THE FERTILITY OF MICE WITH ORTHOTOPIC OVARIAN GRAFTS DERIVED FROM FROZEN TISSUE

DELPHINE M. V. PARROTT*

Division of Experimental Biology, National Institute for Medical Research, London, N.W.7

(Received 12th January 1960)

Summary. Normal offspring were obtained from mice with orthotopic ovarian grafts of tissue that had been frozen and stored at \(-79^\circ\) C. Tissue so treated showed a remarkable capacity for reorganization and function, but the number of oocytes surviving was small and the reproductive life of the females bearing the grafts was curtailed in each of the four strains of mice used.

The most successful method of preservation involved soaking the tissue for 30 to 40 min in a medium consisting of 12% glycerol in horse serum before slow cooling to \(-79^\circ\) C. Oocyte destruction was increased when the concentration of glycerol was reduced to 8%, when the tissue was soaked for 1 to 2 hr in 15% glycerol in horse serum, and when the tissue was stored at \(-79^\circ\) C for longer than 24 hr. Soaking in glycerol solutions at room temperature for 1½ hr without subsequent freezing also eliminated many oocytes. No viable grafts were obtained after ‘two-stage’ rapid cooling.

Preservation of the fertility of mice with grafts of ovarian tissue has proved to be more difficult than maintenance of cyclic cornification of the vagina. The problems involved are discussed.

INTRODUCTION

In 1956, Parkes showed that autografts of mouse ovarian tissue retained their viability after freezing and thawing; he used the methods that had been evolved for the preservation of rat ovarian tissue (summarized by Parkes, 1957). All these experiments were solely concerned with the endocrine function of grafted tissue, but Deanesly (1954, 1957) and Green, Smith & Zuckerman (1956) estimated the numbers of surviving oocytes in preserved tissue; they calculated that more than 90% of the oocytes in rat tissue that had been frozen and stored before subcutaneous autografting were destroyed. However, a varying number of small and primordial follicles survived and came to maturity. More recently, Parrott (1958) and Parrott & Parkes (1960) demonstrated by orthotopic

grafting that mouse ovarian tissue, after freezing and thawing, retained the
ability to produce viable ova although the recipients were not as fertile as
animals that had received fresh material. This paper describes in greater detail
the application and modification of existing techniques of freezing and thawing
to the preservation of the gametophoric capacity of mouse ovarian tissue.

Most of the previous studies on the preservation of ovarian tissue were con-
fined to experiments with autografts so that damage by freezing was the only
variable. The results of a few experiments with orthotopic autografts are
presented in this paper, but it is more practicable to use homografts between
members of the same strain. This, however, introduces another hazard — the
homograft reaction — which may have a considerable yet variable effect upon
the establishment of intrastrain orthotopic grafts and the fertility of recipients
(Parrott & Parkes, 1959). Even when inbred strains of mice were used, grafting
resulted in great loss of oocytes which shortened the reproductive life of graft
recipients to about half of the normal span (Mussett & Parrott, 1961). Moreover,
the average size of litters born to mice with orthotopic ovarian grafts was
small and pseudopregnancies and resorbed pregnancies were more frequent
than in normal mice of the same age (Mussett & Parrott, 1961). In consequence,
all the results obtained from mice with grafts of frozen tissue have been com-
pared with results from other mice of the same strain and age which had
received grafts of freshly donated ovarian tissue. The age of donors of both
frozen and fresh material was the same.

MATERIALS AND METHODS

ANIMALS

Four different stocks and strains of mice have been used in these experiments:

*CBA*. This is an inbred strain supplied by the Laboratory Animals Centre,
Carshalton.

*CBA/Fa-at or G strain*. An inbred strain in which three types of coat colour
are found (Parrott, 1960). The donors and recipients were different pheno-
types so that any offspring deriving from an orthotopic graft would be immedi-
ately distinguishable from the recipient's own offspring.

*TO*. The origin of this stock has been described by Gledhill (1959). A closed
colony has been maintained by line breeding at the National Institute for
Medical Research since 1953.

*R*. A hybrid stock, obtained by courtesy of Dr T. C. Carter of the Radio-
biological Research Unit, Harwell. Male parents are a cross between two inbred
strains (*3H* and *101*). Female parents are from a closed but not inbred colony
known as PCT. R mice are therefore double hybrids (*PCT × [C3H × 101]*)

IRRADIATION AND GRAFTING PROCEDURES

All mice were sterilized by X-irradiation before the grafting operation. Different
doses and methods of irradiation were used, but in every experiment the
treatment of recipients of both fresh and frozen ovarian tissue was the same. In
previous experiments (Parrott & Parkes, 1956; Parrott, 1958), mice were
sterilized with large doses of whole-body irradiation, but more recent work (unpublished) showed that lower doses were equally effective in some strains of mice. Local irradiation to the dorsolumbar region was also successful, and this method for sterilization has been described in experiments with hamsters (Parrott, 1959). Mice to be sterilized by whole-body irradiation were enclosed, ten at a time, in a shallow box to prevent them shielding one another. The dose rate was 50 r/min (potential 80 kV; current 6 mA; filter 1 mm aluminium), and the doses of irradiation given were as follows:

1. Two doses of 450 r whole-body irradiation separated by an interval of at least 2 weeks (TO and R stocks).
2. Two doses of 300 r whole-body irradiation also separated by an interval of at least 2 weeks (TO, R and CBA).
3. One dose of 200 r whole-body irradiation (G strain).
4. One dose of 435 r to the dorsolumbar region alone (G strain).

The operative technique has already been described in detail (Parrott & Parkes, 1956; Parrott, 1959).

METHODS OF PRESERVATION

Techniques for the preservation of rat ovarian tissue were described in detail by Parkes & Smith (1953, 1954), Smith & Parkes (1954) and Parkes (1957, 1958). Similar methods have been used, with only minor modifications, in the present experiments. Donor mice were killed, the ovaries removed and dissected free from fat; tissue from adult donors was divided into four parts. Unless otherwise stated the ovaries were soaked in horse serum containing 15% glycerol for 1 hr at room temperature. The tissue was then sealed in glass ampoules containing 1 ml of medium, cooled in the Lovelock cooler (Polge & Lovelock, 1952) to $-79^\circ$ C and kept at that temperature overnight. Thawing was carried out as rapidly as possible by plunging the ampoule into warm water at $+40^\circ$ C.

The efficiency of each method of preservation was assessed by three criteria: the proportion of recipient mice fertile after grafting, the length of time that the mice remained fertile and the number of oocytes remaining in the grafts recovered at autopsy.

OOCYTE COUNTS

At autopsy, the whole of both ovarian sites was removed from recipient mice. Only surplus fat and the distal part of the Fallopian tube were removed before fixation in Bouin's fluid so that the relative positions of each graft, capsule and sterilized ovary were preserved. All tissue was cut into serial sections of 7 µ thickness and stained with haematoxylin and eosin.

The number of oocytes in each graft was counted by the methods of Mandl & Zuckerman (1950, 1951) and Ingram (1958). The numbers of sections examined for small follicles (Stages 1 and 2) varied according to the total number of oocytes within the graft. When this number was very small, every section was scanned.
HISTOLOGICAL SECTIONS OF STERILIZED OVARIES (SO) AND ORTHOTOPIC OVARIAN GRAFTS (OG) ALL FROM R HYBRID MICE

Fig. 1. Fresh graft. The recipient was killed 133 days after grafting, having had three litters of twelve, two and two offspring. (GOM 492) ×19.

Fig. 2. Frozen graft. The tissue was soaked for 1½ to 2 hr in 15% glycerol in horse serum before freezing. The recipient was killed 42 days after grafting having had one litter of three offspring. (SIM 439) ×16.

Fig. 3. The tissue was soaked for 1½ hr in 15% glycerol in horse serum at room temperature without freezing. The recipient was killed 80 days after grafting having had one litter of three offspring. (SIM 486) ×20.

Fig. 4. Frozen graft. The tissue was soaked for 40 min in 12% glycerol in horse serum before freezing. The recipient was killed 46 days after grafting having had one litter of five offspring. (GOM 517) ×19.5.

(Facing p. 233)
Ovarian Grafts from Frozen Tissue

RESULTS

VARIABLES IN THE METHOD OF FREEZING AND THAWING

The effect of soaking for at least 1 hr in a medium containing 15% glycerol before freezing and thawing

Parkes & Smith (1953, 1954) and Parkes (1956) showed that the preservation of endocrine activity of ovarian tissue after freezing was considerably improved by the addition of glycerol to the suspending media. A medium containing 15% glycerol in horse serum with a soaking time of at least 1 hr at room temperature before freezing was satisfactory for both rat and mouse tissue; the same medium was used in the first experiments with orthotopic grafts (Table 1).

Five of the ten R mice in the first group mated when paired and two showed implantation signs but both pregnancies were resorbed. The results were slightly better when the donor material was soaked for a longer period (1½ to 2 hr) in 15% glycerol in horse serum before freezing and thawing. One of the eight mice had a live litter of three offspring; two others became pregnant but the pregnancies were resorbed. None of the mice in either group mated for a second time and the grafts removed from these animals at autopsy were completely exhausted of oocytes; only corpora lutea remained even in the graft from the mouse which had had a litter (Pl. 1, Fig. 2). In contrast, ten out of thirteen R mice with grafts of freshly donated material were fertile and many of them had successive litters (Pl. 1, Fig. 1). Many oocytes were still present in grafts removed from these animals after 120 days. However, Parkes (1957) showed that there was no difference in the number of ‘takes’ when mouse ovarian tissue was autografted subcutaneously whether the graft material was fresh or preserved by this method. Moreover, the grafts derived from frozen tissue remained viable for at least 12 weeks after implantation.

At this time, it was considered possible that a period of 1 hr was insufficient for glycerol to penetrate the tissue and in the next experiments with TO-stock mice this time was extended. The tissue that was implanted into the first group of mice (Table 1) was soaked for 2½ hr in 15% glycerol in horse serum before freezing. When the mice were killed 25 days later, 1 to 8 medium-sized and large follicles were present in the grafted tissue from four of the ten mice, no small oocytes remained, and none of the grafts was fertile. No oocytes or follicles survived in the grafted tissue that had been soaked for 4 hr in 15% glycerol in horse serum before freezing and subsequent implantation into recipients (Table 1).

Results with the TO-stock mice were complicated by intrastrain homograft reaction, which reduced the number of ‘takes’ to about 40% even in the control mice with grafts of fresh tissue, but it was probable that prolonged soaking in glycerol had increased the destruction of oocytes.

The effect of soaking in a medium containing 15% glycerol without subsequent freezing and thawing

The findings with R and TO mice made it necessary to examine the effect of glycerol media alone upon the fertility of ovarian tissue. R mice received grafts of ovarian tissue that had been soaked for 1½ hr in glycerol solutions at room
### Table 1

**Effect of Time of Soaking in 15% Glycerol on the Fertility of Mice with Ovarian Grafts Derived from Fresh and Frozen Tissue**

<table>
<thead>
<tr>
<th>In vitro treatment of tissue before implantation</th>
<th>Age of donors (days)</th>
<th>No. and strain of grafted mice</th>
<th>No. fertile mice</th>
<th>No. litters</th>
<th>Average litter size</th>
<th>No. resorptions</th>
<th>No. pseudo-pregnancies</th>
<th>No. oocytes in grafts from fertile mice*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>30 to 40</td>
<td>5 R 8 R</td>
<td>4 6</td>
<td>12 6</td>
<td>3.8</td>
<td>3</td>
<td>0</td>
<td>476, 495, 539 (80)†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1 killed pregnant, 2 foetuses in utero)</td>
<td></td>
<td></td>
<td></td>
<td>Many oocytes present but not counted (100 to 127)</td>
</tr>
<tr>
<td>Soaked for 1 hr before freezing to -79°C. Stored overnight</td>
<td>30 to 40</td>
<td>10 R</td>
<td>2</td>
<td>0</td>
<td></td>
<td>2</td>
<td>0</td>
<td>None (48)</td>
</tr>
<tr>
<td>Soaked for 1½ to 2 hr before freezing to -79°C. Stored overnight</td>
<td>30 to 40</td>
<td>8 R</td>
<td>3</td>
<td>1</td>
<td>3.0</td>
<td>2</td>
<td>0</td>
<td>None (44)</td>
</tr>
<tr>
<td>Soaked 1½ hr in 15% glycerol in horse serum. Not frozen</td>
<td>30 to 40</td>
<td>8 R</td>
<td>8</td>
<td>7</td>
<td>3.0</td>
<td>4</td>
<td>3</td>
<td>9, 12, 14, 35 (80)</td>
</tr>
<tr>
<td>Soaked 1½ hr in 15% glycerol in saline. Not frozen</td>
<td>30 to 40</td>
<td>8 R</td>
<td>5</td>
<td>4</td>
<td>2.0</td>
<td>4</td>
<td>5</td>
<td>0, 0, 10, 12 (80)</td>
</tr>
<tr>
<td>No treatment</td>
<td>10 to 16</td>
<td>18 TO</td>
<td>6</td>
<td>1</td>
<td>5.0</td>
<td>0</td>
<td>1</td>
<td>145 (22); 179, 476, 1018 (37)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Five killed pregnant, 4, 5, 6, 8, 9 foetuses in utero)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soaked 2 to 3 hr in 15% glycerol before freezing to -79°C. Stored overnight</td>
<td>10 to 16</td>
<td>10 TO</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soaked 4 hr in 15% glycerol before freezing to -79°C. Stored overnight</td>
<td>10 to 16</td>
<td>10 TO</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Some of the grafts were not recovered in a suitable state for histological examination.
† Figures in parentheses show days after grafting when mice were killed.
temperature but had not been frozen and thawed. This treatment did not affect the initial establishment of the grafts and fertility of the recipients whether the suspension medium was 15% glycerol in 0.9% sodium-chloride solution or 15% glycerol in horse serum (Table 1). However, the reproductive life of the recipient mice was considerably reduced, for no fertile matings occurred in either group later than 39 days after grafting and very few oocytes remained in grafts recovered at autopsy (Pl. 1, Fig. 3).

These results confirmed the observations of Deanesly (1954, 1957) who reported that glycerol solutions alone had a damaging effect upon oocytes. It appeared that approximately 80% of the oocytes in ovarian tissue were destroyed by soaking in a solution of 15% glycerol at room temperature and that, when this was followed by freezing and thawing, almost all the remaining oocytes were eliminated.

Progressive ‘upgrading’ of the percentage of glycerol

An attempt was made to reduce the deleterious effects of glycerol by gradual ‘upgrading’ at a reduced temperature in the following manner:

Firstly, ovarian tissue from TO donors was soaked in a medium containing 2% glycerol for 2 ½ hr at room temperature. The concentration of glycerol was then increased to 6% and the temperature reduced to +4° C. After a further 2 hr, the concentration of glycerol was increased to 15% and the tissues left overnight at +4° C. The following day, the ovarian tissue was sealed in ampoules and cooled slowly to −79° C. It was stored overnight at this temperature and then thawed and grafted into TO mice. All the mice mated on pairing and when they were killed 21 days after operation one mouse was found to be pregnant. Small grafts were present in six out of nine mice and an average of five large or medium-sized follicles was present in each graft. No small oocytes remained. These results suggest that soaking at +4° C rather than at room temperature reduces the deleterious effects of glycerol, but they are not significantly different from those previously obtained.

Reduction of glycerol percentage and soaking time before freezing

Whilst these experiments were in progress, Parkes (1958) observed the effects of reducing the soaking time in glycerol media at room temperature before freezing on the viability of rat ovarian tissue. He showed that the soaking time could be reduced, with advantage, from 1 hr to 15 min and that reduction of the glycerol concentration to 10% only slightly decreased the number of viable grafts. Deanesly (1957) had noted that a preservation medium containing 10% glycerol did not appreciably reduce the numbers of surviving oocytes in subcutaneous grafts. It was decided, therefore, to adopt similar modifications in the preservation of mouse tissue.

Ovarian tissue that had been soaked for an average of 40 min at room temperature in 12% glycerol in horse serum before freezing was grafted orthotopically into ten R mice. There was a marked improvement in both the establishment of grafts and fertility of the recipients, since eight of them had functional grafts and six were fertile (Table 2; Pl. 1, Fig. 4). Five mice had litters of from one to five live offspring and there were still some oocytes
### Table 2

**Effects of Reduction in the Proportions of Glycerol and in the Soaking Time Before Freezing and Storage Overnight at −79°C on the Fertility of Mice with Ovarian Grafts**

<table>
<thead>
<tr>
<th>In vitro treatment of tissue before implantation</th>
<th>No. grafted mice</th>
<th>Age of donors (days)</th>
<th>No. fertile mice</th>
<th>No. litters</th>
<th>Average litter size</th>
<th>No. resorptions</th>
<th>No. pseudo-pregnancies</th>
<th>No. oocytes in grafts from fertile mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>10</td>
<td>30 to 40 (2 mice killed pregnant, 3 and 4 foetuses in utero)</td>
<td>7</td>
<td>20</td>
<td>3.6</td>
<td>3</td>
<td>4</td>
<td>287, 287, 412 (83)*; 405, 599, 935, 1632 (133)</td>
</tr>
<tr>
<td>Soaked 25 to 50 min in 12% glycerol before freezing</td>
<td>10</td>
<td>30 to 40 (1 mouse killed pregnant, 3 foetuses in utero)</td>
<td>6</td>
<td>6</td>
<td>1.8</td>
<td>3</td>
<td>11</td>
<td>288 (46); 53 (63); 28, 39, 42, 178 (94)</td>
</tr>
<tr>
<td>Soaked 20 to 50 min in 8% glycerol before freezing</td>
<td>10</td>
<td>30 to 40 (1 mouse killed pregnant, 1 foetus in utero)</td>
<td>3</td>
<td>2</td>
<td>2.0</td>
<td>1</td>
<td>3</td>
<td>0, 149, 432 (62)</td>
</tr>
</tbody>
</table>

*Figures in parentheses show days after grafting when mice were killed.*
remaining in the grafts removed from mice killed 94 days after operation. In fact the damage caused by soaking ovarian tissue in 12% glycerol for 30 min followed by freezing and thawing was less than that caused by soaking in 15% glycerol for 1/3 hr without freezing.

When the proportion of glycerol in the suspension medium was altered to 8%, the number of R mice with established and fertile grafts was again reduced, although there were oocytes in the grafts removed from two mice at autopsy.

The fertility of mice of three other strains (CBA, G and TO) was restored after the implantation of ovarian tissue, which had been soaked in 12% glycerol in horse serum for 30 to 40 min before freezing and thawing. Established grafts were present in ten out of eleven CBA and six of the mice were fertile (Table 3). By contrast, only one out of ten TO mice that had received grafts from infantile donors was fertile although this one animal was as successful as the control mice (Table 3). None of the ten TO mice that had received frozen ovarian tissue derived from adult donors was fertile and consequently this group is not included in Table 3; some grafted tissue had survived but no oocytes remained.

Two groups of G-strain mice were grafted with tissue that had been soaked in 12% glycerol for 30 to 40 min before freezing (Table 3). The first group received tissue from infantile donors but the results were not as successful as those obtained with the R and CBA strains. Only three of the seventeen recipients were fertile. In a second experiment, when adult donors of ovarian tissue were used, six out of ten mice were fertile. Five mice became pregnant more than once and one mouse that was not killed until 162 days after grafting had three litters. These mice were killed within 12 to 16 hr of mating and the Fallopian tubes searched for ova. Three mice had ovulated and one, two and five fertilized eggs were found, although less than twelve small and large follicles remained in any of the grafts from the three animals. All the offspring from the G-strain recipients were genetically identified as deriving from the grafts; no mixed litters were obtained.

**Two-stage rapid cooling**

Parkes (1958) found that two-stage rapid cooling, that is plunging ampoules into freezing mixture at −30 °C and after 5 min at this temperature transferring to alcohol at −79 °C, was almost as effective in preserving the endocrine function of rat ovarian tissue as the more complicated slow-cooling method. However, no success was obtained when this method was applied to the preservation of fertility of mouse grafts. In only two out of ten R mice did any grafted tissue survive, neither of the mice was fertile and no oocytes remained in the grafts.

**Fertility of mice with grafts of ovarian tissue preserved for long periods**

The homograft reaction in the TO stock is very variable and it was decided to eliminate this hazard by making autografts, although this involved longer storage of the graft material. Weanling mice were spayed unilaterally and the ovaries frozen and stored at −79 °C. When the mice had recovered from the
### Table 3

The fertility of mice with grafts of tissue that was soaked for 30 to 50 min in 12% glycerol before being frozen and stored overnight at —79°C.

<table>
<thead>
<tr>
<th>In vitro treatment of tissue before implantation</th>
<th>No. and strain of grafted mice</th>
<th>Age of donors (days)</th>
<th>No. fertile mice</th>
<th>No. litters</th>
<th>Average litter size</th>
<th>No. resorptions</th>
<th>No. pseudopregnancies</th>
<th>No. oocytes in grafts from fertile mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>9 TO</td>
<td>10 to 16</td>
<td>3</td>
<td>14</td>
<td>2.8</td>
<td>0</td>
<td>1</td>
<td>351, 491, 877 (146)*</td>
</tr>
<tr>
<td>Frozen and thawed</td>
<td>10 TO</td>
<td>10 to 16</td>
<td>1</td>
<td>4</td>
<td>5.0 (Mouse killed pregnant, 7 foetuses in utero)</td>
<td>0</td>
<td>0</td>
<td>69 (143)</td>
</tr>
<tr>
<td>No treatment</td>
<td>8 CBA</td>
<td>56 to 60</td>
<td>7</td>
<td>8</td>
<td>3.1 (5 mice killed pregnant, 1, 2, 3, 4, 4 foetuses in utero)</td>
<td>4</td>
<td></td>
<td>363 (67); 72, 125, 213, 234, 288 (83)</td>
</tr>
<tr>
<td>Frozen and thawed</td>
<td>11 CBA</td>
<td>56 to 60</td>
<td>6</td>
<td>1</td>
<td>3.0 (3 mice killed pregnant, 1, 2, 3, foetuses in utero)</td>
<td>2</td>
<td>1</td>
<td>23 (26); 23, 45, 97, 139 (36)</td>
</tr>
<tr>
<td>No treatment</td>
<td>16 G</td>
<td>19 to 27</td>
<td>12</td>
<td>9</td>
<td>2.8 (3 mice killed pregnant, 2, 2, 7, foetuses in utero)</td>
<td>10</td>
<td>8</td>
<td>343, 412, 591 (39); 136, 309 (60); 167, 170, 251, 259, 248 (90); 29 (103); 137 (45)</td>
</tr>
<tr>
<td>Frozen and thawed</td>
<td>17 G</td>
<td>23 to 30</td>
<td>3</td>
<td>1</td>
<td>1.0 (1 mouse killed pregnant, 3 foetuses in utero)</td>
<td>3</td>
<td>1</td>
<td>54 (37); 12 (42); 58 (48)</td>
</tr>
<tr>
<td>No treatment</td>
<td>8 G</td>
<td>43 to 51</td>
<td>7</td>
<td>10</td>
<td>2.1 (1 mouse killed pregnant, 7 foetuses in utero)</td>
<td>2</td>
<td>7</td>
<td>250 (44); 100, 134, 171, 257 (79); 65 (116); 85 (137)</td>
</tr>
<tr>
<td>Frozen and thawed</td>
<td>10 G</td>
<td>40 to 60</td>
<td>6</td>
<td>5</td>
<td>2.0 (1 mouse killed pregnant, 4 foetuses in utero)</td>
<td>8</td>
<td>9</td>
<td>7 (87); 41 (91); 38 (94); 3, 7 (109); 4 (162)</td>
</tr>
</tbody>
</table>

*Figures in parentheses show days after grafting when mice were killed.
operation, the remaining ovary was sterilized by whole-body irradiation. After an interval of some weeks, the stored ovary was autografted back into each recipient. Three groups of TO mice were treated in this way, but the time of soaking before freezing, the concentration of glycerol in the suspension media and the period of storage were varied (Table 4). There were no ‘takes’ in twelve mice which received tissue that had been soaked for 1 hr in 15% glycerol in horse serum before freezing and storage at \(-79^\circ C\) for 44 days. When the experiment was repeated, three out of ten mice had ‘takes’ and one of these was fertile although the storage time was slightly increased. Reduction in the soaking time and glycerol concentration, but with the storage time again

### Table 4

**The Fertility of 'TO' Mice With Unilateral Autografts of Ovarian Tissue Preserved at \(-79^\circ C\) for Long Periods**

<table>
<thead>
<tr>
<th>Soaking time at room temp. (min)</th>
<th>Conc. of glycerol in suspending media</th>
<th>Storage time (days)</th>
<th>No. grafted mice</th>
<th>No. fertile mice</th>
<th>No. litters</th>
<th>No. oocytes in grafted tissue from fertile mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 to 75</td>
<td>15</td>
<td>44</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>(1 mouse killed pregnant, 1 foetus in utero)</td>
</tr>
<tr>
<td>30 to 75</td>
<td>15</td>
<td>57</td>
<td>10</td>
<td>1</td>
<td>2 (33)*</td>
<td></td>
</tr>
<tr>
<td>25 to 65</td>
<td>12</td>
<td>92</td>
<td>11</td>
<td>1</td>
<td>0</td>
<td>(1 mouse killed pregnant, 3 foetuses in utero)</td>
</tr>
</tbody>
</table>

* Figures in parentheses show days after grafting when mice were killed.

increased, gave a similar result: only one animal was fertile. The number of oocytes in both fertile grafts was very low. Allowance must be made for the fact that mice in this experiment were grafted unilaterally instead of bilaterally as in all the other experiments. However, since the hazard of homografting was eliminated, a higher proportion of mice with established and fertile grafts might have been expected. It seems probable, therefore, that prolonged storage at \(-79^\circ C\) increased the destruction of oocytes.

There was no problem of homograft reaction with G-strain mice so that routine irradiation and grafting procedures were used. Ovaries from weanling donors were soaked for 30 min in 12% glycerol in horse serum before being slowly cooled and stored at \(-79^\circ C\). Twenty days later, the tissue was rapidly thawed and grafted into recipients. Seven out of nine mice had implantation signs indicating the presence of fertile grafts but only one of the animals had a litter of one offspring. Three mice mated again but there was no further evidence of graft activity. As there was a shortage of G-strain mice of the required coat colours, the same animals were used again and each received a second graft, this time of fresh tissue. At the second operation, it was noted that the only recipient with any residual graft tissue was the mouse that had given
birth to a single offspring. Six out of eight of the second grafts were fertile and many of the recipient mice had successive litters.

Ten R mice received ovarian tissue that had been stored at \(-79^\circ\) C for 1 year in 15% glycerol in horse serum. There were no ‘takes’ and no evidence of even transient endocrine activity by the grafted tissue.

All these findings contrast with those of Parkes (1956, 1957) who showed that storage for several weeks had little effect upon the subsequent functioning of either mouse or rat tissue autoplastically grafted under the skin. Moreover, Deanesly & Parkes (1957) reported that sufficient cells survived ultimately to form an endocrinologically active graft, even when ovarian tissue was stored for months under the most unfavourable conditions.

HISTOLOGICAL APPEARANCE OF GRAFTS
In general appearance, all grafts were similar to those from mice which received fresh tissue. However, host reaction, i.e. lymphocyte infiltration in the R and TO mice, was more pronounced when tissue had been frozen before grafting than when it was untreated, and this reaction was most evident in grafts with very small numbers or with no surviving oocytes. As had been observed in other rat and mouse ovarian grafts (Parrott, 1959; Mussett & Parrott, 1961), the corpora lutea were the most resistant ovarian elements to host reaction (Pl. 1, Fig. 2). There was no immune response to the grafts in the CBA and G strains. These facts emphasize the necessity of using either autografts or homografts between members of an inbred strain in this type of experiment so that the hazards are limited to those incurred during the freezing procedures.

DISCUSSION
The results show that slight alterations in the concentration of glycerol in the suspending medium and in the soaking time before freezing and thawing affect the subsequent production of viable ova by ovarian tissue after grafting. Further detailed investigations would therefore be worth while. Pregnancies resulting in the birth of live offspring to recipient mice occurred infrequently and the average litter size was small. There were many pseudopregnancies and resorptions. Only one mouse had successive live litters and this was the only animal in which pregnancy and lactation were concurrent. However, any offspring derived from grafts were normal, they were weaned successfully and some were interbred without any abnormality appearing. Offspring from the G-strain mice were genetically identified as deriving from the grafts. Results from recipients with implants of fresh tissue (Mussett & Parrott, 1961) have shown that the small average litter size was a consequence of the small number of ova reaching the uterus. It is probable that the increased frequency of pseudopregnancies and the decreased number of litters in mice with grafts of frozen tissue were due to the even smaller number of ova implanted. However, normal ovulation, fertilization and pregnancy continued to take place as long as any oocytes remained in the grafted tissue.

Preservation of oocytes in ovarian tissue during freezing and thawing thus proved to be more difficult than maintenance of endocrine activity. However, any comparison of the results presented in this paper with those of other authors
must allow for differences both in animals and experimental technique. For instance, in the experiments of Parkes & Smith (1954), Parkes (1957, 1958) and Deansley (1957) grafts of frozen tissue were placed subcutaneously and it is possible that this site is more favourable than the orthotopic position for the reconstitution and survival of endocrinologically active grafts from damaged tissue. Moreover, only autografts were implanted subcutaneously, whereas in some of the present experiments homografts between non-inbred strains were used which introduced the additional hazard of homograft reaction. Finally, most of the initial work (Parkes & Smith, 1954; Parkes, 1958) was carried out on rats, whereas mice were used in the present experiments because of the ease of orthotopic grafting in this species. Orthotopic grafts have been successfully established in the rat (Parrott, 1958) but the considerable range of immune reaction within the outbred stocks available render them unsuitable for this type of work. This disadvantage does not apply to the golden hamster in which orthotopic grafting is simple and both inter- and intrastrain homograft reaction is slight (Parrott, 1959), but the available techniques for freezing and thawing have proved unsuccessful in preserving the fertility of hamster ovarian tissue (Parrott & Parkes, 1960; Parrott, unpublished data).

ACKNOWLEDGMENTS

I should like to thank Dr. A. S. Parkes for the interest he has taken in this work. Miss A. Wakeford gave invaluable technical assistance and Mr. J. B. Clark prepared the photomicrographs.

REFERENCES