) of F.S.H. were used to determine if the number of ova produced was related to the dose of F.S.H. (2) Capacitators were killed from 12 to 18 hours after mating to determine if these time differences influenced the quantity of sperm recovered from the uterine horns. (3) Hyaluronidase was used in some experiments for removing cumulus masses while

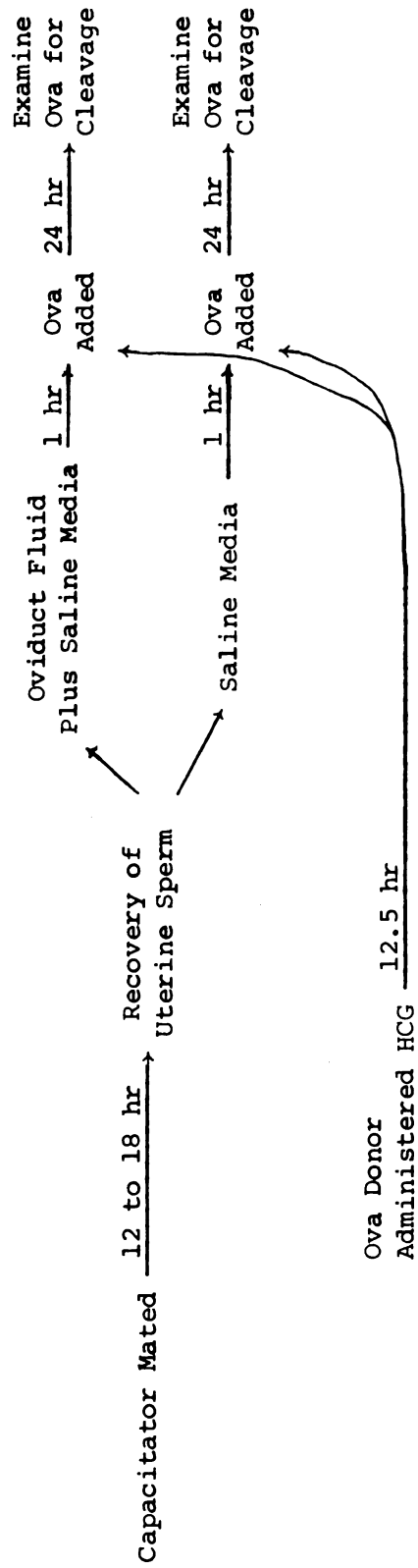


Figure 1. Experimental design for rabbit *in vitro* fertilization experiments.

mechanical means were used to remove masses in other experiments. Two concentrations of hyaluronidase (0.01 and 0.005%) were used to determine what concentration was necessary for cumulus removal.

Two efforts were made to insure equal chances for fertilization: (1) All ova were pooled from the donors and then randomly distributed to the sperm suspensions. (2) Equal volumes of sperm suspension from each uterine horn were distributed to each culture dish.

In some experiments, ova recovered from the oviducts of capacitated rabbits were incubated *in vitro*. This served as a control on the fertilizing ability of the sperm *in vivo* and on the ability of the media to support cleavage.

Results

A total of 27 rabbit *in vitro* fertilization experiments were conducted, the purpose of which was to determine the effect oviduct fluid has upon the fertilizing ability of capacitated sperm in the rabbit.

Six experiments resulted in the *in vitro* fertilization of ova. The numbers of ova and the rates of fertilization for these experiments are shown in Table 1. These figures are also given for all ova incubated in the presence of sperm in all 27 experiments in Table 2. The results indicate that rabbit oviduct fluid had an adverse effect on the fertilizing ability of the sperm.

Table 1

Rates of *In Vitro* Fertilization in Experiments in Which *In Vitro*
Fertilization Occurred

	Sperm Treatment	
	50% Oviduct Fluid- 50% Saline-Serum Medium	100% Saline-Serum Medium
Number of Ova in Exps. in Which <i>In Vitro</i> Fertilization Occurred	25	27
Number of Fertilized Ova	6	16
% Fertilized	24%	59%

Table 2

Rates of *In Vitro* Fertilization for All Ova Incubated in the
Presence of Sperm

	Sperm Treatment	
	50% Oviduct Fluid- 50% Saline-Serum Medium	100% Saline-Serum Medium
Total Number of Ova Incubated in Presence of Sperm	122	126
Number of Ova Fertilized	6	16
% Fertilized	5%	13%

F.S.H. was given at doses of 0.25, 0.50 and 0.75 mg in an attempt to determine the dose which would produce the greatest number of ova. The effects of the different dosages of F.S.H. upon the number of ova recovered after an ovulatory injection of H.C.G. is shown in Table 3. The number of ova produced by superovulation does not seem to be dose dependent within this dose range. The mean number of ova for all doses of F.S.H. was 9.9 ova per rabbit. In experiments involving 6 rabbits which received no F.S.H. prior to an injection of 100 i.u. of H.C.G. the mean number of ova recovered was 4.8 per rabbit, as shown in Table 3. F.S.H. did, therefore, have a superovulatory effect at the dose levels given.

Table 3

Effect of Varying Doses of Follicle Stimulating Hormone Upon the Number of Ova Recovered

Dose of F.S.H. ^a	Number of Ova Recovered per Rabbit ^b	Number of Rabbits
0.00 mg	4.8	6
0.25 mg	10.3	8
0.50 mg	10.0	16
0.75 mg	9.0	8

^a Administered twice daily for 3 days.

^b After administration of 100 i.u. H.C.G.

Sperm were originally aspirated from the uterus 17 to 18 hours after mating. Due to the absence of fertilization in several experiments it was felt that the chances of fertilization might be improved if the number of sperm incubated with the ova *in vitro* were greater. It was thought recovering sperm earlier than 17 or 18 hours would increase sperm numbers by allowing less time for sperm loss. The results of sperm aspiration at different times following mating are shown in Table 4. No more sperm were lost after 17 to 18 hours than were lost by 12 to 13 hours. Large numbers of sperm must be lost rapidly from the uterus after mating, within 12 hours at least, and after that the loss is very gradual. The number of sperm necessary for *in vitro* fertilization is low since fertilization was achieved with a sperm concentration as low as 4×10^3 sperm per ml (sperm concentration in the uterine aspiration was 8×10^3 /ml, but this concentration was halved when either oviduct fluid or culture medium was added to the incubation dish).

Cumulus masses about ova were originally removed mechanically using micro probes and forceps. If the number of ova contained in the cumulus exceeded 4 or 5, however, the time involved in freeing the ova became excessive, considering the short fertilizable life of the rabbit ova, and some of the ova had to be abandoned. Because of this, hyaluronidase was used to remove the cumulus masses by

placing the flushed masses in either a 0.01 or 0.005% hyaluronidase in culture media solution for 10 minutes. At the end of this time the ova were completely freed from their cumulus masses at both concentrations. In the 11 experiments in which hyaluronidase was used, however, fertilization was achieved only once, as opposed to fertilization occurring in 5 of the first 9 experiments in which cumulus removal was mechanical. Finally, therefore, the use of hyaluronidase was abandoned and mechanical means again used.

Table 4

Number of Uterine Sperm Recovered at Varying Times After Mating

Hours After Mating	Number of Sperm per ml	Number of Rabbits
12-13	8.2×10^3	4
15-16	8.4×10^3	5
17-18	10.0×10^3	4

Ova recovered from the oviducts of capacitator rabbits were incubated as a check on the fertilizing ability of the sperm *in vivo* and also of the ability of the media to support development once fertilization had occurred. This control was included in 13 experiments. Fertilization occurred in 5 of these experiments. Table 5 shows the fertilization rates among these ova.

Sperm were found in uterine aspirations in 17 of 27 experiments.

Table 5
Rates of *In Vivo* Fertilization

	Number of Capacitated Ova Recovered	Number of Capacitated Ova Fertilized	% Fertilized
Exps. in which <i>In Vivo</i> Fertilization Occurred	17	14	83%
All Experiments	44	14	32%

Discussion

The results of 6 experiments in which *in vitro* fertilization of ova occurred indicate that oviduct fluid has an adverse effect on the ability of capacitated sperm to fertilize ova in the rabbit. While fertilization by oviduct fluid treated sperm did occur, the frequency of fertilization for control sperm was approximately 2.45 times greater than for treated sperm. The fertilization rate of control sperm was greater than that of oviduct fluid treated sperm in each of the 6 experiments in which fertilization occurred. These results support the suggestion of Dukelow and Williams (1967) that some factor of the oviductal environment is harmful to capacitated sperm fertility. This factor does not require the presence of the oviduct to exert itself; it is an oviduct fluid factor which is effective *in vitro* as well as *in vivo*.

The nature and mechanism of the factor causing the decreased fertilizing ability is unknown. Perhaps it is an antipacitination substance. The factor could coat the acrosome, inhibiting the acrosome reaction. Alternatively, a substance could be present in oviduct fluid which inhibits the acrosomal enzymes' penetration abilities. Also, some substance could affect the metabolism of sperm, rendering them incapable of ovum penetration. The function of this naturally occurring antifertility factor is also unknown.

The nature, natural function and antifertility implications of this oviduct fluid factor can be learned only through further research.

Twenty-one of 27 experiments resulted in no *in vitro* fertilization by either control or oviduct fluid treated sperm. The reasons for this cannot definitely be known in all cases, although several things are known about these experiments which point to factors which may have been responsible.

In 9 of the 21 experiments no sperm were found upon uterine aspiration. Obviously, no *in vitro* fertilization could have taken place in these experiments. All capacitator rabbits were mated at least twice so that sufficient numbers of sperm were introduced into their reproductive tracts. Though rabbits are constant estrus animals, they do have days in which they are more sexually receptive. These animals were selected for mating by observing the color of their external genitalia and checking for a lordosis response. These are subjective observations, however, so perhaps rabbits were chosen which were not truly receptive. These rabbits may not have been in the ideal hormonal state and the sperm may have been phagocytized. Sperm destruction due to pseudopregnancy progesterone dominance may be ruled out since only rabbits which had been isolated for a minimum of 21 days were used.

Hyaluronidase was used for cumulus mass removal in 11 experiments. Bedford and Chang (1962a) and Brackett, Killen and Peace (1971) used this procedure for cumulus removal and still obtained *in vitro* fertilization. In the work reported here, however, of these 11 experiments only one resulted in *in vitro* fertilization. If the use of hyaluronidase was the reason for the lack of fertilization in these experiments, perhaps the hyaluronidase used contained a contaminant which rendered ova unfertilizable.

Capacitator rabbit oviducts were flushed for ova in 20 experiments. Ova were found in 13 of these, and of these, fertilized ova were found in 5. The absence of ova in the capacitator did not indicate unsuitability of the capacitator to capacitate sperm since *in vitro* fertilization did occur in 2 of the 7 experiments in which no capacitator ova were recovered. The presence of *in vivo* fertilized capacitator ova was a good indicator of success of *in vitro* fertilization since of the 5 experiments in which capacitator ova were fertilized *in vivo*, fertilization also occurred *in vitro* in 4 of these experiments. In the 8 experiments in which capacitator ova were found but were not fertilized, *in vitro* fertilization did not occur. Apparently, if sperm were able to fertilize ova *in vivo* they could also do so *in vitro*, and conversely, if sperm were not able to fertilize ova *in vivo*, they were also incapable of *in vitro* fertilization.

RABBIT OVIDUCT FLUID ANALYSIS

Literature Review

The composition of oviduct fluid in the rabbit has been the subject of several investigations in the past few years. Hamner and Williams (1965) developed an intra-abdominal flask which continuously collected oviduct fluid. They reported each oviduct secreted 0.4 to 2.0 ml of fluid per day. The pH ranged from 7.8 to 8.2. They also analyzed the fluid for the presence of several ions, polysaccharide, amino acids and total protein.

Holmdahl and Mastroianni (1965) devised an apparatus for the continuous collection of oviduct fluid at 2 to 4°C. They collected oviduct fluid before and after induced ovulation. They also collected fluid at body temperature, before and after ovulation. Before ovulation glucose and calcium concentrations were higher in the "cold" oviduct fluid than in the "warm." Other components, including total protein, were present in equal amounts of cold and warm fluid. After ovulation the sodium concentration decreased and calcium increased in both cold and warm collections. Protein concentration was unchanged after ovulation in both cold and warm oviduct fluid.

Iritani, Nishikawa, Gomes and VanDemark (1971) collected oviduct fluid using a flask similar to that used by Hamner and Williams (1965). They reported a mean yield of 0.89 ml per animal per day (2 oviducts). They found that the concentration of constituents in oviduct fluid was inversely related to the secretion rate of fluid.

Urzua, Stambaugh, Flickinger and Mastroianni (1970) used the refrigeration apparatus devised by Holmdahl and Mastroianni (1965) to collect oviduct fluid before and after induced ovulation. They analyzed the fluid for protein by acrylamide gel electrophoresis. They reported that the protein pattern was different from that of serum in that a lesser quantity of transferrin was present in the oviduct fluid. It was also different from uterine fluid in that it had no blastokinin. Oviduct fluid, uterine fluid and serum had similar quantities of albumin. Exogenous administration of estradiol had no affect on oviduct or uterine fluid protein patterns. Exogenous progesterone or progesterone plus estradiol administered to castrates produced a postalbumin protein in uterine fluid but had no effect upon oviduct fluid protein patterns.

Shapiro, Jentsch and Yard (1971) studied oviduct fluid proteins using disc gel electrophoresis and immunoelectrophoresis. Gel electrophoresis showed oviduct fluid lacked certain globulins that serum contained, and that serum lacked certain globulins that oviduct fluid contained.

Oviduct fluid had a greater albumin content than serum did. Albumin was the most prominent protein in oviduct fluid. Immuno-electrophoresis showed fewer precipitation bands in oviduct fluid than in serum. After immunization to yeast alcohol dehydrogenase, which is foreign to the rabbit, anti-yeast alcohol dehydrogenase was present in serum but not in oviduct fluid. Antibodies prepared in the hen against rabbit oviduct fluid proteins reacted with rabbit serum. These results all indicated that some proteins present in serum were not present in oviduct fluid, and some proteins present in oviduct fluid were not present in serum, and some proteins were present in serum and oviduct fluid. The authors concluded that oviduct fluid is neither a complete transudate nor a complete secretory product, but instead, a mixture of the two. The means used for oviduct fluid collection in these experiments was not specifically stated, but it was implied that the flask developed by Hamner and Williams (1965) was used.

Materials and Methods

Rabbit oviduct fluid was subjected to gel chromatography in order to achieve fractionation of the oviduct fluid. The resulting fractions were examined with an ultraviolet spectrophotometer at a wavelength of 280 m μ to determine the presence of protein peaks. The peaks' protein character was confirmed by the ninhydrin reaction.

Gel Chromatography

Gel chromatography of rabbit oviduct fluid was performed with Sephadex gel (Pharmacia Fine Chemicals). Sephadex is a dextran bead gel. The beads are available with different sized pores, ranging from pores which will exclude molecules above 700 mw to pores which will include molecules up to 800,000 mw. The sizes of pores used in these experiments were Sephadex G-25 which excludes molecules above 5,000 mw, and Sephadex G-75 which excludes molecules above 70,000 mw (Sephadex, 1970). The eluants used were distilled water and a 0.10 molar sodium chloride solution. The sizes of the columns used were 30 cm x 0.9 cm and 30 cm x 1.5 cm. The quantity of oviduct fluid fractionated in each experiment was 0.25 ml. As the eluant passed out of the column it was collected in tubes with a Gilson model fraction collector, with either 100 or 75 drops per tube. Gel chromatography was done at a temperature of 4°C.

Spectrophotometry

Spectrophotometry of oviduct fluid fractions was done with a Beckman B-D spectrophotometer using a Beckman hydrogen light as the ultraviolet light source. All readings were taken at a wavelength of 280 mμ. The standard used was the same as the column eluant used in that fractionation.

Ninhydrin Test

Whole oviduct fluid and oviduct fluid fractions were subjected to the ninhydrin test for amino acids. The ninhydrin reagent was obtained from Nutritional Biochemicals Corp.

Results

A total of 17 fractionations of oviduct fluid were conducted.

Three oviduct samples were fractionated on Sephadex G-25. Spectrophotometry of each of these revealed that these fractionations resulted in a single peak. The peak appeared within tubes 2 through 5 in all 3 cases. Figure 2 shows the results of a fractionation with Sephadex G-25 on a 30 cm x 0.9 cm column.

Fourteen oviduct fluid samples were fractionated on Sephadex G-75. Spectrophotometry for 12 of these showed the presence of 2 peaks. For one sample a single peak was revealed, and in another sample 3 peaks were present. Eleven of the 14 fractionations using Sephadex G-75 were run on a 30 cm x 0.9 cm column. Among all of these, the first peak appeared within a range of tubes 1 through 5, with 8 runs having the first peak in tubes 2 through 4. The second peak appeared in the range of tubes 5 through 9, with 7 runs having this peak in tubes 6 through 8. Figure 3 shows the results of a fractionation with Sephadex G-75

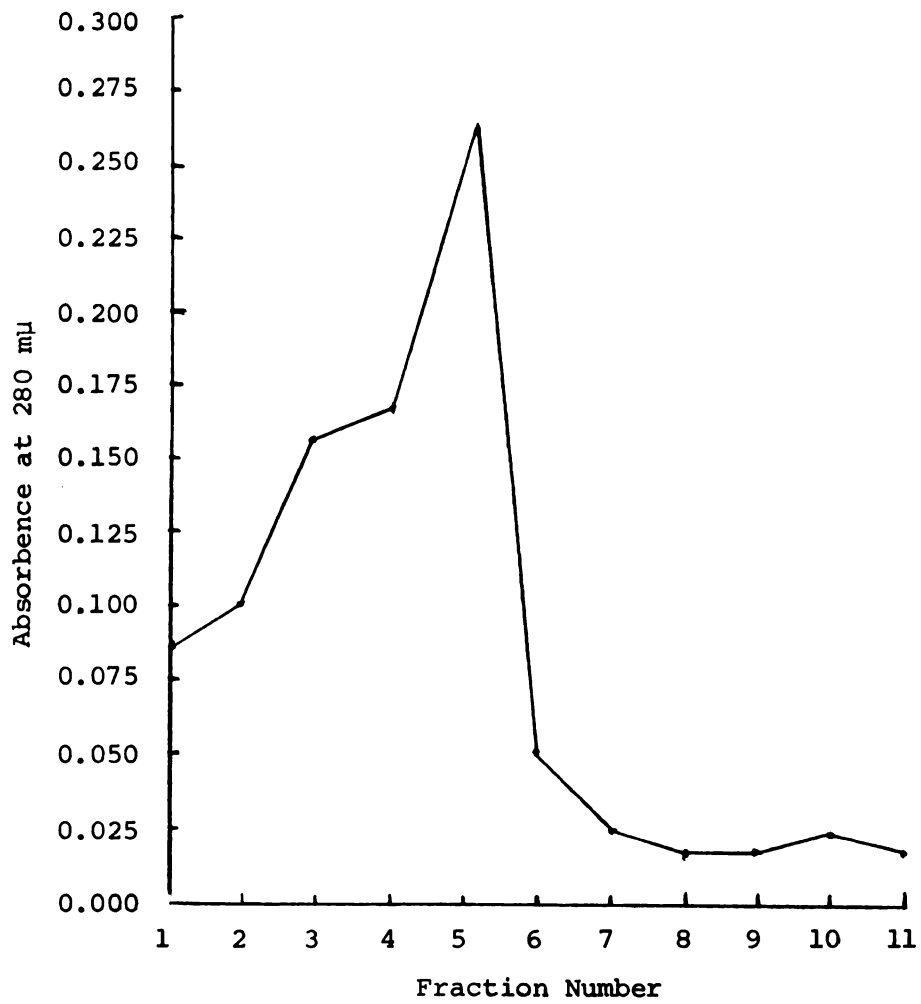


Figure 2. UV spectrophotometry following Sephadex G-25 chromatography on a 30 cm x 0.9 cm column.

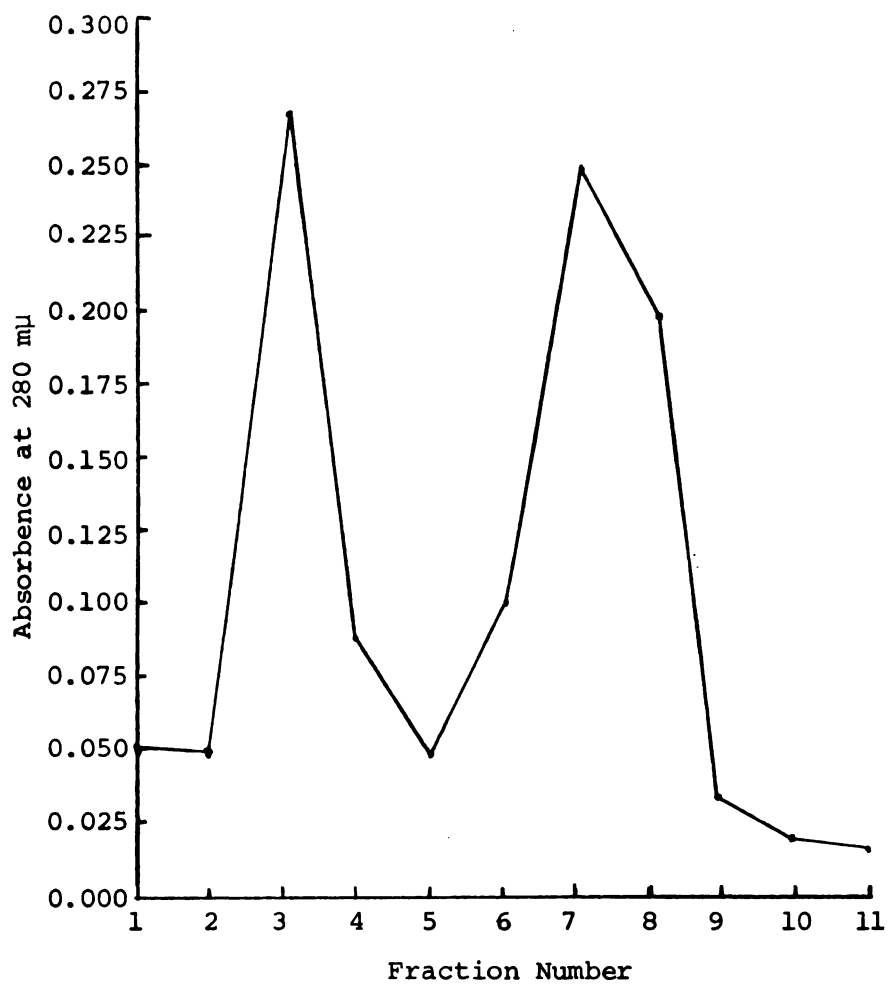


Figure 3. UV spectrophotometry following Sephadex G-75 chromatography on a 30 cm x 0.9 cm column.

on a 30 x 0.9 cm column. Figures 2 and 3 are fractionations of the same oviduct fluid sample run on the same size column using different sizes of Sephadex. The fractionation which resulted in a single peak had that peak in tubes 1 through 4. The fractionation with 3 peaks had the first peak in tubes 2 and 3, the second peak in tubes 5 through 7 and the third peak in tubes 11 and 12.

Three of the G-75 fractionations were run on a 30 cm x 1.5 cm column. In these, the peaks occurred later than in the 30 x 0.9 cm column. The first peak appeared in tubes 7 through 9 and the second peak was in tubes 18 through 20. The height of the peaks obtained with this column was not as great as in the 30 x 0.9 cm column, but the baseline was slightly higher. Figure 4 shows the results of a fractionation with Sephadex G-75 on a 30 x 1.5 cm column.

The eluant used in fractionations with the 30 x 0.9 cm column was distilled water. That used in the 30 x 1.5 cm column was 0.10 molar sodium chloride. The sodium chloride eluant was adopted after it was learned that Sephadex beads contain a small number of carboxyl groups which may cause inclusion of small amounts of cations into the pores and exclusion of small amounts of anions from the pores. Using an ionic eluant provides cations and anions for the carboxyl groups and the travel of the substance being fractionated is less interfered with. The difference

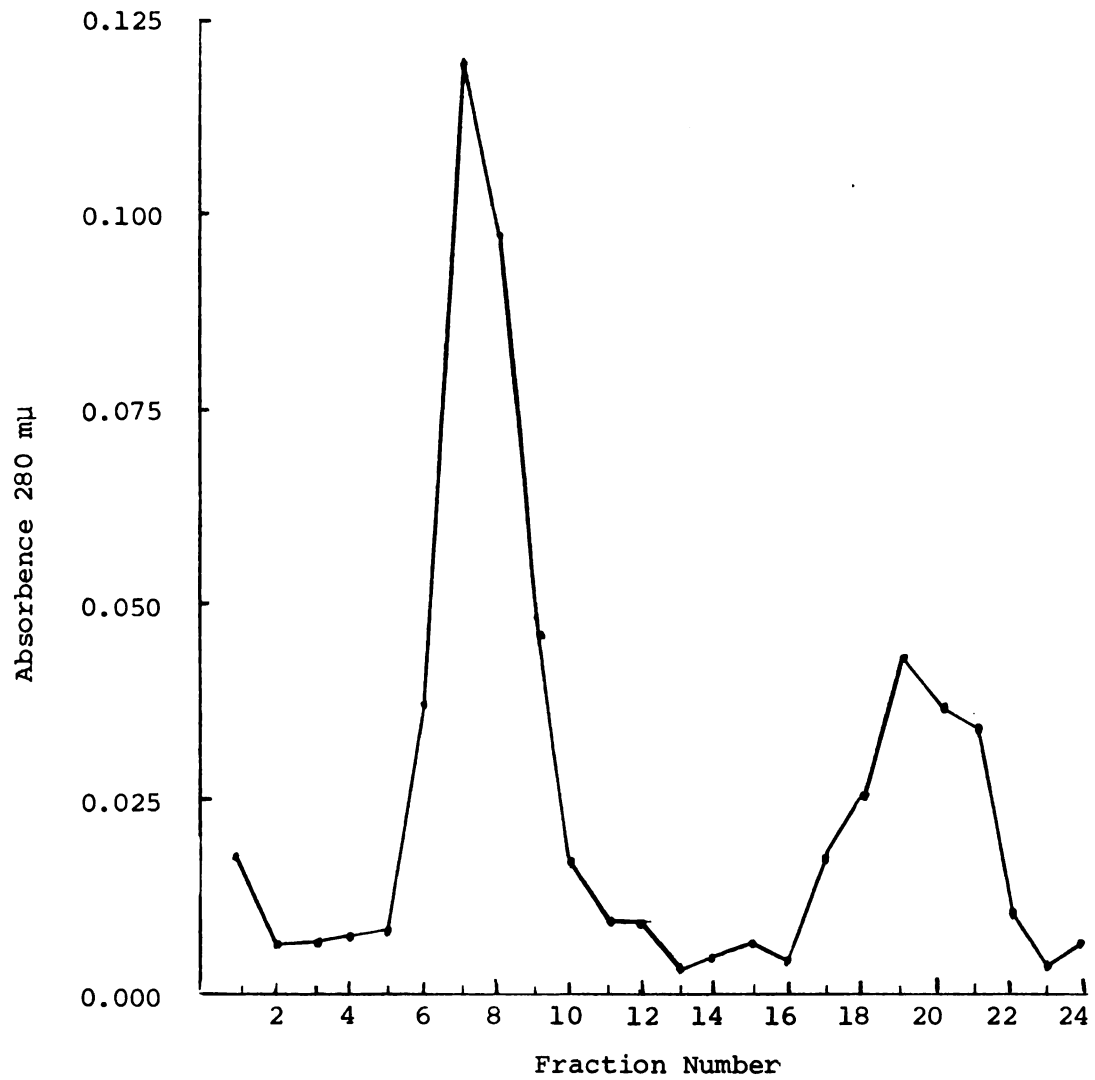


Figure 4. UV spectrophotometry following Sephadex G-75 chromatography on a 30 cm x 1.5 cm column.

between the distilled water and sodium chloride eluants cannot be determined in these experiments since when the change in eluant was made the change in column size was also made, and column size also affects the rate of travel through the column.

Oviduct fluid yield on a daily basis did not follow any pattern, as Figure 5 shows. Daily secretion averaged 1.7 ml (2 oviducts) over 16 weeks, with a range of 1.0 to 2.7 ml. Chromatography and spectrophotometry of oviduct fluid collected on days of light yield (less than 1.9 ml) always revealed the second peak to be greater in size than the first, as shown in Figure 6. In samples from days of heavy yield (1.9 ml or more) the second peak always decreased in size relative to the first peak, as shown in Figure 7. In 40% of the heavy yield samples the second peak was smaller than the first peak, as Figure 8 shows.

To determine the quantity of the substance represented by the peaks present in a day's yield of oviduct fluid the following was done: The area under the absorbance plots was calculated by counting squares on ruled graph paper. Since 0.25 ml of oviduct fluid was applied to the chromatographic column, 0.25 was divided into the volume of the day's yield. This figure was multiplied by the number of squares to find the total number of squares for the day's yield. Values were similarly obtained for individual peaks.

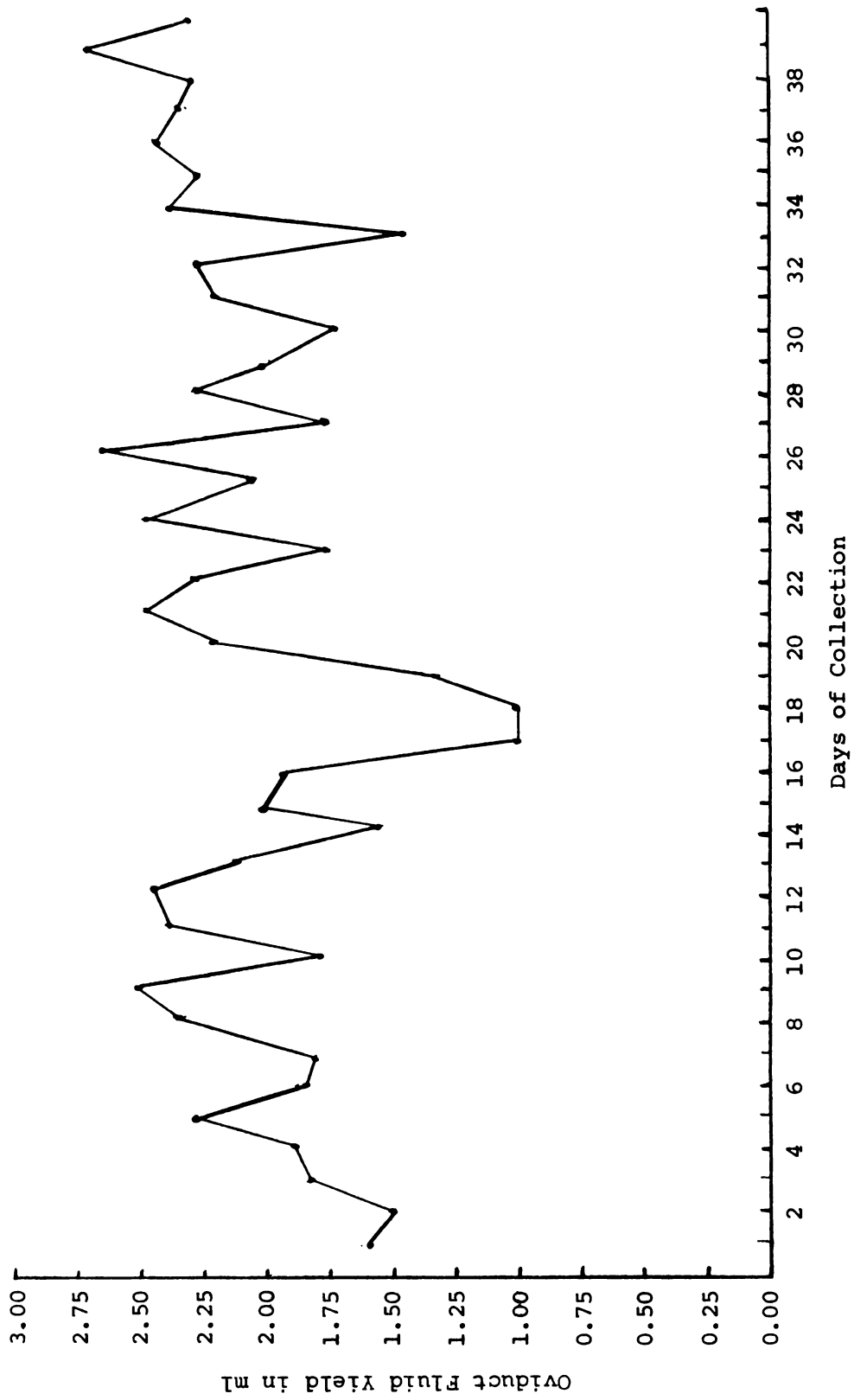


Figure 5. Daily yield of rabbit oviduct fluid.

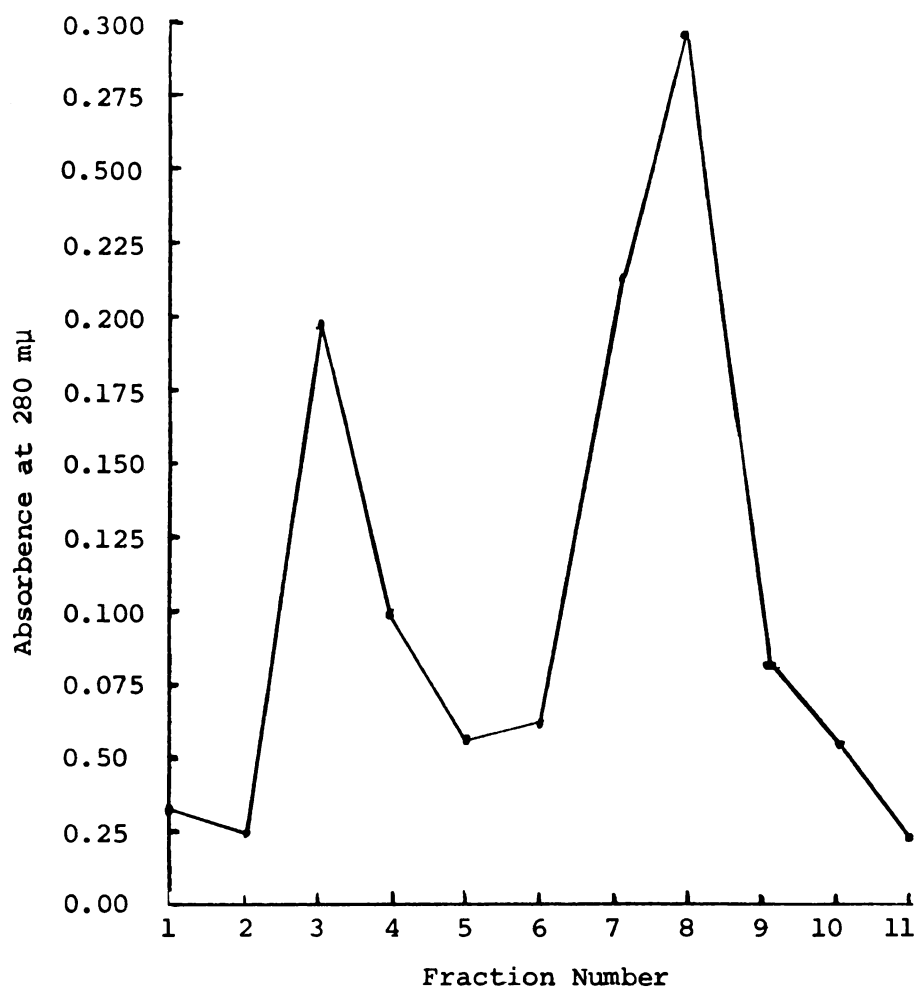


Figure 6. UV spectrophotometry following Sephadex G-75 chromatography on a 30 cm x 0.9 cm column of a sample of light yield.

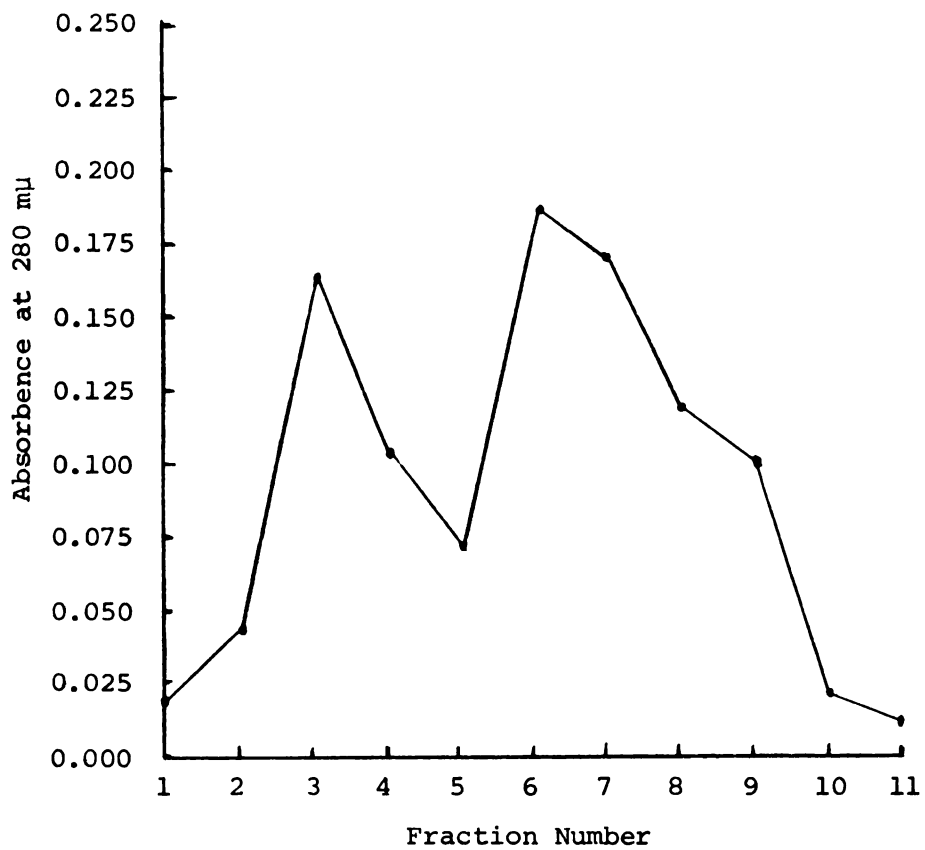


Figure 7. UV spectrophotometry following Sephadex G-75 chromatography on a 30 cm x 0.9 cm column of a sample of heavy yield.

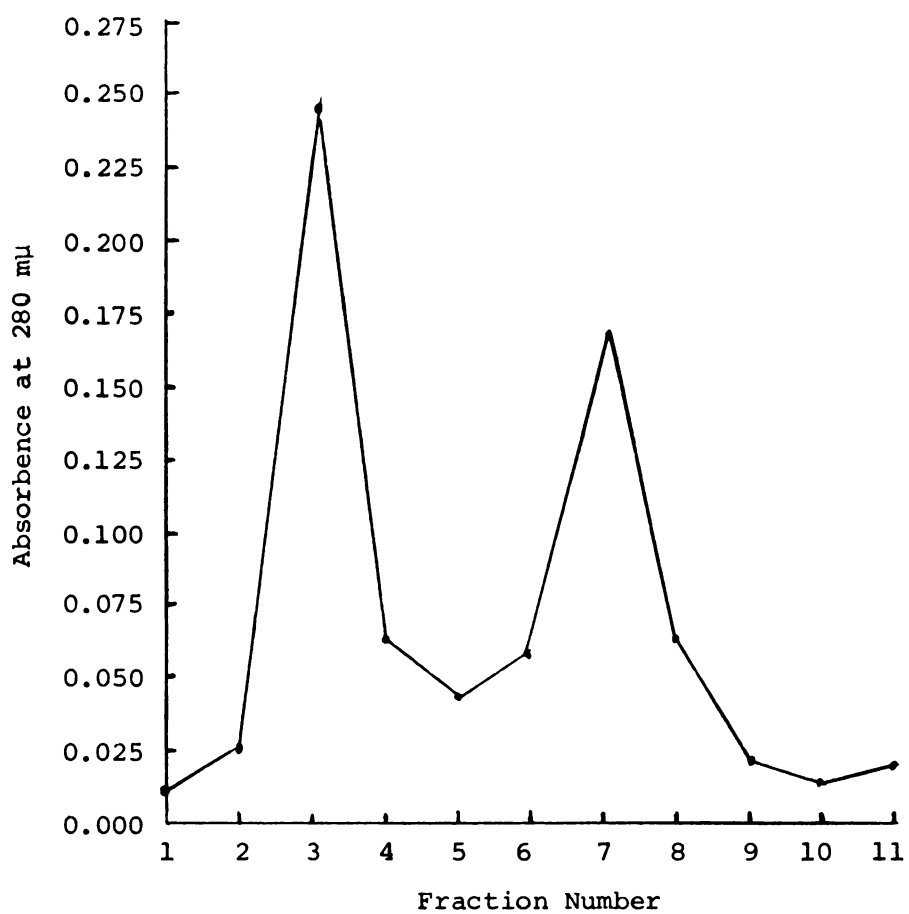


Figure 8. UV Spectrophotometry following Sephadex G-75 chromatography on a 30 cm x 0.9 cm column of a sample of heavy yield.

This was done for 4 oviduct fluid samples, 2 of light yield and 2 of heavy yield. The average values for the daily quantities of substances represented by the peaks for light and heavy yields are shown in Table 6. From these figures it appears the total substances of the peaks did not change as the yield changed. It does seem that as the yield increased the quantity of the substances in peak 2 decreased, while the substances in peak 1 increased.

The 1.9 ml dividing point between light and heavy yields was used since half of the oviduct fluid samples analyzed by chromatography and spectrophotometry were from days of less than 1.9 ml oviduct fluid yield and half were from days of 1.9 ml of greater yield.

Table 6

Relative Quantities of Peak Substances on Days of Light and Heavy Oviduct Fluid Yield

Average Quantity of Yield	Average Quantity of Peak Substance ^a	Total Quantity of Peak Substance ^a	Number of Rabbits
Light--1.75 ml	1st Peak--367	1190	2
	2nd Peak--823		2
Heavy--2.3 ml	1st Peak--520	1189	2
	2nd Peak--669		2

^aAs represented by the number of graph squares.

The substances of the peaks showed the greatest absorbence of u.v. light at 280 m μ . Whole oviduct fluid and the peak fractions gave positive ninhydrin test results. These factors indicated the substances represented by the peaks to be proteins.

Discussion

The substances in the peaks being proteins, as evidenced by positive ninhydrin reactions and peak absorbence at 280 m μ , and given the changes in peak size with changing yields of oviduct fluid, it appears the production of secreted proteins does not parallel the production of oviduct fluid. The total amount of protein remains constant whether oviduct fluid production increases or decreases. And, while total protein does not change quantitatively, it does change qualitatively; as oviduct fluid production increases, the protein represented by peak 1 increases, while the protein represented by peak 2 decreases.

The reasons for the changes in the quantity of oviduct fluid secreted daily are not known. The function of protein in oviduct fluid and the reasons for the changes in the amounts of the two protein peaks which accompany changes in the quantity of oviduct fluid secreted are also unknown. Perhaps there is a relationship between the protein and the oviduct fluid's detrimental effect on the fertilizing ability of capacitated sperm, although there is presently no evidence for this.

SAIMIRI SCIUREUS IN VITRO
FERTILIZATION EXPERIMENTS

Literature Review

Saimiri sciureus is a desirable laboratory primate from the standpoint of its size and the economics of its maintenance. Little is known reproductively about this animal, even though the use of the squirrel monkey in biomedical research ranks second only to *Macaca mulatta*.

Reports on the length of the estrus cycle in *Saimiri sciureus* are not consistent. Rosenblum (1968) reported a cycle length of 6 to 9 days duration. Hutchinson (1970) showed that the cycle length was affected by the environment. Cycles of free ranging squirrel monkeys had a mean length of 13.4 days. Those in 10 feet by 10 feet sized cages had a mean cycle length of 11.3 days. Those in 3 feet sized cages had a mean length of 8.2 days. High rates of pregnancy were reported among the free ranging and large cage enclosed animals, while a very low rate was reported for those in small cages. Three stages of the estrus cycle were characterized: estrous, regression from estrous, and estrogen stimulation at the peak of regression.

Bennet (1967a) was the first to attempt ovulation induction in *Saimiri sciureus*. Using various levels of pregnant mare's serum (P.M.S.) for follicle stimulation and H.C.G. for ovulation induction, he found 10 i.u. P.M.S. administered twice daily for 9 days plus either 250 or 500 i.u. H.C.G. during the last 5 days gave the best results. Recovery of ova was from oviducts 2 to 3 days after the final injections. Dukelow (1970) studied the effects of several follicle stimulators: F.S.H., F.S.H. plus luteinizing hormone (L.H.), and P.M.S. In all his experiments 5 mg of progesterone were administered daily for 5 days before follicle induction was begun, to duplicate the luteal phase of the cycle. He found 1 mg of F.S.H. given for 4 days after the final progesterone injection, plus 500 i.u. of H.C.G. given on the last day of F.S.H. administration with ova recovery 40 hours later gave the best results. He reported that 500 i.u. of H.C.G. produced more ova than 250 i.u., and that intramuscular administration of H.C.G. allowed for more accurate timing of ovulation than did intravenous injection. Cline, Gould and Foley (1972) reported ovulation using 5 mg of progesterone for 3 days followed by 100 i.u. of P.M.S. on day 5 with ova recovery 45 hours after P.M.S. administration. Ova were recovered from both ovarian follicles and oviducts.

Bennet (1967b) reported a method of electro-ejaculation for the recovery of sperm in *Saimiri sciureus*. He used a rectal probe and rhythmically administered pulses every few seconds ranging from 2 to 10 volts. Ejaculation was achieved within 5 minutes.

The need for sperm capacitation has not yet been demonstrated in the squirrel monkey. Dukelow (1969) provided indirect evidence that capacitation is required of rhesus monkey and human sperm. The evidence was based on 4 findings: Rhesus and human seminal plasma contained decapacitation factor; various foreign species' uteri removed a tetracycline-fluorescent layer from rhesus and human sperm after 12 hours of uterine incubation (based on Ericsson's (1967) demonstration of removal of a tetracycline-fluorescent label from ejaculated sperm during uterine incubation); incubation in rhesus oviducts for 18 hours removed the tetracycline-fluorescent label from rhesus sperm; respiration of rhesus sperm increased after 12 hours incubation in hamster uteri.

Seitz, Rocha, Brackett and Mastroianni (1971) also reported evidence for the need of capacitation in man by achieving *in vitro* cleavage of human ova using ejaculated human sperm which were incubated in rhesus uteri for 4 to 5 hours, while washed ejaculated sperm produced no cleavage.

At the time *in vitro* fertilization of *Saimiri sciureus* ova was first attempted in our lab, no reports

of *in vitro* fertilization in this animal had been published. Since then, Cline et al. (1972) have reported successful *in vitro* fertilization of squirrel monkey ova. Their ovulation induction scheme was as earlier mentioned (Cline et al., 1972). A range of 3 to 8 ova were recovered from 2 females used in each experiment. Sperm were collected by electro-ejaculation and no attempt at *in vivo* capacitation was made. Sperm and ova were incubated together in TC 199 medium with 20% agamma newborn calf serum and 50 i.u. penicillin per ml added. Ova were examined for fertilization over a 72 hour period. They reported the presence of 2 pronuclei and 2 polar bodies in 6 ova at 20 to 24 hours after insemination. Three of these ova had cleaved to 2 cells by 42 hours. One ovum cleaved to 8 cells by 42 hours, although lacmoid staining failed to show a nucleus in each cell.

Materials and Methods

Experiments were conducted to develop a system for the *in vitro* fertilization of *Saimiri sciureus* ova. Some of the procedures used in rabbit *in vitro* fertilization were applicable in these experiments also. Some procedures used were similar to those used by other investigators in this field (Cline et al., 1972; Bennet, 1967). Others were designed specifically to fit these experiments.

Animals

Saimiri sciureus (squirrel monkey) were obtained commercially from the Tarpon Zoo, Tarpon Springs, Florida. They were of the Brazilian type. They were in our lab 19 months prior to the beginning of these experiments. A total of 6 squirrel monkeys were used, 4 females and 2 males. The females were housed together along with 2 other females in a stainless steel cage measuring 2 feet x 2 feet x 3 feet. The 2 males were housed together in a similar cage. The animals were fed 10 ounces of monkey pellets (Wayne Monkey Diet, Allied Mills) per cage per day and water was supplied *ad libitum*. The temperature within the colony was maintained at 74°C. The humidity averaged between 65 to 75%. The lighting cycle was 12 hours of light, 12 hours of darkness.

Ovulation Procedure

Several ovulation schemes were used. The first was as follows: 1 mg F.S.H. given intramuscularly daily for 4 days, followed by 500 i.u. H.C.G. intramuscularly on day 5. Ova recovery was attempted 9 hours after H.C.G. administration (Dukelow, 1970). The second scheme was: 200 i.u. P.M.S. (Equinex, Ayerst Labs) intramuscularly both a.m. and p.m. for 4 days, followed by 100 i.u. P.M.S. both a.m. and p.m. on days 5 through 9, with 500 i.u. H.C.G. intramuscularly both a.m. and p.m. on days 6 through 9. Ova recovery

was attempted 13 hours after the final injections of P.M.S. and H.C.G. (Bennet, 1967). The final scheme was: 5 mg progesterone (Mann Research Labs) subcutaneously daily for 5 days, followed by 1 mg F.S.H. intramuscularly on days 6 through 9, with 500 i.u. H.C.G. intramuscularly 12 hours after the final F.S.H. injection. Ova recovery was attempted 8 hours after H.C.G. administration (Dukelow, 1970). The first and third procedures were each used in two experiments. The second was used in one experiment.

Recovery of Sperm

Sperm were collected from 2 male squirrel monkeys. Approximately one hour before surgery was to begin on the females, the males were electroejaculated. This was done using a rectal probe and applying a current of 4 to 8 volts every 2 to 3 seconds until ejaculation was accomplished (Bennet, 1967b). If possible, both males were ejaculated. The ejaculate was collected in a 10 ml beaker. The ejaculate was washed to the bottom of the beaker with culture medium, then this sperm suspension was transferred into a culture dish under silicone oil and then placed into an incubator. A small amount of the ejaculate was placed on a slide and examined for sperm motility.

After the ejaculate was collected in the beaker and after the ova were aspirated, all manipulations of gametes, except when being transferred to the incubator, were done

under a hood at 37 to 38°C. Gametes were placed in culture dishes under 5% CO₂ in air equilibrated silicone oil as quickly as possible. The culture medium and silicone oil were at 37 to 38°C. All equipment other than the electroejaculator was sterilized by autoclaving. The culture medium was sterilized using millipore filters.

Recovery of Ova

After a given time following the final injection of the ovulation procedure, the squirrel monkey was opened with a midline abdominal incision, while under sodium pentobarbital anesthesia. The ovaries were brought to the surface and were examined for follicular development. Those follicles believed to be advanced, or those which appeared to have already ovulated were aspirated using a syringe containing 1 ml of culture medium with a 25 to 30 gauge needle. After the initial aspiration of the follicle a small amount of media was injected into the collapsed follicle and it was aspirated again. This was done in the event that the initial aspiration failed to collect an ovum. The injection and second aspiration were done with a separate syringe to avoid losing an ovum which may have been aspirated the first time. The contents of the syringes were emptied into culture dishes containing culture medium, under silicone oil. Using a stereoscopic dissecting microscope and a Pasteur pipette the ova were recovered from this culture dish and

transferred to the sperm suspension culture dish. This dish was returned to the incubator. The squirrel monkeys' incisions were sutured and the animals were returned to their cage after recovery from the anesthesia. The ova were examined for signs of fertilization 24 to 30 hours after their addition to the sperm suspension.

The Culture Medium

The culture medium used was as follows: Nutrient Mixture F-10 (Grand Island Biological Co.) with 20% heated agamma newborn calf serum (Grand Island Biological Co.), with 25 μ g conjugated estrogens (Premarin, Ayerst Labs) per ml. Fifty ml of the medium were prepared for each experiment.

Experimental Design

The experimental design involved in attempting to develop an *in vitro* fertilization system for *Saimiri sciureus* varied considerably, since there was no proven system to use as a model. Three different ovulation procedures were used to varying degrees of success. Also, the gauge of the needle used to aspirate follicles was changed several times. The general design for the experiments is shown in Figure 9.

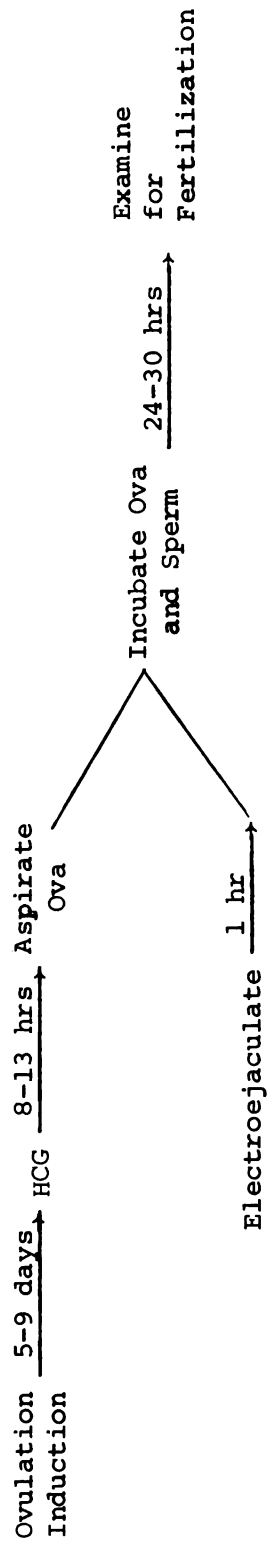


Figure 9. Experimental design for *Saimiri sciureus* in vitro fertilization experiments.

Results

Five experiments were conducted in attempts to *in vitro* fertilize ova of *Saimiri sciureus*. The experiments are summarized in Table 7.

Each of the ovulation induction regimes produced prominent follicles on the ovarian surface. Ova were recovered after induction with both the F.S.H.-H.C.G. and the progesterone-F.S.H.-H.C.G. procedures. No ova were recovered after the P.M.S.-H.C.G. scheme.

Of the 5 experiments, one ended with the incubation of ova and sperm. Of 4 ova in this experiment, 3 had sperm associated with them: 1 had sperm attached to the zona pellucida, 2 had sperm in the cumulus cells about the ova. The 2 ova with sperm in the cumulus cells appeared to be fragmented. One had 2 small bodies and 1 large body contained within zona. The other had 5 small and 2 large bodies. In the ovum with no associated sperm the cytoplasm had shrunken away from the zona.

Four of the experiments resulted in there being no incubation of ova and sperm. In one experiment no ejaculate was obtained. In another an ejaculatory plug was collected but no sperm were present. In another no ova were recovered. And, in another, a single ovum was obtained but was lost while transferring it from the collection culture dish to the incubation dish.

Table 7

Summary of Saimiri sciureus In Vitro Fertilization Experiments

Ovulation Induction	Number of Animals	Number of Follicles Aspirated	Number of Ova Recovered	Results
FSH-HCG	2	13	4	Sperm attached to zona of one ova. Sperm in cumulus masses of two ova.
FSH-HCG	2	6	1	Ovum lost in transfer
PMS-HCG	2	3	0	
Prog.-FSH	2	3	0	
Prog.-FSH- HCG	2	4	4	Ejaculatory plug recovered but no sperm present
Prog.-FSH- HCG	-	-	-	No ejaculation obtained

Discussion

Of the attempts at *in vitro* fertilization of *Saimiri sciureus* ova, only one had a chance of success, since only one resulted in the incubation of ova with sperm. Though fertilization was not achieved in this experiment, a degree of success was obtained in that sperm attachment to the zona pellucida did occur with one ova. Why fragmentation occurred in the 2 ova in which sperm were associated with the cumulus cells is not known. Four ova from an experiment in which ova were incubated in the absence of sperm remained intact throughout incubation. Apparently the culture medium was capable of maintaining the ova.

A factor which may or may not have been dealt with in these experiments is sperm capacitation. If capacitation is required for *Saimiri sciureus* sperm, in order for *in vitro* fertilization to occur *in vitro* capacitation would have had to occur since ejaculated sperm were used. This may be the case, since the *in vitro* fertilization of *Saimiri sciureus* reported by Cline et al. (1972) involved ejaculated sperm. If *in vitro* capacitation of *Saimiri sciureus* did occur, the substances of the media must meet the requirements for capacitation in this species.

MACACA FASCICULARIS PLASMA ANALYSIS

Literature Review

Advances in assay techniques recently have allowed for the measurement of the small quantities of sex steroids present in blood. Neill, Johansson and Knobil (1969) using competitive protein binding techniques reported plasma progesterone concentrations for menstrual cycles of *Macaca mulatta*. For the first 10 days (of 28 day cycles) progesterone levels were less than 1 ng per ml of plasma. There was then a rapid rise and between days 14 and 20 the concentrations were between 3 and 4 ng per ml. After day 20 there was a reduction in plasma concentration until, by day 25, the level was again less than 1 ng progesterone per ml plasma.

Kirton, Niswender, Midgley, Jaffe and Forbes (1970) also used competitive protein binding to measure progesterone concentrations in *Macaca mulatta*. In cycles of 28 to 30 days length, they found progesterone plasma concentrations to be lower than 1 ng per ml for the first 12 to 14 days. There were then rises to between 2 to 9 ng per ml between days 15 to 27. Reduction in plasma

progesterone began from days 20 to 26, and concentrations were less than 1 ng per ml by the end of the cycle.

Hotchkiss, Atkinson and Knobil (1971) measured serum estradiol concentrations in *Macaca mulatta* using a radio-immunoassay. They reported estradiol concentrations to be between 50 to 150 pg per ml serum for the days of the cycle prior to an estradiol peak. The peak had a duration of one day and occurred on days 9 through 18 of the cycle, depending on the animal. The estradiol concentration on the peak day varied from 250 pg per ml to 450 pg per ml serum. After the peak, estradiol levels dropped off rapidly, reaching the lowest level of the cycle within 2 days. The estradiol concentrations varied from 25 to 100 pg per ml serum for the remainder of the cycle.

It must be noted that great variations of these steroids occur between individual monkeys. Kirton et al. (1970) found the peak progesterone level to be as low as 4 ng per ml and as high as 9 ng per ml in different animals. Hotchkiss et al. (1971) reported the estradiol peak to vary between 250 and 450 pg per ml. They found the estradiol peak to occur anywhere from day 9 to 18 of the cycle. Steroid concentrations also vary considerably from day to day within a single animal. Kirton et al. (1970) in one monkey found the progesterone rise in the second half of the cycle to rise to 4 ng per ml, fall to 1.5 ng per ml and then rise to 4 ng per ml before returning to the low

level of the first half of the cycle. Hotchkiss et al. (1971) reported estradiol concentrations to vary as much as 125 pg per ml from day to day before and after the estradiol peak in one animal.

Materials and Methods

Plasma samples were collected daily from adult female *Macaca fascicularis* from the beginning of one menstrual cycle to the beginning of the next cycle. The samples were then radioimmunoassayed by Dr. A. A. Shaikh of the Worcester Foundation, Schremsbury, Mass., for estrone, estradiol, progesterone and 17 α -hydroxyprogesterone.

Animals

Macaca fascicularis, the crab-eating macaque is an old world monkey. Ours were obtained from the Detroit Zoological Park. They were in our lab at least 4 years before use in these experiments. A total of 4 female monkeys were used. All monkeys were housed individually in stainless steel cages 2 feet x 2 feet x 3 feet. Each monkey was fed 10 ounces of monkey pellets per day, and water was supplied *ad libitum*. The temperature within the colony was maintained at 24°C. The humidity averaged between 65 to 75%. The lighting cycle was 12 hours of light, 12 hours of dark.

Blood Drawing Procedure

The monkey was anesthetized with Sernylan (Phencyclidine HCl, Biocentric Labs), administered intramuscularly at 0.15 mg per kg body weight. It was then laid on its back and a hind limb was extended. A Vacutainer needle 22 gauge x 1.5 inches with a 7 ml Vacutainer tube containing an anticoagulant was introduced under the skin on the medial side of the leg, approximately midway between the hip and the knee and slightly dorsal in respect to the ventral-dorsal axis. The vacuum seal was then broken. The container was slowly moved at an angle toward the hip and dorsally. With experience, femoral vein or artery puncture was often made in the first attempt. Once puncture was obtained, it was maintained until approximately 5 ml of blood were withdrawn. The Vacutainer was then withdrawn from the leg and the site of puncture was held between the thumb and forefinger to limit hematoma development. The opposite leg was used the next day.

Plasma Collection

The drawn blood was emptied into a centrifuge tube and spun at 862 x g for 15 minutes. The plasma was then drawn off, placed in a plastic vial and frozen at -15°C. The quantity of whole blood and plasma was recorded.

Plasma Analysis

When a sufficient number of samples had accumulated, they were delivered to Dr. A. A. Shaikh of the Worcester Foundation. He then analyzed the samples for estrone, estradiol, progesterone, and 17 α -hydroxyprogesterone by radioimmunoassay (Shaikh, 1971).

Experimental Design

Samples of blood were collected from menstruating *Macaca fascicularis*. Blood was drawn daily from the first day of a cycle, which was considered to be the first day of menstrual bleeding, through the first day of the next cycle. Plasma was collected and analyzed by radioimmunoassay for estrone, estradiol, progesterone, and 17 α -hydroxyprogesterone.

Results

Plasma samples were collected from 4 female *Macaca fascicularis*. One monkey (number 11) had a cycle length of 27 days, and one (number 14) had a cycle length of 34 days. Two monkeys failed to recycle during plasma collection, the collection continuing for 45 days in one case and 50 days in the other. Plasma samples from the monkeys which did not recycle were not assayed.

The results of the radioimmunoassays of monkeys 11 and 14 for estrone, estradiol, progesterone and 17 α -hydroxyprogesterone are shown in Figures 10, 11, 12, and 13.

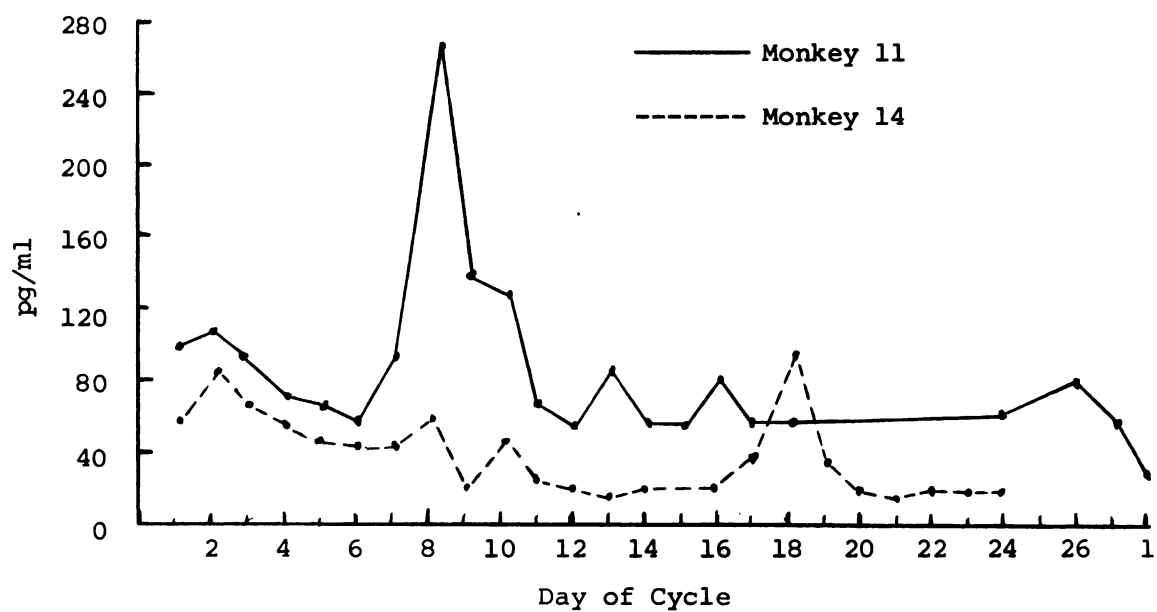


Figure 10. Blood plasma concentrations of estrone.

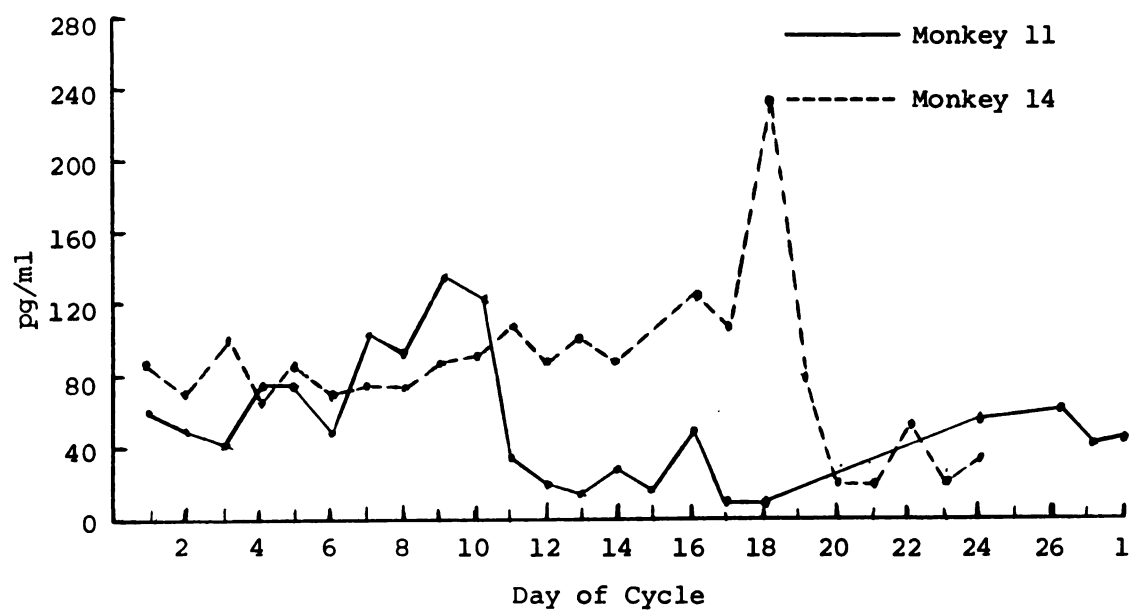


Figure 11. Blood plasma concentrations of estradiol.

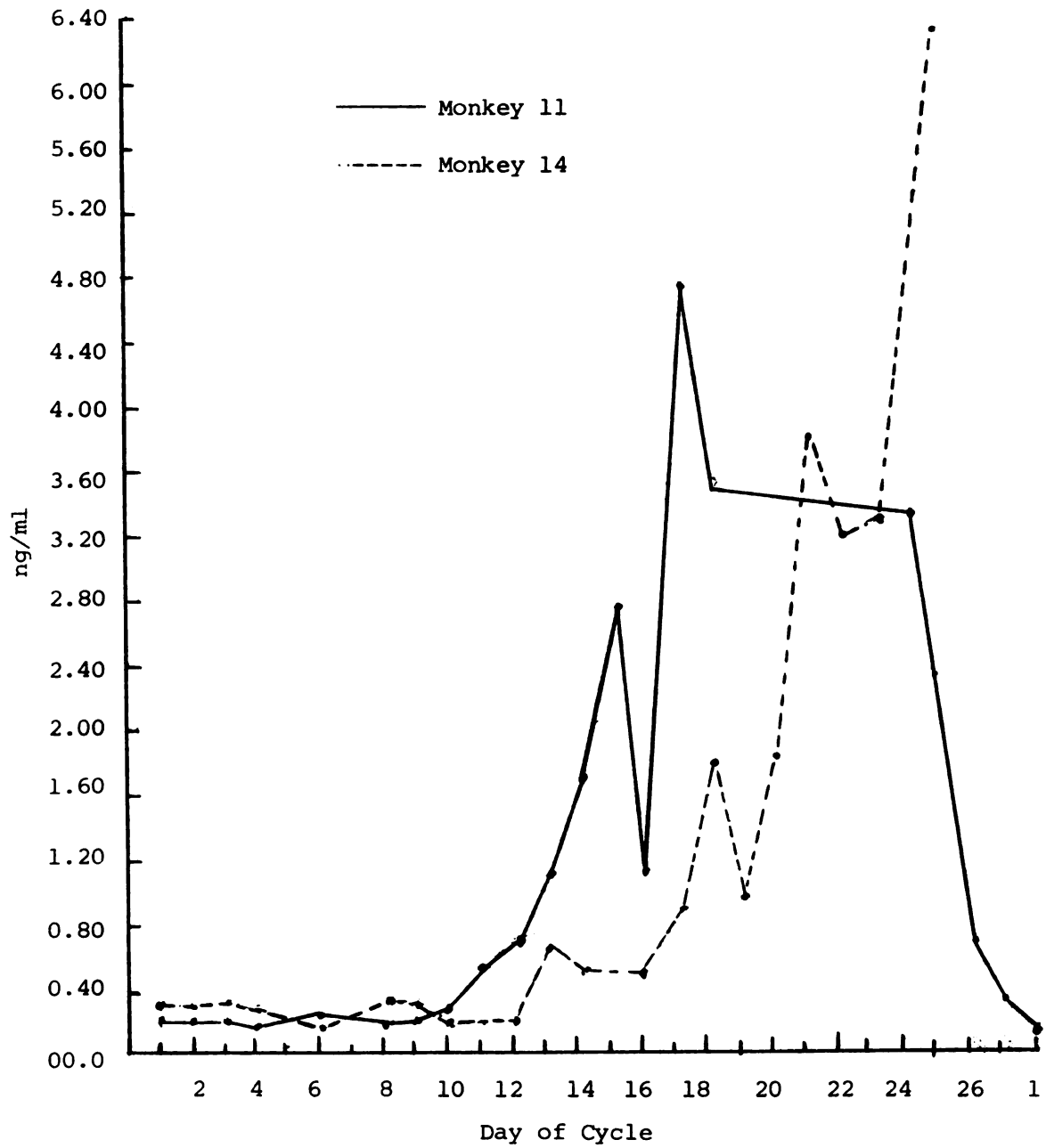


Figure 12. Blood plasma concentrations of progesterone.

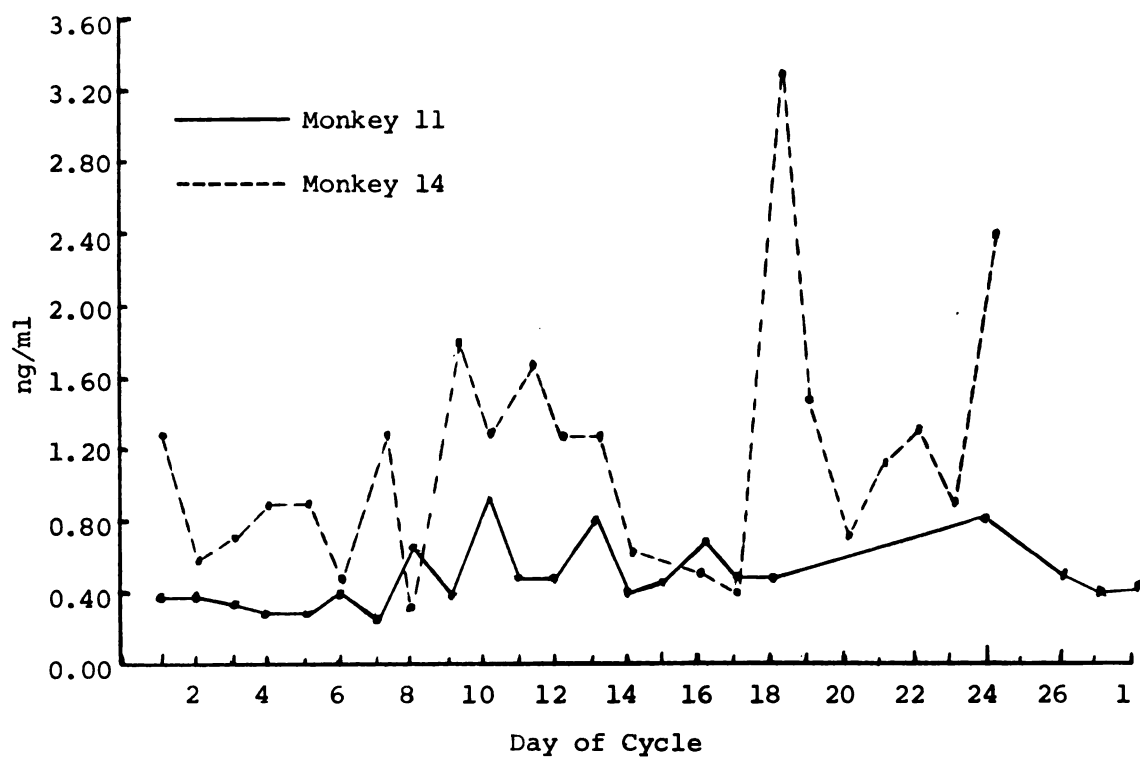


Figure 13. Blood plasma concentrations of 17α-hydroxyprogesterone.

Samples from monkey 11 were assayed for days 1 through 18, days 24, 26, 27 and day 1. Samples from monkey 14 were assayed for days 1 through 14 and days 16 through 24.

Estrone plasma concentrations are shown in Figure 10. Monkey 11 had a level of 70 to 100 pg per ml plasma for the first 7 days of the cycle with a peak of 270 pg per ml occurring on day 8. Following the peak, the concentration returned to near those concentrations present before the peak. Monkey 14 showed no estrone peak, the concentrations varying between 20 and 100 pg per ml throughout the samples assayed.

Estradiol plasma concentrations are given in Figure 11. Monkey 11 showed fluctuations between 40 and 100 pg per ml per the first 8 days of the cycle, followed by a peak of 130 pg per ml on day 9. Following the peak, concentrations decreased rapidly and remained between 10 to 50 pg per ml for the remainder of the cycle. Monkey 14 had estradiol concentrations varying from 60 to 120 pg per ml for the first 17 days. A peak to 230 pg per ml occurred on day 18, followed by a rapid decline to concentrations of 20 to 50 pg per ml for the rest of the cycle.

Progesterone plasma concentrations are given in Figure 12. Monkey 11 had concentrations less than 1.0 ng per ml days 1 through 12. The level then rose to a peak of 4.7 ng per ml over days 13 through 17, and then declined to

less than 1.0 ng per ml by day 26. Monkey 14 had a concentration of less than 1.0 ng per ml for the first 17 days of the cycle, followed by a peak of 6.3 ng per ml by day 24.

17 α -hydroxyprogesterone plasma concentrations are shown in Figure 13. Monkey 11 had no peak, with the concentrations remaining between 0.3 and 0.9 ng per ml throughout the cycle. Monkey 14 had variations between 0.4 and 1.8 ng per ml days 1 through 17. A peak of 3.3 ng per ml occurred on day 18 with a rapid decline to 0.7 ng per ml, followed by another peak to 2.4 mg per ml plasma.

Discussion

The plasma concentrations of estrone are not consistent between the two monkeys sampled. Monkey 14 had a relatively constant plasma levels with no peaks while monkey 11 had a prominent peak with near constant levels before and after the peak. The difference between the animals is not understood.

Estradiol concentrations found are in accordance with those previously reported (Hotchkiss et al., 1971). Both monkeys had a single day peak with a rapid decline after the peak. While monkey 14 had the estradiol peak occur 9 days later than the peak of monkey 11, monkey 14 had a cycle length 7 days greater than monkey 11.

Unfortunately, samples for the last days of the cycle of monkey 14 were not assayed for estradiol or the other steroids.

The plasma concentrations of progesterone are similar to those previously found (Neill et al., 1969; Kirton et al., 1970). The peak for monkey 14 began 6 days later than for monkey 11, but, again, the cycle lengths had a difference of 7 days.

The concentrations of 17α -hydroxyprogesterone also are not consistent among the two animals. Monkey 11 had relatively constant plasma concentrations, while monkey 14 had varying levels with 2 prominent peaks. The difference is not understood.

Examining estradiol and progesterone concentrations for the same monkey (Figures 14 and 15), it can be seen that the estradiol peak occurs within the first part of the cycle and that this peak is followed within a few days by the beginning of the progesterone peak. The estradiol peak is of short duration while the progesterone peak is maintained for a number of days.

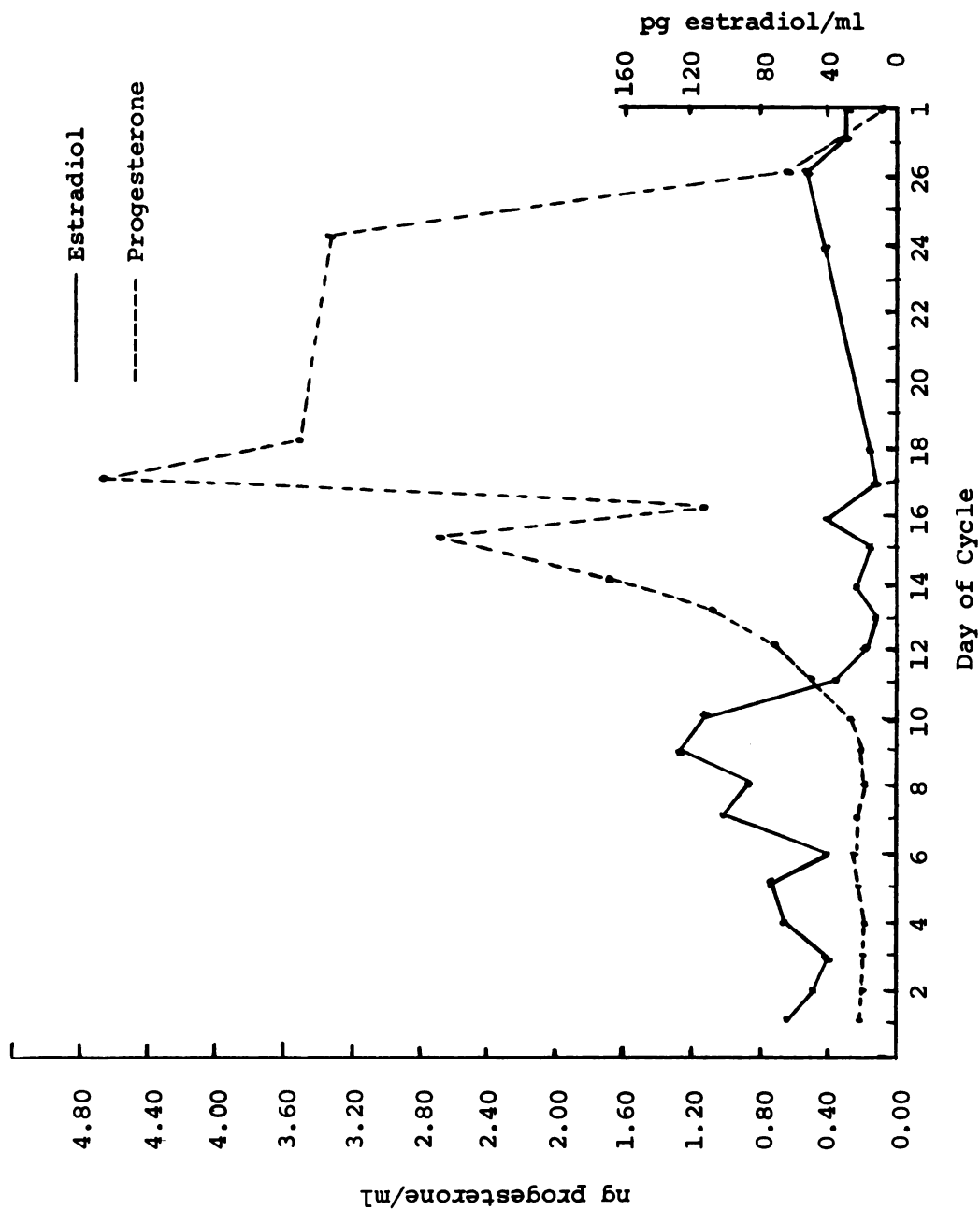


Figure 14. Blood plasma concentrations of estradiol and progesterone for monkey 11.

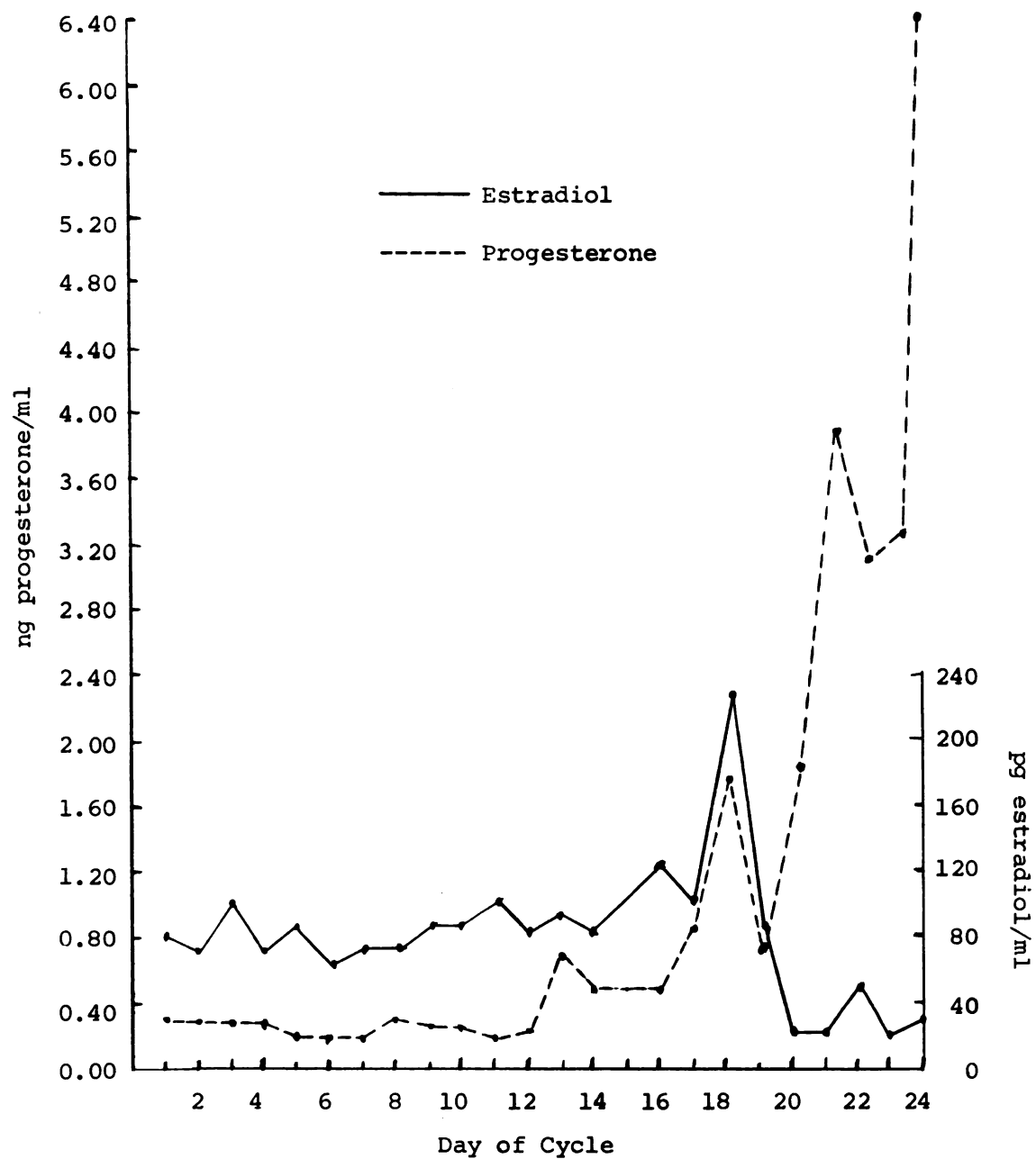


Figure 15. Blood plasma concentrations of estradiol and progesterone for monkey 14.

SUMMARY AND CONCLUSIONS

Experiments using *in vitro* fertilization systems and chemical analysis procedures were conducted to study oviduct fluid and its influence on fertilization in the rabbit, *in vitro* fertilization of squirrel monkey (*Saimiri sciureus*) ova, and blood concentrations of sex steroids in the crab-eating macaque (*Macaca fascicularis*). From the results of these experiments the following conclusions were indicated:

1. Oviduct fluid has a detrimental effect on the fertilizing ability of capacitated rabbit sperm,
2. Gel chromatography and spectrophotometry revealed rabbit oviduct fluid to contain two protein peaks,
3. As oviduct fluid daily yield varies, protein remains constant quantitatively, yet varies qualitatively,
4. Zona pellucida penetration of squirrel monkey ova by sperm was achieved, *in vitro* fertilization was not,
5. Blood plasma concentrations of estradiol and progesterone in the crab-eating macaque are similar to those in the rhesus monkey (*Macaca mulatta*).

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Follicular Morphology Near Ovulation in *Macaca fascicularis*. D.A. Jewett, R.M. Harrison, M.P. Johnson and W.R. Dukelow. *Soc. Study Reproduction Fourth Annual Meeting*, June-July, 1971.

STUDIES ON OVIDUCTAL FLUID AND *IN VITRO*
FERTILIZATION IN RABBITS AND
NONHUMAN PRIMATES

M.P. Johnson, R.M. Harrison and W.R. Dukelow

Rabbit oviductal fluid (OF) was collected with an intraabdominal flask, centrifuged, and frozen until use. Some oviductal fluid was fractionated on Sephadex G-25 and G-75. Capacitated sperm were recovered 17 hr. after mating. The sperm were incubated in 50% OF in saline with 20% rabbit serum for 1 hr. Control sperm were incubated in saline. Ova were recovered 12.5 hr. after HCG and added to the sperm suspensions; then, examined after 24 hr. for cleavage. OF treated sperm caused cleavage in 25% of the ova compared to 53% with controls. These studies suggest the presence of a factor in the OF which effects the fertilizing ability of sperm. Studies on the *in vitro* fertilization of squirrel monkey ova were similar except that we used either 20% heated (55°C. for 20 min) squirrel monkey serum or agamma fetal calf serum in TC-199. In addition, 25 µg/ml estrone sulfate was added. These media supported sperm motility and attachment to the zona pellucida. (Supported by NIH Contract No. 70-2061, USPHS Grant No. 5-)(6-RR00366-04, to the Center for Laboratory Animal Resources, and NIH-CDA 1-K4-HD35, 306-01.)

OVIDUCTAL-UTERINE RELATIONSHIPS TO SPERM CAPACITATION

R.M. Harrison, M.P. Johnson and W.R. Dukelow

An *in vitro* system for the fertilization of rabbit ova was used to study the influence of the oviduct on fertilization. Ova recovered from superovulated does were incubated with sperm from the uterine horns of mated does. The incubation medium was modified acidic saline containing 20% heated rabbit serum. Gametes were incubated at 38°C for 24 hr under a humid atmosphere of 5% CO₂/air. Ova recovered from the ovarian surface 11.75 hr after HCG fertilized at a rate of 56% compared to 50% for ova recovered from the oviduct. Uterine sperm recovered 17 hr after mating fertilized 37% of the ova compared to 30% for sperm recovered 11 hr after mating. Uterine sperm recovered 11 hr after mating from a ligated uterine horn fertilized 24% of the ova compared to 35% using nonligated uterine sperm. With *in vivo* studies, 8 and 10 hr sperm from ligated uterine horns fertilized 67 & 70% of 4 hr old ova respectively. These studies suggest that exposure to the oviduct makes the ova more susceptible to changes in environmental conditions but that the oviduct is not essential for capacitation to occur in 11 hr or less. (Supported by NIH Contract 70-2061, USPHS Grant No. 5-P06-FR00366 and CDA 1-K4-HD-35, 306-1, NIH.)

FOLLICULAR MORPHOLOGY NEAR OVULATION
IN *MACACA FASCICULARIS*

D.A. Jewett, R.M. Harrison, M.P. Johnson
and W.R. Dukelow

In the course of 189 laparoscopic examinations of 31 female cynomolgous macaques (*Macaca fascicularis*), ovarian morphology was observed and photographed for the purpose of determining the characteristics of pre- and post-ovulatory follicular development. Serial observations were begun on day 10 of the menstrual cycle and continued for 6 days at intervals varying from 6 to 24 hr. Generalized swelling and darkening of the ovary at the site of the developing follicle could not be identified until 20-24 hr prior to ovulation. The presence of a relatively large, coiled vessel near the follicular cone, smaller vessels around the base, and a single small vessel transecting the follicular membrane were found to be the most reliable indications of immediate preovulatory development and were not found earlier than 8 hr prior to ovulation. The most significant postovulatory morphological changes were the diffusion of the small vessels at the base of the follicular cone, the occlusion of clear areas in the follicular membrane, and flattening or irregularity in the follicular dome. These changes were clearly evident 10-24 hr following ovulation;

and, therefore, the corpus hemorrhagicum can be easily distinguished morphologically from the preovulatory follicle. This investigation demonstrated that the time requirement for the formation of the follicular dome and related vasculature is not more than 20 hr; that ovulation can be diagnosed by laparoscopy; and that comparative follicular morphology during the late stages of development will permit future accurate prediction of ovulation time by a single laparoscopic examination.

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