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Problems Associated with the Transfer of Ova Between Cattle

P. J. Dziuk, J. D. Donker, J. R. Nichols,
and W. E. Petersen

University of Minnesota
Agricultural Experiment Station

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P. J. Dziuk, J. D. Donker, J. R. Nichols,
and W. E. Petersen[1]

Introduction

TRANSFER OF OVA is the process by which ova are moved from one animal to another for the completion of the gestation period. This technique has been successful in several laboratory animal species and in some domestic species. Successful transfer of ova in cattle could be used as a means to accelerate genetic improvement. The technique of obtaining many ova from a cow by means of superovulation and transferring them to several other cows would be analogous to collection and extensive use of semen from a bull. Transfer of ova offers a means to differentiate early embryonic mortality arising from genetic causes from the mortality caused by an unfavorable uterine environment. Thus far all successful transfers in cattle have involved sacrificing the donor and surgical intervention in depositing the ovum in the host. The necessity of a surgical approach would severely limit the practical usefulness of transferring ova between cattle.

Before a simple technique is developed by which bovine ova are transferred from one cow to many other cows, problems of complex nature must be solved. The major apparent problems involved in successful, practical transfer of ova in the bovine are as follows: superovulation of the donor, collection and isolation of ova from the donor, synchronization of the estrous cycles of the donor and the recipients, and handling and transfer of recovered ova.

This bulletin presents a summary of research carried out on various aspects of this problem. To facilitate presentation of the results, the material is divided on the basis of the major problems. A separate bibliography is given at the end of each section. In addition to this, development and use of an endometrial biopsy technique and endoscopy will be considered.

The primary objective in these experiments has been to develop a practical, completely nonsurgical method to recover and transfer bovine ova.

[1] P. J. Dziuk is a former staff member, now at the University of Illinois; J. D. Donker is Associate Professor of Dairy Husbandry; J. R. Nichols is a former staff member, now at the University of Tennessee; and W. E. Petersen is Professor of Dairy Husbandry.

Part I. Superovulation of the Donor

REVIEW OF LITERATURE

It has been pointed out by Chang (*4*)[2] and Hammond (*6*) that in order to achieve successful large-scale ova transfer it was imperative to be able to superovulate the donor animal. Successful superovulation means being able repeatedly to bring about maturation of many follicles and their rupture with the release of viable ova. Conditions within the genital tract must not prevent the ova from becoming fertilized either *in vivo* or *in vitro*.

Willett (*16*), in his comprehensive review, has shown that superovulation has been effected by various gonadotropic hormone preparations in many species. Rabbits were superovulated with the available gonadotropic hormone preparations (Pincus, *12*). Superovulation has been accomplished in the ewe (Hammond *et al.*, *8*; Casida *et al.*, *3*; Cole *et al.*, *5*; Murphree *et al.*, *11*) and in the sow (Tanabe *et al.*, *14*). Casida *et al.* (*2*) was one of the first to superovulate cattle. He administered a subcutaneous injection of either an unfractionated pituitary extract or a purified follicle-stimulating hormone (FSH) preparation, followed by an intravenous injection of luteinizing hormone (LH).

The time of initiating treatment in relation to the stage of the estrous cycle was found to be very important. Few ova became fertilized as the result of initiating injections in the follicular phase, while there were practically no ova fertilized when the injections were made in the luteal phase. The last discovery has been upheld by subsequent studies (Rowson, *13*; Willett *et al.*, *18*; Brock and Rowson, *1*). Hammond and Bhattacharya (*7*) used pregnant mare serum (PMS) and horse pituitary extracts to cause superovulation in cows. They noted that the presence of a *corpus luteum* during injections lessened the percent of fertilized ova. Brock and Rowson (*1*) administered various preparations of PMS to produce usable ova. Superovulation of many cows was accomplished by Umbaugh (*15*), using a wide variety of treatments. There was a corresponding wide variety of results in numbers and viability of ova. Marden (*9*) was able to superovulate calves with many different gonadotropic hormones.

These studies showed that several different gonadotropic preparations caused stimulation of the bovine ovary, but that there was much variation in response among preparations and from one animal to another, using the same preparation and dosage. It has not been possible to produce a consistent response. Willett, Buckner, and McShan (*17*) found there was a decline in the number of follicles produced in successive superovulations in the same animal. Unfractionated pituitary extracts and PMS were used, and it was thought that the refractoriness may have been due to an immune reaction to foreign proteins and not necessarily due to the production of specific antihormones.

Superovulation is necessary for the application of the ova-transfer technique, and although many successful superovulations have been achieved, none of the treatments gave consistent results. Neither could successful treatment be repeated on the same cow. It was toward the solution of these problems that this study was undertaken.

MATERIALS AND METHODS

Forty-five cows of dairy breeding were used in 76 attempts to superovulate them. Most of these animals were parous and were experiencing normal estrous cycles. Evaluation of response to treatment was at times made by isolating ova from uterine flushings as described in the section on recovery of ova, or more commonly by examination of the ovaries in the intact or slaughtered animal and the flushings of the oviduct and uterus in slaughtered animals.

The materials utilized to bring about follicular development were: pregnant

[2] Numbers in italic refer to Literature Cited, listed separately for each major part of the study in this bulletin.

mare serum (PMS)[3] commercially produced and assayed for potency; sheep pituitary follicle-stimulating hormone (SFSH),[4] prepared and assayed by the method of McShan and Meyer (*10*); unfractionated sheep pituitary gonadotropins, (USGA),[4] (USGS)[5]; and hog pituitary follicle-stimulating hormone (HFSH),[6] experimentally produced by a commercial concern. The materials used in attempts to cause ovulation of the developed follicles were: human chorionic gonadotropin, (HCG),[7] (USGS), (USGA); and hog pituitary luteinizing hormone (HLH),[8] experimentally produced and assayed by a commercial concern. The pituitary gland extracts and HCG used were lyophilized products that were dissolved in sterile distilled water or physiological saline just prior to use. Progesterone[9] was dissolved in corn oil at the rate of 1 percent or 2 percent, or put into an aqueous suspension. Diethylstilbestrol[10] (DES) was used as a 1 percent corn-oil solution.

Subcutaneous injections were made in the area immediately behind a shoulder, intravenous injections into a jugular vein, and the intramuscular injections into a semimembranous muscle.

Days of the estrous cycle were calculated by considering the day of heat as "day one," and so on. An animal was considered in heat only if she allowed other animals to ride her.

The most usual procedure in inducing superovulation was to inject subcutaneously on day 15 either the entire follicle-stimuating dose or a fraction of it, administering equal amounts daily up to five days. The ovulating injection was most often made intravenously on day 20 or 21. Many of the animals were treated once, but several have been treated for superovulation two or more times to ascertain the degree of refractoriness to repeated treatments.

RESULTS AND DISCUSSION

The condensed results of each treatment for each animal are found in tables 1 and 2. Each treatment is chronologically listed in each table. There are some animals listed in both tables, as certain animals had two different follicle-stimulating treatments. This can be seen in table 3, which lists all cows that have been treated two or more times. The treatments of each cow are in chronological order in table 3. Since the fulfillment of the object of this study depended upon the development of a technique for superovulation which was successful, if treatment was not successful it was changed after a few trials and as different hormone preparations became available. Table 4 outlines the potency of the various gonadotropins used in this work. Evaluation of the response when continually changing procedures were employed and the results measured in subjective terms such as obtained by rectal palpation does not give data which lend themselves to statistical analysis. It was found by comparing the number of *corpora hemorrhagica* and the number of follicles at slaughter to estimates made of them by rectal palpation that a smaller number of both was estimated to be present than was actually the case, especially when ovaries were greatly stimulated. Many of the follicles and *corpora hemorrhagica* were so close together and of such a shape as to be indistinguishable by rectal examination. This seriously limits the accuracy of estimated response to the various treatments. Direct observation at slaughter helped to form a standard by which to judge superovulated ovaries per rectum. Examples of superovulated ovaries can be seen in figure 1.

There were fewer ova recovered at slaughter and by flushing the uterus than should have been present, judging from

[3] Product of Cutter Laboratories.
[4] Prepared by one of the authors (JDD).
[5] Product of Searles Laboratories, gonadophysin USGS, lot #147.
[6] Products of Armour Laboratories, lots #R377201, K47109R, and KD4ODS.
[7] Product of E. R. Squibb & Sons.
[8] Product of Armour Laboratories, lot #R377242H.
[9] Product of Glidden Co., crystalline progesterone USP, lot #E-174.
[10] Product of American Bio-Synthetics USP XIII, lot #412-413.

the number of ovulation points present. In those cases in which uterine flushing was used, the effects of the flushing technique would have influenced the numbers of ova recovered, but this should not have been the case in slaughtered animals, as the recovery procedure is simple and reliable.

An example of the discrepancy between numbers of ovulation points and ova recovered is shown from observations of cow 934. The right ovary had 16 ovulation points and the left 15. Both ovaries were the same size and shape, and both were free of any adhesions or detectable abnormalities. Both oviducts were patent and the horns of the uterus appeared to be similar. She was slaughtered 48 hours after the ovulating hormone was given, and each oviduct and horn of the uterus was flushed separately. The left oviduct yielded 11 ova, the right oviduct 2 ova, and the two horns of the uterus, none. This indicated that some important factor in the mechanism of ovum pickup was not functioning. There are several possibilities: the superovulated ovary was so large that the infundibulum could not encompass it; the mobility of the infundibulum and oviduct was not normal; the ova were trapped in the follicles even though they had ruptured; follicles had developed which did not contain ova; or the ova were held in the oviduct and it was impossible to flush them out. The possibility of entrapment of ova in follicles was explored by examining serial sections of supervulated ovaries for trapped ova. None were found in two ovaries examined. Many follicles on superovulated ovaries which had failed to rupture were punctured with a scalpel and the inside lightly scraped. The fluid which was recovered was examined for ova. In all cases there was found one ovum for each follicle. This would indicate that it would be reasonable to expect to find an ovum in every follicle. Until new techniques are developed, it will be difficult to ascertain the effects of the other possibilities of why the ova were not found.

The percent of recovered ova that were fertilized was very low. In many cases none of the recovered ova were fertilized, although it was the policy to inseminate the cows at the time of administering the ovulator and 12 hours later with semen from bulls of known fertility. In those cases in which fertilized ova were found, there were also many ova that had not developed. There are many possibilities as to why they were not developing. Ova produced while the animal was under the influence of progesterone are not thought to be capable of developing (Casida *et al.*, 2). These nonfertilized ova may unknowingly be produced under these conditions. The conditions of the uterus, cervix, and oviduct may be such as to not favor sperm life and or transport. The ova may not be of a favorable physiological age so that they can be fertilized and develop normally.

Follicle development due to PMS administration was quite variable even though the dosage and other factors were equal. Administration of HFSH caused a much more consistent response in numbers of follicles developed. The HFSH was relatively pure, containing about 5 percent impurities; the primary contaminant was LH. Administration of the HFSH followed by intravenous injection of HLH caused follicle development, but it was not followed by ovulation. The follicles were examined at slaughter and found to be very tough and not easily broken, as is the case with normal mature follicles. Intravenous administration of USG following HFSH did cause ovulation, usually in 24 to 36 hours, but also caused the development of a new group of follicles that reached maturity about four to five days after the USG injection. This second group of follicles did not rupture spontaneously, but persisted and became cystic. It was thought that the HFSH did not contain enough LH to permit normal development; therefore, HLH was added to the HFSH to determine whether or not follicles which were more nearly normal could be developed. Judging by the limited cases, it appears that this mixing of the FSH and LH gave a more desirable response than HFSH alone.

Despite considerable variation in results, these studies indicated that under

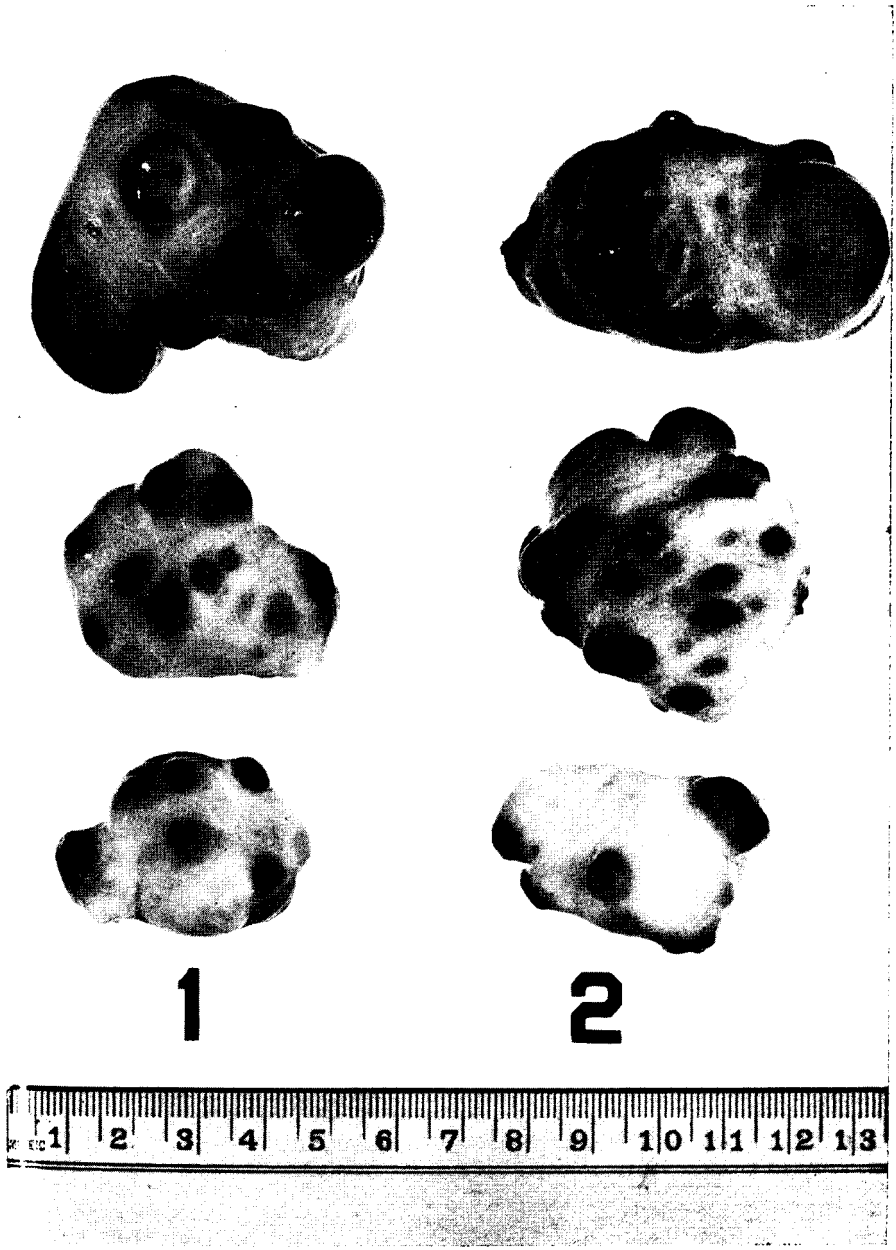


Fig. 1. Examples of ovaries after treating cows with hog follicle-stimulating and luteinizing hormones. (1) Left ovaries. (2) Right ovaries.

our conditions the procedure that was most dependable in inducing the desired superovulation was as follows: initiation of treatment immediately following progesterone treatment for synchronization of the estrous cycles, or on the fifteenth day of a normal cycle; subcutaneous injection of 20 Au. of HFSH and 20 mg. of HLH daily for four or five days; intravenous injection of USG (100 mg. or 1,500 ru.) on the day following the last follicle-stimulating injection. The time and quantity relationships between the two follicle-developing hormones and the timing of the injections in relation to the cycle of the cow are still very uncertain, and need much more detailed work.

No consistent reduction in response was demonstrated in those animals repeatedly superovulated, as listed in table 3. In two cases, anaphylactic reactions, marked by excess salivation, lacrimation, and labored respiration, were noted following injection of PMS which had been preceded by one or more PMS treatments. One of the anaphylactic reactions was noted after an intravenous injection, and the other and more severe reaction followed a subcutaneous injection.

A single animal was given daily subcutaneous injections of PMS (100 to 200 ru. daily) for 59 days. The initial injection started on day 15 of the estrous cycle, at which time the ovaries were normal to palpation. The ovaries became greatly hypertrophied during the first 23 days of treatment but gradually regressed toward normal, even though PMS treatment continued. Essentially normal estrous activity was noted throughout treatments.

Blood samples were taken periodically and assayed in young female rats which were concurrently treated with PMS at low levels, and no inhibition of the effect of PMS on the rats was demonstrated from these blood samples. It was quite apparent that early in the treatment sequence the ovaries were reactive to the PMS; equally apparent, later in the sequence, PMS was not effective, even though increased amounts of PMS were administered. The exogenously administered gonadotropic may have stimulated production of "anti-PMS." This was not demonstrated and neither, if it was present, was it effective against endogenously produced gonadotropin.

The DES injections prior to follicle stimulation were made in an effort to cause the ovaries to regress so that no active *corpus luteum* or developing follicle would be present. It was thought that bringing ovaries to an "equal state" might give more consistent results from one cow to another. An example of very extensive superovulation from ovaries that were not greatly increased in size was seen in the case of cow 859, when she was treated with 6,000 ru. of PMS that was administered after the ovaries had been brought to a quiescent stage by stilbestrol treatment. It was estimated that following administration of USG the cow ovulated 71 times within 32 hours, as evidenced by rectal palpation and direct examination of the ovaries at slaughter 3 days after administering USG. This cow had been given 5.0 mg. of stilbestrol every other day for 36 days prior to receiving PMS. It would appear from such cases of successful superovulation following administration of stilbestrol that further work using this technique is warranted, as it appeared that the PMS used in the later trials after stilbestrol treatment may have lost its potency.

Superovulated ovaries increased to about three to four times the normal size during follicle stimulation. If the follicles were ruptured and many *corpora lutea* formed, the ovaries regressed to nearly normal size in three to four weeks. If several large follicles remained unruptured they became cystic, and irregular estrous cycles resulted. There were too few cases of estrous cycle length of near-normal duration following superovulation to conclude whether or not a completely ovulated ovary would immediately resume cycling normally. Many abnormal cycles followed superovulation but there was no pattern to the alteration.

Many animals which were treated to develop follicles near the time of expected estrus did not exhibit typical estrous symptoms. In many cases the uterine tonus was increased, there was a vaginal mucous

discharge, and the vulva was swollen, but there were no signs of psychic heat such as bawling, riding other cows, or allowing other cows to mount. This may have been due to the formation of a *corpus luteum* as a the result of the first follicle-stimulating injection.

SUMMARY AND CONCLUSIONS

Follicle stimulation can be induced by subcutaneous administration of several gonadotropic hormone preparations which are primarily FSH. Ovulation of the follicles developed can be achieved by intravenous administration of gonadotropic hormone preparations which consist mainly of LH. There was much variation in the number of follicles developed and in the percentage of those developed that were ruptured by the ovulating injection.

The number of ova found in the reproductive tract after superovulation has always been less than the number of ovulation points. The percentage of ova fertilized of those recovered is also quite low, which indicated that some factor or factors that influence fertility were not optimal during superovulation. In most cases the animals did not exhibit normal estrous behavior while being superovulated. The ovaries regressed to normal size quite rapidly following follicle development and complete ovulation. Cystic follicles did occur if ovulation was not complete.

Table 1. Superovulation trials using hog and sheep FSH as follicle stimulators

Cow no.	Follicle-stimulating procedure: Material used	Amt. per inject.	No. of inject.	Days of cycle of inject.	Estimated nos. of follicles developed	Ovulating procedure: Material used	Amount used	Day of cycle of inject.	Estimated nos. of ovulation	Comments
643	HFSH	34 Au.	3	First day postexpulsion of *corpus luteum*	21§	None			2§	HFSH inject. made every 8 hrs., cow slaughtered 5 days post-HFSH
331	HFSH	50 Au.	10	14-18	26§	None			1§	HFSH inject. made every 12 hrs., cow slaughtered 5 days post-HFSH
886	SFSH	Ca. 60 mg. eq.	4	Day 1-4 post-progesterone recycling	3§	USGS	1,000 ru.	5 days post-progesterone	2§	Cow slaughtered 9 days post-USGS
15	SFSH	Ca. 60 mg. eq.	4	17-20	None	None				
680	HFSH	50 Au.	10	Day 1-5 post-progesterone recycling	5‡	USGS	1,500 ru.	5 days post-progesterone	4‡	HFSH inject. given every 12 hours
866	HFSH	50 Au.	10	15-19	2-7	USGA	100 mg.	20	0	HFSH inject. given every 12 hours
						HCG	5,000 iu.	24	0	
14	SFSH	100 mg.	5	16-20	1-2	USGA	100 mg.	21	2	
14	USGA	300 ru.	5	15-19	2-7	None			1	Estrus occurred at day 19 prior to major follicular development
E741	USGA	300 ru.	4	15-18	1-2	None			1	
868	PMS HFSH SFSH	1,500 ru. 200 Au. 460 mg.	1 1 1	18 18 18	51§	USGA	100 mg.	5 of next cycle	43§	Cow slaughtered; 4 nonfertile ova found

Table 1. Continued

Cow no.	Follicle-stimulating procedure: Material used	Amt. per inject.	No. of inject.	Days of cycle of inject.	Estimated nos. of follicles developed	Ovulating procedure: Material used	Amount used	Day of cycle of inject.	Estimated nos. of ovulation	Comments
T18	HFSH	150 Au.	1*†	7 months pregnant	1-2	None				Cow slaughtered 6 days post-HFSH
T19	HFSH	150 Au.	1*†	Recently calved	2-7	None				Cow slaughtered 6 days post-HFSH
351	HFSH	100 Au.	1*†	6	10§	None			5§	Cow slaughtered 6 days post-HFSH
698	HFSH	200 Au.	1*†	30 days pregnant	2-7	None				Cow slaughtered 6 days post-HFSH
864	HFSH	225 Au.	1	Pregnant	22	None				
T9	HFSH	225 Au.	1†	Pregnant	8§	None			1§	Cow slaughtered
T10	HFSH	225 Au.	1†	Pregnant	22	None			1	
A62	HFSH	225 Au.	1†	11	14	USGA	100 mg.	21	11	2 nonfertile ova found by flushing
A62	HFSH	225 Au.	1†	Day 1 post-progesterone recycling	4	None			0	
E740	HFSH	75 Au.	4†	Unknown	2-7	None				
E797	HFSH	20 Au.	4	15-18	6	HLH	60 mg. eq.	20-21	4	Ovulator given in 2 doses on day 20 and 21
T40	HFSH	20 Au.	5	15-19	9	HLH	100 mg. eq.	20	0	
T105	HFSH	20 Au.	5	16-20	4	HLH	100 mg. eq.	21	0	
E799	HFSH	20 Au.	5	15-19	20	HLH	75 mg. eq.	20	0	Ovulator divided into 3 doses 5 min. apart; 3 ova found by flushing, following manual rupturing

Table 1.—Continued

Cow no.	Follicle-stimulating procedure: Material used	Amt. per inject.	No. of inject.	Days of cycle of inject.	Estimated nos. of follicles developed	Ovulating procedure: Material used	Amount used	Day of cycle of inject.	Estimated nos. of ovulation	Comments
T53	HFSH	20 Au.	5	15-19	12	HLH	75 mg. eq.	20	6	Ovulator divided into 3 doses 8 minutes apart
T65	HFSH	20 Au.	5	16-20	10	HLH	200 mg. eq.	21	0	More follicles developed 4-5 days post-USGS
						USGS	2,400 ru.	22	8	5 ova found at flushing; 3 fertilized
E801	HFSH	24 Au.	5	16-20	10	HLH	100 mg. eq.	21	0	
						USGS	1,500 ru.	22	8	
876	HFSH	20 Au.	5	Day 1-5 post-progesterone recycling	12	USGS	1,500 ru.	6 days post-progesterone	4	5 nonfertilized ova found at flushing
						USGS	1,500 ru.	7 days post-progesterone	6	Many follicles developed 5 days post-USGS
E799	HFSH	25 Au.	4	18-21	12	HLH	40 mg. eq.	21		
						HLH	60 mg. eq.	22	2	
958	HFSH	25 Au.	3	16-18	8	HLH	100 mg. eq.	20	0	Manually ruptured on day 22, recovered 1 nonfertilized ovum by flushing on day 27
E800	HFSH	20 Au.	4	17-20	14	USGS	1,500 ru.	21	10	Many new follicles 4 days post-USGS
	HLH	20 mg. eq.	2	18-19						
T65	HFSH	20 Au.	4	16-19	8	USGS	1,000 ru.	20	0	
	HLH	20 mg. eq.	2	18-19		USGS	1,000 ru.	22	6	
T109	HFSH	100 Au.	1	16	16	HLH	100 mg. eq.	20	0	Many new follicles 4 days post-USGA
						USGA	75 mg.	21	14	

Table 1.—Continued

Cow no.	Follicle-stimulating procedure: Material used	Amt. per inject.	No. of inject.	Days of cycle of inject.	Estimated nos. of follicles developed	Ovulating procedure: Material used	Amount used	Day of cycle of inject.	Estimated nos. of ovulation	Comments
T65	HFSH	20 Au.	5	15-19	8	USGS	1,500 ru.	20	6	6 nonfertilized ova found at flushing
934	HFSH	20 Au.	5	15-19	34§	USGS	1,500 ru.	20	31§	13 (1 fertilized) ova found at slaughter on day 22
876	HFSH	20 Au.	5	15-19	16	USGS	1,500 ru.	21	14	8 new follicles 8 days post-USGS
958	HFSH	20 Au.	6	16-21	14	USGS	1,500 ru.	22	14	20 *corpors lutea* on day 26

* Intramuscular injection.
† Armour vehicle 40 used as carrier.
‡ One ovary only.
§ Actual visual count.
Abbreviations:
HFSH—Hog Follicle-Stimulating Hormone.
HLH—Hog Luteinizing Hormone.
SFSH—Sheep Follicle-Stimulating Hormone.
USGS—Unfractionated Sheep Gonadotropin—Gonadophysin.
USGA—Unfractionated Sheep Gonadotropin prepared in laboratory.
HCG—Human Chorionic Gonadotropin.
ru.—rat unit.
Au.—Armour unit.
mg. eq.—milligram equivalent.
iu.—international unit.

Table 2. Superovulation trials using PMS as a follicle stimulator

Cow no.	Follicle-stimulating procedure: Units (ru) per inject.	No. of inject.	Days of cycle of inject.	Estimated nos. of follicles developed	Material used	Amount used	Ovulating procedure: Day of cycle of inject.	Estimated nos. of ovulation	Comments
A59	1,500	1	11	0	None			None	Slight ovarian hypertrophy
789	1,500	1	20	0	None			None	Ovaries not affected
789	1,500	2	16-17	0	None			None	Ovaries not affected
595	3,000	1	19	0	None			None	Very slight ovarian hypertrophy on day 23
A59	1,500	1	17	0	None			1	
	1,000*	1	18						
	1,500	1	19						
A59	4,500	1	14	2-7	None			1 or 2	
325	1,400	1	16	36†	None			19†	Cow slaughtered
686	1,500	2	16-17	25†	USGA	50 mg.	21	None†	Cow slaughtered 8 days post-USGA
293	1,500	2	16-17	5†	USGA	50 mg.	21	4†	Cow slaughtered 8 days post-USGA, only 1 ovary stimulated
828	1,500	2	16-17	8†	USGA	50 mg.	21	1†	Cow slaughtered 8 days post-USGA; 1 nonfertilized ovum found
859	1,500*	1	17	1-2	None			0	Follicles developed after day 21
	1,500*	1	20						
V866	1,000*	1	15	1	HCG	1,000 iu.	21	1	
T51	1,000*	1	15	1	HCG	1,000 iu.	21	1	Ovulator was divided into two doses
E740	1,000*	1	15	1	HCG	2,000 iu.	21	1	Ovulator was divided into two doses
A62	1,500*	1	15	2-7	HCG	3,000 iu.	21	4	Ovulation delayed long after estrus

Table 2.—Continued

E741	1,500	1	15	1	None			1	
T51	3,000	2	15-16	20	USGA	100 mg.	21	18	Results were satisfactory
903	3,000	2	11-12	15†	USGA	100 mg.	18	15†	Cow slaughtered 8 days post-USGA; 4 fertilized ova found
A62	3,000	2	15-16	20	USGA	100 mg.	21	20	Results were satisfactory
699	3,000	2	15-16	18†	USGA	100 mg.	21	0†	Cow slaughtered 30 hours post-USGA
859	3,000	2	DES treated	71†	USGA	100 mg.	8 days post-DES	71†	Cow slaughtered 3 days post-USGA; 18 ova found
866	3,000	2	DES	22	USGA	100 mg.	6 days post-DES	22	3 fertilized ova found at flushing
15	2,250	2	DES treated	15	USGA USGS	100 mg. 1,200 ru.	10 days post-DES	8	
E741	3,000	1	12	20	USGA	100 mg.	19	10	Part of ovulator given subcut; 7 nonfertilized ova recovered
	3,000	1	14						
891	3,000	1	During pregnancy	28	None				
A62	3,000	1	DES treated	4	None			4	Anaphylactic reaction to injections
692	3,000	2	DES treated	20†	USGA	100 mg.	6 days post-PMS	20†	Cow slaughtered; 2 ova found
E740	2,250	2	DES and progesterone treated	8-18	HLH	100 mg.	6 days post-PMS	9	
T77	2,250	2	Day 1 and 2 post-progesterone recycling	16	USGA	100 mg.	6 days post-PMS	16	

Table 2.—Continued

T77	3,000	2	Day 1 and 2 post-progesterone re-cycling	8†	USGS	1,500 ru.	5 days post-PMS	8†	Slaughtered 8 days post-USGS
T71	3,000	1	18	2-7	None				
T53	5,000	2	Post-DES	2-7	USGA	100 mg.	3 days post-PMS	2-7	1 fertilized ovum found at flushing
T65	5,000	2	Post-DES	2-7	USGA	100 mg.	3 days post-PMS	2-7	
T54	5,000	2	Post-DES	0					
T740	5,000	2	Post-DES	0					
T106	5,000	2	Post-DES	0					
A62	5,000	2	Post-DES	0					
T65	9,000	2	Day 1 and 2 post-progesterone re-cycling	4	USGS	1,500 ru.	6 days post-progesterone	4	
E798	9,000	2	Day 1 and 2 post-progesterone re-cycling	4	USGS	1,500 ru.	6 days post-progesterone	4	

* Intravenous injection.
† Actual visual count.
‡ One ovary only.
Abbreviations:
HFSH—Hog Follicle-Stimulating Hormone.
HLH—Hog Luteinizing Hormone.
SFSH—Sheep Follicle-Stimulating Hormone.
USGS—Unfractionated Sheep Gonadotropin—Gonadophysin.
USGA—Unfractionated Sheep Gonadotropin prepared in laboratory.
HCG—Human Chorionic Gonadotropin.
DES—Diethylstilbestrol.
PMS—Pregnant Mare Serum.
ru.—rat unit.
Au.—Armour unit.
mg. eq.—milligram equivalent.
iu.—international unit.

Table 3. Results of repeated superovulations

Cow no.	Follicle-stimulating material used	Amount used	Estimated no. of follicles developed	Estimated no. of ovulations	Days since last treatment	Comments
15	SFSH	Ca. 60 mg.	0			
15	PMS	4,500 ru.	15	8	337	
A59	PMS	1,500 ru.	0			
A59	PMS	4,000 ru.	1	1	77	
A59	PMS	4,500 ru.	2-7	1-2	49	
859	PMS	3,000 ru.	1-2	0		Follicles developed after heat
859	PMS	6,000 ru.	71*	71*	115	18 ova found at slaughter--mild anaphylaxis
866	HFSH	500 Au.	20-50	0		
866	PMS	6,000 ru.	22	22	357	3 ova found at flushing
14	SFSH	500 mg.	2	2		
14	USGS	1,500 ru.	8-18	1	157	
E741	PMS	1,500 ru.	1	1		
E741	USGS	1,200 ru.	1	1	124	
E741	PMS	6,000 ru.	20	10	70	7 ova found at flushing
A62	PMS	1,500 ru.	4	4		
A62	PMS	6,000 ru.	20	20	67	
A62	PMS	3,000 ru.	0	0	182	Anaphylactic response to PMS subcutaneous
A62	HFSH	225 Au.	14	11	43	2 ova found at flushing
A62	HFSH	225 Au.	4	0	60	
A62	PMS	10,000 ru.	0		83	

Table 3.—Continued

Cow no.	Follicle-stimulating material used	Amount used	Estimated no. of follicles developed	Estimated no. of ovulations	Days since last treatment	Comments
E740	PMS	1,000 ru.	1	1		
E740	PMS	4,500 ru.	8-18	9	364	
E740	HFSH	300 Au.	2-8		122	
E740	PMS	10,000 ru.	0	0	325	
E799	HFSH	100 Au.	20	0		3 ova found at flushing following manual rupturing of follicles
E799	HFSH HLH	100 Au. 40 mg. eq.	12	2	145	
T53	PMS	5,000 ru.	7-8	7-8		1 fertilized ovum found at flushing
T53	HFSH	100 Au.	12	6	195	
T65	PMS	5,000 ru.	2-7			
T65	PMS	9,000 ru.	4	4	77	
T65	HFSH	100 Au.	10	8	191	5 ova found at flushing
T65	HFSH HLH	80 Au. 40 mg. eq.	8	6	123	
T65	HFSH	100 Au.	8	6	64	6 ova found at flushing
876	HFSH	100 Au.	12	8		5 ova found at flushing
876	HFSH	100 Au.	16	14	95	
958	HFSH	75 Au.	8	0		1 ovum found at flushing following manual rupturing of follicles
958	HFSH	120 Au.	14	14	60	
789	PMS	1,500 ru.	0	0		

Cow no.	Follicle-stimulating material used	Amount used	Estimated no. of follicles developed	Estimated no. of ovulations	Days since last treatment	Comments
789	PMS	3,000 ru.	0	0	17	
T77	PMS	4,500 ru.	16	16		
T77	PMS	6,000 ru.	8	8	97	

* Actual visual count.
Abbreviations:
HLH—Hog Luteinizing Hormone.
HFSH—Hog Follicle-Stimulating Hormone.
PMS—Pregnant Mare Serum.
mg. eq.—milligram equivalent.
ru.—rat unit.

Table 4. Assay evaluation of materials used to bring about follicular development and ovulation

Material used	Units employed	Value of a unit
HFSH	Au.	1.0 Au. will increase ovarian weight of hypophysectomized immature Sprague-Dawley rat by five times over control
HLH	mg. eq.	Amount of material equal in potency to 1.0 mg. of a standard preparation (Armour Laboratories)
SFSH	mg.	7 mg. increased ovarian weight (increase due to clear follicles) of 21-day-old Sprague-Dawley rats by three times over control (av. 3 preps)
USGA	mg.	5 mg. increased ovarian weight (increase due to heavy luteinization) of 21-day-old Sprague-Dawley rats by five times over controls
USGS	ru.	1.0 ru. will increase average uterine weight 50 percent over controls in immature rats 100 hours after first of 6 injections given over 3 days
HCG	iu.	1.0 iu. is least amount of material that will bring about cornification in vaginas of immature rats
PMS	ru.	1.0 ru. will bring about average maturation of 3 mature follicles in ovaries of 21-day-old Sprague-Dawley rats at fifth day after first of 3 subcutaneous injections

LITERATURE CITED

1. Brock, Helen, and Rowson, L. E. The production of viable bovine ova. Jour. Agr. Sci. *42*:479. 1952.

2. Casida, L. E., Meyer, R. K., McShan, W. H., and Wisnicky, W. Effects of pituitary gonadotropins on the ovaries and the induction of superfecundity in cattle. Amer. Jour. Vet. Res. *4*:76. 1943.

3. Warwick, E. J., and Meyer, R. K. Survival of multiple pregnancies induced in the ewe following treatment with pituitary gonadotropins. Jour. Anim. Sci. *3*:22. 1944.

4. Chang, M. C. The problems of superovulation and egg transfer in cattle. Proc. First National Egg-Transfer Breeding Conference, p. 39. 1949.

5. Cole, H. H., Hart, G. H., and Miller, R. I. Studies on the hormonal control of estrous phenomena in the anestrous ewe. Endocrinology *36*:370. 1945.

6. Hammond, J. The possibility of artificial pregnancy in cattle. Jour. Minis. of Agr. *57*:67. 1950.

7. Hammond, John Jr., and Bhattacharya, P. Control of ovulation in the cow. Jour. Agr. Sci. *34*:1. 1944.

8. Hammond, John Jr., Hammond, J., and Parkes, A. S. Hormonal augmentation of fertility in sheep. I. Induction of ovulation, superovulation and heat in sheep. Jour. Agr. Sci. *32*: Part 3. 1942.

9. Marden, W. G. R. The hormone control of ovulation in the calf. Endocrinology *50*:456. 1952.

10. McShan, W. H., and Meyer, R. K. The preparation and properties of pituitary follicle-stimulating fractions made by trypsin digestion. Jour. Biol. Chem. *135*:473. 1940.

11. Murphree, R. L., Warwick, E. J., Casida, L. E., and McShan, W. H. Potential fertility of ova from ewes treated with gonadotropins. Jour. Anim. Sci. *3*:13. 1944.

12. Pincus, G. Superovulation of rabbit. Anat. Rec. *77*:1. 1940.

13. Rowson, L. E. Methods of inducing multiple ovulation in cattle. Jour. Endocrinology. *7*:260. 1951.

14. Tanabe, T. Y., Warnick, A. C., Casida, L. E., and Grummer, R. H. The effects of gonadotropins administered to sows and gilts during different stages of the estrual cycle. Jour. Anim. Sci. *8*:550. 1949.

15. Umbaugh, R. E. Superovulation and ovum transfer in cattle. Amer. Jour. Vet. Res. *10*:295. 1949.

16. Willett, E. L. Egg transfer and superovulation in farm animals. Iowa State Jour. Sci. *28*:83. 1953.

17. Willett, E. L., Buckner, P. J., and McShan, W. H. Refractoriness of cows repeatedly superovulated with gonadotropins. Jour. Dairy Sci. *36*:1083. 1953.

18. Willett, E. L., McShan, W. H., and Meyer, R. K. Relation of stage of cycle and source of luteinizing hormone to superovulation in dairy cattle. Soc. Expt. Biol. and Med. Proc. *79*:396. 1952.

Part II. Collection and Isolation of Ova

REVIEW OF LITERATURE

The usual procedure used to obtain mammalian ova has involved sacrifice of the donor, removal of the reproductive tract, and flushing of the ova from the tract. Pincus (*10*) found that flushing was most efficient if the position of the eggs in the tract was taken into account. Reciprocal ova transfers were made by Nicholas (*9*) by performing a laparotomy, excising and flushing one oviduct from each animal, and isolating the ova by examining the recovered fluid under a dissecting microscope. Fekete (*5*) isolated mouse ova by excising the oviducts. They were then sectioned into small pieces and the ova either came free or were forced out. Ova from the cow were isolated by Hartman *et al.* (*6*) from an oviduct recovered from a slaughtered cow.

Allen *et al.* (*1*) was one of the first to isolate an ovum from an intact individual. They recovered an unfertilized ovum from a human by flushing the oviduct of a woman during a laparotomy. Cow ova were recovered by Umbaugh (*13*) by flushing the oviducts at laparotomy. He inserted a blunt needle at the tubouterine junction and flushed a few drops of fluid toward the infundibulum. Chang (*2*) and Dowling (*3*) isolated ova from animals during laparotomies by flushing into a watch glass. In successful bovine ova transfers by Willett *et al.* (*15*) the ova were recovered by flushing the oviducts of uteri of slaughtered animals with homologous serum. Similar transfers on other species have been reported by Hunter *et al.* (*7*), Kvasnickii (*8*), West (*14*), and Runner and Palm (*12*).

Rowson and Dowling (*11*) devised a rubber, three-lumen tube that was inserted into the uterus by way of the cervix. A stiff rod in one lumen held the tube rigid while it was being introduced into the cervix. This lumen served as an outlet for the fluid used in flushing. One lumen was for introduction of the fluid and the other led to a rubber balloon that was inflated to aid in retaining the instrument in place. A report by Dowling (*3*) indicated recovery of ova in 50 percent of the cases with this instrument. Another nonsurgical approach to recovery was effected by Dracy and Petersen (*4*). A steel probe surrounded by a cannula was introduced into the uterus. The probe was withdrawn to allow a plastic tube to be introduced to the tip of the uterine horn. Fluid was introduced by the plastic tube and recovered as it came out by way of the cannula. The ova were allowed to settle and the final isolation was done by a search with a low-power miscroscope. Ova were recovered 12 times in 37 attempts by this method.

Since slaughter of the donor very seriously limits the practical application of ova transfer in the bovine, the following study was carried on in an attempt to develop further an efficient, nonsurgical method for the recovery and isolation of bovine ova.

MATERIALS AND METHODS

The experiments were conducted on dairy cattle and with dairy cattle ova, except in those cases mentioned later, in which rabbit ova were used to evaluate recovery methods.

Various modifications of the Dracy-Petersen method were used. A balloon was constructed on the end of the cannula by gluing to it a section of penrose rubber tubing. This was connected to the outside for inflation by a long section of fine Koroseal tubing. This permitted inflation of the balloon formed on the end of the cannula, so that the cannula would be more easily retained.

Another modification consisted of sealing up the outside end of the cannula so that negative pressure could be applied in an attempt to recover the introduced fluid. In some cases, the tube going through the cannula to the tip of the uterine horn was eliminated. Fluid was introduced and recovered by way of the cannula.

The Rowson-Dowling type of flushing instrument was modified to have only two

lumina (figure 2). One lumen led to a retaining balloon designed to hold as much as 30 cc. The other led to the tip of the catheter. There were 10 holes about one mm. in diameter, equally distributed throughout the length of that part introduced into the uterus. The diameter of the catheter was 20 fr. The catheter was introduced into the uterus with the aid of a stainless-steel wire which was withdrawn as the catheter went into the uterus. The retaining balloon was inflated with about 10 cc. of either air or water to keep the catheter in place and to seal one horn from the other. The insertions were all made by using recto-vaginal manipulations. Infusion of fluid into the uterine horn was made by a 50-cc. syringe which forced the fluid in and supplied negative pressure for withdrawal. Sometimes the same fluid was flushed in and out twice rather than once. Rectal manipulation of the uterus helped to force the fluid back.

A phase of this study involved the determination, as nearly as possible, of the volume of fluid that the nonpregnant uterus can hold at the pressures normally used in flushing without rupturing the uterine wall. Uteri of slaughtered cows from a local packing house were isolated and filled with water at a pressure of six feet. The uteri had been removed from the cows no more than a few minutes before volume measurements were taken. Water was first infused into the uterus, then poured into a graduated cylinder, and the volume measured and recorded.

A limited number of cows were slaughtered following normal ovulation or superovulation. The reproductive tract was recovered and the oviducts and horns of the uterus were flushed with either physiological saline solution or blood serum.

The estimated number of potential ova was determined in most cases by rectal palpation just prior to flushing. In the case of slaughtered animals, the estimated number of ova, as seen in table 5, was the actual number of ovulation points observed on the ovaries.

In those cases in which ova were expected to be found and the fluid volume recovered was 500 cc. or more, a separatory funnel was used to remove most of the fluid, as described in the Dracy-Petersen technique. The remainder of the fluid was divided into 50-cc. lots placed in Petri dishes about three inches in diameter. When the recovered fluid was less than 500 cc., it was divided immediately into 50-cc. lots and placed in Petri dishes. The bottom of the dishes had been previously marked off in squares by a diamond-point pencil. The squares were very nearly the size of one field of the binocular stereoscopic microscope at the 18X magnification. The ova very quickly settled to the bottom of the fluid, so focusing was usually limited to that level at the bottom of the fluid. It was found that, if the light was transmitted through the fluid being searched, identification of ova was accomplished more easily than if the light was reflected from the material. For this

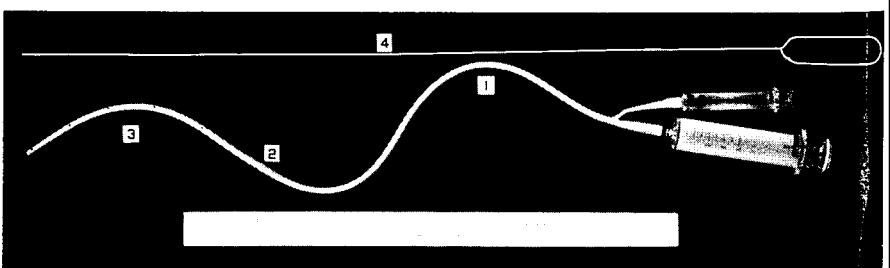


Fig. 2. Uterine flushing apparatus. (1) Body of flushing tube. (2) Retention balloon (deflated). (3) Perforated distal end of tube. (4) Wire which is inserted in tubing while making entrance to cow's uterus.

reason, the fluid had to be nearly transparent. When trauma was sufficient to cause bleeding, the red blood cells obscured vision and made the search for ova difficult. After experience had been gained, identification and isolation became relatively easy.

The ova were picked up from the Petri dishes by capillary pipettes using capillary flow or by inseminating pipettes with an attached syringe to create suction.

RESULTS AND DISCUSSION

The uteri of 66 animals with unknown histories were used to ascertain the volume of fluid the uterus would hold at six feet of water pressure. Nine of these uteri were ruptured by this much pressure. The most common rupture sites in these uteri were at the attachments of the broad ligaments and at the bicornual ligaments. The average volume of the remaining 58 uteri was 193 cc., with a range of 60 to 325 cc. The larger, more flaccid uteri tended to hold a larger volume than those with a higher tonus. There was no loss of fluid through the oviduct, even when the pressure was sufficient to rupture the uterus.

There are 84 cases of flushing the uterus (table 5). Ten of these flushings were made on reproductive tracts which were removed and flushed following slaughter, and the other 74 cases were performed on intact animals. Bovine ova were recovered in 11 of the trials which were done with intact animals. From the slaughtered animals, ova were recovered in four cases out of 10.

Many of the animals had also been superovulated prior to flushing, but since there were so few cases in which ova were recovered there was little relationship between estimated ova expected and those recovered. Neither can there be a comparison made between flushing methods on the basis of ova recovered, because the instances of recovery were so few and the number of ova present was unknown. Of those cases in which the animal was treated to bring about superovulation and was then slaughtered, not all yielded ova, even by careful flushing of the oviducts and the horns of the uterus. As noted in the section on superovulation, each ovulation point does not indicate the presence of an ovum which may be recovered.

Seventy-one percent of the total fluid infused into uteri by the plastic tube and cannula was recovered in 21 trials where recovery was effected and recorded. An average of 855 cc. of fluid was used and an average of 245 cc. was not recovered at each flushing. When the steel cannula was used alone or the short rubber catheter was used, an average of 77.5 percent of the total volume infused was recovered. The average amount of fluid used for a flushing was 112 cc., of which 88 cc. was recovered, losing 24 cc. While using the long catheter with the multiple perforations, 77 percent of the total fluid infused was recovered. The amount used, recovered and lost averaged 39, 30, and 9 cc. respectively for 122 individual flushings carried out in 37 trials of this type.

Care had to be taken to be certain that the long catheter followed the lumen of the uterus when being inserted. In some cases, the catheter became doubled up as it was slipped off the stiff wire and into the horn of the uterus. This could usually be detected by rectal palpation. If fluid was infused when it was in this position, no recovery could be made, since the catheter sealed itself when negative pressure was applied.

In some instances, with all types of flushing techniques, the wall of the uterus was punctured by the equipment. In several cases, especially when large volumes of fluid were used, the uterus was thought to be ruptured during infusion of the fluid, since no recovery of fluid was possible.

In one case, 10 six-day-old rabbit ova were introduced into the uterus of a living cow prior to flushing (trial 33, table 5). Five broken-up remnants of the zona pellucida were recovered, but no intact ova. Ova can tolerate quite severe physical treatment, but perhaps the flushing treatment was too violent and they were destroyed in the recovery attempt.

In another trial, eight two-day-old rabbit ova were deposited prior to recovery

attempts (trial 40, table 5). In this trial, eight consecutive 10 cc. portions were flushed in and out and examined. Only one ovum, which was found in the seventh portion, was recovered.

Since it is impossible to ascertain accurately the number of bovine ova present in the uterus of a superovulated cow, it would seem that the technique of depositing known numbers of rabbit ova prior to recovery attempts would have considerable merit in evaluating flushing techniques.

Chances for recovery of fertilized ova seemed to be best when conditions not within the scope of the flushing technique were optimum. The conditions which seemed to create an optimum situation were as follows: moderate ovarian stimulation by the follicle-stimulating preparation, so that about 10 or less palpable follicles were present on each ovary; very decided estrous signs in the cow on the expected day; breeding at or near the end of heat; and complete rupturing of follicles within 24 hours after the end of heat by the luteinizing hormone preparation. This set of conditions possibly insures that a maximum number of ova will be present in the uterus five days later.

A 28-day-old embryo with intact fetal membranes was recovered in one case, using the steel cannula alone. This technique of recovering small bovine embryos may aid embryologists in obtaining material for study without recourse to slaughter of an animal for each embryo.

Diethylstilbestrol was administered subcutaneously one or two days prior to flushing, in six cases, to facilitate passage through the cervix. Although there did seem to be some cervical relaxation following this treatment, there was the possibility of creating an undesirable environment for ova in the uterus. In only six cases in which flushing was attempted was it impossible to gain entrance to the uterus because the cervix could not be penetrated. The cervix is an obstacle but not a serious one, once experience in penetrating it has been gained.

Two cows that had been flushed were bred and subsequently calved. One had been flushed three times. No attempts were made to determine the fertility of animals following flushing and the two that conceived did so as a result of another experiment.

SUMMARY AND CONCLUSIONS

Ova were recovered in 11 of 74 trials by flushing the uteri of intact cows. From the standpoint of ova recovered, there was no particular advantage of one method over another. Considering the volume of fluid remaining in the uterus or lost, the method utilizing the long catheter by itself was most efficient. The small number of ova recovered indicated that the present methods of collections are not entirely satisfactory, assuming that ova are present in the uterus prior to flushing. A method of consistently obtaining ova by nonsurgical means still needs to be developed.

Excised uteri contained an average volume of 193 cc. when filled at a pressure of six feet of water. The uterus is a relatively delicate organ and can easily be ruptured by pressure or punctured by various instruments.

Ova settled rather rapidly to the bottom of a container in which the fluid and ova were placed, and were identified rather easily by a low-power stereoscopic microscope.

The placement of ova in known quantities from superovulated rabbits within cows' uteri prior to flushing attempts may lead to an objective evaluation of methods of ova recovery.

Table 5. Attempted ova recovery by flushing techniques

Trial no.	Type of flushing instrument	No. of portions	Total volume used in cc.	Total volume recovered in cc.	Percent recovery of fluid	Estimated no. of potential ova	No. of ova recovered	Comments
1	*	1	1,000	.....	.....	Unknown	0	
2	*	1	1,000	.....	.....	4	0	
3	*	1	1,000	.....	.....	0	0	
4	*	1	1,000	610	61	4	0	
5	*	1	890	760	85	1	0	
6	*	1	1,000	250	25	Unknown	0	
7	*	1	1,000	0	0	2	0	
8	*	1	1,000	820	82	Unknown	0	
9	*	1	800	500	63	1	0	
10	*	1	1,000	850	85	1	0	
11	*	1	1,000	850	85	1	0	
12	*	1	1,000	900	90	4	0	
3	*	1	1,000	750	75	1	0	
4	*	1	1,000	900	90	Multiple ovulation	0	
15	*	1	1,000	900	90	Multiple ovulation	0	
16	*	1	1,000	900	90	Multiple ovulation	0	
17	*	1	1,000	900	90	Multiple ovulation	0	
18	*	1	1,000	950	95	Unknown	0	
19	*	1	500	300	60	1	0	
20	*	1	500	0	0	1	0	
21	*	1	480	350	73	Unknown	0	
22	*	1	480	390	81	Unknown	0	
23	*	1	455	250	55	Unknown	0	
24	*	1	500	310	62	6-7	0	
25	*	1	500	325	65	Unknown	0	
26	*	1	450	50	18	Unknown	0	
27	†	1	250	.....	.....	0	0	Recovered volume not recorded
28	†	3	375	323	86	Unknown	1	
29	†	3	375	270	70	0	0	
30	†	3	375	210	51	0	0	28-day embryo with intact membranes recovered

Table 5.—Continued

Trial no.	Type of flushing instrument	No. of portions	Total volume used in cc.	Total volume recovered in cc.	Percent recovery of fluid	Estimated no. of potential ova	No. of ova recovered	Comments
31	†	4	400	336	84	1	0	
32	†	4	400	371	93	1 or 2	0	
33	†	4	400	313	78	10	0	10 rabbit ova intro. prior to flushing
34	†	4	400	362	91	2	0	
35	†	4	500	340	68	1	0	
36	†	1	150	130	87	8-10	7	Short rubber self-retaining catheter
37	‡	5	250	250	100	Unknown	0	
38	‡	2	40	30	75	Unknown	0	
39	‡	1	50	.....	.....	Unknown	0	Recovered volume not recorded
40	‡	8	80	80	100	8	1	8 rabbit ova introduced into uterus prior to flushing
41	‡	4	80	80	100	22	1	
42	‡	3	90	85	95	22	2	
43	‡	4	200	135	63	1	0	
44	‡	4	200	180	90	Unknown	1	Had moderate follicular development with all follicles ruptured
45	‡	4	200	170	85	4	0	
46	‡	4	200	105	53	Unknown	0	
47	‡	4	210	175	87	Unknown	0	
48	‡	4	180	125	69	Unknown	4	
49	‡	4	100	85	85	6	0	
50	‡	4	190	150	79	8	5	
51	‡	4	95	35	37	Unknown	0	
52	‡	4	90	80	89	8	5	
53	‡	4	80	75	94	8	1	Follicles manually ruptured
54	‡	4	175	105	60	6	6	
55	‡	4	150	120	80	8	0	
56	‡	4	85	75	88	8	0	Done the day following Trial No. 55 on same cow
57	‡	4	200	90	45	Unknown	0	

Table 5.—Continued

Trial no.	Type of flushing instrument	No. of portions	Total volume used in cc.	Total volume recovered in cc.	Percent recovery of fluid	Estimated no. of potential ova	No. of ova recovered	Comments
58	‡	1	50	35	70	4	0	
59	‡	1	50	42	84	Unknown	0	
60	‡	1	60	50	83	Unknown	0	
61	‡	1	50	45	40	14	0	
62	‡	4	80	0	0	10	0	
63	‡	4	200	140	70	4	0	
64	‡	4	130	95	73	14	0	
65	‡	2	90	70	78	4	0	
66	‡	4	130	124	95	2	0	
67	‡	2	100	85	85	4	0	
68	‡	1	60	50	83	Unknown	0	
69	‡	2	105	80	76	12-14	0	
70	‡	2	100	100	100	12	0	
71	‡	4	240	210	88	12	0	
72	‡	1	50	40	80	10	0	
73	‡	4	155	110	71	4	0	
74	‡	4	170	135	79	10	3	1 ovum nonfertile, 2 in 8-16 cell stage
75	‡	2	100	65	65	14	0	
76	§	1	10	10	100	2	0	
77	§	1	5	5	100	19	0	
78	§	1	5	5	100	2	0	
79	§	1	5	5	100	1	0	
80	§	1	5	5	100	4	0	
81	§	1	5	5	100	1	0	
82	§	1	5	5	100	12-16	1	
83	§	1	5	5	100	15	4	
84	§	1	5	5	100	71	18	
85	§	1	5	5	100	43	4	

* Steel cannula and Koroseal tubing.
† Steel cannula alone or short rubber catheter.
‡ Long rubber catheter.
§ Slaughtered and flushed.

LITERATURE CITED

1. Allen, E., Pratt, J., Newell, O. V., and Gland, L. Recovery of the human ova from the uterine tubes, time of ovulation in the menstrual cycle. Jour. Amer. Med. Assoc. *91*:1018. 1928.

2. Chang, M. C. Transplantation of rabbit blastocysts at late stage: Probability of normal development and viability at low temperature. Science *111*:544. 1950.

3. Dowling, D. F. Problems of the transplantation of fertilized ova. Jour. Agr. Sci. *39*:374. 1950.

4. Dracy, A. E., and Petersen, W. E. Isolation of ova from the living bovine. Jour. Dairy Sci. *33*:797. 1950.

5. Fekete, Elizabeth. Differences in the effect of uterine environment upon development in the DBA and C57 black strains of mice. Anat. Rec. *98*:409. 1947.

6. Hartman, C. G., Lewis, W. H., Miller, F. W., and Swett, W. W. First findings of tubal ova in the cow, together with notes on oestrus. Anat. Rec. *48*:267. 1931.

7. Hunter, G. L., Adams, C. E., and Rowson, L. E. Successful inter-breed transfer of ova in sheep. Nature *174*:890. 1954.

8. Kvasnicku, A. V. Inter-breed transplantation of ova. Sovet. Zootekh. *1*:36; abs. in Anim. Breeding. Abs. *19*:224. 1951.

9. Nicholas, J. S. Development of transplanted rat eggs. Soc. Expt. Biol. and Med. Proc. *30*:1,111. 1933.

10. Pincus, G. Observations on the living eggs of the rabbit. Roy. Soc. London, Proc., Ser. B. *107*:132. 1930.

11. Rowson, L. E., and Dowling, D. F. An apparatus for the extraction of fertilized eggs from the living cow. Vet. Rec. *61*:191. 1949.

12. Runner, M. N., and Palmi, Joy. Transplantation and survival of unfertilized ova of the mouse in relation to post ovulatory age. Jour. Expt. Zool. *124*:303. 1953.

13. Umbaugh, R. E. Superovulation and ovum transfer in cattle. Amer. Jour. Vet. Res. *10*:295. 1949.

14. West, G. P. Successful transplantation of sheep's ova. Vet. Rec. *66*:403. 1954.

15. Willett, E. L., Buckner, P. J., and Larson, G. L. Three successful transplantations of fertilized bovine eggs. Jour. Dairy Sci. *36*:520. 1953.

Part III. Synchronization of Estrous Cycles

REVIEW OF LITERATURE

Ova are relatively delicate structures completely dependent on their environment. Transferred ova, in order to survive, must be placed in an environment that corresponds as closely as possible to the one in which they normally develop. Because the uterus constantly undergoes change and the hormonal levels which influence the environment of the ova fluctuate with the estrous cycle, synchronization of ovulations of donor and host animals becomes an important factor in assuring an environment in the host similar to the one prevailing in the donor. It has been found that the interval between the time of ovulation of the donor and the time of ovulation of the recipient is limited to a matter of hours if transferred ova are to survive (Chang, *6*, and Beatty, *1*). Successful transfer of rabbit ova was accomplished by Heape (*15*) when the transfered ova were ovulated approximately 30 hours before ovulation occurred in the host. Chang (*4, 5, 7*) has attempted to store both fertilized and unfertilized rabbit ova for various periods of time at various temperatures. Storage life of ova was limited to about 72 hours.

Because storage life of ova is limited and ovulation time between donor and recipient must closely coincide, it becomes necessary to alter the time of ovulation in either the donor or the recipient or both.

It appears that secretion from the active *corpus luteum* of the cow normally inhibits estrus and ovulation between heat periods. Payne *et al.* (*20*) obtained an unsaponifiable, cholesterol-free, phosphatid-free fraction from the *corpus luteum* of the cow. This extract inhibited estrus while it was being administered and for a few days after its withdrawal. When hormones of the *corpus luteum,* including progesterone, were purified and synthesized, more studies could be made relating their influence on the estrous cycle.

Christian and Casida (*8*) administered progesterone to four heifers by subcutaneous injection. Fifty mg. of progesterone per day inhibited heat and ovulation during administration and for five to six days following cessation of treatment. Further trials to ascertain the level of progesterone needed to inhibit estrus and ovulation were carried out by Ulberg *et. al.* (*22*). They concluded that 50 mg. per day would inhibit estrus and ovulation if injections were started no later than day 15. If the level was lower, inhibition was not complete. If injections were started at a later stage, follicle development was not always inhibited. Fertility proved to be lower at the first estrus following progesterone administration, in a study by Trimberger and Hansel (*21*). The relative infertility of the thirty cows did not persist beyond the first estrus, even though ovarian abnormalities were noted at the first estrus.

The fertility of heifers, given progesterone starting on day 15 or 16 of the estrous cycle, was found by Willett (*25*) to be normal. Dracy (*10*) indicated that readjusting the heat periods of cows by progesterone had no ill effects on fertility, as judged by the recovery of fertilized ova. Successful ova transfer in the bovine was made by Willett *et al.* (*26*), in which the estrous cycles of both the donor and the host were synchronized by progestationally active material.

Progesterone administration for recycling has been used by many workers in other species such as the sheep, pig, and dog. (Dutt and Casida, *13*; O'Mary *et al. 19*; Hunter, *17*; Ulberg *et al. 23*; Ulberg *et al. 24*; Murray and Eden, *18*).

Other compounds, such as *Lithospermum ruderale* (Drasher *11*), prolactin (Dresel, *12*), gonadotropic hormones (Hisaw, *16*; Zawadowsky and Eskin, *27*; Casida *et al. 3*), atropine (Hansel and Trimberger *14*), and stilbestrol (Asdell *et al. 1*) have been used to adjust the time of estrus and ovulation. Dowling (*9*) is one of many workers who have used expression of the *corpus luteum* as a means of changing the time of estrus and ovulation.

The study that follows is limited to progesterone as a recycling agent. The

objective of this study was to attempt to further establish and evaluate progesterone as a means of synchronizing estrous cycles in dairy cattle.

MATERIALS AND METHODS

There were 34 different animals used in 60 trials. Eighteen of these animals had had one or more calves and the other 16 were virgin heifers. All animals were experiencing normal estrous cycles and had no reproductive abnormality that could be detected by rectal palpation and examination. The animals were of dairy breeding.

The source of the progesterone used in this study was Glidden's crystalline progesterone USP lot No. E-174. It was dissolved in corn oil at the level of 10 or 20 mg. per ml. with the aid of a little heat, never exceeding 100° C. Aqueous suspensions of progesterone were prepared in physiological saline at the level of 10 mg. per cc. These preparations were mixed in such quantities that they were exhausted in a few weeks. The preparations were stored at refrigerator temperatures. The subcutaneous injections were made just behind an elbow or shoulder.

Injections were started about the time it was estimated that the *corpus luteum* was becoming inactive. A cow was considered to be in estrus only when she allowed other animals to ride her. All animals were observed while in the exercise lot.

Days of the cycle were numbered by using the day of heat as day 1, and so on.

RESULTS AND DISCUSSION

There were 60 attempts to adjust the estrous cycle with daily injections of progesterone in this study. Of this number, 12 failed to come into heat in 20 days or less after termination of treatment, due to some failure in the complex that causes a complete expression of heat. In many of these cases, the vulva became moist and edematous with reddening of the vestibule and a mucous discharge was present, but the animals did not allow other animals to mount them. Of these 12 cases, all but two, which were slaughtered a few days after expected heat, resumed cycling normally within three months.

The range in the number of days elapsing between cessation of treatment was three to nine in those cases which were less than 20 days. The average length of time was 5.3 days. The treatments and results for each animal are shown in table 7; table 6 shows the frequency of occurrence of certain intervals between cessation of treatment and heat.

There are too few cases to compare the saline suspension to the oil solution, but it can be stated that both vehicles were effective carriers. It appeared that saline suspensions of progesterone become effective more slowly, as one of the animals treated in this manner came into heat during treatment and subsequently ovulated. However, she again came into estrus after termination of injections. The duration of effectiveness of the saline suspension of progesterone may be prolonged, as among those animals which received the suspension as a last injection are those with intervals between last injection and estrus of the longest duration. It was observed that generally the more mature animals, which also tended to be in a higher condition of flesh, seemed to be the most susceptible to failure in recycling. None of the virgin heifers ever failed to come into heat within the three- to nine-day range after stopping treatment. The failures were limited to cows that had at least one calf and were generally in a higher condition of flesh. This would indicate that it would be best to limit recycling to young virgin heifers to increase the chances of heat on a predicted day.

Ovulation was determined by rectal palpation in most cases and was normally related to heat, except in the cases in which the animals became cystic or in some of the cases when the animals failed to come into heat. One animal ovulated while in heat and displayed metrorrhagia concurrent to standing heat.

In the small number of cases in which the animals were superovulated following synchronization, it seemed as though the superovulation treatment tended to reduce the incidence of detectable heat.

Table 6. Frequency distribution of the interval between cessation of progesterone treatment and estrus

No. of days between end of treatment and estrus	No. of cases
3	5
4	9
5	15
6	10
7	5
8	3
9	1
20 or more	12
Total	60

In two instances, heifers E797 and E799, which had been recycled on two previous occasions, came into heat and ovulated nine and eight days after treatment was stopped, and then came into heat and ovulated a second follicle three and four days respectively after the first heat. As nearly as could be ascertained by rectal palpation, no *corpus luteum* was formed following the rupture of the first follicle, but there was one formed in each case following the second ovulation. It was thought that this may have been due to the depression of the secretion of a pituitary luteotropic hormone by repeated long periods of progesterone administration at a relatively high level. Both animals subsequently came in heat and ovulated after a normal period of time.

Nine animals were bred at the first heat following treatment. Two of these conceived to the first service. The remaining seven and two other animals were bred at the second heat and so on until pregnancy resulted. The over-all conception rate was two services per conception on all animals and all breedings. Of the 34 animals used in these trials, 20 became pregnant and produced calves subsequent to progesterone treatment. There was a great variation in the time between cessation of treatment and the first breeding date, and no effort was made to record the number of services required for conception in all cases. Consequently, no conclusions can be reached on the effect on fertility except that, if progesterone treatment impaired fertility, it appeared to be temporary. This was true, even in cases with abnormal cycles following progesterone treatment.

SUMMARY AND CONCLUSIONS

Attempts were made to adjust the estrous cycles of a group of cows by daily subcutaneous injection of progesterone. Only one of the treated cows came in heat during treatment, and in 71 percent of the cases the animals came in heat either the fourth, fifth, or sixth day after the injections were stopped.

In some cases, especially with mature animals, cystic ovaries developed and the animals became nymphomaniac or failed to show any signs of heat for long periods of time.

There were no detectable permanent effects of progesterone administration.

Although progesterone treatment does not cause estrus on a specific day, the usual range is narrow enough to be useful in adjusting the estrous cycle for most needs.

Table 7. Synchronization of estrous cycles

Cow number	Day of cycle injection started	Dose in mg. per day	Number of days treated	No. of days between end of treatment and heat	Comments
686‡	14	50	11	5	
886‡	17	70	6	5	
T57	16	50	1	3	
355‡	16	50*	4	8	
686‡	15	30*	6	5	
T42‡	15	50	7	5	
708‡	16	50	7	6	
706‡	14	50	8	6	
680‡	14	50	4	7	
355‡	14	50	8	7	First 4 injections were saline, no injection 5th day, last 3 injections corn oil
705‡	15	25*	9	8	50 mg. given every other day
355‡	14	30*	7	6	
T89	14	30*	12	6	
T79	14	40	10	5	30 mg. per day in saline first 5 days; 50 mg. per day in corn oil last 5 days
T80	14	50	5	4	
T89	14	30*	10	5	
T88	13	30*	10	5	
E799	14	50	10	5	Was cystic for 2 months following treatment
E799	15	50	9	4	
E799	15	60	11	9	Came into heat again 4 days after this recorded heat
E798	14	50	8	4	
E798	14	50	9	5	Superovulated
E797	13	50	11	4	
E797	15	50	9	4	
E797	15	60	14	8	Came into heat again 3 days after this recorded heat
E797	14	60	10	7	
E800	14	50	9	5	
E800	15	50	7	6	
E800	15	60	6	6	

Table 7.—Continued

Cow number	Day of cycle injection started	Dose in mg. per day	Number of days treated	No. of days between end of treatment and heat	Comments
T65‡	14	50	10	5	Superovulated
T53‡	15	50	10	3	
T53‡	14	50	13	4	
T53‡	15	60	9	†	
T54‡	15	50	4	3	Conceived at next heat
951‡	14	50	10	†	
T7‡	15	50	11	†	
T7‡	15	40	7	4	
T7‡	16	60	4	†	Estrus not observed for 30 days
T8‡	15	40	6	†	
T8‡	17	60	4	3	
T40‡	15	60	7	†	
T105‡	15	60	15	†	
929‡	15	60	18	5	Was cystic for 3 months following treatment
929‡	15	60	5	†	
T141	15	60	16	6	
876‡	15	60	5	†	Superovulated
T140	15	60	5	5	
T140	17	60	4	4	
E801	15	50	13	3	
E798	14	50	9	5	Superovulated
E801‡	12	50	4	†	Superovulated
T65‡	14	50	10	5	Superovulated
876‡	15	50	5	6	Superovulated
876‡	11	50	4	†	Superovulated
958‡	12	50	4	†	Superovulated and slaughtered
T109‡	16	60	5	4	
T11‡	Not cycling	50	6	6	Conceived at next heat
929‡	16	50	2	7	
T121	9	50	5	7	
T121	18	100	3	6	

* Physiological saline suspension of progesterone.

† Estrus was not observed for at least 20 days.

‡ Animal at least 2½ years of age and in good condition of flesh at the time of treatment.

LITERATURE CITED

1. Asdell, S. A., de Alba, J., and Roberts, J. S. The levels of ovarian hormones required to induce heat and other reactions in the ovariectomized cow. Jour. Anim. Sci. *4*:277. 1945.

2. Beatty, R. A. Transplantation of mouse eggs. Nature *168*:995. 1951.

3. Casida, L. E., Dutt, R. H., and Meyer, R. K. Alteration of the estrual cycle by pituitary gonadotropins and persistence of the effects upon reproductive performance in ewes. Jour. Anim. Sci. *4*:24. 1945.

4. Chang, M. C. Normal development of fertilized rabbit ova stored at low temperature for several days. Nature *159*:602. 1947.

5. Chang, M. C. Probability of normal development after transplantation of fertilized rabbit ova stored at different temperatures. Soc. Expt. Biol. and Med. Proc. *68*:680. 1948.

6. Chang, M. C. Development and fate of transferred rabbit ova or blastocyst in relation to the ovulation time of recipients. Jour. Expt. Zool. *114*:187. 1950.

7. Chang, M. C. Fertilization and normal development of follicular oocytes in the rabbit. Science *121*:867. 1955.

8. Christian, R. E., and Casida, L. E. The effects of progesterone in altering the estrous cycle of the cow. Jour. Anim. Sci. *7*:540. 1948.

9. Dowling, D. F. Problems of the transplantation of fertilized ova. Jour. Agr. Sci. *39*:374. 1950.

10. Dracy, A. E. Progesterone and relaxin as aids in ova transfer. So. Dakota Agr. Expt. Sta. 66th Annual Report, p. 130. Abs. in Jour. Dairy Science *37*:664. 1954.

11. Drasher, M. S. The mechanism of action of *Lithospermum ruderale*. Endocrinology *45*:120. 1949.

12. Dresel, I. The effect of prolactin on the estrous cycle of nonparous mice. Science *82*:173. 1935.

13. Dutt, R. H., and Casida, L. E. Alteration of the estrual cycle in sheep by use of progesterone and its effect upon subsequent ovulation and fertility. Endocrinology *43*:208. 1948.

14. Hansel, W., and Trimberger, G. W. Atropine blockage of ovulation in the cow and its possible significance. Jour. Anim. Sci. *10*:714. 1951.

15. Heape, W. Preliminary note on the transplantation and growth of mammalian ova within a uterine foster mother. Roy. Soc. London, Proc. *48*:457. 1890.

16. Hisaw, F. L. The placental gonadotropin and luteal function in monkeys (Macaca Mulatta). Yale Jour. Biol. and Med. *17*:119. 1944.

17. Hunter, G. L. Attempts to synchronize the occurrence of oestrus in sheep as a preliminary to ovum transplantation. Jour. Endocrinology *10*: Soc. for Endocrinology, Proc. XIII. 1954.

18. Murray, G. H., and Eden, E. L. Jr. Progesterone to delay estrum in bitches. Vet. Med. *47*:467. 1952.

19. O'Mary, C. C., Pope, A. L., and Casida, L. E. The use of progesterone in the synchronization of the estrual periods in a group of ewes and the effect on their subsequent lambing records. Jour. Anim. Sci. *9*:680. 1950.

20. Payne, W. B., Van Peenan, H., and Cartland, G. F. The distribution of the estrus-producing and estrus-inhibiting hormones in the ovary of the cow. Amer. Jour. Physiol. *86*:243. 1928.

21. Trimberger, G. W., and Hansel, W. Conception rate and ovarian function following estrus control by progesterone injections in dairy cattle. Jour. Anim. Sci. *14*:224. 1955.

22. Ulberg, L. C., Christian, R. E., and Casida, L. E. Ovarian response in heifers to progesterone injections. Jour. Anim. Sci. *10*:752. 1951.

23. Ulberg, L. C., Grummer, R. H., and Casida, L. E. The effects of progesterone upon ovarian function in gilts. Jour. Anim. Sci. *8*:646. 1949.

24. Ulberg, L. C., Grummer, R. H., and Casida, L. E. The effects of progesterone upon ovarian function in gilts. Jour. Anim. Sci. *10*:665. 1951.

25. Willett, E. L. The fertility of heifers following administration of progesterone to alter the estrual cycle. Jour. Dairy Sci. *33*:381. 1950.

26. Willett, E. L., Buckner, P. J., and Larson, G. L. Three successful transplantations of fertilized bovine eggs. Jour. Dairy Sci. *36*:520. 1953.

27. Zawadowsky, M., and Eskin, I. New data on regulation of the sexual cycle in the cow. Bul. Biol. Med. Expt. U.S.S.R. *4*:200. (Anim. Breeding Abs. *7*:122.) 1939.

Part IV. Handling and Transfer of Ova

REVIEW OF LITERATURE

In the interim between collection and transfer, the ova must be maintained *in vitro* in a viable condition. Many studies have been made on rabbit ova, and findings may or may not apply to other species. As pointed out by Willett (*43*), rabbit eggs differ from eggs of other species in that they pick up a thick layer of material in their passage down the oviducts. This layer may serve as a protective mechanism for the egg and so could render it less subject to injury in handling.

A number of different media have been used for holding and culture of eggs *in vitro*. Hammond (*18*) used a saline and hen-egg extract medium at a pH of 7.2-7.4 to culture mouse eggs at 37° C. for 24 hours. Eight-cell ova developed to the 16-cell stage under these conditions. No 2-cell ova developed past the 4-cell stage.

Blood serum has been used extensively and found to be quite satisfactory by most workers. Chang (*6*) demonstrated an ovicidal factor in the blood serum of several species but not in others. This ovicidal factor was present in cow's blood serum, but was inactivated by heating at 55° C. for 39 minutes or storing at ordinary refrigerator temperatures (3° C.) for 48 hours.

Brock and Rowson (*5*) attempted to culture bovine ova in both blood serum and in follicular fluid. No divisions of the ova were obtained when follicular fluid was the medium. In serum, 3- or 4-cell-stage eggs divided to the 8-cell stage. Pincus (*30*) reported cleavage of bovine ova when cultured in blood serum. These ova were removed from the oviducts at two, three, five, and six days following breeding and the injection of an intravenous ovulating dose of pituitary gonadotropin.

The use of certain semen diluents, such as egg-yolk-buffered phosphate solution, were not satisfactory, in that visualization of ova in this medium was most difficult (Hammond, *16*). In successful mouse ova transplantations, a mixture of half bovine semen diluent and half Locke's solution was used as the medium (Runner and Palm, *36*).

Lewis and Gregory (*23*) cinematographed rabbit eggs which were contained in blood plasma. One-cell eggs were kept alive for two days and developed into the 8-cell stage. Two eggs, recovered 67 hours after breeding the doe, lived and developed for eight days.

Defries (*10*) attempted, unsuccessfully, to culture rat eggs in a number of media, including liquid collected from the uterus of an animal during estrus. He found that they were very senstitive to temperature variations, osmotic pressure, and hydrogen ion concentration.

Boell and Nicholas (*4*) studied respiration of rat eggs *in vitro*, and effects of various media on eggs. They found an 0.8 percent sodium- chloride medium buffered with phosphate to pH 7.4 was most satisfactory. In none of the media tested, however, did eggs cleave more than once.

Willett (*43*) reviewed the major work on this question of media and points out that solutions which are in current use are fairly satisfactory for holding eggs up to a few hours. He cites the work on *in vitro* culture by many workers and points out that only a few cell divisions can be obtained when eggs are cultured using present media and techniques.

Chang and Pincus (*8*), in a comprehensive review of the physiology of fertilization in mammals, cover the work on *in vitro* fertilization, activation, parthenogenesis, and other aspects of this subject.

Odor and Blandau (*28*) reported *in vitro* fertilization and the first segmentation division of rat ova using Ringer-Locke's solution as the medium. Menkin and Rock (*26*) exposed human ova to human sperm suspension, preceded and followed by culture in human blood serum and obtained two eggs in the 2-cell stage and two in the 3-cell stage.

Recently, Shettles (*37*) reported the development of an isolated human ovum to a morula stage of approximately 32 cells

within 79 hours. He aspirated the ovum from a mature follicle and discharged it into a sterile Petri dish along with the follicular fluid. Some minced fragments of fallopian tubal mucosa were added along with fresh human semen, and the mixture was incubated at 37° C. for 72 hours. It was thought that the tubal mucosa, through its fibrinolytic enzyme, would aid in the denudation of the ovum, thus facilitating sperm penetration.

After the ova have been collected and located, they must be identified as fertilized or unfertilized. The criteria commonly used to ascertain fertilization are apparently normal segmentation for the age of the ovum, and/or the appearance of sperm head in the zona pellucida (Winters, *et al., 45*); (Laing, *21*). Segmentation is not an absolute gauge since degenerative fragmentation superficially resembling cleavage of fertilized eggs may occur in unfertilized mammalian eggs, both *in vivo* and *in vitro* (Smith, *38*, and workers cited by him; Spalding *et al., 39*).

Pincus (*29*) related that parthenogenic development may also be initiated by abnormal environmental conditions. Pincus and Enzmann (*31*) exposed rabbit eggs to supernormal temperatures or treatment with hypertonic solution and got activation in unfertilized tubal rabbit ova. Pincus (*29*) obtained the first maturation division which was occasionally followed by cleavage. Courrier (*9*) described an embryo with trophoblast in the ovary of an immature guinea pig, and Krafka (*20*) found a 4-cell egg in the human ovary from a seven-year-old child.

Mann (*24*) observed cleavage in nine of 252 unfertilized rat eggs, two to the 4-cell stage. Austin (*1*) observed that twenty percent of unfertilized rat eggs studied were divided. Chang (*7*) recovered 41 unfertilized tubal ferret ova, 12 of which had fairly equally cleaved into two to six cells, and which appeared quite normal. A 3-cell mink ovum was also obtained, out of 15 unfertilized eggs studied.

Direct examination under a phase microscope is the most suitable method to ascertain if an ovum is fertilized, according to Austin (*1*).

Brock and Rowson (*5*) described a test designed to indicate the viability of ova. A dilute solution of Evan's Blue in Mammalian Ringer at 37° C. was found to penetrate into the vitellus of dead, but not of living, eggs. Rowson (*33*) indicated that none of the ova stained were transferred to test the effects of staining on subsequent viability.

Successful ova transfers reported in the literature, with only one exception known to these authors, employed surgery to obtain the ova and make the transfer. Beatty (*2*) reported successful nonsurgical ova transfers in mice.

As Hammond (*17*) pointed out, surgical transplantation in small animals usually involved the following technique: exposure of the uterus and insertion of the ova by a fine capillary glass pipette through the muscular wall into the lumen. The pipette was inserted through the outer muscular coat and then passed laterally before the inner muscular coat and mucosa was penetrated. When the pipette was withdrawn, the opening was closed and the eggs did not escape. Noyes (*27*) described a similar method of the surgical transfer of follicular ova.

In Willett's three successful bovine-ova transplantations, the recipients were anesthetized, the uterus exteriorized after midventral laparotomy, and the eggs inserted into the uterus near the tubo-uterine junction by means of a glass micro-pipette. As pointed out by Willett (*43*) and Kile (*19*), anesthesia may affect fertility and embryonic mortality.

A completely nonsurgical technique would, if successful, greatly augment the practicability of ova transplantations. A number of nonsurgical, but unsuccessful, transfers have been reported in cattle by Umbaugh (*40*), Dowling (*11*), Rowson (*32*), Hammond (*16*), Dracy (*12*), and Dziuk and Petersen (*13*).

The nonsurgical technique for transferring fertilized ova has followed, to a large extent, that used in artificial insemination. This technique calls for the passage of the pipette through the cervix and into the uterus itself, many times attempt-

ing deep uterine deposition of the ovum into the tip of the horn.

Considering the fact that bovine ova enter the uterus at about the fourth day post-ovulation (Hamilton and Laing, *15*; Winters *et al.* *45*), nonsurgical ova recovery was attempted on the fifth day. Conditions in the uterus of both the donor and the recipient must closely approximate each other, as is discussed elsewhere in this bulletin. Thus if fertilized ova were recovered, they were transferred into the uterus of the host during the luteal phase of the estrous cycle.

The uterus is more susceptible to infection at the stage of the cycle while under the influence of high quantities of progesterone, according to Rowson (*32*), Rowson *et al.* (*35*), Black *et al.* (*3*), and other workers.

A postulation advanced by Brock and Rowson (*5*) and Fry *et al.* (*14*) points to the susceptibility of the uterus to infection when under the influence of a *corpus luteum* as being the main reason for the failure of implantation of ova transplanted via the cervix. Fallopian tube washings to which penicillin had been added were injected into the uteri of cows three to four days following estrus. Upon slaughter two days after injection, all showed obvious signs of uterine infection. It was assumed that this infection was sufficient to prevent conception.

Black *et al.* (*3*) point out that any bacterial infection of the uterus may lead to an inflammatory response which would interfere with placentation. Bacteria introduced in such a manner may attack the embryo directly, as their result indicated.

Dowling (*11*) suggested that irrigation of the uterus with penicillin prior to transplantation may help to overcome harmful infection. Rowson *et al.* (*35*) found antibacterial agents in semen diluents reduce or eliminate the severity of metritis.

Marden and Chang (*25*) successfully transferred fertilized rabbit ova in a medium which had penicillin added to it.

Various attempts have been made to overcome the problem of infection. Lamming and Rowson (*22*) suggested a method in which they embedded 24-hour tubal eggs in gelatin and, by use of a specially designed "dart gun," deposited the egg into the ovarian ligament. The success of this method has not been reported.

Lamming and Rowson (*22*) also attempted the transfer of 4-cell-stage eggs by means of a hypodermic needle connected to a syringe inserted into the lumen of the uterus by way of the vagina.

Vandemark *et al.* (*42*) deposited semen into the uteri of eight pregnant cows (day 64 to day 151 of gestation), and interrupted all pregnancies. However, when penicillin and streptomycin were added to the semen and diluent, only two of six pregnancies were interrupted.

Tanabe *et al.* (*40*) inseminated into the uterine body of pregnant cows and heifers, interrupting 7.5 percent of the pregnancies when antibiotics were used, and 75 percent when antibiotics were omitted.

MATERIALS AND METHODS

The following is the procedure used to handle and transfer ova which were collected either from slaughtered animals or from flushing the uteri of living cows. The collection procedure is related elsewhere in this bulletin. Collections were made at ordinary barn temperatures and the ova held at these temperatures from 10 minutes to two hours before the transfer was made. The collection medium was usually also the transfer medium used. Following collection, the fluid was examined for ova under low-power magnification (9x to 27x) after it was placed in Petri dishes. If fertilized ova were found, they were aspirated into ordinary inseminating pipettes and transferred into the uteri of recipient cows, using the recto-vaginal artificial-insemination technique. The ova were deposited as near the tip of the horn as possible. Two pipettes cemented together so as to allow for deeper deposition of the ova were sometimes used.

Precautions were taken to be as aseptic as possible but it was not very probable that the collection, *in vitro* handling, and subsequent transfers were completely aseptic.

Five cows served as donors and 13 cows as recipients. One cow, T65, served as a recipient on two occasions and as the donor in five instances. Most of the cows had previously been subjected to various experimental treatments in connection with ova-transplant work. These included synchronization of the estrous cycle with progesterone, superovulation, etc.

Grade Holstein and grade Guernsey cows were used, all of which had calved at least once.

A study of the detrimental effect upon conception caused by the nonsurgical technique of collection and transfer was made. This work exactly duplicated the techniques currently used by workers at the University of Minnesota in attempted nonsurgical ova transfers in the cow, as related previously. This involved cervical intervention (fifth day post ovulation) with the deposition of about 1 cc. of blood serum which had been flushed into and recovered from the uterus of another cow that was in the same stage of the estrous cycle as the bred recipient. This was the material in which the ova were usually transferred and this technique will be referred to as "inserumation" for the sake of brevity.

Whether inserumation would or would not prevent the development of the fertilized ovum in the uterus was of primary concern. The possible prevention of harmful effects through the addition of penicillin to the material deposited in the uterus was studied.

Twenty-two cows were artificially bred at estrus. On the fifth day post breeding, the time at which fertilized ova would normally be transferred, and while the cows were under the influence of the *corpus luteum* (progesterone), the cows were inserumated. The flushing material was first observed for ova before being transferred, and usually was held at room temperature for approximately one hour's time prior to transfer. The transfer was made in the same manner as the routine recto-vaginal insemination technique.

Fourteen inserumations were made without addition of antibiotic to the flushing material. Eight were made with penicillin (1,000 units/ml. of serum) which was added at the time the serum was placed under refrigeration, approximately 48 hours prior to the flushing of the uterus.

All cows in each of two groups used in this experiment had calved at least once. Most of them had been dry for approximately 12 months, and most were in higher condition of flesh than is generally recommended for cows prior to parturition. They had previously been used as ova-transplant experimental animals, and most had been subjected to a variety of treatments. These included synchronization of the estrous cycle with progesterone, superovulation, etc.

The cows were bred with frozen semen routinely used to breed the University dairy herd. Inexperienced technicians made the inseminations just past the second cervical ring or into the uterus itself during the second half of estrus. The probability of conception as determined from previous history and experimentation was not ideal but was comparable between the two groups of cows used in this experiment.

RESULTS AND DISCUSSION

The 16 nonsurgical transfers of bovine ova resulted in no pregnancies (table 8). This should not be interpreted, however, as conclusive evidence against the possibility of successful nonsurgical transplants. In only four cases were the transferred ova considered normal. The cows used as recipients were probably below average in their reproductive potential, due to the various experimental treatments to which they had been subjected and to the obesity of many.

The results of inserumation are shown in table 9. Two of 14 cows inserumated on the fifth day post breeding calved after a normal gestation in the "no penicillin" group. The remaining 12 cows exhibited estrus on the average of 26.4 days subsequent to breeding. Four of these showed estrus 42, 38, 32, and 30 days after being bred. This suggests the possibility of conception followed by embryonic mortality in these cows. When these four animals

are removed from the group the average period between breeding and the subsequent estrus was 21.0 days. One cow (T141) was inserumated seven days following breeding and showed evidence of uterine infection, as manifested by pus-containing secretions from the reproductive tract 13 days following inserumation.

Eight cows composed the "penicillin" group, and they were subjected to exactly the same treatment as the "no penicillin" animals, except for added penicillin. Five of the eight cows conceived to first service and normal parturitions followed. The remaining three cows exhibited estrus again on approximately the expected date. None of the cows in the penicillin group showed gross evidence of uterine infection as manifested by pus-containing secretions from the reproductive tract.

Application of the nonsurgical transfer technique (serum without ovum) described here failed to demonstrate that cervical intervention (fifth day postestrum) with the deposition of the material used in transferring ova into the uterus was sufficiently harmful in all cases to prevent the fertilized ovum in the uterus from attachment or of normal development until parturition occurred.

SUMMARY AND CONCLUSIONS

Handling and transfer procedures pertaining to ova transplantation in the cow are discussed.

Sixteen nonsurgical transfers of bovine ova resulted in no pregnancies. Of the 16 transfers made, only four of the ova used were considered to be normal. This number is inadequate to conclude that successful nonsurgical transfers will not be made in the future.

Seven pregnancies were effected in 22 cows, in which breeding was followed in five days by uterine deposition of blood serum which had been flushed into and recovered from the uterus of another cow. Five pregnancies resulted in the eight cows which were inserumated with uterine flushings to which penicillin had been added. Two out of 14 inserumations resulted in pregnancies when penicillin was omitted from the flushings.

The results of this and other work indicate that cervical intervention did not always interrupt pregnancy. Evidence presented points to the addition of penicillin to the transfer medium as a possible aid to nonsurgical transfers.

Table 8. Nonsurgical ova transfers in the cow

Trial no.	No. of ova transferred	Fertility	Transfer medium	Remarks
1	1	.....	.....	.....
2	2	Doubtful	Ringer's Solution	Recipient and donor 5th day of cycle
3	4-5	Follicular ova	Follicular fluid	Recipient bred and transferred into horn day 3
4	4-5	Follicular	Follicular fluid	Recipient bred and transferred into horn day 3
5	4-5	Follicular	Follicular fluid	Recipient bred and transferred into horn day 3
6	1	Blastula stage	Physiological saline 4% PVP (polyvinylpryolidone) 40% blood serum (24 hours of age)	Recipient in heat 3 days following transfer and then not observed in heat for 70 days
7	2	Doubtful	Ringer's solution	Recipient and donor 5th day of cycle
8	4-5	Follicular ova	Follicular fluid	Recipient bred and transferred into uterine horn day 3 of cycle
9	4-5	Follicular ova	Follicular fluid	Recipient bred and transferred into uterine horn day 3 of cycle
10	4-5	Follicular ova	Follicular fluid	Recipient bred and transferred into uterine horn day 4 of cycle
11	2	16-32-cell stage	Homologous blood serum	Recipient in heat 2 days previous to donor; donor, 5th day
12	1	16-32-cell stage	Homologous blood serum	Recipient and donor 5th day of cycle
13	1	Doubtful	Homologous blood serum	Recipient 4th day of cycle donor 5th
14	2	None dividing normally	Homologous blood serum	Donor and recipient 5th day of cycle. Eggs deposited with long pipette deep into uterine horn.
15	2	None dividing normally	Homologous blood serum	Recipient 6th day of cycle. Eggs deposited as above.
16	2	Egg 1—2 cell stage Egg 2—Not dividing	Homologous blood serum	Recipient 6th day of cycle. Donor 5th.

Table 9. The effect upon conception of luteal-phase cervical intervention and uterine deposition of blood serum flushed into and recovered from the uterus of another cow

I. With the addition of penicillin (1,000 units/cc. of serum)

Trial no.	Cow no.	Date bred	Date inserumated	Results	Comments
1	T141	1/28/55	2/2/55	Pregnancy	Parturition, 11/6/55
2	E800	1/27/55	2/2/55	Estrus, 2/20/55	
3	E799	2/8/55	2/12/55	Estrus, 3/6/55	
4	929	2/8/55	2/12/55	Pregnancy	Parturition, 11/16/55
5	T140	3/16/55	3/19/55	Pregnancy	Parturition, 12/22/55
6	E800	3/16/55	3/19/55	Estrus, 4/8/55	
7	1114	5/4/55	5/9/55	Pregnancy	Parturition, 2/6/56
8	E801	5/4/55	5/9/55	Pregnancy	Parturition, 1/27/56
14	E799	12/27/54	12/31/54	Estrus, 1/17/55	

II. Without penicillin added to the serum deposited

Trial no.	Cow no.	Date bred	Date inserumated	Results	Comments
1	1114	9/20/54	9/25/54	Estrus, 10/22/54	
2	T4	9/23/54	9/28/54	Pregnancy	Parturition, 7/7/55
3	945	10/1/54	10/6/54	Estrus, 10/22/54	
4	T7	10/2/54	10/6/54	Pregnancy	Parturition, 7/7/55
5	T141	10/21/54	10/28/54	Estrus, 11/12/54	Pus from vagina, 11/10/54
6	945	10/22/54	10/28/54	Estrus, 12/16/54	
7	T140	10/22/54	10/28/54	Estrus, 12/7/54	
8	E797	11/17/54	11/20/54	Estrus, 12/7/54	
9	E800	11/22/54	11/27/54	Estrus, 1/3/55	
10	T140	12/7/54	12/11/54	Estrus, 1/15/55	
11	T107	12/11/54	12/17/54	Estrus, 1/7/55	
12	1114	12/12/54	12/17/54	Estrus, 12/11/54	
13	1113	12/16/54	12/21/54	Estrus, 1/9/55	

LITERATURE CITED

1. Austin, C. R. The fragmentation of eggs following induced ovulation in immature rats. Jour. Endocrinology *6*:104. 1949.
2. Beatty, R. A. Transplantation of mouse eggs. Nature *168*:995. 1951.
3. Black, W. G., Otto, G. and Casida, L. E. Embryonic mortality in pregnancies induced in rabbits of different reproductive stages. Endocrinology *49*:237. 1951.
4. Boell, E. J., and Nicholas, J. S. Respiratory metabolism of the mammalian eggs. Jour. Expt. Zool. *109*:267. 1948.
5. Brock, Helen, and Rowson, L. E. The production of viable bovine ova. Jour. Agr. Sci. *42*, (No. 4):479. 1952.
6. Chang, M. C. Effects of heterologous sera on fertilized rabbit ova. Jour. Gen. Physiol. *32* (No. 3):291. 1949.
7. Chang, M. C. Cleavage of unfertilized ova in immature ferrets. Anat. Rec. *108* (No. 1):31. 1950.
8. Chang, M. C., and Pincus, G. Physiology of fertilization in mammals. Physiol. Revs. *31*:26. 1951.
9. Courrier, R. Vesicule blastodermique parthenogenetique dans un ovarie de et cobaye impubere. Arch. d' Anat., d' Hist. et d' Embryol. *2*:455. 1923.

10. Defries, A. Some observations on the living eggs and the blastulae of the albino rat. Anat. Rec. *57*:239. 1933.

11. Dowling, D. F. Problems of the transplantation of fertilized ova. Jour. Agr. Sci. *39*:374. 1949.

12. Dracy, A. E. The future of ova transfer. Iowa State Col. Jour. Sci. *28*, (No. 1):101. 1953.

13. Dziuk, P. J., and Petersen, W. E. Attempt at non-surgical transfer of bovine ova. Jour. Agr. Sci. *13*:1,019. 1954.

14. Fry, R. M., Lamming, G. K., and Rowson, L. E. A. (unpublished), as quoted by Lamming, G. E. and Rowson, L. E. A. Superovulation and ovum transplantation in cattle. Second Internatl. Cong. of Physiol. and Path. of Anim. Reproduction and of Artificial Insemination. p. 144. 1952.

15. Hamilton, W. T., and Laing, J. A. Development of the egg of the cow up to the stage of blastocyste formation. Jour. Anat. *80*:193. 1946.

16. Hammond, J. The possibility of artificial pregnancy in cattle. Jour. Min. Agr. *57*:67. 1950.

17. Hammond, J. Problems concerning the transplantation of fertilized ova or "artificial pregnancy." A parthado de los "Anales de la Facultad de Medicina." Montevideo Tomo *35* (Nos. 5-6):810. 1950.

18. Hammond, J. Recovery and culture of tubal mouse ova. Nature *163*:28. 1949.

19. Kile, J. C. Jr. An improved method for the artificial insemination of mice. Anat. Rec. *109*:109. 1951.

20. Krafka, J. Parthenogenic cleavage in human ovary. Anat. Rec. *75*:19. 1939.

21. Laing, J. A. Observations on the survival time of the spermatozoa in the genital tract of the cow and its relation to fertility. Jour. Agr. Sci. *35*:72. 1945.

22. Lamming, G. E. and Rowson, L. E. A. Superovulation and ovum transplantation in cattle. Second Internatl. Cong. of Physiol. and Path. of Anim. Reproduction and of Artificial Insemination. p. 144. 1952.

23. Lewis, W. H. and Gregory, P. W. Cinematographs of living developing rabbit eggs. Science *69*:226. 1929.

24. Mann, M. C. Cytological changes in unfertilized tubal eggs of rat. Biol. Bul. *46*:316-327. 1924.

25. Marden, W. G. R., and Chang, M. C. The aerial transport of fertilized mammalian ova. Second Internatl. Cong. of Physiol. and Path. of Anim. Reproduction and of Artificial Insemination. p. 140. 1952.

26. Menkin, M. F., and Rock, J. *In vitro* fertilization and cleavage of human ovarian eggs. Amer. Jour. Obst. and Gynec. *55*:440. 1948.

27. Noyes, R. W. Fertilization of follicular ova. Fert. and Steril. *3* (No. 1). p. 1. 1952.

28. Odor, D. L., and Blandau, R. J. Observations on fertilization and the first segmentation division in rat ova. Jour. Amer. Jour. Anat. *89*:29. 1951.

29. Pincus, G. The comparative behavior of mammalian eggs *in vivo* and *in vitro*. IV. The development of fertilized and artificially activated rabbit eggs. Jour. Expt. Zool. *82*:85. 1936.

30. Pincus, G. Observations on the development of cow ova, *in vivo* and *in vitro*. Proc. Nat. Egg Transfer Breeding Conference I, Foundation of Applied Research, San Antonio, Texas. 1949.

31. Pincus, G., and Enzmann, E. V. The comparative behavior of mammalian eggs *in vivo* and *in vitro*. II. The activation of tubal ovarian eggs. Jour. Expt. Med. *62*:665. 1936.

32. Rowson, L. E. Methods of inducing multiple ovulations in cattle. Jour. Endocrinology *7*:260. 1951.

33. Rowson, L. E. Personal communication. 1956.

34. Rowson, L. E., and Lamming, G. E. Uterine infection. Nature *171*:749. 1953.

35. Rowson, L. E., Lamming, G. E., and Fry, R. M. The relationship between ovarian hormones and uterine infections. Vet. Rec. *65*:335. 1953.

36. Runner, M. N., and Palm, J. Transplantation and survival of unfertilized ova of the mouse in relation to post ovulation age. Jour. Expt. Zool. *124*:303. 1953.

37. Shettles, L. B. A morula stage of human ova developed *in vitro*. Fert. and Steril. *6* (No. 4):287. 1955.

38. Smith, A. U. Fertilization *in vitro* of the mammalian egg. The biochemistry of fertilization and the gametes. Biochem. Soc. Symposia *7*:3. 1951.

39. Spalding, J. F., Berry, R. O., and Moffit, J. O. The maturation process of the ovum of swine during normal and induced ovulation. Jour. Anim. Sci. *14* (No. 3):609. 1955.

40. Tanabe, T. Y., Heist, C. E., and Almquist, J. O. Factors affecting pregnancy interruption in artificial insemination of dairy cattle. Jour. Dairy Sci. *38* (No. 6):601. 1955.

41. Umbaugh, R. E. Superovulation and ovum transfer in cattle. Amer. Jour. Vet. Res. *10* (No. 37):295. 1949.

42. Vandemark, N. L., Salisbury, G. W., and Boley, L. E. Pregnancy interruption and breeding techniques in the artificial insemination of cows. Jour. Dairy Sci. *35* (No. 3):219. 1952.

43. Willett, E. L. Egg transfer and superovulation in farm animals. Iowa State Col. Jour. Sci. *28* (No. 1):83. 1953.

44. Willett, E. L., Buckner, P. J., and Larson, G. L. Three successful transplantations of fertilized bovine eggs. Jour. Dairy Sci. *37* (No. 5):520. 1953.

45. Winters, L. M., Green, W. W., and Comstock, R. E. Prenatal development of the bovine. Minn. Agr. Expt. Sta., Tech. Bul. 151. 1942.

Part V. Use of the Biopsy Technique in Evaluating the Bovine Endometrium During Normal and Altered Estrous Cycles

REVIEW OF LITERATURE

The uterus has been studied histologically by many workers. Many of these studies have been made in an attempt to correlate certain histological pictures to a certain stage of the estrous cycle. In most cases, the tissue used in evaluation was obtained by sacrificing the animal. This limits, by reason of economy, the number of samples that can be obtained. The difference in histological sections studied previously arose from differences in stage of cycle and differences between animals at the same stage of cycle. It was the objective of the study that follows to attempt to establish a normal pattern of change in the endometrium from tissues obtained from the same animal by the biopsy technique. A study was also made to ascertain the influence that progesterone recycling would have on the endometrium.

Marshall (*9*) divided the estrous cycle into four periods: rest, growth, destruction, and recuperation. During growth, the stromal cells multiplied and the mucosa increased slightly in thickness. Destruction caused some of the epithelial cells to be torn off. The epithelium was renewed at this time, the congestion gradually diminished, and the mucosa underwent a gradual shrinkage. Postestrous glandular hypertrophy was not definite, but otherwise the changes were suggestive of a recently initiated pregnancy.

Murphey (*11*), in his study of the estrous cycle, stated that there were both microscopic and macroscopic changes constantly taking place in the uterus. Another study by Murphey (*12*) also showed the continuous change taking place in the uterus. He maintained that height and pseudostratification may be explained by volume changes in the mucosa, due to congestion and edema. Zupp (*19*) made smears of the vestibular fluid in an attempt to correlate changes with the stages of the estrous cycle. An extensive study was made on 31 cows slaughtered at known stages of the sexual cycle to correlate changes in the vaginal mucosa to stages in the estrous cycle (Cole, *4*). This led to the conclusion that there is no marked cyclic change in the vagina of the bovine. Cole did, however, note the following uterine changes. Estrus and the first day postestrus were quite similar; from the second to the eighth days the glands were developing; and by the eighth day, there was a marked glandular hypertrophy. A very gradual regression from this peak of activity takes place from about the eighth day on.

Weber *et al.* (*16*) made a more comprehensive study of cyclic uterine changes. They found that the endometrial edema began during early pro-estrus and reached a maximum during the first day postestrus near the time of ovulation. The height of the glandular epithelium increased, beginning at estrus, while the surface epilthelium decreased in height after this period. Caruncular areas remained intact but the intercaruncular areas had many sites of epithelial disruption. Mitotic activity began during the first day postestrus prior to ovulation and continued beyond the second day. Asdell and co-workers (*1*) contributed further to the knowledge on the cyclic changes of the endometrium. They found the epithelial cells were tall during pro-estrus and estrus, were secreting actively at heat and the first day after heat, and were lowest on the second day after heat. After the second day, the cells increased in height throughout the cycle. It was thought that compression due to edema may have caused the elongation of the cells on the first day after heat. The ratio of the cell to nuclei length varied in an orderly manner throughout the cycle. The ratio was about four to one just before

heat; this was reduced to about two to one and continued to decrease for the next two days. The ratio then gradually increased throughout the remainder of the period.

The epithelial cells of an ovariectomized heifer were small and were nearly filled by coarse granular nuclei. Estrogens produced larger cells with more rounded, finely granular nuclei. Progesterone produced tall cells with large, coarsely granular nuclei. Ovariectomy reduced the size of the glands, but did not prevent secretion. Estrogen enlarged the glands and the lumen, but the amount of secretion varied while the cell-to-nuclei ratio did not change. In some trials with estrogen and progesterone alone and together, at various levels it appeared that glandular growth required both hormones in proper balance. In some cases there was very little change due to treatment.

Weeth and Herman (*17*) made a histological and histochemical study of the uteri of several pregnant, nonpregnant, and recently parturient cows. It was indicated that there was very little cyclic change in the endometrium. All changes were very gradual and there was no definite period of regression during the cycle.

Foley and Reece (*6*) made some general observations on the changes in the uterus during certain stages of the estrous cycle. They suggested that the larger glands found in older cows and pregnant cows were indicative of substantial secretory activity in these cases.

Herrick (*7*) utilized the biopsy technique in studying the reproductive tract, but limited his source of tissue to the cervix. Miller (*10*) took successive biopsies of the uterus with an instrument especially designed for taking bovine endometrial samples. He reported no injuries or hemorrhage following 40 operations. He reported that the surface epithelium was sometimes denuded by the biopsy technique or by fixing and sectioning. The castlike invaginations of epithelial cells into the gland cavity were referred to as artifacts resulting from the technique. Ten Thije (*13*), using the endometrial biopsy technique, found that mares had less evidence of inflammation than cows. The samples obtained were one cm. long and one mm. thick. Brus (*3*), Ver der Kaay, and Ten Thije (*14*), and Kampelmacher (*8*) have used the biopsy technique in evaluating the bovine uterus primarily from a pathological viewpoint.

Donker (*5*) devised an instrument similar to that of Miller, and used it to obtain 22 endometrial samples. An instrument identical to that used by Donker was also used by Wrenn *et al.* (*18*) to obtain tissues for alkaline phosphatase and glycogen distribution studies in ovariectomized cows.

MATERIALS AND METHODS

Five of the animals used in these experiments, E797, E798, E799, E800, and E801, were grade Holstein heifers about two years old. They were purchased at a local auction as virgin heifers about a year old. It was discovered after two months that four of the five animals were pregnant. Two of the animals were aborted by the administration of 20 mg. of diethylstilbestrol subcutaneously every other day. This occurred nine days following the initial injection and after a total of 80 mg. of diethystilbestrol had been administered. A third animal received the same treatment, but aborted 15 days following the initial injection. A fourth animal received 20 mg. of diethylstilbestrol every other day for five injections, followed by 30 mg. every other day for three injections. She aborted 24 days following the final injection and after a total of 190 mg. of diethylstilbestrol had been administered. The aborted feti were judged to be about four months of age. All animals resumed regular cycles quite promptly and were normal as determined by rectal palpation. No biopsies were taken from these animals until there had been at least six normal cycles. One other animal, A59, was a cow that had been ovariectomized about one year previous as a amture cow. E728 was a purebred Holstein with a normal reproductive history and was normal as determined by rectal palpation.

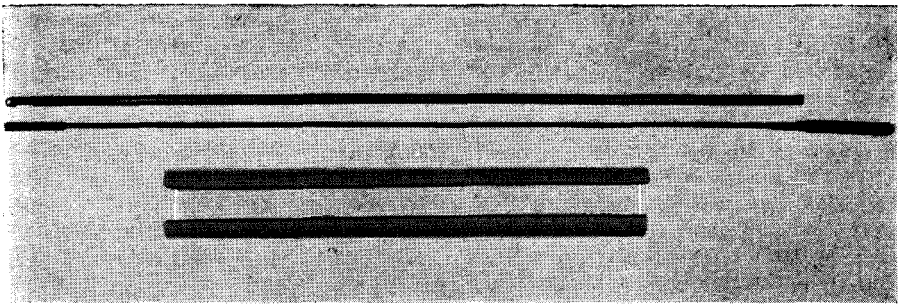


Fig. 3. Disassembled biopsy instrument and speculum used in the biopsy operations.

The progesterone for synchronization was administered subcuteaneously in the region just behind an elbow or shoulder, using corn oil as the solvent. The technique was as described in the section on synchronization.

The endometrial biopsies were taken with the instrument shown in figure 3. When not in use, the instrument was stored in a chemical sterilizing solution composed of the following: 40 gms. of Na_2CO_3, 40 gms. of $NaNO_2$, one gallon of distilled water to which was added 40 cc. of a 10 percent solution of Zephiran. The instrument was always rinsed with sterile distilled water before being used. An artificial insemination speculum was altered by removing a strip one-half inch wide along one side. This allowed the speculum to be removed from the vagina without removing the biopsy instrument. In preparing the cow for the biopsy operation, the rectum was cleaned of feces and the vulva and adjacent regions were wiped as clean as possible with paper towels. The speculum, which was previously rinsed in 70 percent alcohol solution, was lubricated with glycerol and inserted into the vagina up to the cervix. The biopsy instrument was then carefully introduced, avoiding as much as possible any contact with any part except the cervix. After the instrument had been inserted into the cervix, the speculum was withdrawn. Rectal manipulations were continued until the instrument was in the proper place in the desired horn of the uterus. The inner section of the instrument was then withdrawn a little to allow the uterine tissue to go into the opening in the outer section. The inner section was then turned and pushed in to cut off the tissue in the opening. After the tissue was free, the instrument was withdrawn and the tissue carefully removed with a pair of fine forceps and placed direcly into the fixative. The tissue sample was usually about 10 to 15 mm. long and 3 to 5 mm. in diameter.

The fixative used was Helly's (Baker, *2*). The stock solution was stored in the refrigerator and the tissue was fixed either in the refrigerator or at room temperature. The procedure used in the remainder of the histological technique was as follows: The tissues were fixed for 24 hours, rinsed in distilled water, and then dehydrated in dioxan. The tissues were then imbedded in Altmann's mixture (Baker, *2*) in a vacuum-embedding apparatus.

The tissues were sectioned by a microtome at six microns thickness. The tissue sections were floated on a warm albumin solution bath. The best-appearing ones were then fixed on a glass slide and stained in Weber's modification of Shorr's triple stain (Weber, *15*). Usually, six to eight serial sections were included on each slide. After an initial survey to ascertain the plane at which the sections were taken, there were six or seven slides made of each tissue sample.

Examinations of the sections were made by the senior author, and extended over a period of less than two weeks to minimize the differences in subjective observations which might arise from observations being made over a long time interval. The slides were numbered as they were made,

and the card on which were recorded the observations for each slide was coded, so that it was not known at the time of examination what the source of the tissue was. The slides were mixed and the examinations were made in no particular order, so that bias was reduced. The repeatability of observations was checked by making random duplicate observations on 10 slides and then comparing readings. There was a high degree of repeatability even on the subjective measurements, and there were almost identical readings on actual distance measurements, so it was felt that the observations were reasonably accurate and could be depended on as being repeatable.

RESULTS AND DISCUSSIONS

There are a total of 161 separate biopsy samples that will be considered in this study. These were taken from seven different cows at all stages of the estrous cycle. In most cases, the studies on an individual animal were initiated at or near heat, to make it easiest to make the initial entrance through the cervix with the biopsy instrument. After a little experience had been gained, there were relatively few instances in which it was impossible to gain entrance to the uterus, regardless of the stage of cycle. In the cases in which entry was not successful, the biopsy was attempted the next day.

The technique previously described was used in all cases. The usual procedure was to obtain a biopsy sample every other day for one complete cycle; then during the portion of the time in which progesterone was given, the interval between biopsies was sometimes lengthened to three or four days, since it was believed that the changes would not be rapid under constant treatment. Tissue samples were taken from alternate uterine horns. This resulted in a biopsy sample being taken every fourth day from a single horn. Attempts were made to alter the places from which the samples were taken by varying the distance of the sample site from the os uteri, and also taking samples from different planes such as dorsal, ventral, and the two lateral surfaces. This was done to avoid as much as possible, sampling from the same area, or so near a recent sample site that the tissue might have been affected by the previous biopsy operation.

The operation itself usually caused no evident discomfort to the animal. After the animals became accustomed to regular rectal palpation, the entire procedure could be carried out while the animal continued to eat or ruminate. No restraint other than the usual barn stanchion was used. In some cases, especially with the ovariectomized cow, there was a slight show of blood at the vulva the day following the operation. This was not a regular or usual occurrence, nor was there any evidence that it disturbed the animal. At the time at which the sample was actually cut from the uterus, often a strand or two of tissue was not completely severed. When the biopsy instrument was removed, these strands were torn. At this time the animal usually exhibited some discomfort by what might be termed flinching, stepping around, and switching her tail. This indicates the possibility that there are stretch or pressure-sensitive nerve endings in the uterus.

From the limited number of estrous cycles that were coincidental to, and immediately following a series of biopsies, it is indicated that the taking of the biopsies had no appreciable effect on the length of the estrous cycle (table 10).

Table 10. Estrous cycle lengths during the time biopsies were performed

Cow	Length of cycles in days	
E800	24	
E801	26	21
E728	22	18
E799	19	23
E797	21	22
E798	21	

One animal, E728, was subjected to 19 biopsies, taken every other day. She was slaughtered on the fifth day of the cycle, 48 hours after the last biopsy sample was taken. The uterus was removed and examined, and photographs taken. There was a slight noninflamed wound at the site of the last operation. There was no

evidence of any scar tissue from any of the previous operations, nor was it possible to distinguish the site of the biopsy operation 96 hours prior to slaughter from any of the other biopsy sites (figure 4). Each operation left a barely perceptible dimple in the uterine surface. If it had not been known that biopsies had been made and the location known, the scars would not have been recognized, even after a critical examination. This indicates a remarkable recuperative power of the uterus, and also how little effect the operation had on the uterus as a whole.

There were two cases, E799 and E797, which developed a detectable uterine infection at the time at which the biopsies were taken. A pus discharge from the cervix was seen on the vaginal speculum from E799, the most severe case. There was also evidence of pus on the biopsy instrument and the tissue sample. This was first noticed at the twenty-second biopsy operation. This observation followed a period of progesterone administration for recycling and happened to be the sample which was to have been the last sample taken; no more biopsies were taken. The

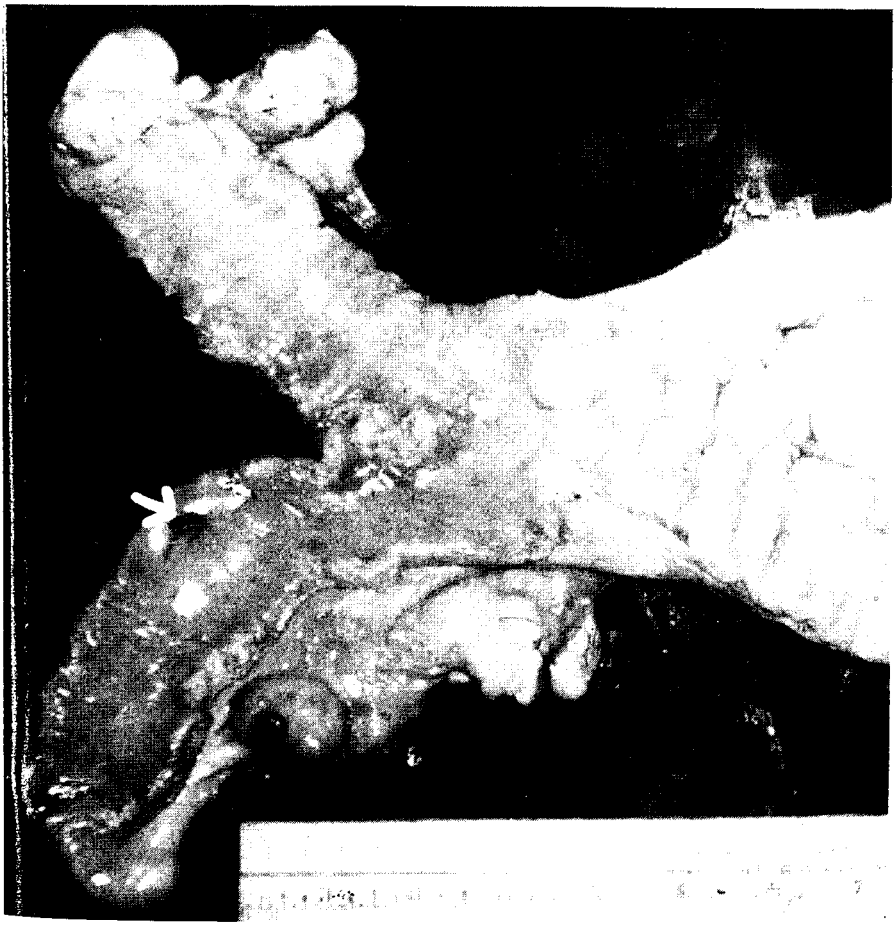


Fig. 4. Uterus of animal slaughtered 48 hours after the last of 19 biopsies had been taken. Arrow indicates site of last biopsy.)

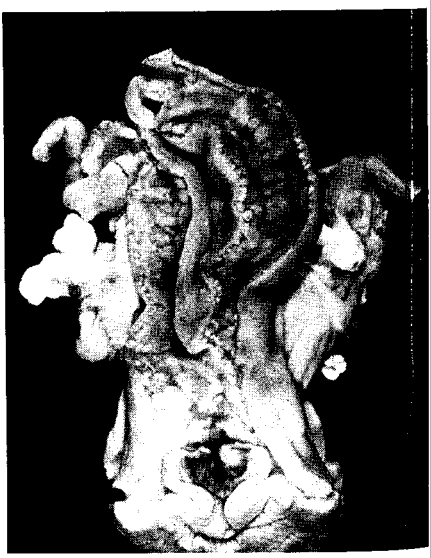
Fig. 5. Biopsy animal; uterus of animal 799.

animal subsequently recovered uneventfully, continued to have cycles of normal length, and has been used in other experiments since. E797 had slight evidence of pyometra, and the mucus following a biopsy operation did show some evidence of pus. This was the sixteenth biopsy and was following a progesterone recycling treatment. It was the last biopsy scheduled, so no further biopsies were performed. This animal recovered uneventfully and was used in subsequent experiments. Both cases of uterine infection followed progesterone treatment. This would tend to substantiate other work, which indicated the uterus was most susceptible to infection when subjected to the influence of either exogenous or endogenous progesterone. However, there was no difference readily detected in the biopsies taken at this time as compared to others.

The two animals mentioned above were both slaughtered in a subsequent experiment. The uteri were examined and failed to reveal any site of biopsies. The uterus of E799 is shown in figure 5 and that of E797 in figure 6. E800 was also slaughtered in a later experiment and no evidence of biopsies taken was found. One of the cows, E801, was subsequently bred and calved normally.

A59, which was ovariectomized, developed a vaginal infection coincidental with a severe case of mastitis during the period in which biopsies were being performed. The severity of the vaginal infection paralleled the severity of the mastitis and both diminished at the same time. The biopsy operations were continued during the vaginal infection and there was no evidence that the uterus became infected. The uterus never became swollen or inflamed, the biopsy samples retained the same character, and there were never any other evidences of uterine infection. A59 had been treated with diethylstilbestrol and progesterone, alone and in combination. It was given 10 mg. of diethyltsilbestrol every other day during the time of treatment. Progesterone was given at the level of 50 mg. per day. The combination of these two levels was given as the combination treatment. There was no evidence that the uterus was changed by any of the treatments as concerns its resistance to infection.

Although attempts were made to perform the biopsy operations under as asep-



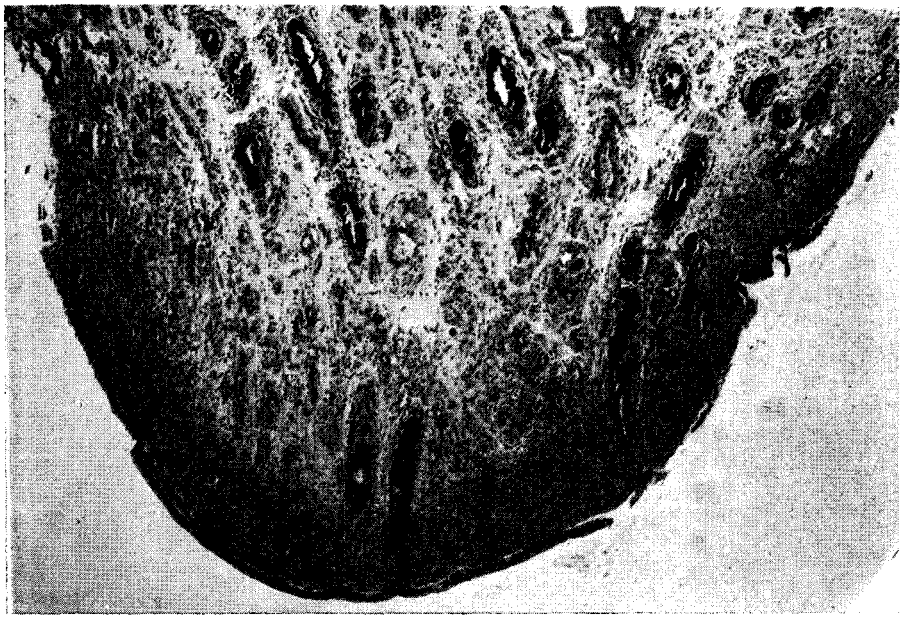


Fig. 7. Biopsy section found listed in table 11. (50X)

tic conditions as possible, it was practically impossible to enter the uterus, with an instrument under the conditions described, without introducing infection. In view of this and the reports of other workers on the lowered resistance to infection of the progestational uterus, it would seem likely that more evidence of infection should have resulted. Wrenn and Sykes, who have used the same instrument, stated privately that they found no difficulties in performing the operations nor did they, in any detectable manner, affect the uterus. They had had no infection due to the biopsy operation.

The histological evaluation of the changes in the endometrium was subjective except for measuring epithelial height. All other evaluations were based on a scale of 0-5, 0 the least and 5 the highest.

All evaluations of a single section were recorded on a card. The evaluations were as follows: surface epithelial height, activity of the surface epithelium, ratio of the height of the gland epithelial cells to the nucleus, over-all gland activity, gland lumen size, amount of cellular debris found in the gland lumen, amount of edema, number of mitoses per section, and other details. Examples of over-all evaluation can be seen by examining table 11 and figures 7, 8, 9, and 10. Grades from individual biopsies were averaged to obtain a number for graphing, as seen in the accompanying graphs.[11] There are many variations in the number of samples that went into a final average grade. Some of the averages contained very few numbers, so they tended to vary widely from the over-all mean (graph 1).

Progesterone administration for recycling was always initiated after the fourteenth day of the cycle. The biopsies taken during progesterone administration were not recorded as part of the regular cycle. There were comparatively fewer samples that made up each average after the fourteenth day of the cycle, with the result that there was more variation shown in that stage of the cycle.

[11] All graphs referred to in this text will be found in the Appendix.

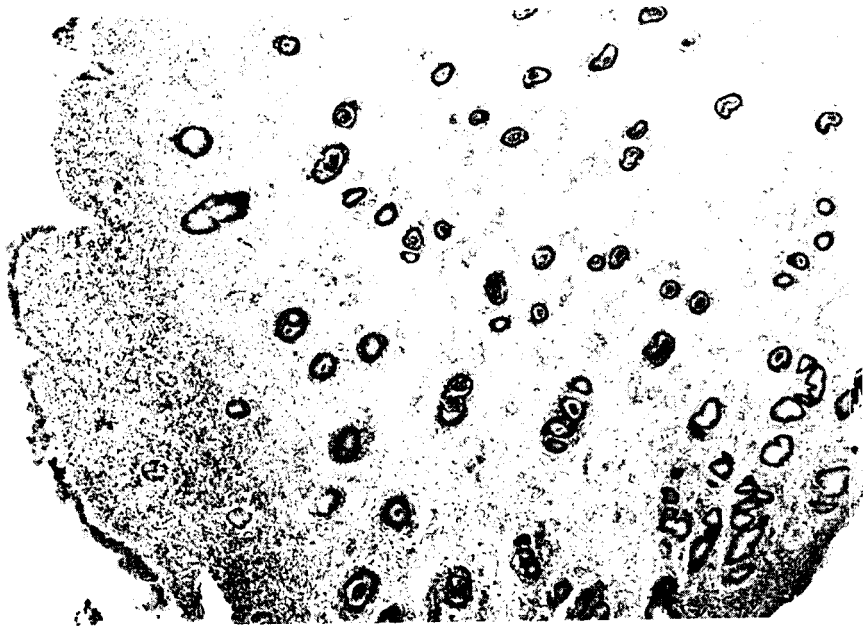


Fig. 8. Biopsy section found listed in table 11. (50X)

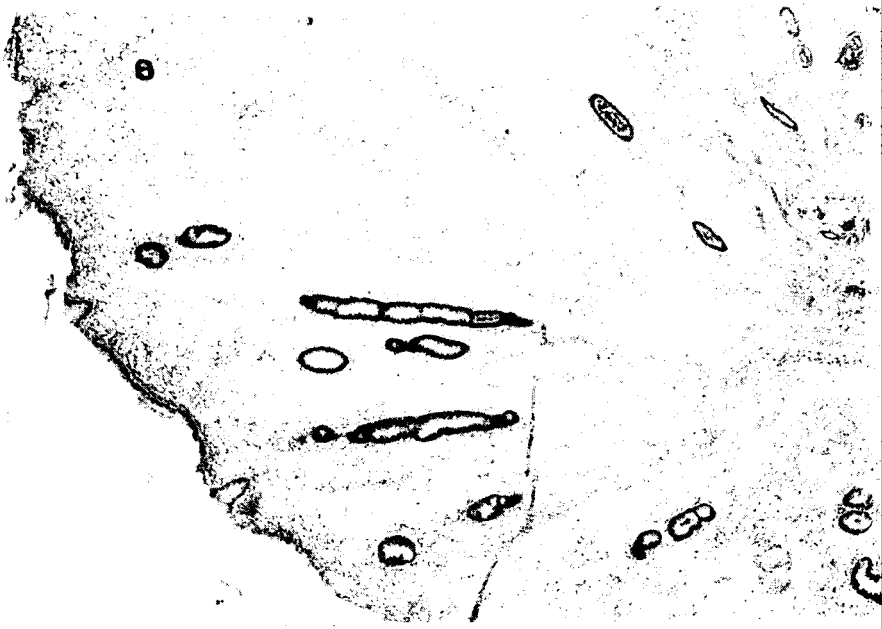


Fig. 9. Biopsy section found listed in table 11. (50X)

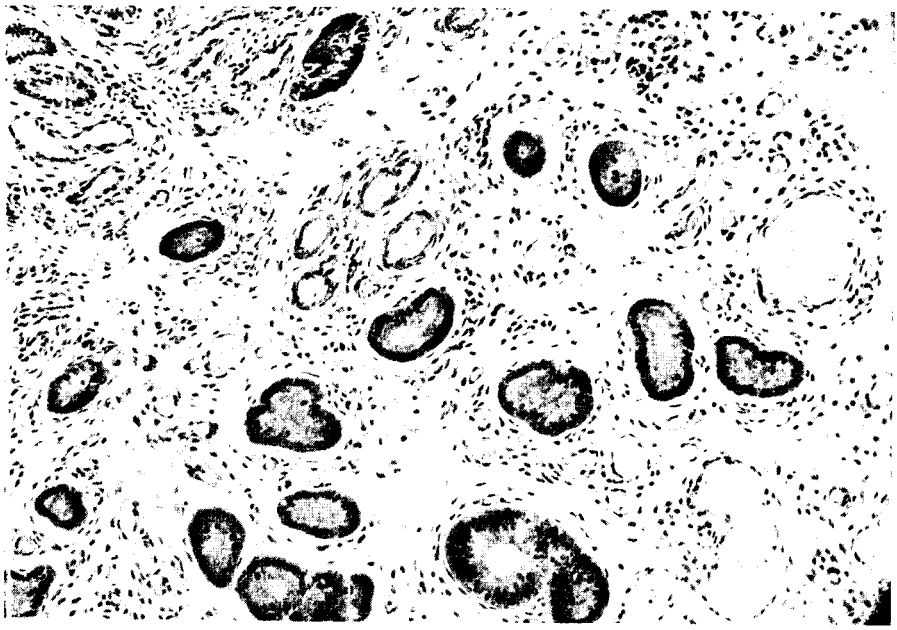


Fig. 10. Biopsy section found listed in table 11. (180X)

The surface epithelial height was measured in microns by means of a calibrated eyepiece micrometer at a magnification of 900X, using an oil-immersion objective. The activity grade was based on size and shape of cells, staining of nuclei and cells, evidence of division, and active secretion. For example, a section with round plump cells and very light-staining nuclei was noted as active (figure 11), while a section with degenerate cells and dark nuclei was noted as relatively inactive (figure 12).

The gland nucleus-to-cell ratio was based on an estimate of the ratio of the length of the gland epithelial cell to the nucleus height. There were variations in the ratio from one area to another, but an effort was made to put a representative grade on a particular section by observing all gland areas and coming to a single conclusion. Gland activity was rated in the same way as the surface epithelial activity; that is, cell size and shape, and nucleus size, shape, and staining. The amount of cellular debris was rated on the total amount of degenerate nuclei and other dark staining material found in the gland lumen. In this case again, an estimate was made after observing all gland areas.

Table 11. Samples of ratings given to certain uterine biopsy sections

Figure number	7	8	9	10
Biopsy number	167	129	130	158
Day of cycle	*	2	17	11
Surface epithelial height in microns	18	20	20	18
Surface epithelial activity	1	2	3	0
Gland cell to nucleus height ratio	1.75	1.25	1.75	2.50
Gland activity	2	2	3	3
Gland debris	1	4	2	1
Gland secretion	1	2	4	2
Gland lumen size	2	3	4	1
Cell concentration	2	2	4	1
Number of mitoses	0	1	0	0

* 4th day post progesterone.

The secretion grade was based on the amount of clear globular substance found in the lumen of the glands. This globular material appeared to be the normal secretory material of the uterine glands (figure 11). The gland lumen size would be in-

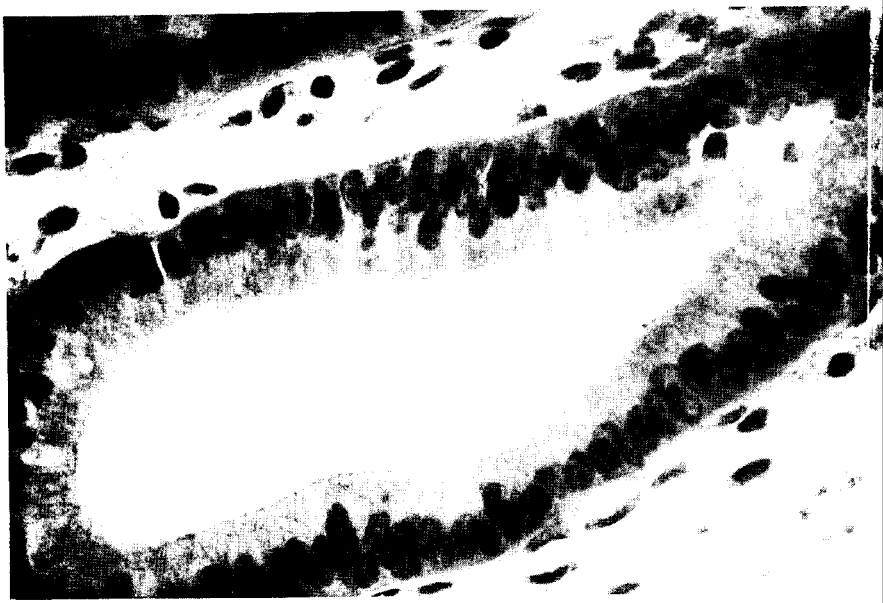


Fig. 11. Uterine gland showing a relatively high degree of activity. (730X)

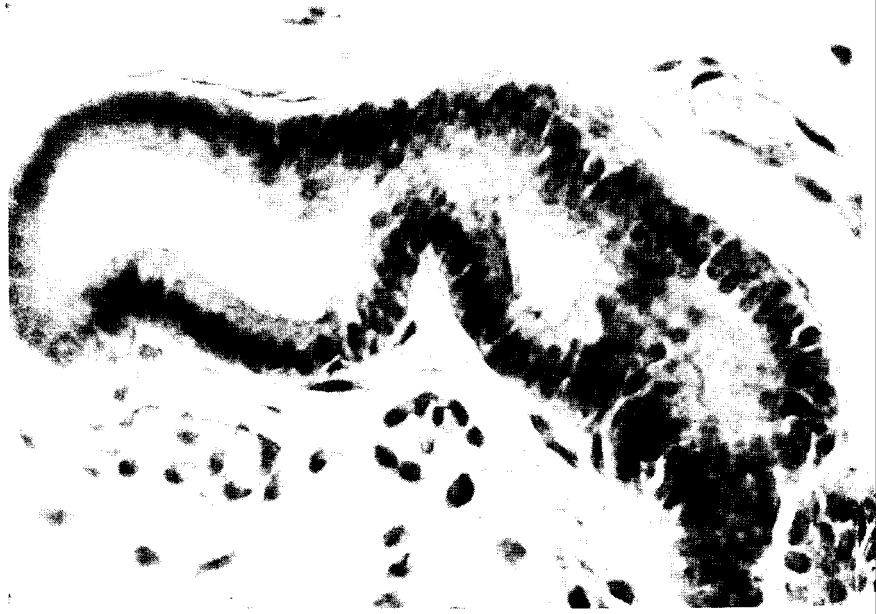


Fig. 12. Uterine gland showing a relatively low degree of activity. (730X)

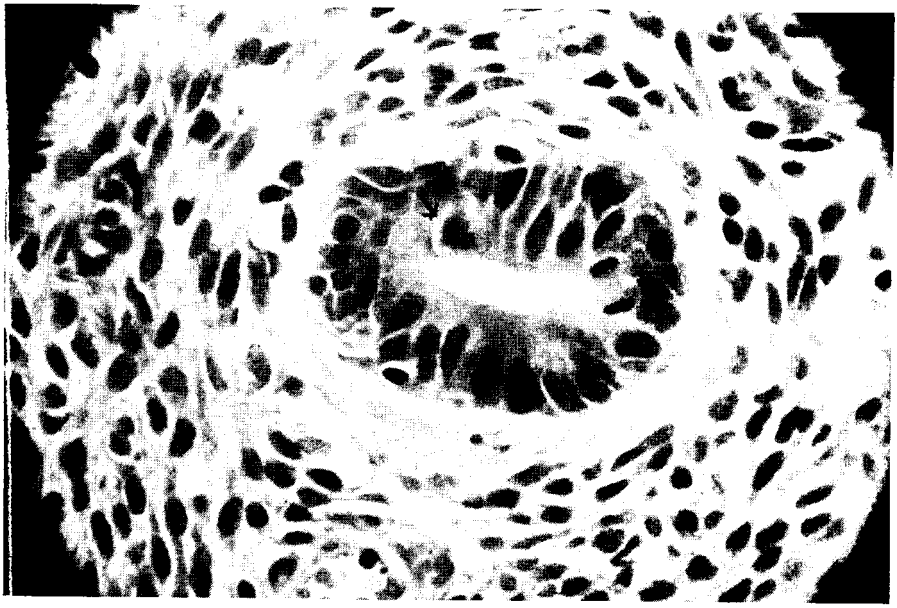


Fig. 13. Uterine gland showing two miotic figures which are indicated by arrows. (730X)

fluenced by the amount of debris and secretion found in it. In addition to this, the lumen size varied more than could be accounted for on the basis of stainable debris or secretion (figure 8). It would seem logical that the gland had been stretched by something, posibly fluid, that was removed by the fixing and staining procedures. It is indicated that the glands are stretched when called upon to accommodate a larger volume than is the usual case. This is evidenced by the much thinner gland walls of those glands that are distended by debris or secretion.

Edema was graded by estimating the cell density in the endometrium nearest the surface epithelium. The numerical grade was inverse to the density of the cells. The rating was limited to that section of the endometrium, since it was found that the area nearest the myometrium did not vary so markedly.

The mitotic figure rating is based on an actual count of the mitotic figures found in the gland or surface epithelium of a section. There is the possibility that some mitotic figures might have been missed, consequently the rating is only a relative indication of cellular division (figure 13).

Another observation that was made concerns the loss of the surface epithelium prior to examination. It is not known whether the loss of epithelium occurred in the sampling operation, in fixing, or in the staining procedure, or whether it was not present at the time the biopsy was taken (figure 14). The surface epithelium was completely absent in those sections in which the height was not recorded. It was indicated, from the number of occurrences of those sections without surface epithelium, that the surface epithelium was more adherent from about the seventh through the tenth day, and perhaps for even longer in most cases (graph 1). This would indicate that when the uterus is under the influence of progesterone and while it would normally be called upon to supply the demands of the fertilized ovum, it is most likely to have an intact epithelium.

The few sections that were taken after cessation of progesterone administration and prior to occurrence of heat are not

included, since nothing different was observed in them nor do they logically fit into either the normal cycle category or the progesterone treatment category.

The ratings for a single animal were arranged according to the stage of the cycle, and observation made to note trends. There was so much variation from one sample to another and from one day to another in any one evaluation grade that it was impossible to denote trends in a single rating in a single animal.

All samples of all the cows were grouped according to date of cycle and averaged. The data for each individual criterion which was utilized to evaluate the endometrium is presented in a series of graphs (graphs 1 through 14). Due to the great variation in the data no great significance can be attached to trends which certain of the graphs suggest. It appears from graph 3 that the ratio of cell length to nucleous length in the glandular epithelium increased during the first half and then decreased in the remainder of the cycle.

Measurements of epithelial height varied as much as 20 microns in some cases when the measurements were taken only 40 microns apart (figure 15). This was especially true in those cases in which the uterus was most rapidly changing. Some areas seemed to develop faster than others. Measurements were made of the lowest intact group of epithelial cells, and no effort was made to average or select a representative height. The surface epithelial height was quite clear cut and uniform in most, but not all, cases. The surface epithelial height did not follow any definite pattern in this study, as indicated in graph 4.

The data suggest (graphs 6 and 7) that the debris and secretion accumulated in the lumen at the time when the gland was the least active was discharged in the early stages of activity during the period following estrus.

Logically, secretion of the glands would be at a maximum at the time the fertilized ovum would need nourishment. Since the ovum does not reach the uterus for four or five days after heat, it is logical that uterine gland activity would be gradually increasing from heat onward, and that the

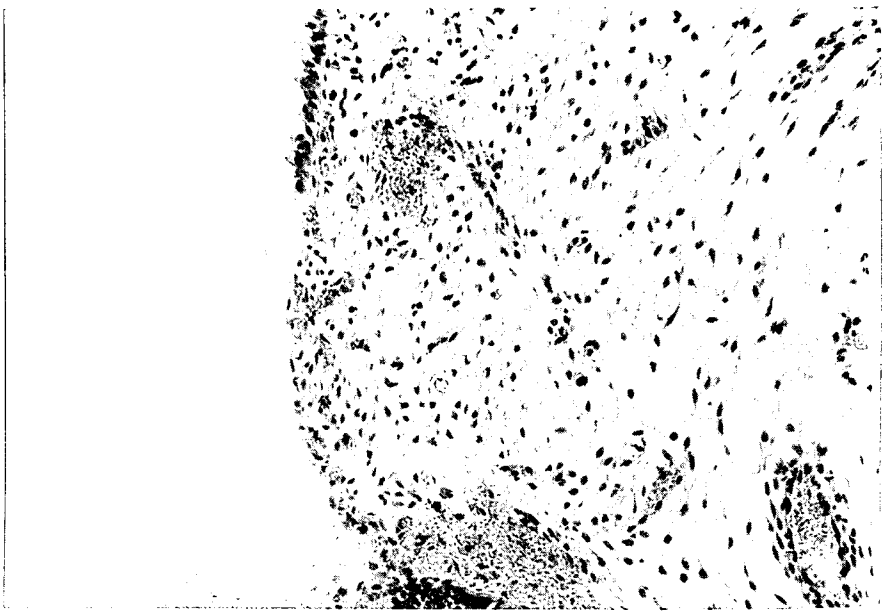


Fig. 14. Uterine section from which the surface epithelium was partially removed. (180X)

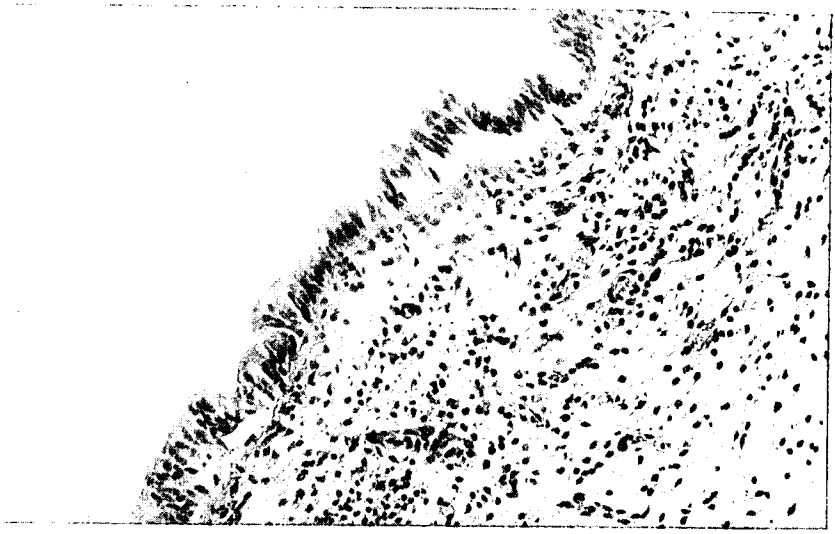


Fig. 15. Uterine section showing high degree of variability in epithelial height. (180X)

uterine cycle of activity would lag behind the ovarian cycle by about four or five days. The gland activity rating suggested this trend (graph 2). During progesterone treatment the activity seemed to be quite low.

The significance of the occurrence of degenerate nuclear material in the gland lumen is not understood. The only mention of it in the literature indicates that it is an artifact brought on by the method of sampling. This does not seem to be entirely true in view of the cyclic nature of the quantity found in the gland lumina, its quite universal occurrence, and its characteristic appearance. In many cases the nuclei were arranged in an orderly fashion, much as if a group of cells had been pushed out at the same time (figure 16). This nuclear debris had the staining properties and general appearance of the inactive, degenerate gland epithelial cells found especially in the basal region in the later stages of the estrous cycle. From this it is strongly indicated that the gland epithelial cells were the source of the nuclear debris found in the gland lumina.

The surface epithelial activity rating varied so much that no trend could be noted, and so no conclusions can be arrived at except that the picture was quite variable (graph 5).

In some cases, the secretion from the surface epithelial cells was quite obvious and resembled goblet cell type of secretion (figure 17). It appeared that cell density in the upper endometrium was lowest at the time when the over-all activity of the uterus was at a maximum (graph 9). The proximity of the biopsy sample to a caruncular area no doubt influenced the variation of tissue samples, based on cellular density, as the greatest density of cells was observed near the caruncules in the endometrium.

The number of mitotic figures was thought to indicate the activity in the section being examined. There were no mitotic figures found after the twelfth day of the cycle nor during progesterone treatment. There was no peak observed during the first 12 days of the cycle, but there was active cell division during the first half of the estrous cycle. When mitotic figures were found, the epithelial cells of both the surface and the glands were usually quite round, plump, light staining, and had the appearance of being quite young. The endometrium, later in the

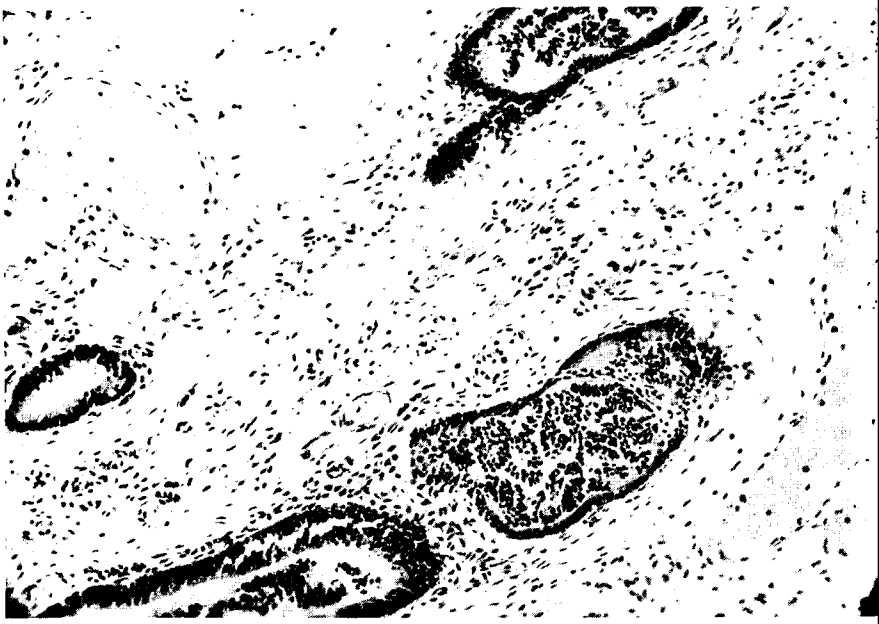


Fig. 16. Uterine section in which the glands show accumulation of nuclear debris, some regularly arranged. (165X)

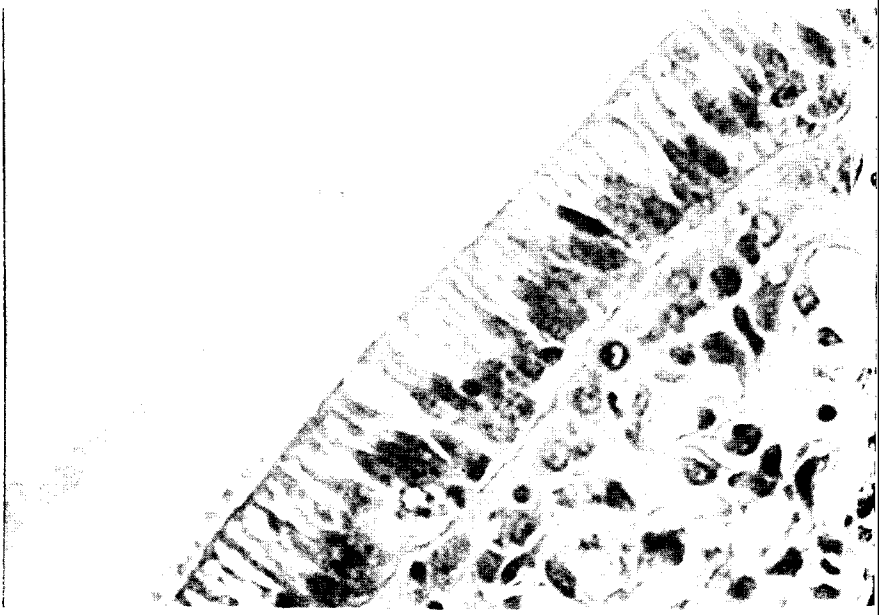


Fig. 17. Uterine surface epithelium showing evidence of secretion emerging from surface. (730X

estrous cycle and during progesterone treatment, did not have this type of cell.

Treatment with progesterone in synchronization of the estrous cycles did not seem to alter the histology of the uterus from that of the normal luteal phase. The usual changes in the endometrium that occurred at heat did not occur during progesterone treatment, but did occur at the time of heat following cessation of progesterone treatment. Although there were only a limited number of comparisons between normal cycles and cycles following progesterone treatment, the two did not seem to differ. Following progesterone recycling, mitotic figures were found and the secretion and nuclear debris decreased. The over-all activity increased, which is further indication of the normalcy of the cycle following progesterone treatment (figure 7).

The biopsy samples from the ovariectomized cow (graphs 10, 11, 12, 13, and 14), while she was untreated, had a very thin layer of endometrium. A large section of myometrium was removed with the tissue at the time of sampling in nearly every case. The gross appearance of the tissue samples was quite different from that of normal cows. The surface was hard, rough, and white, as opposed to the pink, smooth appearance of normal endometrium. The surface epithelium was consistently quite intact, inactive, and very low in height. The cell concentration in the endometrium remained very high and did not change (figure 18).

Treatment with 10 mg. of diethylstilbestrol every other day did not bring about any marked change in the histological picture (figure 19). The uterus was very flaccid, very thin walled, and was not motile, as determined by rectal palpation. Administration of diethylstilbestrol did not change detectably any of the characteristics studied. The administration of progesterone did not bring about any detectable changes in either the gross or microscopic anatomy of the uterus (figure 20). Neither was the motility pattern altered. A combination of 10 mg. of diethylstilbestrol every other day and 50 mg. of progesterone per day did seem to cause

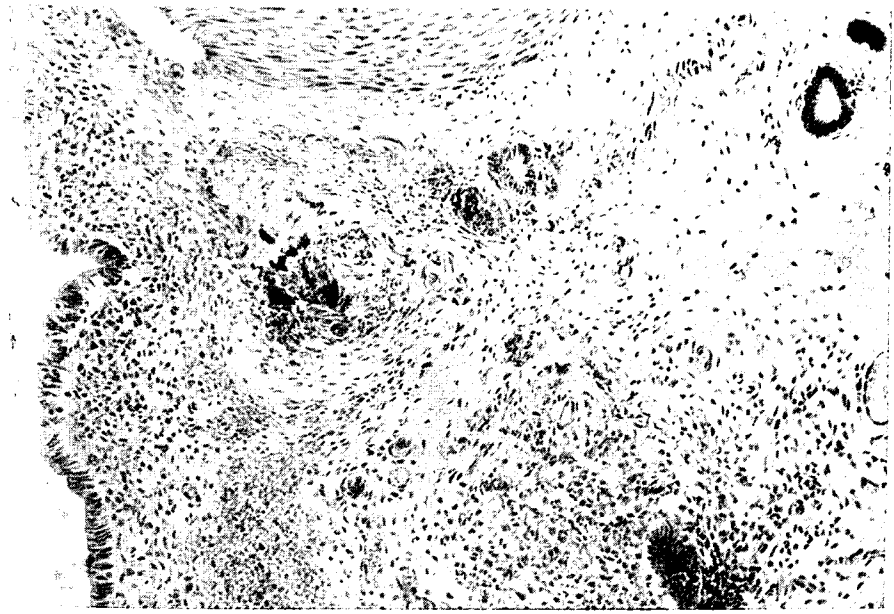


Fig. 18. Uterine section from ovariectomized animal. Control. (180X)

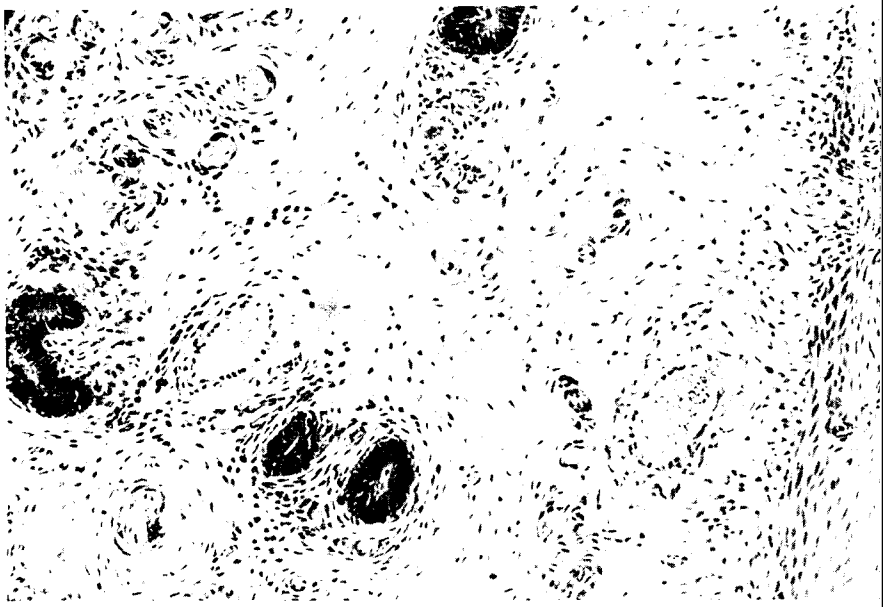


Fig. 19. Uterine section from ovariectomized animal. Diethylstilbestrol treated. (180X)

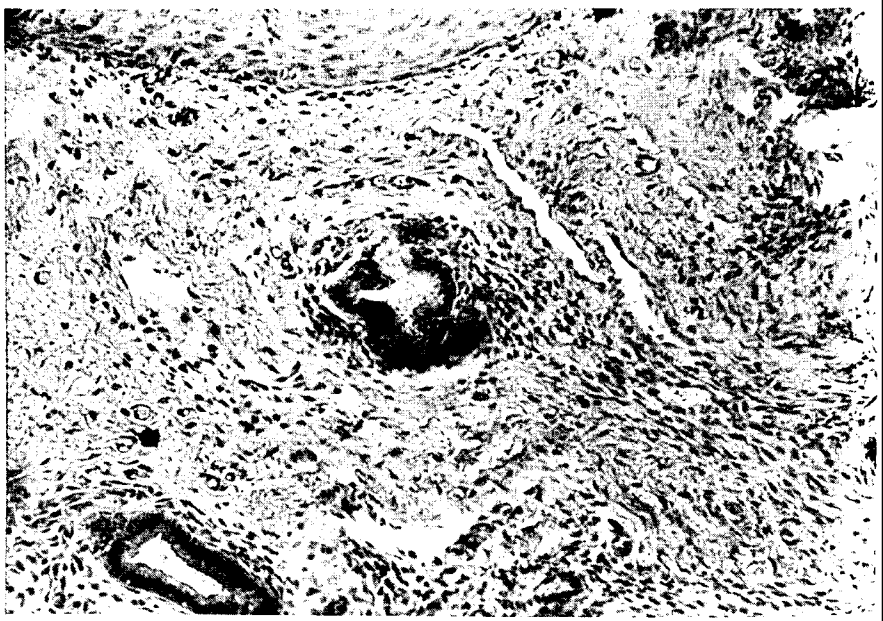


Fig. 20. Uterine section from ovariectomized animal. Progesterone treated. (180X)

an increase of activity of the uterus. The amount of debris and secretion present decreased, the gland cell-to-nucleus ratio increased, and the surface epithelial height increased (figure 21).

All of these things indicated an increase in the mitotic division and secretion rate, especially since the level was quite consistently low preceding this treatment. There are too few sections over too short a treatment period to reach any conclusions as to the effect such a combination of progesterone and diethylstilbestrol might have over a longer period of time. The gross appearance did not change under any of the treatments (see appendix, graphs 10-14).

SUMMARY AND CONCLUSIONS

The biopsy technique provides a method of obtaining uterine tissue suitable for experimental histological studies. The histological changes are not precise enough to identify daily changes, since there was much variation between animals and from one cycle to another in the same animal. There was not shown, in this study, a day-to-day pattern of change. There were changes, but they were gradual. Surface epithelial height varied more from one location to another in the same section than the range which is usually accepted for the entire cycle. The endometrium at heat was very similar to that in the proestrus phase. The over-all activity of the uterus started to increase at estrus or a day or two after, and continued to a peak about 8 to 10 days after estrus. There was a very gradual decline in activity from that point on.

The uterus of the ovariectomized animal was very inactive, tended to be atrophic, and was very thin walled. There were glands present but they did not appear to be actively secreting. The surface epithelium was intact, but was very low in height. The epithelium did not appear to be secreting and there was an accumulation of debris and secretion in the lumina of the glands. Neither diethylstilbestrol nor progesterone alone, under the dosage given, had a detectable effect on the uterus of the ovariectomized animal. A combination of the two preparations did seem to cause a slight increase in activity, which indicated that both preparations were necesary for any degree of response.

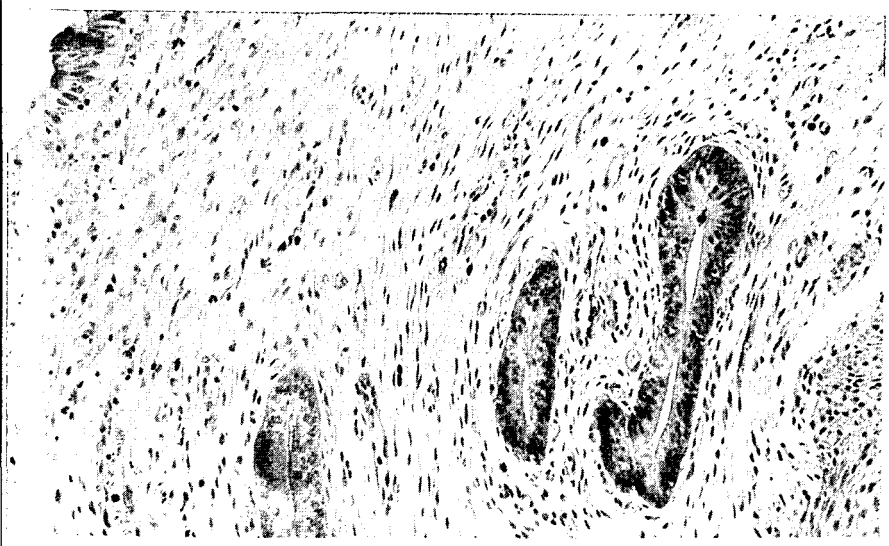


Fig. 21. Uterine section from ovariectomized animal. Diethylstilbestrol and progesterone treated. (180X)

LITERATURE CITED

1. Asdell, S. A., Alba, J. D., and Roberts, J. S. Studies on the estrous cycle of dairy cattle: cycle length, size of corpus luteum and endometria changes. Cornell Vet. *39*:389. 1949.
2. Baker, J. R. Cytological technique. John Wiley and Sons, Inc. 1950.
3. Brus, D. Biopsia Uteri of Cows. Second International Cong. of Physiol. and Path. of Anim. Reproduction and Artificial Insemination *2*:175. 1952.
4. Cole, H. H. A study of the mucosa of the genital tract of the cow, with special reference to the cyclic changes. Amer. Jour. Anat. *46*:261. 1930.
5. Donker, J. D. Ph.D. thesis. University of Minnesota. 1952.
6. Foley, R. C., and Reece, R. P. Histological studies of the bovine uterus, placenta and corpus luteum. Mass. Agr. Expt. Sta. Bul. 468. 1953.
7. Herrick, J. B. The cytological changes in the cervical mucosa of the cow (Bos taurus) throughout the estrous cycle. Amer. Jour. Vet. Res. *12*:276. 1951.
8. Kampelmacher, E. H. Microbiological and histopathological examination of sterile cows. Second Internatl. Congr. of Physiol. and Path. of Anim. Reproduction and of Artificial Insemination *2*:188. 1952.
9. Marshall, F. H. A. The Physiology of Reproduction. Longmans, Green and Co. 1922.
10. Miller, J. G. A technique of endometrial biopsy in the bovine animal. Jour. Amer. Vet. Med. Assoc. *119*:368. 1951.
11. Murphey, H. S. Studies of the estrous or genital cycle of the ox. Jour. Amer. Vet. Med. Assoc. *65*:598. 1924.
12. Murphey, H. S. Studies of the oestrus or genital cycle of the ox. Iowa State Col. Vet. Prac. Bul. *8*:153. 1926.
13. Ten Thije, J. H. De betekenis der biopsie methode bij het steriliteitsonderzoek. (The biopsy method of diagnosis of sterility). Tijdschr. v. Diergeneesk. *76*:599. (Vet. Bul. Abstr. *24*:404. No. 2557). 1951.
14. Ver der Kaay, J. C., and Ten Thije, J. H. Investigations into the causes of sterility of cows and mares. Second Internatl. Cong. of Physiol. and Path. of Anim. Reproduction and Artificial Insemination *2*:199. 1952.
15. Weber, A. F. Personal communication. 1953.
16. Weber, A. F., Morgan, B. B., and McNutt, S. H. A histological study of metrorrhagia. Anat. *83*:309. 1948.
17. Weeth, H. J. and Hermian, H. A. A histological and histochemical study of the bovine oviducts, uterus and placenta. Mo. Agr. Expt. Sta. Bul. 501. 1952.
18. Wrenn, T. R., Moss, S., and Sykes, J. F. The effect of estrogen and progesterone on the distribution of alkaline phosphatase and glycogen in the bovine uterus. Jour. Dairy Sci. *37*:671. 1954.
19. Zupp, B. A. The cyclic secretory phenomena of oestrus in the cow (Bos taurus). Iowa State Col. Vet. Pract. Bul. *8*:123. 1926.

Part VI. In Vivo Observations of the Internal Genital Organs in the Cow

Direct visualization of the internal genital organs of the cow would be most useful in studying various aspects of the physiology of reproduction. Observations of the ovaries in superovulation experiments, uterine motility studies, and perhaps even a detailed study of the actual process of ovulation itself are all possibilities if a suitable technique can be worked out.

REVIEW OF LITERATURE

Decker (*2*) and Decker and Cherry (*4*) devised a cul de scope for use in the diagnosis of pelvic diseases in women. Similar instruments have been developed and used quite successfully in other areas of diagnostic procedures in humans (Schindler, *9*; Gibson, *5*; Nesbit, *8*; Barnes, *1*; and many others).

Decker (*3*) reported that the visualization of the pelvic organs in gynecologic diagnostic procedures was now in common usage and had long since passed the experimental stage.

Hegan (*6*) made observations of the internal reproductive organs of the cow, using an instrument similar to some of those now used in human medicine. His technique was not satisfactory because of the heat produced by the light source of his viewing instrument. This light burned the tissues it contacted, causing the bulb to burn out.

Umbaugh (*10*) reported that he viewed the process of ovulation in a series of superovulated cows by means of laparotomies.

Megale (*7*) points out the benefits of a technique to view the internal genitalia of the cow. The study of physiology of reproduction, diagnostic tool for the clinician, and an aid for the teacher were reported to be possibilities. He employed three different surgical approaches to gain visual access to the internal genitalia. Puncture of the vaginal fornix, puncture of the flank, and observations through a retained cannula in the flank were tried.

His instrument was a precision-made telescope with an outside diameter of 9.9 mm. and a working length of 60.9 cm. Vision was at right angles to the long axis of the instrument. The light source was an incandescent lamp operated on six volts. He found the retained cannula approach to be best for a study of ovulation, motility, and as an aid in teaching.

MATERIALS AND METHODS

Seven trials were conducted in efforts to observe the reproductive tract *in situ*. In the first case, surgical procedures were utilized to place a permanent window into the region of the paralumbar fossa on the right side in a position slightly posterior to the center of the fossa. This window was constructed of lucite. It was adapted from a model which was used in a study of rumen function in sheep at the University of Wisconsin. It consisted of a plate which was placed within the abdominal cavity. Centrally located in this plate was a threaded hole into which a tube was inserted. There was a washer and lock nut going over the outside of the tube down against the skin to hold the apparatus in position. There was a cap on the end of this tube which could be removed for observations. It was anticipated that a proctoscope would be the means to visualize the genitalia. The plastic tube was of such bore as to admit a standard proctoscope.

Temporary endoscopy was resorted to, in the remaining trials, to attempt a direct observation of the genitalia in their natural position. A modified peritoneoscopy would be the most applicable term describing the operation. The animal was restricted in such a position that the front feet were approximately one foot lower than the hind feet. This was done to free the genital organs from the proximity of other visceral organs, which fall forward when the cow is placed in such a position. There was no need of an induced pneumoperitoneum.

The left paralumbar fossa was chosen as the site of entrance in four trials, as there were less viscera to contend with. A localized area was anesthetized with procaine containing added adrenaline and the skin was sectioned through in a short vertical incision. Into this was placed the trocar and cannula, which were worked through the subcutaneous, muscularis, and peritoneal layers by blunt dissection. The trocar was withdrawn and a peritoneoscope inserted into the cannula to make the observations.

In the third trial, a human proctoscope was used as the viewing instrument. This instrument was approximately 14 inches in working length. The total time for the operation from initiating the anesthesia until a final suture was completed was approximately three hours.

It became apparent that a specially designed viewing instrument was necessary. One was designed that had a total length of 62 cm., a working length of 59 cm., and an outside diameter of 2.8 cm. The scope was made from a stainless-steel cylinder with an inside shield into which the light carrier was inserted for protection. The bulb was encased in a plastic cover recessed about one-half inch from the end, which kept the light from being a source of heat pain to the cow. The light operated on two ordinary flashlight batteries connected to the light carrier at the proximal end of the scope. Electrical voltage was insufficient to cause electrical shock to the animal or operator. The instrument had no lens system and was without glass or plastic covers over either end so no fogging-over would be experienced. The area viewed was adjustable only by moving the instrument closer to or further away from the object. The line of vision was with the longitudinal axis of the scope. The instrument is shown in figure 22. The light shield inside the scope is not apparent in the photograph, but when in operation it contained the light carrier, which is shown detached and just below the scope in the photograph.

The fourth and fifth trials were conducted using this instrument, which we shall designate in this paper as an "ovaryscope" for the sake of brevity. The vaginal approach was used in the sixth and seventh trials. The vagina was first irrigated with a warm saline solution. Two percent procaine was infused into the epidural space, followed by a short incision in the anterior one-third of the vagina and through the dorsal surface. The ovaryscope was then inserted through this incision. The ovaries were picked up via the rectum and guided to the end of the scope for viewing. Following the operation, penicillin in oil was administered to the animal intramuscularly as a precautionary measure against subsequent infection.

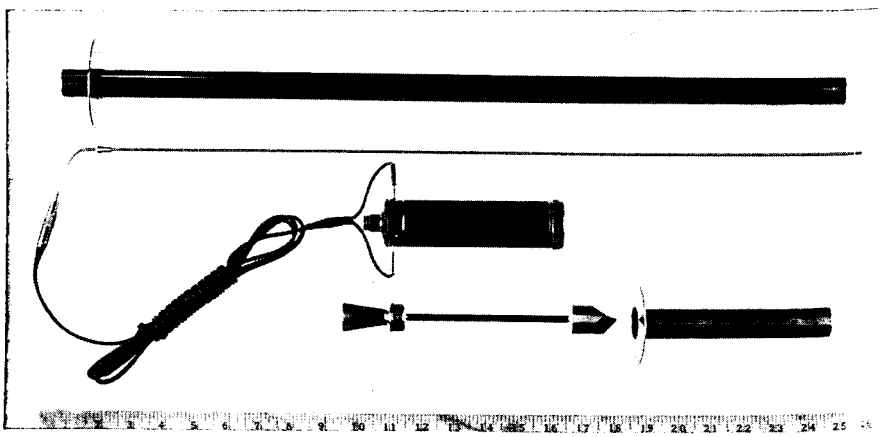


Fig. 22. "Ovaryscope" showing scope, light source, and the trocar and cannula.

In the seventh trial, the viewing instrument was altered with the addition of a mirror placed onto the end of the scope and at such an angle as to allow right angle vision, rather than parallel, with the long axis of the scope. The attached mirror is shown in figure 23. This alteration was considered especially necessary so as to view the ovaries from directly beneath the end of the scope with this vaginal approach. It was hoped that they could be viewed without requiring removal from their natural position.

RESULTS AND DISCUSSION

In the first trial to view ovarian and uterine functions, the "window" was placed into the paralumbar fossa quite successfully. After having been in position for seven days, an exploratory examination was made. By this time, tissue had grown completely over the end of the plate and tube. It was impossible to gently probe through to effect an entrance to the abdominal cavity. Five weeks after the primary operation, cautery was utilized to penetrate through this tissue, which was highly vascularized and approximately one and one-half to two inches in thickness. It seemed that this first penetration through this tissue mass had gone only into the omental bursa. Shortly after the operation, there was considerable bleeding, after which the cow was depressed for several days. Eventually, she appeared quite normal again. There was a constant discharge of fetid purulent material from the liquefying blood constituents which had pooled within the omental bursa. The genitalia could not be viewed through this window and it was subsequently removed.

In the second trial to view the ovaries, a modified peritoneoscopy was performed. The facilities were not adequate to restrain the animal. She was given only a local anesthetic and it was very evident that there was a wide and dense distribution of sensory pain endings among the viscera and on the surface of the peritoneum itself. This fact was established by bringing the hot bulb of the viewing instrument into contact with these tissues. With the necesary modifications, this method of visualization showed promise, as the operation was fairly simple.

The third trial to view the ovaries was unsuccessful in that the scope was not of sufficient length to reach the ovaries. The vision was good, however, of other areas which could be reached. The cow showed no subsequent ill effects of the experiment and was fairly cooperative during the trial.

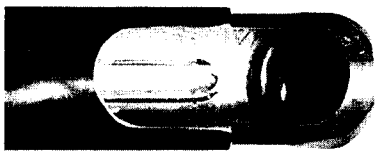


Fig. 23. "Ovaryscope" showing right-angle mirror and light source.

In the fourth trial, the first with the "ovaryscope," the attempt was successful in that both ovaries were seen reasonably well. It required about 30 minutes to find the reproductive tract, but subsequently the uterus, oviducts, and both ovaries were seen from the left flank approach. About 18 to 20 inches of the length of the scope was necessary. The right ovary was more easily seen from the left side than the left ovary from the left side, probably due to the more desirable approach. This cow was slightly depressed the following day, but soon completely recovered and subsequently showed no ill effects.

In the fifth trial, difficulty was experienced with the light source. Little pools of blood collected near the end of the scope and these constantly blocked out the light. The uterus was viewed. The ovaries were not seen; they seemed to be "behind" the mesosalpinx, the surface of

which appeared to be rough and purplish. Attempts were made to guide the ovaries to the scope via rectal manipulation, but they still seemed to be inside the "bursa."

The sixth trial was attempted from the vaginal approach and the field of vision was good. A *corpus hemorrhagicum* on the right ovary of this cow was seen very well. It was necessary to pick up the ovaries manually via the rectum and guide the scope end to them, since the line of vision ran with the long axis of the scope. Results indicated a need for a larger field of vision and a means of looking down on the ovaries from this approach instead of looking directly out the end of the scope.

With the angled mirror attached to the scope, another vaginal approach was made in the seventh trial. The cow was very nervous and unstable on her hind legs, due to the anesthetic. She was constantly trying to lie down, and it was necessary to try and keep her hind feet well forward to keep her standing. In such a position, with her hind feet forward under her body, the abdominal organs pushed out against the pelvis and it was difficult to view. The scope seemed to work quite well except for the fact that blood, fat, etc. pushed up against the mirror when the cow strained, and the material subsequently dried and made vision difficult. It is thought that this can be corrected with a warm saline rinse onto the mirror.

SUMMARY AND CONCLUSIONS

Attempts were made to view the internal genital organs of the cow *in vivo*.

An attempt to place a retained cannula in the cow for repeated observations failed because omental tissues quickly covered the part of the instrument inside the peritoneum.

An instrument was designed for the purpose of viewing the genitalia which, with further alterations, may well serve the purpose.

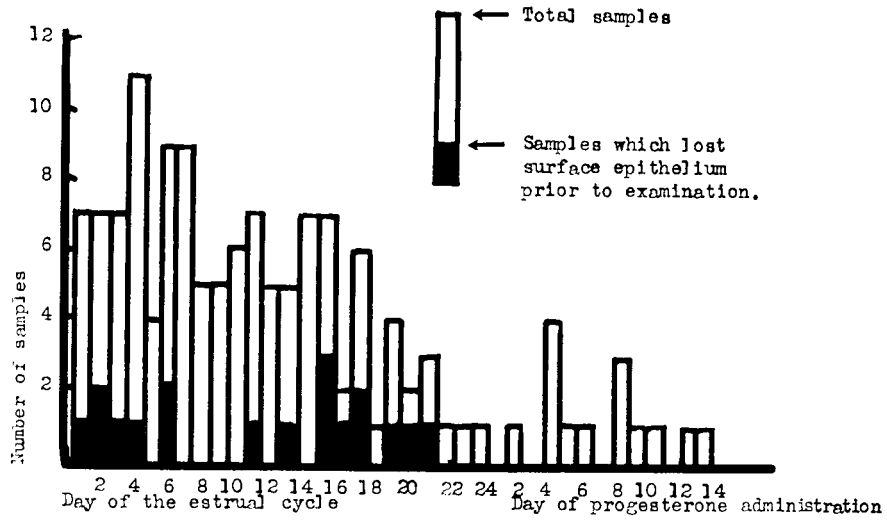
The ovaries, oviducts, and uterine horns were viewed with this instrument, and vision was fairly satisfactory.

Further research is warranted.

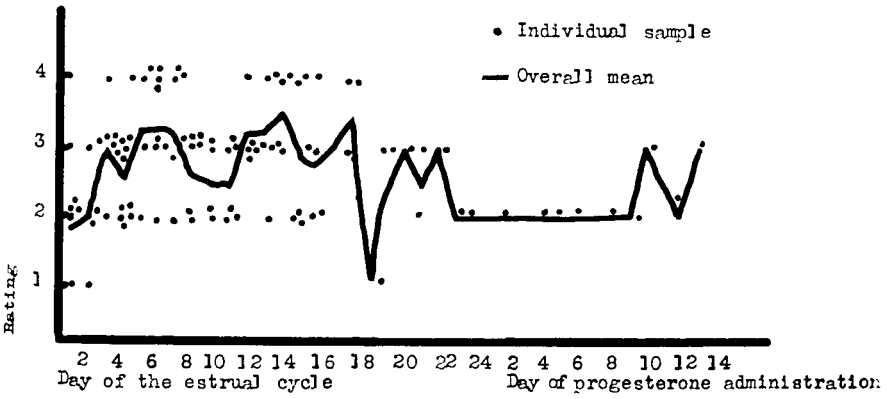
LITERATURE CITED

1. Barnes, R. W. Endoscopic prostatic surgery. The Stern-McCarthy electrotom and its modifications, p. 97. 1943.
2. Decker, A. Pelvic culdoscopy. Progress in gynecology. Grune and Stratton, Inc. 1946.
3. Decker, A. Culdoscopy, a new technique in gynecology and obstetrics diagnosis. Saunders, Philadelphia. 1952.
4. Decker, A. and Cherry, T. H. Culdoscopy, a new method in the diagnosis of pelvic disease. Preliminary report. Amer. Jour. Surg. *74*: (No. 1), 40. 1944.
5. Gibson, T. E. Modification of the Stern-McCarthy resectoscope designed to increase its utility. Jour. Urol. *47*:200. 1942.
6. Hegan, W. A. Peritoneoscopy in the cow. (K. McEntee). Report of the New York State Veterinary College, pp. 26. 1950.
7. Megale, Francisco. Peritoneoscopy in the cow. M.S. thesis. Cornell University. 1955.
8. Nesbit, R. M. A modification of the Stern-McCarthy resectoscope permitting third dimensional perception during transurethral prostatectomy. Jour. Urol. *41*:646. 1939.
9. Schindler, R. Gastroscopy, p. 43. The University of Chicago Press, Chicago, Illinois. 1937.
10. Umbaugh, R. E. Superovulation and ovum transfer in cattle. Amer. Jour. Vet. Res. *10*: (No. 37), 295. 1949.

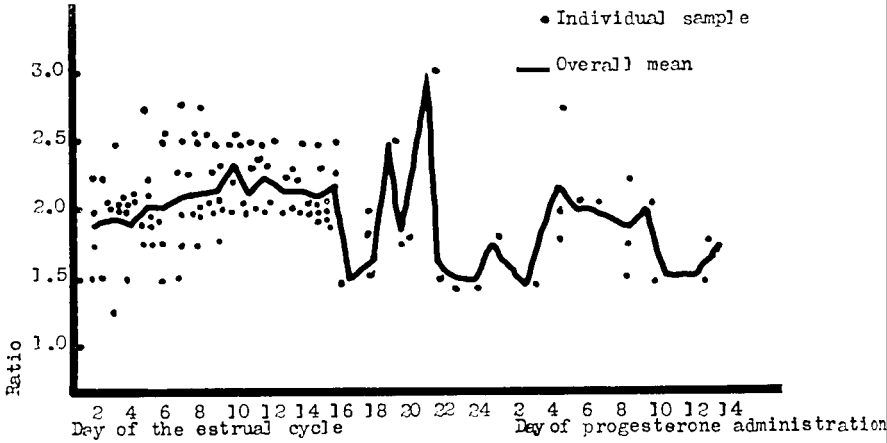
Appendix



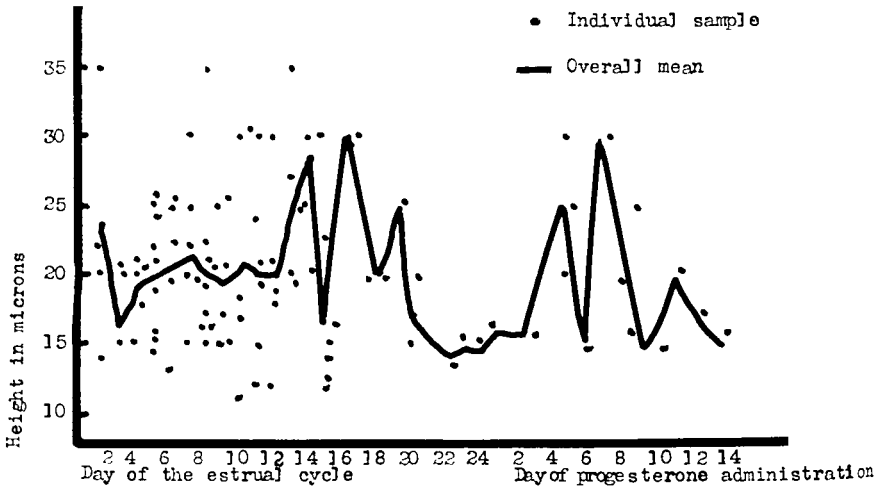
Graph 1. Number of samples obtained on a single day of the cycle, with the number of these which had lost the epithelium prior to examination.



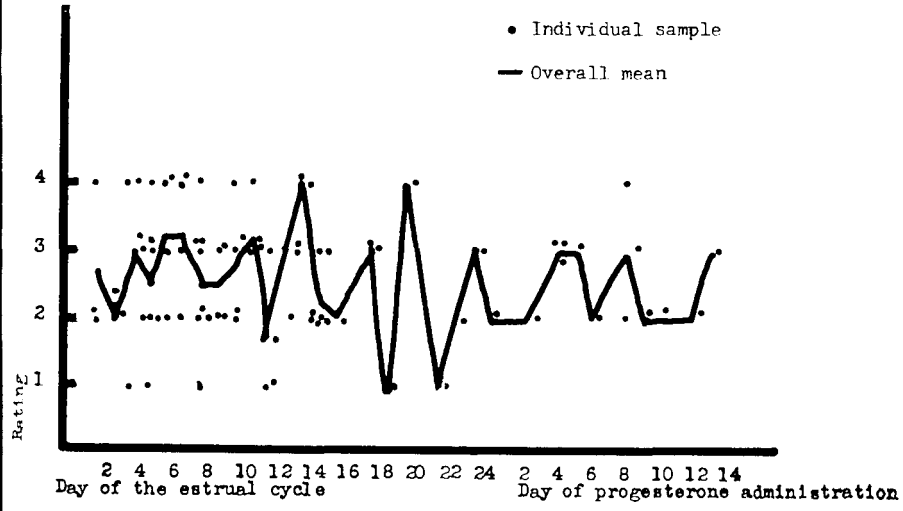
Graph 2. Uterine gland activity rating.



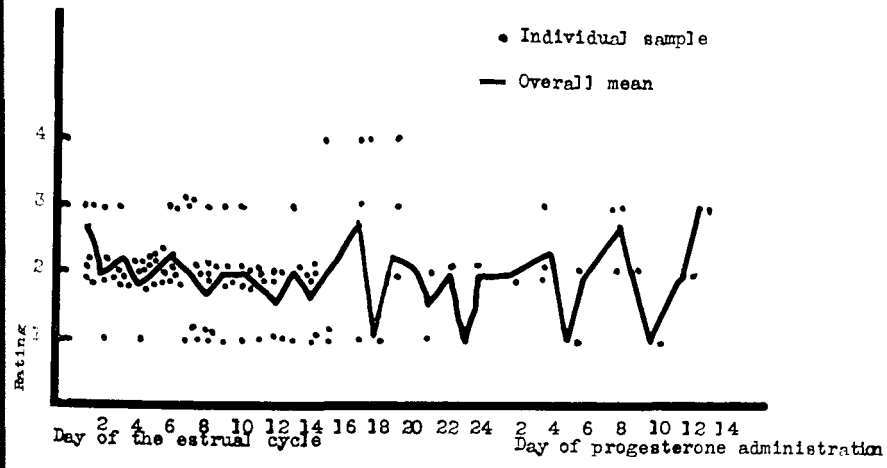
Graph 3. Ratio of uterine gland cell to nuclei height.



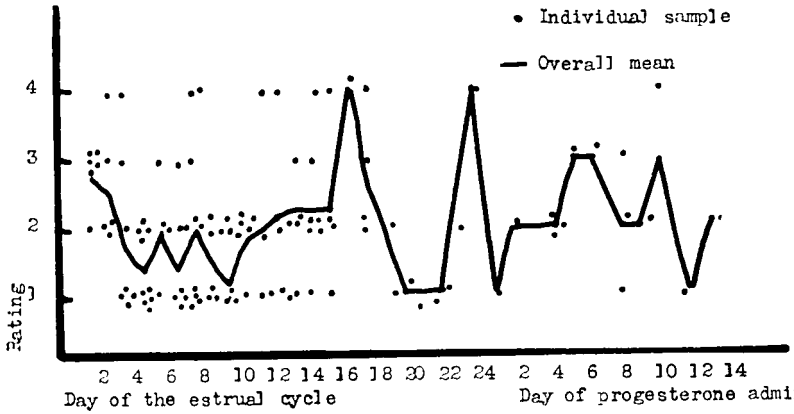
Graph 4. Height of surface epithelium of endometrium.



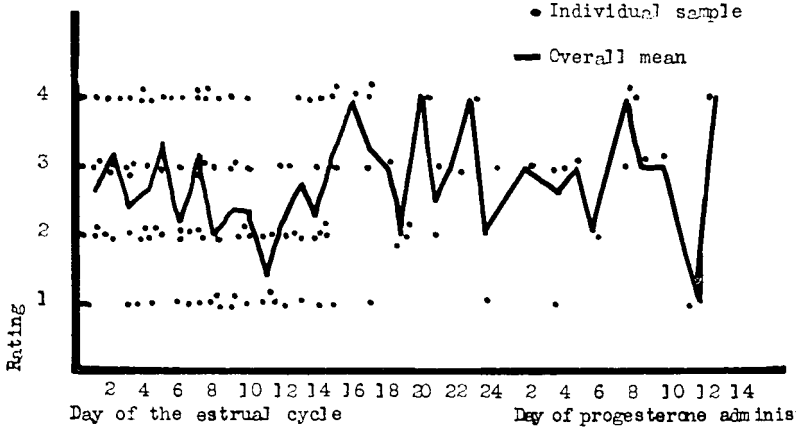
Graph 5. Endometrial surface epithelium activity rating.



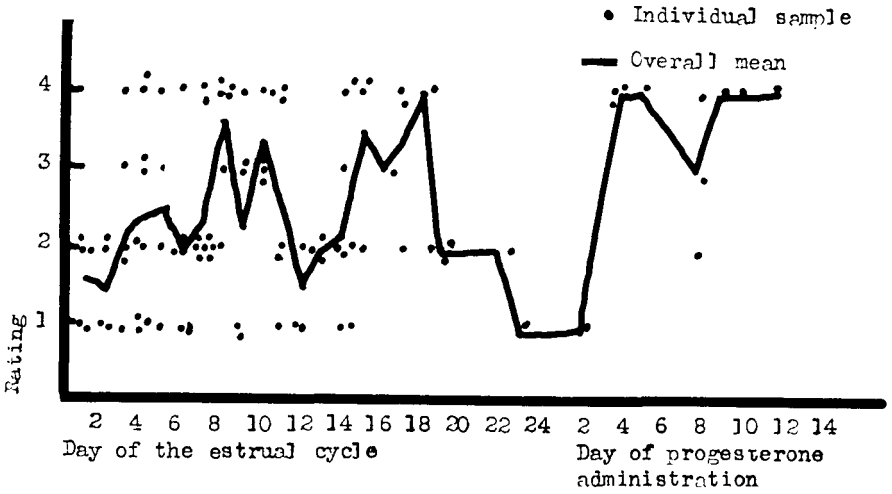
Graph 6. Rating of the quantity of uterine secretion.



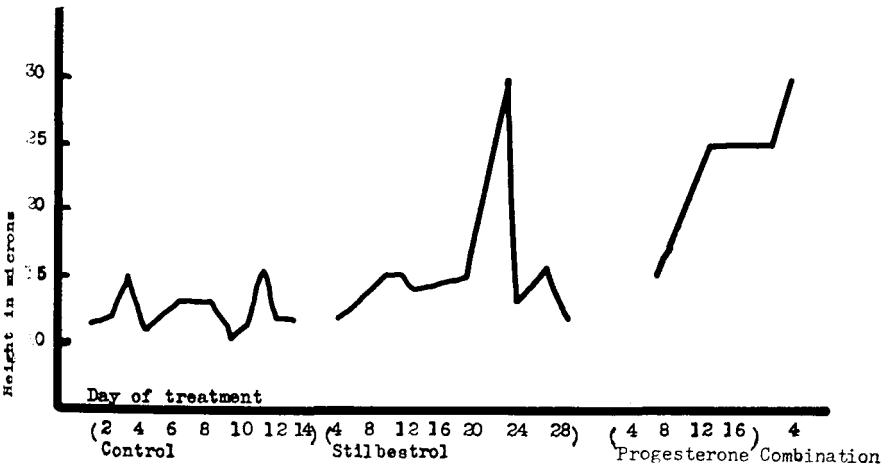
Graph 7. Rating of the quantity of nuclear debris in the uterine glands.



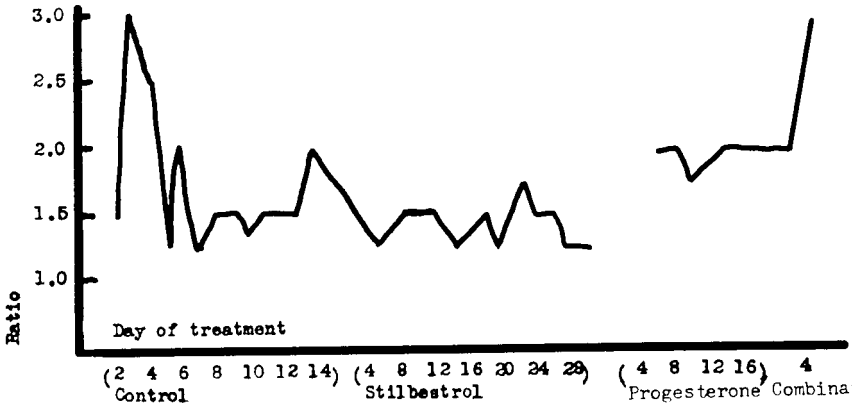
Graph 8. Rating of the size of the uterine gland lumen.



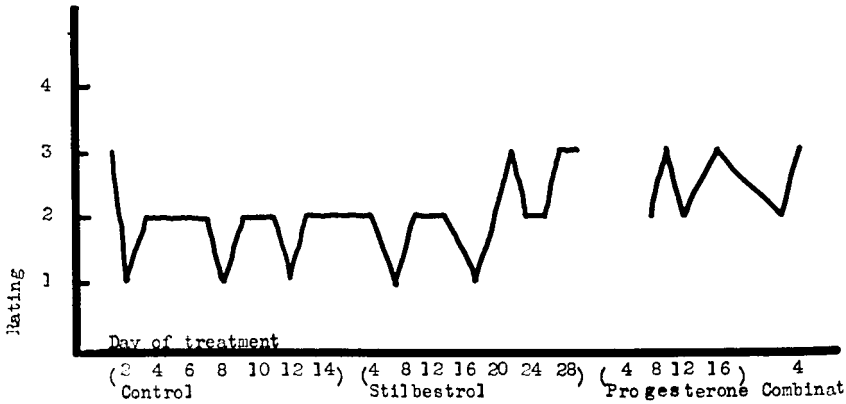
Graph 9. Rating of cell concentration in upper endometrium.



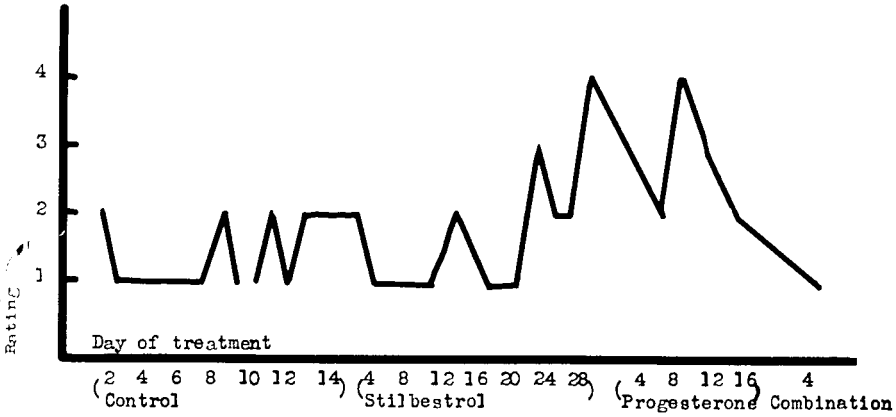
Graph 10. Height of the endometrial surface epithelium of the ovariectomized animal.



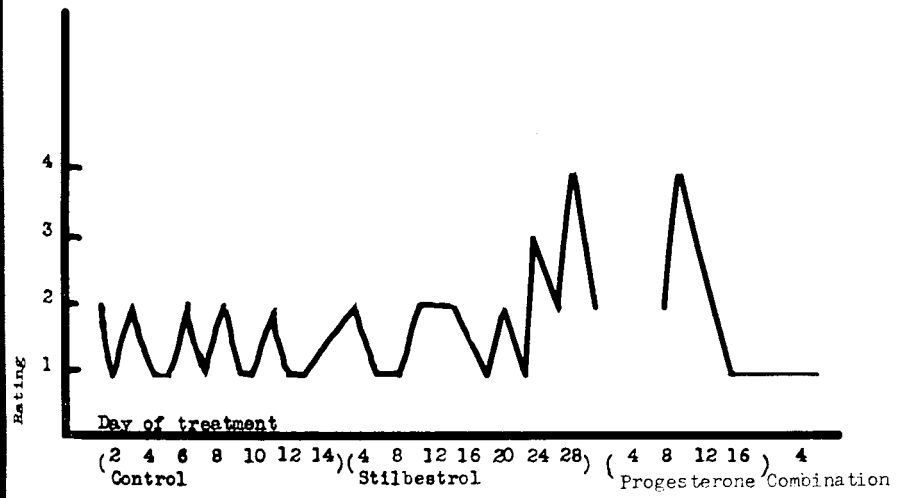
Graph 11. Uterine gland cell to nuclei height ration of the ovariectomized animal.



Gland 12. Uterien activity rating of the ovariectomized animal.



Graph 13. Rating of the quantity of uterine gland secretion of the ovariectomized animal.



Graph 14. Rating of the quantity of nuclear debris in the uterine glands of the ovariectomized animal.