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The Effects of Different Methods of Freezing on the Viability of Rabbit Ova

Mansur Ferdows

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THE EFFECTS OF DIFFERENT METHODS OF FREEZING
ON THE VIABILITY OF RABBIT OVA

by

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A thesis submitted
in partial fulfillment of the requirements for the
degree Doctor of Philosophy, South Dakota
State College of Agriculture
and Mechanic Arts

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EXPERIMENTS were conducted on a method of recovering fertilized ova from donor rabbits, freezing and storing the ova, and transplanting these fertile ova into recipient rabbits.

By flushing the fallopian tube, 614 ova were recovered from 77 donors. This represented about 90 percent recovery based on the number of ovulation points on the ovaries. Over 90 percent of the ova recovered were in the two-cell stage (fertile).

Two kinds of media were used in these experiments as follows: a physiological saline solution, glycerol, and skim milk medium; and a blood serum, glycerol medium. All ova were cooled by refrigeration to 1°C in a period of eight to 10 hours, and were frozen in an alcohol bath to -79°C at many different rates. Of the 14 freezing rates used, two (plans 7 and 8) appeared quite superior, as measured by visual observations of the condition of the ova after freezing, storing and thawing.

By observing the condition of the frozen ova under a microscope it appeared that the blood serum-glycerol medium was superior to the saline-milk glycerol medium for freezing and storage. Storage for more than two days caused apparent rapid degradation of the ova, even in the serum medium.
The viability of the frozen ova stored in the blood serum media, was further tested by transplanting the ova into does either mated with a sterile mate or injected with gonadatropin. Of all the frozen ova transplanted none produced living young. However, when the recipient does were sacrificed 24 hours after the ova were transplanted, some of the ova had further cleaved signifying development. Apparently the freezing process was extremely detrimental to the cells, since transplanting unfrozen fertilized ova into mated or injected does resulted in the birth of young, suggesting that recovery and transplanting techniques were satisfactory.
THE EFFECT OF DIFFERENT METHODS OF FREEZING

ON THE VIABILITY OF RABBIT OVA

This thesis is approved as a creditable, independent investigation by a candidate for the degree, Doctor of Philosophy, and acceptable as meeting the thesis requirements for this degree; but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.
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M. F.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>LITERATURE REVIEW.</td>
<td>3</td>
</tr>
<tr>
<td>Ovar Transplantation.</td>
<td>3</td>
</tr>
<tr>
<td>Basic Physiology.</td>
<td>4</td>
</tr>
<tr>
<td>Estrus and Ovulations.</td>
<td>6</td>
</tr>
<tr>
<td>The Effect of Low Temperature on Ovarian Tissue.</td>
<td>11</td>
</tr>
<tr>
<td>Viability of Erythrocytes in Low Temperature.</td>
<td>13</td>
</tr>
<tr>
<td>Techniques for Isolating Ova.</td>
<td>14</td>
</tr>
<tr>
<td>Storage of Ova.</td>
<td>17</td>
</tr>
<tr>
<td>Media</td>
<td>19</td>
</tr>
<tr>
<td>Synchronization of Estrus</td>
<td>22</td>
</tr>
<tr>
<td><strong>EXPERIMENTAL</strong></td>
<td></td>
</tr>
<tr>
<td>Recovery of Ova from Donor.</td>
<td>24</td>
</tr>
<tr>
<td>Procedure</td>
<td>24</td>
</tr>
<tr>
<td>Results</td>
<td>27</td>
</tr>
<tr>
<td>Discussion of Results</td>
<td>29</td>
</tr>
<tr>
<td>Freezing and Storage of the Ova</td>
<td>31</td>
</tr>
<tr>
<td>Procedure</td>
<td>31</td>
</tr>
<tr>
<td>Results</td>
<td>36</td>
</tr>
<tr>
<td>Discussion of Results</td>
<td>38</td>
</tr>
<tr>
<td>Transplantation of the Ova.</td>
<td>42</td>
</tr>
</tbody>
</table>
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>THE NUMBER OF FERTILIZED AND NONFERTILIZED OVA RECOVERED FROM NORMAL RABBITS</td>
<td>28</td>
</tr>
<tr>
<td>II.</td>
<td>RATE OF TEMPERATURE DROP OF ALCOHOL IN THE REFRIGERATION.</td>
<td>32</td>
</tr>
<tr>
<td>III.</td>
<td>EFFECTS OF MEDIUM ON CONDITION OF OVA AFTER FREEZING TO -79°C.</td>
<td>37</td>
</tr>
<tr>
<td>IV.</td>
<td>EFFECT OF LENGTH OF STORAGE AT -79°C IN SERUM MEDIUM ON CONDITION OF OVA.</td>
<td>38</td>
</tr>
<tr>
<td>V.</td>
<td>THE NUMBER OF OVA RECOVERED AFTER STORAGE AT -79°C.</td>
<td>39</td>
</tr>
<tr>
<td>VI.</td>
<td>RESULTS OF TRANSPLANTATION STUDIES.</td>
<td>45</td>
</tr>
</tbody>
</table>
INTRODUCTION

During the period between 1940 and 1960 artificial insemination of dairy cows grew from the experimental stage to an extensive business enterprise in the United States. According to the Dairy Herd Improvement Letter (Vol. 36, No. 3, A.R.S., U.S.D.A.) only 7,359 cows and heifers were artificially inseminated in 1939, but by 1959 this number had increased to 6,932,249. Along with the extended use of superior sires, a logical conclusion was that some attention should be directed toward increasing the use of good female germ plasm. If this could be accomplished, a more rapid improvement in the quality of dairy cattle would be possible.

The problems of increasing the use of female germ plasm are many. The fact that females usually shed only one ovum at a time makes the problem very complicated when compared to insemination where millions of sperm are released per ejaculate of the male. The techniques of gathering the ovum from the donor and transferring it into the uterus of the recipient have been investigated (35, 36, 41, 99, 115, 123). However, this process is in the experimental stage and if it is to become of practical value, methods must be developed for storing ova over long periods of time.

The early experiments by Heape (61, 62, 63), Pincus and others (85-93) have shown that ova can be removed from the female reproductive tract and stored at refrigerator temperatures for as long as one week, but if ova transfer is to become as economically feasible
as artificial insemination has, longer and better storage facilities will need to be discovered.

Since frozen semen stored for many months is being used extensively, it seemed logical that similar techniques might be used with ova. Because long time storage is so desirable many attempts were made to find the most suitable conditions for freezing and storing ova. The rabbit was the experimental animal chosen for these experiments with the hope that the techniques developed could be applied to dairy cattle. The procedures and results of these experiments are described in this manuscript.
LITERATURE REVIEW

The literature reviewed in this manuscript deals primarily with information concerning the problem of transplanting ova. Major emphasis is given to the problems of freezing and storing ova at low temperatures, of isolating and transplanting ova, and to the basic physiology that directly affects the ova.

Ova Transplantation

Many workers have reported on the successful recovery of ova from one female and transferring them into the reproductive tract of another female. Between 1890 and 1897 Heape (61, 62) succeeded in transplanting fertilized rabbit ova from the rabbit of a small breed into the uterus of a large breed and produced normal young. Pincus (85) and Pincus and Enzmann (92) obtained normal young by transplanting rabbit ova fertilized \textit{in vitro} and \textit{in vivo} into the pseudopregnant rabbit.

Hammond (57) and Noyes (79) reported on surgical transplantation of ova in small animals. They exposed the uterus of the animal and with a fine capillary glass pipette, they inserted the ova through the muscular wall into the lumen. The pipette was inserted through the outer muscular coat and then passed laterally, before the inner muscular and mucosa were penetrated. When the pipette was withdrawn, the opening closed preventing the egg from escaping.

Umbaugh \textit{et al.} (115), Dowling (35), Rowson (100), Dracy (36) and Dzuik and Petersen (41) reported a number of nonsurgical, unsuccessful
ova transfers in cattle. Willett et al. (123) reported three successful bovine ova transplantations. In these experiments the uterus was exteriorized after midventral laparotomy. The egg was inserted into the uterus near the uterine and oviduct junction by means of a glass micropipette.

Dracy (38), Dracy et al. (39), Hammond (57), Millett (121), and Dziuk et al. (42) reported some of the problems which must be considered before ova transfer becomes of practical importance in agriculture. They are as follows:

1. Superovulation or the production of many ova per estrus period.
2. Successful application of a technique for recovering the ova.
3. Synchronization of the estrus periods of the cow.
4. Further studies on the physiology of bovine ova in vitro and in vivo.
5. Simple methods for introducing ova into the recipient cow.

Dracy (36) suggested that the following factors should also be taken into consideration:

1. Economic aspects of the technique.
2. Simplicity of procedure involved.
3. Assurance that the transferred offspring born is the correct offspring and not from the development of the recipient's gamete.

Basic Physiology

Observations and experiments have demonstrated that many
endocrine mechanisms are involved in controlling the reproduction of the female.

Hammond and Marshall (58) demonstrated the presence of a substance in the blood stream which stimulates the formation and development of follicles during estrus and that the basic activity of the ovary is dependent upon this substance. Smith and Engle (108) have reported that this substance in the blood stream was a gonadotrophic principle from the anterior pituitary gland. Ascheim and Zondek (5) demonstrated that two of the gonadotrophic hormones which are involved in ovarian stimulation are commonly known as the follicle stimulating hormone (F.S.H.) and the luteinizing hormone (L.H.).

The follicle stimulating hormone is a product of the basophilic cells of the anterior hypophysis. This is necessary for the growth of the graafian follicles in the ovary and is essential for the maintenance and growth of the epithelium of the seminiferous tubule and for spermatozoan production. The luteinizing hormone, which is a product of the delta basophilic cells of the anterior pituitary gland, initiates ovulation and growth of the corpus luteum from the granulosa cells after the graafian follicle ruptures. Under natural conditions the rabbit ovulates only after copulations; however, when the rabbit is injected with human pregnancy urine, ovulation and pronounced luteinization occurs.

In addition to pituitary F.S.H. and L.H., there is another hormone, luteotrophin, which is secreted by the carminophil cells of the anterior pituitary gland. After being formed under the influence
of LH, this hormone is responsible for activating the build-up of the corpus luteum. This hormone has been isolated in highly purified form.

Friedman (49) reported that there were gonadotrophic hormones in the pituitary gland of 10 day old rabbits. At the age of 15 to 21 days, the follicle stimulating hormone was detected. At the age of 28 days, luteinizing hormone also was present. Friedman and Hall (50) demonstrated that there was no detectable change in the prolactin content of the pituitary gland after mating. Saxton and Greene (104) found that the hypophysis from rabbits 4 to 48 hours after mating produced less follicle maturation and less luteinization in the ovaries of test animals than did those from normal unmated female rabbits of the same age. Meites and Turner (75) have shown the content of the prolactin in the pituitary gland of immature female rabbits to be 14 microgram per gram of tissue with an increase to 42 microgram at the time of maturity. The suckling usually has 66 percent more prolactin than those that are not nursing. Furthermore the prolactin and luteotrophic hormones are now considered to be the same substance, but their physiological role is not yet fully understood.

Estrus and Ovulations

The special period of sexual desire for the female is known as estrus. This terminology has been universally accepted. During this period there is both a willingness of the female to accept the male and a series of physiological changes. In the rabbit ovulation occurs soon after copulation. Following coitus and even before ovulation the
uterine mucosa is prepared for the zygote.

Since there is no regular estrus cycle in the female rabbit, she can be in the state of estrum whenever conditions are suitable. A certain minimal size of the ovarian follicle is responsible for keeping the female rabbit in heat. In the absence of the buck, the female remains in heat for a long period. Hammond and Marshall (58) reported that this condition existed for 36 days.

Final enlargement of the follicle and ovulation depend upon sexual excitement, either by actual coitus or by intense nervous stimulation. Asdall (6) reported that the nervous stimulus initiates a sudden release of anterior pituitary hormones in the rabbit.

Following the stimulus of coitus in rabbits, the follicles swell and during the final one or two hours of the ovulation period they exhibit their greatest increase in size, and form a stigma on their free surfaces. Markee and Hinsey (74) reported that prior to ovulation, these areas thin out and that a vascular zone dilates in an hour and finally ruptures.

The exact mechanism of ovulation is unknown, but there are some theories in regard to the mechanism of development of intrafollicular pressure in ovulation. Clark (28) suggested that congestion of the blood vessels within the ovary accompanied by increased arterial tension, ruptures the follicle. Similarly Walton and Hammond (118) stated that enlargement had to be fairly rapid since the walls of the follicle could not accommodate a gradual accumulation of fluid. If the accumulation were slow, the follicles became unusually large
and failed to rupture. Markee and Hinsey (74) reported that ovulation occurred after a rise in intra-follicular pressure, accompanied by some local changes in the wall of the ovary, where a portion was removed at the time of rupture. Furthermore, Reynolds (97) demonstrated that the local regulation of blood pressure within the ovary by the helicine arteries caused the rupture of the follicle. Apparently the structure of arteries, veins, capillaries, and lymphatic vessels of the ovary and their relationship to one another, are well adapted for promoting swelling in the ovary during periods when increased permeability is essential for ovulation. As a result of increased capillary permeability, fluid accumulates in the ovarian tissue causing the follicle to swell and rupture.

There are three forms of estrogen. Estradiol is the principal hormone produced by the ovary. Estrone is ordinarily found in the urine of pregnant women and in the human placenta. Estrone is likewise a less active form of estrogen produced by the ovary. Using uterine motility as a standard measure, Reynolds (99, 96) found that estradiol was the most potent; estriol the least potent; and estrone intermediate in potency.

The three forms of estrogen are about equal in their ability to stimulate motility in rabbits. Small quantities of estrone administered daily produced some uterine growth. When the optimal quantity was injected, uterine enlargement was several times its size in the resting state. Allen and Corner (3) and Burdick and Pincus (15) reported that when the daily injection of estrone was begun one or two days after
copulation, degeneration in the early blastocyst stage was observed. Similarly, when Pincus and Kirsch (93) injected estrone at various periods before and after ovulation, their observations confirmed earlier reports on blastocyst degeneration.

Beersteiner (12) reported that the estrus rabbit excreted five R.U. of estrogen per liter of urine. The excretion values during pseudopregnancy rises to 25 Rat unit (R.U.) between the 11th and 15th days. Similarly during pregnancy estrogenic substances rise to 15 Rat unit (R.U.) between 16 and 20 days of pregnancy. At the time of parturation the level of estrogen declines rapidly to 10 Rat unit (R.U.).

As the corpus luteum is formed progesterone is secreted in varying quantities. From all the corpora lutea Reynolds (98) has reported that the rabbit secretes from 1.4 to 1.6 mg. of progesterone per day. Correspondingly one corpus luteum secretes about 0.2 mg. of progesterone daily. This more than supplies the daily minimum requirements of 0.5 to one mg. necessary to maintain pregnancy.

The factors which determine the length of time a corpus luteum may remain active are not completely understood. Asdell and Hammond (7), Lock and Smith (67), and Chu, Lee and You (27), found that in hysterectomized pseudopregnant rabbits the corpus luteum functioned 30 days. Also in non-hysterectomized rabbits the corpus luteum remained active. Green (53) and Chu et al. (27) demonstrated that the removal of the uterus of pregnant rabbits causes a shortening of the active life of the corpus luteum.

Pincus (87) reported that estrogen is necessary for the
maintenance of corpus luteum. Ordinarily one microgram of estradiol maintained luteal functions in rabbits. Also 0.5 milligrams of progesterone twice daily was necessary for implantations after ovariectomy and one milligram twice daily at 11 days supported pregnancy to term. Allen (2) concluded that two I.U. daily were needed for the first 11 to 15 days and after that only four I.U. were necessary.

The potential fecundity of the female rabbit is determined by the number of follicles which ripen at each estrus. Hammond (55) and Asdell (6) reported that the number of ova shed at each ovulation varies with the genetic strain. With domestic rabbits which ranges from one to 12 ova with an average of seven. However with wild rabbits, Brembell (13) found that the ova shed ranged from one to nine with an average of five.

The number of ovulations which were obtained by injecting follicle stimulating hormones is not yet fully understood. In the case of rabbits, coitus stimulates the anterior pituitary gland, thus secreting the luteinizing hormones which initiates ovulation. Pincus (89) reported that by injecting three small daily doses of the follicle stimulating hormone followed on the fourth day by intravenous injections of luteinizing hormones, multiple ovulation occurred in the rabbit. On the other hand when Pincus (90) studied the ovulating effect of extracts from pregnant mare's serum, he found superovulation occurred occasionally but never to the extent initiated by the use of pituitary extracts. The pituitary extract stimulated the shedding of as many as 80 eggs.
Hammond and Marshall (58) reported that the atrophy of large follicles in the rabbit began nine days after the initiation of lactation and continued throughout the entire lactation. In cases where the ova were shed and fertilized before lactation the follicles atrophied and the fetii were resorbed. Apparently a special type of atrophy, which causes the cavity to become filled with blood, exists in the rabbit.

Correspondingly many other factors are essential for pregnancy. Worthy of mention are age and nutrition. In addition, breeding season is important for the fertilization of the ova and embryonic development.

The Effect of Low Temperature on Ovarian Tissue

The majority of mammalian cells and tissue including eggs and ovaries show no signs of viability when cultured in vitro or implanted in vivo after rapid freezing to very low temperatures and storage at those temperatures.

Parkes and Smith (83) reported on rat ovaries which were treated with 15 percent glycerol saline, cooled and stored at -190°C for a period of 117 days. These were then grafted into ovarectomized rats, which after two to four weeks demonstrated positive vaginal smears. The best results were obtained when the ovaries were stored in 15 percent glycerol and 0.85 percent NaCl solution at -190°C for nine days. After these ovaries were thawed, no normal eggs or oocytes were detected in the ungrafted ovaries. Following grafting, all eggs and follicles of all stages were degenerated.

In further studies on rats, Daanesly (29) reported on freezing
and thawing the normal ovary of the seven to nine-day old rat which was packed with follicles and primordial ova. All the large follicles and primordial ova were damaged, but some small follicles appeared normal. Apparently exposure to very low temperature did not necessarily destroy all the oocytes. Parkes and Smith (84) reported that the rat ovarian tissue was preserved for a long time by chopping it into small pieces, freezing it slowly to -79°C, and storing it in liquid nitrogen at -190°C. The tissue preparation, after being stored for a period of one year, has been shown to stimulate the vaginal cycle in approximately two weeks.

In continued studies Deanesly (30) reported on the implantation of one group of rat ovaries which were chopped and equilibrated in 15 percent glycerol and saline. Another group was treated in the same way but frozen to -79°C for an hour before implantation. Thirteen to 34 days after grafting, three out of five of the glycerol treated controls and seven out of 10 of the frozen grafts had ovulated normally. From this it seems that ovulation does occur regularly in most autografts, but only in half the homografts which were treated the same as the autografts. Deanesly and Parkes (32) reported on ovariectomized rats receiving autografts of ovarian tissue frozen under unfavorable conditions in glycerol saline, glycerol horse serum, or glycerol egg yolk, and being stored for one week and one month at -79°C. There was no trace of oocyte in any of these grafts. Greene et al. (52) have shown that the number of oocytes in rat ovarian autografts which had been frozen to -190°C was considerably smaller than the number in autografts.
which, before implantation, had been maintained at room temperature. This indicates that freezing to very low temperatures and thawing from very low temperatures have damaging effects on the germinal element in ovarian tissue.

Deanery (31) reported that immature rat ovaries, when grafted subcutaneously after freezing in 15 percent glycerol rat serum and 15 percent glycerol saline, showed a heavy loss of eggs, also that ovaries stored eight days showed better egg survival than ovaries stored 10 to 12 days. Parke (81) reported on the viability of rat ovarian tissue grafted subcutaneously after freezing to -190°C and storing for seven days. The initial damage caused by freezing and thawing was not increased by prolonging the storage period to one year.

Parke (82) reported on freezing ovarian tissue and stated that rapid cooling from -20°C to -79°C destroys tissue. Rapidly freezing the tissue from room temperature to -20°C and then transferring it to alcohol at -79°C for 24 hours was less damaging to the tissue than was the one-stage rapid-cooling method.

Viability of Erythrocytes at Low Temperatures

Smith (107) demonstrated that red cells mixed with an equal volume of 30 percent glycerol in a saline solution could be frozen to -79°C and thawed without appreciable hemolysis. Further, Mallison and Slowiter (77) showed that human red cells mixed with glycerol could be frozen to -79°C, stored for six months, and not be metabolically affected. Also the behavior was normal upon transfusion.
Chaplin and Mollison (20) found that by using higher concentrations of glycerol, the amount of hemolysis during freezing and thawing was reduced until 95 percent of the human red blood cells remained intact after storage for two months at \(-79^\circ\text{C}\). Lovelock (68, 69) demonstrated that a destructive process took place during the freezing and thawing of red blood cells. He concluded that the red blood cells needed a 2.5 molar solution of glycerol to prevent hemolysis.

Lovelock (70) reported that protection occurred only when the solution was able to permeate the cell and also that the concentration of solute required for protection must be proportional to its molecular weight. In addition Lovelock (70, 71) showed that when cells were stored between \(0^\circ\text{C}\) and \(-79^\circ\text{C}\), a considerable portion of the membrane was lost with resultant hemolysis.

Chaplin and Mollison (26) reported the electrolyte composition of a 2.7 molar media was important when cells were stored at \(-79^\circ\text{C}\). Also when the red cells were stored at \(-20^\circ\text{C}\), the electrolyte composition of the suspending media became increasingly important. When the red cells were stored in a glycerol saline solution for a few weeks, complete hemolysis occurred. However, when the red cells were stored in sodium citrate, only 10 percent hemolysis occurred after one year.

Techniques for Isolating Ova

The techniques for isolating fertilized ova are many. For this discussion only two major categories are described. The removal of the ova from the sacrificed female is the more common technique;
however, the recovery of fertilized zygotes from the intact female is gaining popularity and is obviously the more desirable.

The most common and easiest way of recovering the fertilized ova is to sacrifice the female. When this is done, she is mated long enough prior to sacrifice to allow fertilization and cleavage. Pincus and Engmann (92), Chang (17), Willett (123), Rummer and Palm (103), Hunter et al. (64), and West (119) are among the pioneers in this work and explain their technique simply as removing the oviducts after death. The oviducts are then washed with a medium and the ova are recovered in a watch glass. Because the ova are so small, they must be found under a microscope and handled with a micropipette.

The surgical technique for isolation is essentially the reverse flushing of the oviduct. Immediately after a laparotomy was performed through the para lumbar fossa the reproductive tract was exposed so that a tube was inserted into the uterus at the utero tubule junction. As soon as the tube was in place, a medium usually blood serum, was forced through the oviduct in such quantity that a few drops were collected in a watch glass. Because of the construction of the ova as well as lack of motility, the ova follows the course of the fluid and are collected with a few drops of media. Umbaugh (115), Chang (22), and Avis (10) have demonstrated the feasibility of using this method. There are some limitations in so far as practical applications are concerned.

To recover the ova is not enough; the health and the well-being of the female must be considered. Surgery at best is a shock.
Furthermore, entrance into the uterus provides a chance for infection. Salpingitis, metritis, and endometritis are extremely common whenever the body cavity is opened for these experiments.

The isolation of ova non-surgically from a living animal has been done by many workers. Rowson and Dowling (101) devised a rubber three-lumen tube that was inserted into the uterus through the cervix. This catheter was used for flushing the uterus with a small quantity of fluid. A stiff rod was inserted in lumen to hold the tube rigid while it was being introduced in the cervix. The second lumen was for introducing fluid into the uterus, and the third lumen was connected to a rubber balloon to stabilize the apparatus just anterior to the cervix. Dracy and Petersen (40) and Donker (34) reported on the recovery of fertilized ova from living animals by a different non-surgical technique. A steel probe surrounded by a steel cannula was introduced in the uterus. After the cannula was in place the probe was withdrawn to allow a plastic tube to be introduced through the cannula to the tip of the uterine horn. Pressure was then applied to force the fluid inside the uterus. The fluid was recovered as it came out by the way of the cannula around the plastic tube. The fluid was poured into a series of French separatory funnels. The ova were allowed to settle and the final isolation was done by recovering the ova in a watch glass under observation with a low-power microscope. Dracy and Petersen (40) were able to recover ova 12 times in 37 trials with this technique. Nicholas (78) and Dziuk et al. (42) demonstrated various modifications of the Dracy and Petersen method. They were able to recover eggs in 11 out of 74 trials by
flushing the uteri of intact cows. Evidently there was no particular advantage of one method over another.

When comparing the recovery of fertilized ova by \textit{in vitro} and \textit{in vivo} techniques, Nicholas (78) and many other workers have stated that the percentage of ova recovered by the \textit{in vivo} method was always lower than that of ova recovered \textit{in vitro}. Therefore the small number of ova recovered \textit{in vivo} compared with \textit{in vitro} recovery indicated that the present method of collection is not entirely satisfactory.

\textbf{Storage of Ova}

The practical significance of the preservation of mammalian spermatozoa at very low temperatures has been given considerable interest during recent years because of the important practical application to \textit{artificial insemination} in farm animals. Observations have been made on the survival of mammalian cells and tissue of many types at low temperature. For a number of years mammalian spermatozoa have been kept at low temperatures with negligible loss of fertilizing capacity.

Hammond and Asdell (59) reported that the vitality of rabbit spermatozoa in the female tract after copulation is approximately 30 hours. Hammond (54) shows that rabbit spermatozoa are capable of remaining alive \textit{in vitro} for 96 hours at 10°C or 168 hours at 15°C. Therefore the life span of spermatozoa is shorter at body temperature \textit{in vivo} or \textit{in vitro} than at low temperatures \textit{in vitro}.

Rowson and Polge (102) reported that it was probably necessary to keep sperm at temperatures lower than -79°C for best results. When semen was refrigerated at -192°C for a period of 15 months, the motility
was similar to semen thawed after only one week of storage. Elliott et al. (44) reported that more sperm cells were lost when semen was stored at -195°C than when stored at -79°C. Etgen and Ludwick (45) and Etgen et al. (46) found that frozen semen stored at -96°C with mechanical refrigerator showed higher survival rates than semen stored with dry ice. Larson and Graham (66) reported that frozen bovine spermatozoa exhibited greater motility when stored at -196°C had no beneficial or harmful effects on livability.

For a number of years the conclusion has been that the fertilized ovum may possibly be kept in vitro at low temperatures for a certain period of time without losing vitality. Pincus and Enzmann (92) and Pincus (88) obtained normal young by transplanting rabbit ova which were activated by means of high and low temperature in vitro. Chang (18) reported normal development of ova stored at 0°C, 5°C, 10°C, and 25°C. The optimal temperature for the survival of ova was found to be 10°C. Chang (19) studied fertilized ova which were stored at 38°C, 30°C, 10°C and 0°C for 24 hours at various stages of development.

Adams (1) reported that when the ova were incubated in the two to 16 cell stage at 37°C for one or two days, there was approximately the same cleavage rate as would be expected in vivo. Averill (8) demonstrated that fertilized sheep ova could be kept in a viable condition when stored at temperatures between 5°C and 8°C for a period of 72 hours.

Smith (106) attempted to store rabbit ova at -79°C with little success. Ferdow (48) reported that from 98 ova, which were transplanted after being stored at -79°C, only 19 fertil were recovered. They
mentioned also that when eggs were stored at -20°C, none of the ova were alive. Likewise Averill and Rowson (9) were unable to get satisfactory results from storing fertilized sheep ova at -79°C.

Media

There have been many studies made using a number of different media for holding and culturing eggs in vitro. Pincus (86, 90) and Chang (18) reported that equal combinations of Ringer-Locke solution and homologous blood serum and also equal combinations of physiological saline and blood serum, were good media for flushing ova from the fallopian tube as well as the storage of fertilized ova. Hammond (56) employed a saline and hen egg extract media at a pH of 7.2 to 7.4 to culture mouse eggs at 37°C for 24 hours. These experiments revealed that eight cell ova developed to the 16 cell stage but under similar conditions the two cell stage did not develop to the four cell stage.

The use of either homologous or heterologous blood serum has been a successful media for culturing ova. Hunter et al. (65) demonstrated this in two experiments with sheep. In the first experiment out of seven ova transferred, four produced lambs. In the second experiment out of eleven ova transferred, four produced lambs. Pincus (90) and Chang (20) demonstrated that serum was a good medium for rabbit ova. Later Pincus (91) showed that bovine ova would divide when cultured in blood serum. Willott et al. (122) used blood serum for recovering and transferring his successful transplant.

Defries (33) reported negative results when culturing rat eggs.
in a number of media including liquid collected from the uterus of an animal during estrus. Brock and Rowson (14) attempted to culture bovine ova in both blood serum and in follicular fluid. The blood serum medium yielded positive results, but only negative results were recorded from using follicular fluid.

Experimental evidence has shown that the tissues undergo dramatic changes in both physical and chemical properties during the freezing and the thawing process. Rey (95) reported that when crystallization took place, the salt concentrations inside the tissue increased. When the water froze, the ice separated into a pure form, the crystals grew, and the volume of the interstitial liquid phase diminished at the same time the salt concentration increased. This increased concentration may have caused a change within the tissue.

The practical significance of the glycerol action in protecting spermatozoa during freezing has been reported by many workers. Lovelock (68, 69) found that the destruction of red blood cells during freezing and thawing was caused by a high concentration of salt which resulted from the conversion of water to ice. He also mentioned that in the presence of glycerol, the rise in electrolyte concentration effect by freezing was sufficiently reduced to abolish this effect. Lovelock and Polge (70) demonstrated the relationship which established the degree of damage caused by freezing at various temperatures below zero degree centigrade. Probably the addition of glycerol reduced the amount of damage caused by freezing. Spermatozoa from different species seem to have a difference in their resistance to increased electrolytic concentrations and the amount of glycerol necessary to protect them at different sub zero temperatures varies accordingly.
Parkes and Smith (83) reported that the storage of ovarian tissue up to one year in glycerol saline at -190°C does not increase the damage done by freezing and thawing, but at -79°C tissue will begin to degenerate very rapidly. Deanesly (31) found that the egg survival in immature rat ovaries (grafted after freezing and thawing) in 15 percent glycerol rat serum, gave slightly better results than in a 15 percent glycerol saline solution. Sherman (105) demonstrated that the presence of glycerol within unfertilized mouse eggs does not prevent their fertilization and subsequent normal embryonic development.

Various observations are recorded concerning the effect of rapid freezing rates on cell preservation. Ray (95) reported that during the course of rapid freezing and rapid thawing the biochemical equilibrium of the cell breaks down, therefore, slow freezing and thawing are necessary for adaptation into the new environment. However, a very rapid freezing blocks all metabolic function. Merryman (76) found that in order to form extremely small ice crystals, it was necessary to increase the rate of freezing. Furthermore the thawing must be extremely rapid to prevent denaturation due to an increased electrolyte concentration resulting from water crystallization in the solid state.

Other workers have reported on the effects of rapid freezing rates on the progressive motility of frozen bovine sperm. Swanney (109) reported that when semen was frozen quickly, the appearance was satisfactory just after freezing, but as the storage time approached 24 hours, the motility decreased rapidly. Van Demark (116) found that cooling spermatozoa slowly to -79°C at the rate of 0.25°C or 0.5°C per
minute was inferior to rates of 1°C to 4°C per minute. Van Demark (117) observed that when semen was cooled at the rate of 2°C per minute, from +5°C to -19°C and 4°C per minute from -19°C to -79°C, the survival of spermatozoa was increased. O'Dell (80) reported that when semen samples were frozen very rapidly (70°C per minute) to -79°C, the rate of temperature drop was detrimental to spermatozoa. Graham (51) indicated that below -45°C the rapid lowering of temperature of semen to -79°C in dry ice and to -196°C in liquid nitrogen showed no detrimental effect on the semen.

Synchronization of Estrus

Synchronizing the ovulation of both donor and host animal is an important factor which must be considered in ova transplantations. The uterus constantly undergoes change and the hormonal levels which influence the environment of the ova fluctuate during the estrus cycle. Chang (23) reported that the successful transfer of ova depend on the interval between the time of ovulation of the donor and the time of ovulation of the recipient. Chang (24) found that synchronization of the estrus of the donor and recipient is necessary in transplantation of ova.

Casida et al. (16) were able to change the time of estrus in sheep by injecting follicle stimulating extracts. However, little reliability could be placed on predicting the time of estrus.

Dutt and Casida (43) showed that by subcutaneously injecting 50
mg. of progesterone per day estrus and ovulation were prevented until five or six days after termination of the treatment. Ulberg et al. (112) and Ulberg (111) reported on the level of progesterone which was needed to inhibit estrus and ovulation. They found that the subcutaneous injection of 12.5 to 100 milligrams of progesterone daily could control the estrus cycle. Ulberg (113), Ulberg et al. (114) and Baker (11) showed in a series of studies that gilts require more progesterone to control ovulation than ewes or dairy heifers.

Willett (120) found that the fertility of heifers was normal after subcutaneous injections of progesterone at the 15th and 16th days of the estrus cycle. Dracy (37) reported that readjusting the estrus cycle had no ill effect on fertility.

Ulberg (111) reported that the response to progesterone for controlling estrus is different for each species. Gilts require larger quantities of progesterone to control estrus than do other animals. Some groups will develop cystic follicles after one injection, while another group will not become cystic. It appears that the higher levels will best control the time of estrus, but that the lower levels will permit a higher conception rate. In cattle there appears to be no harmful effect from treating with progesterone.

Dziuk et al. (42) reported that 75 percent of the animals treated with progesterone came into heat either on the fourth, fifth, or sixth days after the injections were stopped. Cystic ovaries developed in mature females while some became nymphomaniac, and others failed to show any signs of heat for long periods of time.
EXPERIMENTAL

Although there are many problems relating to ova transfer, the literature reveals but little in the way of solutions to these problems. Primary emphasis has been placed on the recovery of ova from the donor and on storage without freezing.

Three phases of work have been investigated and are reported here. These include: (1) recovery of ova from fertilized donor rabbits, (2) freezing and storage of the ova, and (3) transplantation of the ova. This three-fold approach allows for an assessment of the importance of several steps in the technique to successful ova transplant work.

Recovery of Ova from Donor

Procedure

The rabbits used in these experiments were purchased from several commercial sources. The general health and condition of the animals was good upon arrival. These rabbits received a prepared rabbit chow that contained all the known essential factors for good nutrition and normal reproduction. All of the rabbits were similarly managed throughout the experiments.

There were 100 attempts made to recover ova from rabbits by flushing the uterus in vitro. Non-pregnant rabbits from one to three years old of mixed breeding were mated. All rabbits were killed 24 to 26 hours after mating. In these experiments all donor
rabbits were bred twice. Approximately 30 minutes before sacrifice, the donor rabbits were bled by heart puncture to obtain donor serum. The tubes and uterus of the donor rabbit were removed separately immediately after sacrifice. Either a physiological saline solution or a pure rabbit serum was used for flushing the ova from oviduct and uterus as soon as possible after slaughtering the rabbits, usually within 10 to 20 minutes. Volumes of flushing solutions varied from two to ten milliliters.

All recovered ova were contained in the first few drops of solution, which was placed on a watch glass, and observed under a low power dissecting microscope. The ova have a greater specific gravity than either the physiological saline or serum; therefore, after the ova settled to the bottom of the dish, they were located, and stage of development was identified.

Frequently an air cell may be mistaken for an ovum. The difference between them may be detected by focusing the microscope upon the object. Dracy and Petersen (39) reported that the reflection of the ovum tends to have a dark center with a light outer ring, whereas the air cell has a light center and a dark outer ring.

Two kinds of media, a physiological saline solution plus pasteurized skim milk and glycerol, and a blood serum plus glycerol were used during these experiments. The physiological saline solution used for freezing and storing the ova was prepared according to the following formula:
Solution A: Skim milk heated for 10 minutes in a double boiler to a temperature between 92°C and 96°C and then cooled to room temperature.

Solution B: Two hundred milliliters of 0.9 percent sodium chloride solution sterilized in an autoclave at a temperature of 248°F for 16 minutes.

Solution C: Four milliliters of skim milk from solution A and 96 milliliters from solution B mixed thoroughly.

Solution D: Thirty milliliters of glycerol added to 70 milliliters of solution B and mixed thoroughly.

When equal quantities of solutions C and D were combined, the resulting solution had the following composition: 83 percent of 0.9 percent sodium chloride solution, 15 percent glycerol, and 2 percent skim milk.

The blood serum used in this experiment was obtained by centrifuging the whole blood for 20 to 30 minutes at 2000 R.P.M. Either homologous or heterologous serum was used for flushing and storing frozen ova, since Chang (18, 19) showed that both worked satisfactorily. The blood serum used in the trials on frozen ova consisted of the following proportions and was prepared in the following manner:

Solution E: 70 milliliters of blood serum and 30 milliliters of glycerol were mixed thoroughly.

Solution F: 100 milliliters of blood serum.

By combining equal quantities of Solutions E and F the following composition was arrived at: 85 percent blood serum and 15 percent.
glycerol. In addition a small amount of procaine penicillin was added to some of the transfer media.

**Results**

The purposes of these experiments were to recover ova from donor does, freeze them at a different rate to -79°C and determine the viability after storage at -79°C.

The data in Table I show the number of ovulation points and the number of ova recovered. It appeared that the number of ovulation points and the number of ova recovered might correlate with the health and the weight of the animals, but the data with respect to this were not conclusive and have not been included here. An average of 89.7 percent of the ova produced, based on the number of ovulation points observed, were recovered from the donor rabbits.

For the does exhibiting ovulation points there was considerable variation in the number of such points observed. The number ranged from 8 to 19 per animal. Correspondingly, there was a range from 7 to 17 ova recovered from these animals.

Fertilized ova (two cell stage or more) were recovered from 77 of the 100 mated rabbits. In the case of some of these animals, unfertilized (one cell stage) ova were recovered along with those that were fertilized. As the data in Table II indicate, 90.5 percent of the recovered ova were fertilized.

Of the 23 donor rabbits from which fertilized ova were not recovered, seven copulated but showed no ovulation points, six
TABLE I. THE NUMBER OF FERTILIZED AND NONFERTILIZED OVA
RECOVERED FROM NORMAL MATING OF RABBITS

<table>
<thead>
<tr>
<th>Plan No.</th>
<th>No. of Rabbits</th>
<th>Number of Ova Recovered</th>
<th>Ovulation Points</th>
<th>% of Ova Recovered</th>
<th>No. of Ova Unfertilized</th>
<th>No. of Ova Fertilized</th>
<th>% of Ova Fertilized</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>28</td>
<td>32</td>
<td>87.5</td>
<td>-</td>
<td>28</td>
<td>100.0</td>
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<td>2</td>
<td>2</td>
<td>19</td>
<td>21</td>
<td>90.5</td>
<td>-</td>
<td>19</td>
<td>100.0</td>
</tr>
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<td>3</td>
<td>2</td>
<td>16</td>
<td>20</td>
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<td>-</td>
<td>16</td>
<td>100.0</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>38</td>
<td>42</td>
<td>90.5</td>
<td>-</td>
<td>38</td>
<td>100.0</td>
</tr>
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<td>-</td>
<td>30</td>
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<td>25</td>
<td>96.0</td>
<td>-</td>
<td>24</td>
<td>100.0</td>
</tr>
<tr>
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<td>10</td>
<td>119</td>
<td>127</td>
<td>93.7</td>
<td>5</td>
<td>114</td>
<td>95.8</td>
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<tr>
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<td>16</td>
<td>181</td>
<td>187</td>
<td>96.8</td>
<td>5</td>
<td>176</td>
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<td>37</td>
<td>41</td>
<td>90.0</td>
<td>10</td>
<td>27</td>
<td>73.0</td>
</tr>
<tr>
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<td>4</td>
<td>45</td>
<td>49</td>
<td>91.8</td>
<td>3</td>
<td>42</td>
<td>93.3</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>95</td>
<td>120</td>
<td>79.2</td>
<td>31</td>
<td>64</td>
<td>67.4</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>95</td>
<td>115</td>
<td>82.6</td>
<td>9</td>
<td>86</td>
<td>90.5</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>28</td>
<td>32</td>
<td>87.5</td>
<td>8</td>
<td>20</td>
<td>71.4</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>59</td>
<td>61</td>
<td>96.7</td>
<td>6</td>
<td>53</td>
<td>89.8</td>
</tr>
</tbody>
</table>

Total 814 907 89.7 77 737 90.5
copulated and showed ovulation points but yielded no ova on flushing, and 10 copulated twice each and ovulated, but yielded no fertilized eggs.

Discussion of Results

The most successful method for recovering ova from the donor animal appears to involve sacrificing the donor, removing the reproductive tract, and flushing the ova from the tract. Hammond and Bhattacharya (60) and Dzuik et al. (42) were able to recover ova from 42 out of 93 (44.2 percent) and four out of 10 (40 percent) cows respectively. Chang (21) reported a recovery of 80 percent of the ova from donor rabbits, and Ferdows and Dracy (48) reported a recovery of 74%, again from rabbits. In this work, therefore, the recovery of 89.7 percent appears very good.

Several factors may explain the failure of seven of the rabbits, which copulated, to ovulate. While the rabbits used in this work were born in the spring and were used during the late summer, it is possible that some of them may not have reached sexual maturity. Hammond and Marshall (58) reported that does born in the fall reach puberty in about five and one-half months, but those born in the spring require about eight and one-half months. Perhaps breed difference must be considered here also. They also mention that as does pass into the anestrus condition, copulation may occur but Graafian follicles do not reach the state at which they are capable of rupturing. Furthermore, Makepeace and Winslow (73) have demonstrated that copulation may occur with the pseudopregnant rabbit without ovulation.
Finally, it seems reasonable that the state of nutrition may be important in the matter of ovulation. Which of these factors were responsible for the failure of the seven does in this experiment to ovulate cannot be definitely stated.

As stated previously, six rabbits copulated and showed ovulation points, but yielded no ova on flushing. It is assumed that in the case of these rabbits the failure to recover ova most probably was the result of faulty technique.

In this experiment ten recipient does were copulated and ovulated but all their ova were unfertilized. Some of the factors that might contribute to this are (1) destruction of sperm within the uterine body because of some infection, (2) anatomical abnormality of the reproduction tract preventing the sperm from entering the fallopian tubes, and (3) failure to use the male for a long period of time, resulting in a majority of the sperm being dead. Also there is evidence that the rabbit is able to ovulate after sexual excitement without copulation. Fee and Parke (47) reported that when the recipient does jump upon each other, ovulation may occur spontaneously.

During these experiments there was a rather large variation in the number of ovulation points observed. Several factors might account for this, such as, gonadotrophic hormone injection (91), genetic strain (58, 6), weight of the animal (58), or perhaps nutritional deficiency or the natural secretion of hormones. The data obtained from this work did not allow for stating which factor or factors were responsible. The same is true for the failure to obtain 100 percent fertilization.
when many factors may be involved (4, 63, 89, 24, 25, 72, 17).

In spite of the previous discussion, it nevertheless appears that the procedures and techniques used in this work for the care and breeding of the rabbits and for the recovery of the ova were reasonably sound and quite satisfactory for a study of this nature.

Freezing and Storage of the Ova

Procedure

After the ova had been flushed into a watch glass, they were picked up by means of fine capillary pipette and dropped into two-milliliter vials. Each vial contained either one milliliter of solution C (which consisted of a physiological saline solution and milk) or one milliliter of solution F (blood serum).

An equal volume of solution D (physiological saline solution and glycerol) was added in two different portions of 0.3 and 0.7 milliliters at five minute intervals to the vial that contained the ova in solution C (physiological saline and skim milk). Mixing was accomplished by rotating the vials after each addition of solution D. This gave volume of two milliliters.

The solution E of blood serum and glycerol was added to the vial that contained blood serum and ova in the same manner as with the physiological saline solution. The final dilution of blood serum and glycerol was also two milliliters. All vials were allowed to stand for 10 minutes at room temperature and then were put into a small beaker which contained 100 percent alcohol. The beaker was placed at
refrigerator temperatures for eight to ten hours. The temperature of the alcohol was measured by placing a thermometer inside the beaker in the refrigerator. The temperature of the alcohol was checked each 30 minutes for a period of 10 hours. The rate of temperature drop is presented in Table II.

**TABLE II. RATE OF TEMPERATURE DROP OF ALCOHOL IN THE REFRIGERATOR**

<table>
<thead>
<tr>
<th>Time temperature was checked</th>
<th>Temperature Reading at °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>7:30 a.m.</td>
<td>27.0</td>
</tr>
<tr>
<td>8:00 a.m.</td>
<td>18.0</td>
</tr>
<tr>
<td>8:30 a.m.</td>
<td>13.0</td>
</tr>
<tr>
<td>9:00 a.m.</td>
<td>10.0</td>
</tr>
<tr>
<td>9:30 a.m.</td>
<td>8.0</td>
</tr>
<tr>
<td>10:00 a.m.</td>
<td>6.0</td>
</tr>
<tr>
<td>10:30 a.m.</td>
<td>5.0</td>
</tr>
<tr>
<td>11:00 a.m.</td>
<td>4.0</td>
</tr>
<tr>
<td>11:30 a.m.</td>
<td>3.5</td>
</tr>
<tr>
<td>12:00 a.m.</td>
<td>3.0</td>
</tr>
<tr>
<td>12:30 a.m.</td>
<td>2.5</td>
</tr>
<tr>
<td>1:00 p.m.</td>
<td>2.0</td>
</tr>
<tr>
<td>1:30 p.m.</td>
<td>2.0</td>
</tr>
<tr>
<td>2:00 p.m.</td>
<td>1.5</td>
</tr>
<tr>
<td>2:30 p.m.</td>
<td>1.5</td>
</tr>
<tr>
<td>3:00 p.m.</td>
<td>1.0</td>
</tr>
<tr>
<td>3:30 p.m.</td>
<td>1.0</td>
</tr>
<tr>
<td>4:00 p.m.</td>
<td>1.0</td>
</tr>
<tr>
<td>4:30 p.m.</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The vials that contained the fertilized ova in either the blood serum or physiological saline solutions were frozen in an alcohol bath by 14 different procedures. These procedures were as follows:

1. Vials of fertilized ova at a temperature of 25°C were put
directly into alcohol, which was at a temperature of -79°C.

2. Vials of fertilized ova were cooled by refrigeration to 1°C, as described previously.

3. Vials containing fertilized ova were cooled to 1°C by refrigeration, then they were put directly in alcohol, which was at a temperature of -79°C.

4. Vials containing fertilized ova were cooled to 1°C by refrigeration, then they were cooled to -5°C (at the rate of approximately 1°C per 10 minutes). These vials were placed directly in alcohol, which was at a temperature of -79°C.

5. Vials containing fertilized ova were cooled to 1°C by refrigeration, then they were cooled to -10°C (at the rate of approximately 1°C per 10 minutes). These vials were then placed directly in alcohol, which was at a temperature of -79°C.

6. Vials containing fertilized ova were cooled to 1°C by refrigeration, then they were cooled to -15°C (at the rate of approximately 1°C per 10 minutes). Vials were then placed directly in alcohol which was at a temperature of -79°C.

7. Vials containing fertilized ova were cooled to 1°C by refrigeration. Then they were cooled to -20°C (at the rate of approximately 1°C per 10 minutes from 1°C to -10°C, 1°C per five minutes from -10°C to -20°C). These vials were then placed directly in alcohol which was at the temperature of -79°C.

8. Vials containing fertilized ova were cooled to 1°C by refrigeration then they were cooled to -25°C (at the rate of
approximately 1°C per 10 minutes from 1°C to -10°C, 1°C per five minutes from -10°C to -25°C). These vials were then placed directly in alcohol which was at the temperature of -79°C.

9. Vials containing fertilized ova were cooled to 1°C by refrigeration, then they were cooled to -30°C (at the rate of approximately 1°C per 10 minutes from 1°C to -10°C, 1°C per five minutes from -10°C to -30°C). These vials were then placed directly in alcohol which was at the temperature of -79°C.

10. Vials containing fertilized ova were cooled to 1°C by refrigeration, then they were cooled to -79°C at the following approximate rates:

   1°C per 10 minutes from 1°C to -15°C.
   1°C per five minutes from -15°C to -35°C.
   2°C per five minutes from -35°C to -79°C.

11. Vials containing fertilized ova were cooled to 1°C by refrigeration, then they were cooled to -79°C at the following approximate rates:

   1°C per 10 minutes from 1°C to -10°C.
   1°C per five minutes from -10°C to -20°C.
   2°C per five minutes from -20°C to -34°C.
   3°C per five minutes from -34°C to -79°C.

12. Vials containing fertilized ova were cooled to 1°C by refrigeration, then they were cooled to -79°C at the following approximate rates:

   1°C per 10 minutes from 1°C to -5°C.
1°C per five minutes from -5°C to -15°C.
1°C per three minutes from -15°C to -25°C.
1°C per two minutes from -25°C to -35°C.
1°C per one minute from -35°C to -45°C.
2°C per one minute from -45°C to -79°C.

13. Vials containing fertilized ova were cooled to 1°C by refrigeration, then they were cooled to -79°C at the following approximate rates:

1°C per 10 minutes from 1°C to -5°C.
1°C per five minutes from -5°C to -10°C.
1°C per three minutes from -10°C to -15°C.
1°C per one minute from -15°C to -20°C.
2°C per one minute from -20°C to -30°C.
3°C per one minute from -30°C to -79°C.

14. Vials containing fertilized ova were cooled to 1°C by refrigeration, then they were cooled to -79°C at the following approximate rates:

1°C per 10 minutes from 1°C to -10°C.
1°C per five minutes from -10°C to -15°C.
1°C per three minutes from -15°C to -20°C.
1°C per two minutes from -20°C to -30°C.
1°C per one minute from -30°C to -35°C.
2°C per one minute from -35°C to -45°C.
3°C per one minute from -45°C to -79°C.

All the vials were stored in a dry ice refrigerator at a constant
temperature of -79°C until used for observation and transplantation.

During these experiments 737 fertilized ova were frozen to a temperature of -79°C. The effects of the different rates of freezing were observed under a low power microscope after 20 minutes of storage. The stage of development and condition of nuclei, cytoplasm, and zona pellucida and disintegration of ova were recorded. Those ova which appeared normal and regular, without signs of fragmentation or of being misshapen, were considered to be in good condition.

Results

Ova were frozen in two kinds of media, one consisting largely of physiological saline solution and the other largely of blood serum, as described previously. The data in Table III compare the suitability of the two media as measured by the condition of ova after freezing and short-term storage. Because the other freezing methods yielded very few ova in good condition, regardless of the medium used, data for plans six through nine only are included in the table. Even in the case of plan six, the yield of ova in good condition was small and the data are perhaps of little consequence. It appears, however, from the results shown for the four plans that the blood serum medium was more satisfactory from the standpoint of preserving the ova than was the saline solution. Blood serum was, therefore, used in a large majority of the tests conducted.

Using plan two the ova were not frozen, but were stored at 1°C. Five ova were stored under this plan in physiological saline solution and 14 in blood serum. Three of four ova recovered from physiological
TABLE III. EFFECTS OF MEDIUM ON CONDITION OF OVA AFTER FREEZING TO -79°C

<table>
<thead>
<tr>
<th>Plan</th>
<th>No. of ova stored</th>
<th>Time of storage</th>
<th>No. of ova recovered</th>
<th>No. of ova in good condition</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>12</td>
<td>2 Hr.</td>
<td>12</td>
<td>0</td>
<td>Saline</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2 Hr.</td>
<td>12</td>
<td>3</td>
<td>Blood</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
<td>2 Hr.</td>
<td>9</td>
<td>2</td>
<td>Saline</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>2 Hr.</td>
<td>11</td>
<td>11</td>
<td>Blood</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>2 Hr.</td>
<td>11</td>
<td>3</td>
<td>Saline</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>2 Hr.</td>
<td>24</td>
<td>24</td>
<td>Blood</td>
</tr>
<tr>
<td>9</td>
<td>12</td>
<td>2 Hr.</td>
<td>7</td>
<td>1</td>
<td>Saline</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2 Hr.</td>
<td>19</td>
<td>9</td>
<td>Blood</td>
</tr>
</tbody>
</table>

Saline were in good condition after ten hours storage. Twelve of 13 ova recovered from the blood serum medium were in good condition after a similar storage period. These limited data showed no particular advantage for storage at temperatures above freezing for either medium.

Limited studies were made to establish whether or not the length of storage at -79°C had an effect on the recovery of ova in good condition. The results are given in Table IV. These results indicate a rather severe adverse effect on condition of the ova by storage for more than two days.

From 737 ova that were frozen by the various plans and stored for various lengths of time, 696 were recovered after storage, the
### TABLE IV. EFFECT OF LENGTH OF STORAGE AT -79°C IN SERUM MEDIUM ON CONDITION OF OVA

<table>
<thead>
<tr>
<th>Plan and rabbit no.</th>
<th>No. of ova Stored</th>
<th>Time of Stored</th>
<th>No. of ova recovered</th>
<th>No. of ova in good condition</th>
<th>Percent of recovered ova in good condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 (2)</td>
<td>26</td>
<td>2 Hrs.</td>
<td>25</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>(5)</td>
<td>56</td>
<td>2 days</td>
<td>54</td>
<td>45</td>
<td>83</td>
</tr>
<tr>
<td>(1)</td>
<td>11</td>
<td>3 days</td>
<td>11</td>
<td>5</td>
<td>45</td>
</tr>
<tr>
<td>(1)</td>
<td>14</td>
<td>6 days</td>
<td>14</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>8 (2)</td>
<td>24</td>
<td>2 Hrs.</td>
<td>24</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>(4)</td>
<td>53</td>
<td>4 Hrs.</td>
<td>45</td>
<td>44</td>
<td>98</td>
</tr>
<tr>
<td>(2)</td>
<td>22</td>
<td>5 Hrs.</td>
<td>20</td>
<td>17</td>
<td>85</td>
</tr>
<tr>
<td>(5)</td>
<td>48</td>
<td>2 days</td>
<td>41</td>
<td>34</td>
<td>63</td>
</tr>
<tr>
<td>(1)</td>
<td>13</td>
<td>3 days</td>
<td>13</td>
<td>5</td>
<td>38</td>
</tr>
<tr>
<td>(1)</td>
<td>9</td>
<td>6 days</td>
<td>9</td>
<td>3</td>
<td>33</td>
</tr>
</tbody>
</table>

remaining 41 probably being lost as a result of their clinging to the surface of the vials. The results are summarized in Table V. Of those recovered, 300 were in good condition, and 210 of these had been frozen by plan 7 or 8. Actually, therefore, 77 percent of the recovered ova from plans 7 and 8 were found to be in good condition, while only 21 percent from all other plans combined were in good condition. While not shown in the table, even when the effects of length of storage time and the medium are taken into account, these two plans appear far superior to any of the others as measured by recovery of ova in good condition.

### Discussion of Results

Chang (18, 19) found no difference between homologous and
<table>
<thead>
<tr>
<th>Plan No.</th>
<th>No. of rabbits</th>
<th>No. of ova stored</th>
<th>Age of ova before storage hours</th>
<th>Length of storage</th>
<th>No. of ova recovered after storage</th>
<th>% recovered after frozen</th>
<th>No. of ova in good condition</th>
<th>% of ova in good condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>28</td>
<td>24 to 25</td>
<td>1 hr.</td>
<td>24</td>
<td>85.7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>19</td>
<td>24 to 24.5</td>
<td>10 hr.</td>
<td>17</td>
<td>89.5</td>
<td>16</td>
<td>94.1</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>16</td>
<td>24 to 24.5</td>
<td>2 hr.</td>
<td>15</td>
<td>93.8</td>
<td>2</td>
<td>13.3</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>38</td>
<td>24 to 24.5</td>
<td>2 hr.</td>
<td>37</td>
<td>97.4</td>
<td>4</td>
<td>10.8</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>30</td>
<td>24 to 24.5</td>
<td>2 hr.</td>
<td>29</td>
<td>96.7</td>
<td>4</td>
<td>13.8</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>24</td>
<td>24 to 24.5</td>
<td>2 to 4 hr.</td>
<td>24</td>
<td>100.0</td>
<td>3</td>
<td>12.5</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>114</td>
<td>24 to 25</td>
<td>2 hr. to 6 days</td>
<td>108</td>
<td>94.7</td>
<td>80</td>
<td>74.1</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>176</td>
<td>24 to 25</td>
<td>2 hr. to 6 days</td>
<td>164</td>
<td>93.2</td>
<td>130</td>
<td>79.3</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>27</td>
<td>24</td>
<td>2 hr.</td>
<td>26</td>
<td>96.3</td>
<td>10</td>
<td>38.5</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>42</td>
<td>24 to 27</td>
<td>2 hr.</td>
<td>40</td>
<td>95.2</td>
<td>12</td>
<td>30.0</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>64</td>
<td>24 to 25</td>
<td>2 hr. to 26 days</td>
<td>62</td>
<td>96.9</td>
<td>20</td>
<td>32.3</td>
</tr>
<tr>
<td>12</td>
<td>10</td>
<td>86</td>
<td>24 to 25</td>
<td>2 hr. to 21 days</td>
<td>81</td>
<td>94.2</td>
<td>10</td>
<td>12.3</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>20</td>
<td>24 to 27</td>
<td>2 hr. to 27 days</td>
<td>19</td>
<td>95.0</td>
<td>4</td>
<td>21.1</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>53</td>
<td>24 to 26</td>
<td>2 hr.</td>
<td>50</td>
<td>94.3</td>
<td>5</td>
<td>10.0</td>
</tr>
</tbody>
</table>
heterologous sera, as measured by the recovery of normal ova, for the storage of ova at temperatures between 0°C and 35°C. He also found little, if any difference between a physiological saline solution and serum for storage media in this temperature range. The limited data from the experiments presented here for storage of unfrozen ova appear to bear out Chang's findings.

Since the freezing process itself can cause cell destruction, it would seem reasonable to expect that effects of the medium should express themselves to a greater extent when ova are frozen than when they are not. In these experiments, serum appeared to exert a protective effect when the ova were frozen. No study was made of the effect of adding glycerol to the media, but the work of Lovelock and Polge (70), Parkes and Smith (83), and Desmolsly (31) on the freezing of tissue, and the work on the freezing of erythrocytes discussed earlier in the Literature Review made it seem advisable to include this alcohol in the medium.

Chang (18, 19, 20, 22, 23, 25) found that ova could be stored in either physiological saline or serum for at least 24 hours at 5°C or 15°C without damage. At 0°C some cells in the two-cell stage were viable at 96 hours, but not all. No cells in the four- or six-cell stage remained viable under these conditions. Pincus (89) found that ova could survive at 0-10°C for 24 hours. The data in Table IV show that cells can be recovered in apparent good condition after several days of storage at -79°C, but that destruction does occur almost continuously during the storage period and the rate of destruction is
rather rapid after two days. It would seem, therefore, that considerable improvement in the medium used for storage is necessary if ova transplants are ever to become practical.

The method of freezing and of thawing a cellular material is very important in the preservation of the cell viability. Ice crystal formation can, itself, apparently affect the physiological status of tissue being frozen. Rate of freezing would be important here, as would the rate of thawing. Yet, our knowledge of the best rates of freezing and thawing is limited, and some reports offer apparently conflicting views on this matter. Meryman (76), for instance, reported that the rate of freezing and thawing must be extremely rapid in order to prevent large ice crystals. Rey (95), on the other hand, found that it is necessary to freeze slowly to prolong the period of adaptation of the cell to its new environment. Taylor (110) demonstrated that a rapid rate of thawing was essential for the survival of frozen tissue. The data in Table V indicate that an intermediate rate of freezing was best, as measured by the criteria used in this study. No data on the rate of thawing were obtained.

Plans 7 and 8 gave a good yield of cells in good condition. It would appear, therefore, that these freezing methods might be suitable for use in a program of ova transplants. There seems little doubt that they are much superior to the other freezing methods used. Yet, the method of assessing the condition of the cells was not such that the results could be used to predict the actual viability of the ova, and the results of transplant studies must also be considered.
That there is a critical region of temperature in which cell damage is most severe during freezing has been well demonstrated. Lovelock (70), for instance found this critical region to be between \(-3^\circ C\) and \(-40^\circ C\) for human erythrocytes. Folge (94) found it to be between \(-15^\circ C\) and \(-20^\circ C\) for bull spermatozoa. In this work, the most critical temperature region seemed to be \(0^\circ C\) to \(-15^\circ C\).

Transplantation of the Ova

**Procedure**

The recipient rabbits used in the transplantation work were similar to those used in the studies on the recovery of ova. Seven of the recipient does were injected with 50 units of gonadotrophin hormone (Cutter Laboratories pregnant mare serum or P.M.S.) 24 hours before implanting the ova. At about the same time, 14 of the does were mated to a vasectomized rabbit. These treatments were used to prepare the does physiologically to receive the fertile ova. The three vasectomized rabbits used for the preparatory mating had previously been mated to does to establish that the males were sterile.

About 24 hours after hormone injection or mating with the sterile male, the recipient was anesthetized with 30 milligrams nembutal per kilogram of body weight. The abdomen was shaved. The shaved area was washed with soap and water and then with 70 percent alcohol. A midline incision was made and the ovary, fallopian tubes, and infundibulum were exposed.

The vials containing ova for transplant, in the meantime, had been
quickly thawed (two to three minutes being required) and poured into a sterile watch glass. The number condition and stage of development of the ova were determined by microscopic examination. Using a fine capillary pipette, the ova were removed from the watch glass and the pipette was then inserted into the infundibulum. The fluid and ova in the pipette were forced into the fallopian tube and the pipette was examined microscopically to make sure all ova had been forced out. In all rabbits, ova were transferred into the right oviduct. In rabbits 11A, 21A, 22A, and 23A, ova were also transferred into the left oviduct.

Following the transplant, the abdominal cavity opening was sutured and the recipient doe was injected with 200,000 units of procaine penicillin.

The ova used in this work had all been recovered by the sterile technique as previously described. They had been frozen and stored under various conditions (see Table VI), except that the medium used was in all cases the serum medium and the storage temperature was −79°C. All of the ova transferred were in the two-cell stage.

In order to establish whether or not the transplantation techniques used in this work were satisfactory, 21 fertilized ova recovered from donor rabbits in a serum medium, as previously described, were implanted in two does. These recipients were allowed to go for 31 days, the normal term of pregnancy, to determine whether the ova would develop into young.

Other recipient does were either sacrificed early within the normal gestation period and the ova were recovered as previously
described, or the does were allowed to go past full term to determine whether young would be born. Ova recovered from the sacrificed animals were observed for stage of development. Three of the does which had been allowed to go full term were also sacrificed for examination for ova after full term had been passed.

**Results**

The results of this experiment are summarized in Table VI.

Rabbits 1A and 2A, which had been implanted with fertile ova immediately after their recovery from a donor rabbit, developed young which were born in a normal term of pregnancy.

Young were not born in any of the other rabbits allowed to go full term (rabbits 3A, 5A, 6A, 7A, 8A, 9A, 10A, 11A, 13A, 14A). Three of these rabbits (3A, 5A, 6A) were sacrificed on the thirty-first day and no ova were found.

No ova were recovered from rabbits sacrificed on the eighth (12A) or twelfth (4A) day. However, from those rabbits sacrificed on the first day after the transplants were made there was good recovery of the ova and a few showed development (four- and six-cell stage ova in rabbits 18A through 23A).

**Discussion of Results**

The results obtained for rabbits 1A and 2A indicate that technique for recovering fertile ova from donor rabbits and for transplanting these ova to recipient does were satisfactory. These rabbits (1A and 2A) were mated to vasectomized males, pre-tested for sterility.
<table>
<thead>
<tr>
<th>Recipient doe No.</th>
<th>Pre-transplant treatment*</th>
<th>Plan of freezing</th>
<th>Time stored at -79°C</th>
<th>No. of ova transferred**</th>
<th>Time between implanting and examination</th>
<th>Observations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>Mated</td>
<td>Not frozen</td>
<td>=</td>
<td>9</td>
<td>31 days</td>
<td>5 young born</td>
</tr>
<tr>
<td>2A</td>
<td>Mated</td>
<td>Not frozen</td>
<td>=</td>
<td>12</td>
<td>31 days</td>
<td>7 young born</td>
</tr>
<tr>
<td>3A</td>
<td>Mated</td>
<td>10</td>
<td>27 days</td>
<td>7</td>
<td>31 days</td>
<td>No young born***</td>
</tr>
<tr>
<td>4A</td>
<td>Mated</td>
<td>10</td>
<td>15 days</td>
<td>11</td>
<td>31 days</td>
<td>No ova recovered</td>
</tr>
<tr>
<td>5A</td>
<td>Mated</td>
<td>11</td>
<td>34 days</td>
<td>12</td>
<td>31 days</td>
<td>No young born***</td>
</tr>
<tr>
<td>6A</td>
<td>Mated</td>
<td>11</td>
<td>26 days</td>
<td>6</td>
<td>31 days</td>
<td>No young born***</td>
</tr>
<tr>
<td>7A</td>
<td>Mated</td>
<td>11</td>
<td>23 days</td>
<td>3</td>
<td>Over 31 days</td>
<td>No young born</td>
</tr>
<tr>
<td>8A</td>
<td>Mated</td>
<td>12</td>
<td>21 days</td>
<td>7</td>
<td>Over 31 days</td>
<td>No young born</td>
</tr>
<tr>
<td>9A</td>
<td>Mated</td>
<td>12</td>
<td>12 days</td>
<td>8</td>
<td>Over 31 days</td>
<td>No young born</td>
</tr>
<tr>
<td>10A</td>
<td>Mated</td>
<td>12</td>
<td>32 days</td>
<td>3</td>
<td>Over 31 days</td>
<td>No young born</td>
</tr>
<tr>
<td>11A</td>
<td>P.M.S.</td>
<td>8</td>
<td>2 days</td>
<td>10</td>
<td>Over 31 days</td>
<td>No young born</td>
</tr>
<tr>
<td>12A</td>
<td>P.M.S.</td>
<td>8</td>
<td>6 days</td>
<td>8</td>
<td>Over 31 days</td>
<td>No ova recovered</td>
</tr>
<tr>
<td>13A</td>
<td>P.M.S.</td>
<td>8</td>
<td>6 days</td>
<td>8</td>
<td>Over 31 days</td>
<td>No young born</td>
</tr>
<tr>
<td>14A</td>
<td>P.M.S.</td>
<td>8</td>
<td>2 days</td>
<td>12</td>
<td>Over 31 days</td>
<td>No young born</td>
</tr>
<tr>
<td>15A</td>
<td>Mated</td>
<td>7</td>
<td>16 days</td>
<td>14</td>
<td>1 day</td>
<td>12 two-cell stage ova recovered</td>
</tr>
<tr>
<td>16A</td>
<td>P.M.S.</td>
<td>7</td>
<td>2 days</td>
<td>15</td>
<td>1 day</td>
<td>14 two-cell stage ova recovered</td>
</tr>
<tr>
<td>17A</td>
<td>Mated</td>
<td>7</td>
<td>2 days</td>
<td>10</td>
<td>1 day</td>
<td>8 two-cell stage ova recovered</td>
</tr>
<tr>
<td>18A</td>
<td>Mated</td>
<td>7</td>
<td>2 days</td>
<td>12</td>
<td>1 day</td>
<td>10 two-cell and 1 four-cell stage ova recovered</td>
</tr>
<tr>
<td>19A</td>
<td>P.M.S.</td>
<td>8</td>
<td>2 days</td>
<td>20</td>
<td>1 day</td>
<td>14 two-cell and 2 four-cell stage ova recovered</td>
</tr>
<tr>
<td>20A</td>
<td>P.M.S.</td>
<td>8</td>
<td>2 days</td>
<td>6</td>
<td>1 day</td>
<td>4 two-cell and 1 four-cell stage ova recovered</td>
</tr>
<tr>
<td>21A</td>
<td>Mated</td>
<td>8</td>
<td>4 hours</td>
<td>24</td>
<td>1 day</td>
<td>20 two-cell and 1 four-cell stage ova recovered</td>
</tr>
<tr>
<td>22A</td>
<td>Mated</td>
<td>8</td>
<td>5 hours</td>
<td>20</td>
<td>1 day</td>
<td>14 two-cell and 1 six-cell stage ova recovered</td>
</tr>
<tr>
<td>23A</td>
<td>Mated</td>
<td>8</td>
<td>4 hours</td>
<td>22</td>
<td>1 day</td>
<td>16 two-cell and 1 four-cell stage ova recovered</td>
</tr>
</tbody>
</table>

*Mated to vasectomized male or injected with P.M.S.  **All in two-cell stage and in good condition.

*** Killed and observed for ova after 31 days. None found.
and used later on other does without young being born, quite conclusively establishing that the young born were from the transplanted ova.

Since young were not born to any of the other rabbits, it is evident that freezing, storage, or thawing techniques are unsatisfactory. Whether the preparation of the doe (to receive ova) by hormone treatment or by mating to a vasectomized male had any effect cannot be established from the data in Table VI with surety, but it appears that neither method had any advantage.

Neither can any conclusions be drawn as to which plan of freezing was best. As determined by observation of the condition of ova recovered, plans 7 and 8 appeared considerably superior to the other (see "Freezing and Storage of the Ova"). However, observed condition of the ova has not been correlated with viability. Therefore, while plans 10, 11, and 12 yielded few ova in good condition and while the data in Table VI for these plans are negative, they cannot be assumed less suitable than plans 7 and 8. All ova used from these freezing plans were stored for a rather long period and were also allowed to remain in the recipient doe for a rather long period (rabbits 3A through 10A). Ova stored under plan 8 even for periods of only two days, but allowed to remain in the recipient for long periods (rabbits 11A through 14A) also yielded negative results.

In view of what has just been discussed, the somewhat encouraging results obtained with rabbits 15A through 23A cannot be ascribed to freezing method. The fact that ova were recovered no doubt was the result of their rather short term stay in the recipient doe.
Comparing results for rabbits 11A and 14A with those for rabbits 19A and 20A rather clearly establishes this. However, there is perhaps some encouragement in the data for rabbits 18A through 23A where some cell division was found. Nevertheless, it is obvious that ova frozen, stored and thawed as they were in these experiments are not undamaged, and while they may indeed divide to some extent when transferred they soon are dead and disappear.

In view of what was found in this experiment, it is apparent that major emphasis in future studies should be placed on freezing and thawing methods, using short term (less than two days) storage. Studies on the effects of length of storage could then follow.
SUMMARY

1. Studies were conducted on methods of recovery of fertile ova from donor rabbits, on methods of freezing and on storage of the ova, and on the transplantation of fertile ova to recipient rabbits.

2. A technique was developed by which almost 90 percent recovery of ova was obtained. Over 90 percent of these recovered ova were in a two-cell stage (fertile).

3. Of the many freezing methods used, two (plans 7 and 8) appeared quite superior, as measured by visual observation of the condition of the ova after freezing, storing, and thawing. A blood serum-glycerol medium appeared superior to a saline-milk-glycerol medium for freezing and storage. Storage for more than two days caused apparent rapid degradation of the condition of the ova, even in the serum medium.

4. Transplantation studies demonstrated that while techniques for recovering and implanting the ova were satisfactory, freezing, storage, and thawing techniques as used in the work were not satisfactory.
LITERATURE CITED


(49) Friedman, M. H. Seasonal Variation in the Gonadotropic Hormone Content of the Rabbit Pituitary. Endocrinology, 24: 626. 1939.


