FERTILIZATION IN THE PIG

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Summary. Observations were made on ova recovered from mated, from inseminated and from unmated sows killed at varying intervals up to 144 hr after the onset of heat. The morphological findings on penetrated, pronucleate and cleaved ova are described.

The timing of the events that precede and follow fertilization is examined. The cumulus cell mass (egg-plug) was found around ova only from sows killed less than 8 hr after mating. Spermatozoa were not found in the cumulus until 5½ hr after mating. Penetration and pronucleus formation occurred not earlier than 6 hr after mating. The first cleavage in normal mated pigs occurred 21 hr after mating. Some cleaved ova were recovered earlier than this from sows after ‘delayed’ mating. The distribution of ova at different stages of cleavage is recorded for sows killed at varying intervals after mating or insemination.

Three of a total of 1677 ova resembled primarily oocytes; all other detectable abnormalities could have arisen in originally normal ova.

The number of spermatozoa on the zona pellucida tended to increase with advancing stages of development, but counts of spermatozoa on ova at the same stage of cleavage recovered at different intervals after mating furnished no evidence that cleavage rate is increased by increasing sperm numbers.

In unfixed ova from unmated sows, spontaneous fragmentation was observed with increasing frequency after 72 hr from the onset of heat; degenerative changes were identified in fixed and stained ova 48 hr after the onset of heat. Cleaved (fragmented) ova from unmated pigs never showed a normal nuclear pattern.

The findings are discussed.

INTRODUCTION

The observations recorded here deal with three aspects of fertilization in the pig:

(1) The morphology of fertilized and unfertilized pig ova.
(2) The changes that occur with time in fertilized and in unfertilized pig ova.
(3) The numbers of spermatozoa on the zona pellucida of pig ova.

The observations were made on material collected in the course of experiments on artificial insemination of pigs in which the success of insemination was judged by the proportion of fertilized ova recovered at autopsy 2 to 4 days.
after insemination and on material from sows mated and killed at times that were chosen specifically to provide data on some aspect of fertilization.

MATERIAL AND METHODS

The animals used in this work were surplus sows from the Organization’s Pig Breeding Unit. Management and the procedures used for the detection of heat, for mating, for insemination and for the slaughter and recovery of ova have been dealt with previously and need not be considered in detail here (Hancock, 1957, 1959). Heat was determined by trial once or twice daily with a vasectomized boar.

In this paper, ‘onset of heat’ means the time when a sow first accepted the boar. Not all sows regarded as accepting the boar were served by the boar; where service did not take place this was because the boar was restrained. Day 0 here means the day of first acceptance (or the first day of heat) and Day 1 means the day after (or the second day of heat). In experiments designed to give information specifically about the timing of events at fertilization, the time of each mating was recorded and the precise interval between mating and, for example, recovery of ova is known. For routine insemination trials, the sows were tried once daily for evidence of heat and were mated and inseminated either on Day 0 or on Day 1. In experiments designed to study the effects on fertility of time of mating relative to the onset of heat, sows were tried twice daily and matings were made (1) at the onset of heat, (2) 24 hr later, (3) 30 hr later or (4) 48 hr later. ‘Delayed matings’ here means matings made 30 hr or more after the onset of heat. ‘Normal matings’ are those made earlier.

The interval between autopsy and recovery of ova was recorded to the nearest half hour for sows killed less than 41 hr after mating. For sows killed after this time, the interval between autopsy and recovery was recorded to the nearest 24 hr. Because, for routine purposes, pigs were slaughtered between 8 a.m. and 2 p.m., and were mated or inseminated between 9 a.m. and 1 p.m., the stated interval between mating or insemination and slaughter is subject to a variable error (± 6 hr).

Estimation of the stage of cleavage of fresh unfixed ova was made as a result of examination with a dissecting microscope at a magnification of 20 to 50 diameters. No attempt was made to count accurately the numbers of blastomeres in ova with more than four cells. The ova were classed as 1-cell, 2-cell, 3-cell, 4-cell, 5- to 8-cell or 9- to 12-cell; ova developed beyond this stage were classed as morulae or blastocysts according to whether or not the blastocoele could be identified.

Stained uncleaved ova were classified according to their nuclear morphology and stained cleaved ova were classified according to the numbers of nuclei. It was possible to count without difficulty the numbers of nuclei present when there were not more than twelve. Ova with more than sixteen nuclei counted are grouped here into two classes: those with between sixteen and thirty-two nuclei and those with more than thirty-two nuclei.

The following methods were used for the examination of ova:

(1) Direct microscopy of whole ova either in 0-9 % sodium-chloride solution
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(living ova) or after fixation in buffered (pH 6.8) formal-saline solution (Hancock, 1957).

(2) Phase-contrast microscopy of ova fixed and cleared in acetic-alcohol and stained in aceto-carmine (Hancock, 1958). These preparations were also used for counting the numbers of spermatozoa present.

(3) Sectioned ova. These were fixed in either buffered formal-saline solution or in Helly’s solution, were embedded in ester wax and cut serially at 4µ (Wigglesworth, 1959), or embedded in paraffin wax and cut at 7µ. Sections were stained by the conventional method with haematoxylin and eosin, with Weigert’s iron haematoxylin and eosin, by the periodic acid-Schiff (PAS) method followed by counter-staining with methyl green, or by Feulgen’s method followed by counter-staining with Orange G.

(4) ‘Egg-plugs’ with their ova were fixed in Helly’s solution and cut at 7µ after paraffin embedding.

RESULTS

MORPHOLOGY

Most of the observations were made on whole ova cleared of their yolk content by treatment with acetic-alcohol and stained for examination (Method 2 above). This treatment destroys most of the cytological components of the cell including its membranes. Nucleoli are still detectable as vesicles within the nucleus which is well preserved. Removal of the yolk platelets leaves the cytoplasm marked with vacuoles; the size and disposition of these vacuoles depends presumably on the size and disposition of the removed platelets and it is possible in these preparations to see evidence of the changes that occur in the size of the yolk platelets. These were found to be inconspicuous in freshly shed ova and became increasingly obvious with increasing age; the changes have not been followed in detail and the observation recorded here deals primarily with the nuclear morphology.

Fertilization and cleavage

Some recently shed ova were found grouped within a mass of cells (the ‘egg-plug’) formed by the adhesion of several ova with their surrounding cumulus cells. The appearance of an ovum as seen in paraffin section of an egg-plug is shown in Pl. 1, Fig. 1. The cumulus cells are loosely held together by a very scanty cement substance. The ovum shows in lateral view the metaphase chromosomes on the second maturation spindle with the first polar body just outside the vitellus. The second maturation spindle itself is shown more clearly in Pl. 1, Fig. 2. The long axis of the spindle is radial to the surface of the ovum; this was the case in virtually all ova with intact spindles. A few ova were recovered showing some of the changes that take place immediately after penetration and that precede pronucleus formation. Two whole stained ova have been seen in each of which a spermatozoon, apparently in contact with the vitellus, showed an enlarged sperm head. One of these two ova had a second spermatozoon present in the zona pellucida, the relative sizes of the two
spermatozoa is shown in Pl. 1, Figs. 4 and 5. It is noticeable that the spermatozoa on the zona pellucida, as well as being smaller, retains some of the prominent morphological features of the ejaculated spermatozoon—e.g., the equatorial segment is distinctly visible. In contrast, the enlarged head of the penetrated spermatozoon has lost this feature. Pl. 1, Fig. 6 shows the nuclear apparatus of the same ovum. What is apparently the formative female pronucleus appears as a compact intensely stained body lying to the right of the second polar body. The cytological findings here resemble closely those recorded by Odor & Blandau (1951) for the rat ovum at the same stage. In pronucleate ova, it was possible in only a small proportion of ova to distinguish by the presence of the sperm tail the male from the female pronucleus. The sperm tail was visible in ten of 175 pronucleate ova examined as whole mounts and in three of these ova the sperm tail could be used to identify the male pronucleus. In all three ova, the female pronucleus showed a coarsely granular thickening of the nuclear membrane on the face opposite the male pronucleus.

In a few exceptionally clear preparations, the remains of the second matura
tion spindle could be seen attached to the second polar body (Pl. 1, Fig. 3). Structures that may have been the centrosomes derived from the penetrating spermatozoon were identified in one ovum (Pl. 1, Fig. 10). It is possible that these structures were visible and not recognized in other preparations.

Twenty-one pronucleate ova were examined after embedding and sectioning. In fifteen of these, conjugation had begun and in seven of the fifteen the forma
tion of the first cleavage spindle appeared to be nearly complete. The remaining six ova were from a sow killed 8 hr after mating; here the pronuclei were still some distance apart.

It was possible to identify the tail of the penetrating spermatozoon in only one sectioned ovum and even in this case it was impossible to say to which of the two pronuclei it belonged; it was therefore impossible to distinguish the male from the female pronuclei in these sectioned ova. However, morphological differences between the two pronuclei were evident: usually one pronucleus showed a coarsely granular clump of chromatin on the nuclear membrane which was not shown by the second pronucleus (Pl. 1, Fig. 7). In all of the six pronucleate ova where conjugation had not yet begun, the chromatin aggregations were on the face of the nucleus opposed to the second pronucleus. In these ova, it was not possible to identify the male and female pronuclei.

No morphological distinction of the sort described above could be made between conjugating pronuclei, possibly because it was difficult to define the boundaries of the two nuclei at their interface. There is some evidence of chromatin aggregations at the interface of two conjugating pronuclei in Pl. 1, Fig. 8 and these aggregations seem to belong to the nucleus on the right but this is open to doubt; however, in view of the possibility that this feature is asso
ciated with the female pronucleus only, it is of interest to note that of the three pronuclei in Pl. 1, Fig. 9, the two smaller show some evidence of similar features. This observation is discussed in more detail below since it has an important bearing on the interpretation of the findings. The difference in the size of the pronuclei shown by the ovum in Pl. 1, Fig. 9 was shown by a total of three of nine pronucleate ova (seven of which had three or more pronuclei) recovered
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from the same sow; these are the only ova that showed a size difference between pronuclei.

The number and size of nucleoli varied widely between different pronuclei in the same ovum. These could be separated into two main classes: large nucleoli of about one-third of the diameter of the nucleus and smaller nucleoli half to one-third the size of the larger. Both classes were seen within single nuclei; morphological differences between pronuclei could not be associated with distinct differences in either size or number of nucleoli, but the nucleoli of presumptive male pronuclei tended to be fewer and of larger size than those of the presumptive female pronuclei. It was noticeable that the nucleoli were not identifiable in ova nearing the completion of the formation of the first cleavage spindle.

No detailed observations were made on the nuclear changes during cleavage. There were noticeable differences in size between the nuclei of some cleaved ova. Cleaved ova showed rounded nuclei with one to five prominent nucleoli; often these nucleoli were bounded on one side or encircled by chromatin masses similar to those described in rat ova by Austin (1953). In some ova examined as whole stained preparations, the nuclear membrane showed distinct chromatin masses resembling the 'sex chromatin' which can be seen in the motor neurones of the spinal ganglia of the pig (Cantwell, Johnston & Zeller, 1958) (Pl. 2, Fig. 17). No corresponding structure was clearly demonstrated in sectioned ova after staining with Feulgen's method although the chromatin associated with the nucleoli was clearly evident in these preparations.

Although most nuclei were uniformly rounded in outline, it was noticeable that nuclei with angular outlines were prominent among some older ova; this might be evidence of morbid changes in the older embryos but in the absence of clear evidence on this point they were regarded here as normal. A few ova were found with lobulated nuclei. Mitotic figures were observed in nearly all morulae and older embryos. Spindle remnants were identified in a few 2-cell ova.

Unfertilized and abnormal ova

Unfertilized ova showed a very variable sequence of morphological changes with time. In unfixed ova, there was a marked loss of optical contrast shown by the zona pellucida and the general outline of the ovum tended to become irregular. The changes in the nuclei were even more variable. The first evidence of degeneration was seen in the scattering of the chromosomes from the spindle. In some ova, the chromatids separated into two more or less equal groups so that the appearance was that of an ovum about to complete the second maturation division (Pl. 2, Fig. 12); in other ova, the chromosomes scattered along the circumference of the ovum (Pl. 2, Fig. 13). Some unfertilized ova contained structures that resembled normal nuclei more or less closely. Pl. 2, Figs. 14 and 15, show ova from unmated sows with one presumably diploid nucleus (Fig. 14) and with two presumably haploid nuclei (Fig. 15). These nuclear changes were found usually in ova that showed no evidence of cytoplasmic changes. Ova showing cytoplasmic fragmentation usually showed only one nucleus or none. The nuclear membrane of these nuclei was frequently incomplete. In some

D*
apparently normal 2-cell ova and in some fragmenting ova, two or more nuclei were observed in a single ‘blastomere’ (Pl. 2, Fig. 18). Four morphological classes of apparently unfertilized ova or abnormal fertilized ova could be distinguished according to the existence of one or more of the features described above. These were:

2. Degenerate ova.
   b. Cytoplasm cleaved (often irregularly, i.e. fragmented) with some or all of blastomeres anucleate.
   c. Cytoplasm cleaved. One or more blastomeres having more than one nucleus.

The distribution of these classes is analysed below. For this purpose, ova in Class 1 are called ‘normal’ unfertilized ova. Ova in Class 2 are called ‘degenerate’. It may be important that Class 2c (ova with blastomeres having more than one nucleus) was found only among ova from mated or inseminated pigs; they may therefore be degenerate fertilized ova. Examples of other classes were all found among ova from unmated sows; they are therefore known to be degenerate unfertilized ova.

Immature ova

Of a total of 1677 ova examined and classified after staining, three were judged on morphological grounds to be primary oocytes. All three showed a large vesicular nucleus with a large nucleolus and one of the three was clearly abnormal in other respects. It was smaller (diameter 135µ) than other normal ova from the same clutch (diameter 150µ). The other two oocytes observed were not noticeably different in size from normal ova in the same clutch.

Timing of Fertilization and Cleavage

Unfixed living ova

Of six sows, that had ovulated when killed less than 32 hr after the onset of heat (less than 8 hr after mating), four had ova still embedded in the egg-plug. Egg-plugs were never observed in sows killed more than 8 hr after mating, although a few cumulus cells were found attached to isolated ova. Table 1 shows the stage of cleavage of ova recovered at varying intervals after natural matings to fertile boars.

Of 293 ova from all sows killed less than 24 hr after mating, thirty-nine (13·3 %) were cleaved, thirty-seven were at the 2-cell stage and two were 3-cell ova. This group consists of sows mated at varying intervals up to 48 hr after the onset of heat. Of the thirty-nine ova recorded as cleaved, twenty-three 2-cell ova and both 3-cell ova were from ‘delayed’ matings. The remaining fourteen 2-cell ova were from two sows both killed 21 hr after mating. This is therefore the earliest record of the occurrence of cleavage other than among ova from...
### Table 1

Cleavage Classes of Unfixed Ova from Sows Killed at Varying Intervals after Natural Mating

| Class            | 0 to 24 |        | 24 to 41 |        | 48     |        | 72     |        | 96     |        | 120    |        | 144    |        | Totals |        |
|------------------|---------|--------|----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|                  | n     | %      | n     | %      | n     | %      | n     | %      | n     | %      | n     | %      | n     | %      |        |        |
| Sows             | 24    | 6      | 9     | 24     | 8     | 6      | 7     | 23     |        |        |        |        |        |        |        |        |
| Fragmented 1-cell| 254   | 53     | 40    | 66     | 1     | 10     | 7     | 22     |        |        |        |        |        |        |        |        |
| 2-cell           | 37    | 94-8   | 25    | 89-3   | 40    | 48-2   | 22    | 8-4    | 0      | 0      | 0      | 0      | 0      | 0      |        |        |
| 3-cell           | 2     | 5-1    | 2     | 7-1    | 7     | 8-4    | 10    | 3-8    | 0      | 0      | 0      | 0      | 0      | 0      |        |        |
| 4-cell           | 1     | 3-6    | 30    | 36-1   | 190   | 72-2   | 46    | 45-5   | 1      | 1-9    | 0      | 0      | 0      | 0      |        |        |
| Ova              |        |        |       |        | 6     | 7-2    | 40    | 15-2   | 1      | 1-0    | 12     | 22-6   | 0      | 0      |        |        |
| 5- to 8-cell     |        |        |       |        | 6     | 7-2    | 40    | 15-2   | 1      | 1-0    | 12     | 22-6   | 0      | 0      |        |        |
| 12-cell          |        |        |       |        | 1     | 0-4    | 4     | 3-9    | 22     | 41-5   | 16     | 36-4   |        |        |        |        |
| m                |        |        |       |        |       |        |       |        |        |        |        |        |        |        |        |        |
| b                |        |        |       |        |       |        |       |        |        |        |        |        |        |        |        |        |
| Total            | 293   | 81     | 123   | 329    | 103   | 70     | 89    | 1087   |        |        |        |        |        |        |        |        |
| Cleaved          | 39    | 13-3   | 83    | 263    | 101   | 53     | 44    |        |        |        |        |        |        |        |        |        |

**M** = morula.  
**b** = blastocyst.
'delayed' matings. The apparently premature cleavage of ova from delayed matings is discussed below.

The 2-cell ova recorded from delayed matings were recovered 6 hr (one ovum), 8 hr (one ovum), 17 hr (six ova) and 21 hr (fifteen ova) after mating. No direct comparison can be made of the findings on individual ova before and after staining but it was found after staining that all ova among litters recovered 17 hr or earlier before mating were abnormal. In some 2-cell ova from these litters, two nuclei were present in one of the two cells. A sperm tail was attached to the nucleus of one 'blastomere' in one ovum, the nuclei may therefore have been pronuclei of prematurely cleaved ova. All litters containing such abnormal cleaved ova also contained some 1-cell ova with more than two pronuclei.

The precise interval between mating and slaughter was recorded for six sows killed between 24 and 41 hr after mating. A single 3-cell and a single 4-cell ovum were recovered from one sow killed exactly 24 hr after mating; the remaining 3-cell ovum was from a sow killed 41 hr after mating. The evidence that the second cleavage may take place only 24 hr after mating consists therefore of findings on only two ova from one sow and may be open to doubt; the recorded frequencies of 2-cell ova (48·2%) and of 4-cell ova (36·1%) recovered at 48 hr are evidence that the second cleavage normally occurs about this time.

The earliest records for recovery of ova at other cleavage stages are: 5- to 8-cell, 48 hr; 12-cell, 96 hr; morulae, 72 hr (one ovum, only); blastocysts, 120 hr. No 2-cell ovum was recovered later than 72 hr after mating. One 3-cell and one 4-cell were recovered more than 120 hr after mating. The frequencies of 4-cell ova at 72 hr (72·2%) and 96 hr (45·5%) show that the 4-cell stage is of relatively long duration.

A comparison of the frequencies of ova at different stages of cleavage from sows inseminated or mated on Day 0 and from sows inseminated on Day 1 shows differences that are clearly due to the different interval between mating and the time of ovulation (Table 2).

The more advanced stage of cleavage of ova from sows inseminated on Day 1 is shown clearly for ova recovered 48 hr after insemination. By this time, 67·8% of ova from sows inseminated on Day 1 are at the 4-cell stage; for sows inseminated or mated on Day 0, the percentages of ova at the 4-cell stage are 28·9 and 36·6. This difference is not apparent 24 hr later. The proportion of 2-cell ova for all classes declines in the next 24 hr so that at 72 hr in all three groups the majority of ova are 4-celled. This is also the case at 96 hr, although substantial data are available only for pigs inseminated on Day 1. Comparing the results of matings and inseminations made on Day 0, there is little evidence of any difference in cleavage rate between the two groups, although at 96 hr only thirteen of seventy-three cleaved ova from inseminated pigs have developed beyond the 4-cell stage, compared with forty-seven of eighty-six from mated pigs.

Both mated and inseminated sows yielded some apparently cleaved ova which could not be classified with certainty. These were ova in which irregularities of cleavage were present but were insufficiently marked to be called 'fragmented'. The ova are not included in Tables 1 and 2. Of a total of 2294 ova recovered 48 to 96 hr after insemination, 125 (5·4%) were unclassifiable in this sense. Of these, thirty-five resembled 2-cell ova, sixteen resembled 3-cell
### Table 2

**NUMBERS OF FRAGMENTED (FR) AND 1-CELL OVA AND NUMBERS AND PERCENTAGES* OF OVA AT VARYING STAGES OF CLEAVAGE**

<table>
<thead>
<tr>
<th>Interval: mating to slaughter (hr)</th>
<th>Sow Class</th>
<th>fr</th>
<th>1-cell</th>
<th>2-cell</th>
<th>3-cell</th>
<th>4-cell</th>
<th>5- to 8-cell</th>
<th>9- to 12-cell</th>
<th>m</th>
<th>n</th>
<th>%</th>
<th>b</th>
<th>Totals</th>
<th>All</th>
<th>Cleaved</th>
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<tr>
<td>48</td>
<td>AI₀</td>
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<td>54</td>
<td>68-4</td>
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<td>11</td>
<td>28-9</td>
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<td>0</td>
<td>0</td>
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</tr>
<tr>
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<td>30</td>
<td>36-6</td>
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<td>11-8</td>
<td>8</td>
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<td>63-1</td>
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<td>9-2</td>
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<tr>
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<td>0</td>
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<td>1-2</td>
<td>4</td>
<td>4-6</td>
<td>88</td>
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</table>

Ova were recovered at varying intervals after mating or insemination from sows inseminated on the first (AI₀) and second (AI₁) day of heat and from sows mated on the first day of heat (M₀).

* Percentages of ova at each stage of cleavage are percentages of cleaved ova.

Figures in parentheses refer to numbers of sows.
ova and twenty-two resembled 4-cell ova; the remainder were not classified even to this extent. Most of the unclassified ova were from sows inseminated with $1 \times 10^9$ spermatozoa or less. Of 407 ova recovered 48 to 96 hr after natural mating, five (1.2%) were unclassified.

In view of the doubts that existed about some 2-cell ova, it may be significant that there is evidence in Table 2 of a difference between mated and inseminated sows in the frequencies of 2-cell ova recovered at 96 hr after mating. No 2-cell or 3-cell ova were recorded as late as this among eighty-six cleaved ova from mated sows. Of a total of eighty cleaved ova from inseminated sows, ten were 2- or 3-cell ova.

The occurrence of cleavage among unfertilized ova was examined among ova from unmated sows and from sows mated to vasectomized boars. Table 3 summarizes the findings as regards the relative proportions of 1-cell (un-cleaved) and fragmented (cleaved) ova among this material. It is shown that there is an increase in the frequency of fragmented ova, with increasing intervals between onset of heat and slaughter.

### Table 3

<table>
<thead>
<tr>
<th>Interval: onset of heat to autopsy (hr)</th>
<th>48 to 72</th>
<th>72 to 96</th>
<th>96 to 120</th>
<th>120 to 144</th>
<th>&gt;144</th>
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<tbody>
<tr>
<td>Ova 1-cell</td>
<td>33</td>
<td>147</td>
<td>65</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>Fragmented</td>
<td>0</td>
<td>27</td>
<td>30</td>
<td>23</td>
<td>63</td>
</tr>
<tr>
<td>Total [Ova]</td>
<td>33</td>
<td>174</td>
<td>95</td>
<td>41</td>
<td>66</td>
</tr>
<tr>
<td>Total [Sows]</td>
<td>3</td>
<td>13</td>
<td>8</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

**Fixed ova**

The results of observations on fixed and stained ova enable one to examine more closely the changes that occur with time in fertilized and in unfertilized ova. A total of thirty-three ova found embedded in egg-plugs from four sows killed 10, 70, 90 and 330 min after mating were examined in serial paraffin sections and all showed the second maturation spindle at the stage of arrested metaphase. Fertilization had therefore not begun in any of these ova.

Ova were examined after fixation and staining from 186 mated, inseminated and unmated sows which shed a total of 3159 ova. Of the total of 2502 recovered (79.1% of ova shed), 2002 were available for examination after fixation and staining (80.0% of recovered ova, 66.5% of all ova shed). For various reasons, it was impossible to classify all available fixed and stained ova. Most of the failures to classify ova were due to technical difficulties such as persistence of yolk platelets which obscured nuclear details or to mechanical damage to ova during mounting, but a proportion was due to difficulties of interpretation particularly of findings on ova recovered within a few hours after mating before pronucleus formation had occurred. Data are available on 163 mated or
inseminated sows with complete records. These yielded 1747 fixed ova of which 1677 were classifiable after examination. Twenty-four ova from four sows were all unclassifiable; the data as regards ova from the remaining 159 sows are summarized in Tables 4 and 5.

Distinct pronuclei were found in all of nine ova from a sow killed 6 hr after mating; this is the earliest evidence of fertilization recorded here. A total of thirty-seven pronucleate ova were recovered from five sows killed less than 12 hr after mating. Of these, seven ova were from a sow killed 11 hr after mating on Day 0 and two were from a sow killed 8 hr after mating on Day 1; none of these ova showed conjugating pronuclei but it is possible that in these sows ovulation had not occurred at the time of mating. The remaining twenty-eight ova were from sows killed 8 hr (two sows) and 6 hr after mating on Day 2; fifteen of the ova showed conjugating pronuclei. The evidence here, then, is that syngamy occurs 6 to 8 hr after mating or within an hour or two after fertilization but it may be significant that the evidence refers to ova recovered after ‘delayed’ matings.

**Table 4**

**DISTRIBUTION OF OVA ACCORDING TO STAGE OF DEVELOPMENT, FROM MATED SOWS KILLED AT VARYING INTERVALS WITHIN 24 HR AFTER MATING**

<table>
<thead>
<tr>
<th>Class</th>
<th>Interval: mating to autopsy (hr)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 to 5½</td>
<td>6 to 11½</td>
</tr>
<tr>
<td>Unfertilized</td>
<td>n</td>
<td>d</td>
</tr>
<tr>
<td>Penetrated</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Pronucleate</td>
<td>0</td>
<td>14</td>
</tr>
<tr>
<td>Cleaved</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>Ova</td>
<td></td>
</tr>
<tr>
<td></td>
<td>33*</td>
<td>81</td>
</tr>
</tbody>
</table>

Stage of development judged by examination of aceto-carmine stained whole preparations.

* Ova examined in paraffin sections of egg-plugs.

n = normal, d = degenerate (see text).

Table 4 summarizes data on the results of observations on whole mounts of all stained ova recovered within 24 hr of mating. It is shown that fertilization had begun or was complete in fifty-two of eighty-one ova recovered within 12 hr of mating and thirty-seven of these fifty-two were at the pronucleate stage; one ovum had cleaved, the rest were recently penetrated ova. There is a sharp increase in the numbers of cleaved ova among ova recovered between 18 and 24 hr after mating. It is shown that, of a total of 277 ova recovered within 24 hr of mating, 158 were fertilized; of these, sixteen were recently penetrated, 120 were pronucleate and twenty-two were cleaved. One 2-cell ovum is recorded as having been recovered from a sow killed less than 12 hr after mating. The nuclei differed morphologically from those of other 2-cell ova, being angular in outline. This ovum was recovered from a sow mated 48 hr after the onset of
## Table 5
DISTRIBUTION OF UNFERTILIZED, PENETRATED, PRONUCLEATE AND CLEAVED OVA FROM SOWS KILLED AT VARYING INTERVALS AFTER NATURAL MATING OR INSEMINATION

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Interval: mating or insemination to autopsy (hr)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 to 41</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>f</td>
</tr>
<tr>
<td>Unfertilized</td>
<td>49</td>
<td>89</td>
</tr>
<tr>
<td>Penetrated</td>
<td>26</td>
<td>60</td>
</tr>
<tr>
<td>Pronucleate</td>
<td>1</td>
<td>2-3</td>
</tr>
<tr>
<td>Cleaved ova</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-cell</td>
<td>16</td>
<td>3-7</td>
</tr>
<tr>
<td>3-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12- to 15-cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16- to 31-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertilized ova</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleaved</td>
<td>16</td>
<td>288</td>
</tr>
<tr>
<td>Total</td>
<td>43</td>
<td>307</td>
</tr>
<tr>
<td>All ova</td>
<td>92</td>
<td>429</td>
</tr>
<tr>
<td>Sows</td>
<td>9</td>
<td>38</td>
</tr>
</tbody>
</table>

Phase-contrast microscopy of aceto-carmine stained ova.

Unfertilized: n = normal; d = degenerate.

% f = percentage of fertilized ova.

% c = percentage of cleaved ova.
heat; this was also the case with the one degenerating unfertilized ovum in the same group.

Table 5 summarizes data on the stages of cleavage of ova recovered 24 hr or more after mating or insemination and examined after fixation and staining. No attempt is made to separate the results according to the interval between the onset of heat and mating but most of the inseminations were made on Day 1. The findings on these fixed and stained cleaved ova are in general agreement with those recorded for fresh ova but precise data are provided for some new classes. Of 288 cleaved ova recovered 48 hr after mating (or insemination), three were 5-cell (1.0%) and three were 7-cell (1.0%). At 72 hr, the frequencies of these classes were 2.0% and 2.6%. Inspection of the separate data for matings and inseminations shows little or no evidence of differences in rate of cleavage between the two classes of material but substantial data are available only for the periods 48 hr and 72 hr after mating.

Because cleavage is frequently used as a criterion of fertilization, it is of interest to know what proportion of fertilized ova are likely to be uncleaved at any particular time after mating. Of 330 uncleaved ova from sows killed about 72 hr after mating or insemination, fifteen were found to be pronucleate; thirteen of these were from one sow killed 66 hr after mating.

No direct comparison is possible between estimates made by the two methods on the frequencies of fertilized ova because with the procedure used it was impracticable to relate findings on an individual ovum after fixation, but there is evidence that errors occurred in judging fresh ova. When unaware of the source of the ova, six of seventy-six ova from unmated sows were judged to be fertilized; in the same circumstances, none of these ova was judged to be fertilized after fixation. Of one litter of eleven ova judged to be uncleaved, three that were examined after staining were shown to be normal morulae. Ova of this age had not previously been seen and their age was unknown when they were examined. In all other cases of ova from mated sows, the findings on ova judged to be cleaved when examined unfixed were in accord with findings after fixation and staining. These remarks do not apply to findings on ova from inseminated sows. Some apparently normal cleaved ova from inseminated sows judged to be at the 2-cell and 4-cell stage were found to have more than one nucleus in one or more blastomeres. Similar findings were recorded for ova judged to be unclassifiable before fixation.

The cytological changes that occur with time among unfertilized ova were examined again among stained ova from unmated sows and from sows mated to vasectomized boars. The findings are summarized in Table 6. The morphological criteria of 'normal' and degenerate ova are described above (see Morphology). The table shows that degenerative changes which precede fragmentation can be detected in ova; these changes were already evident in nine of twenty-three unfragmented ova recovered 48 hr after the onset of heat. Ninety-six hours after heat, fifty-four of sixty ova recovered showed degenerative changes although only twenty-three of these showed fragmentation.

The detection of the onset of morbid changes in embryos is of considerable interest. It might be expected that death of an embryo will be heralded by a lag in development, and that evidence of this might be found in an increase in
the differences in stage of development to be observed between ova within litters for ova recovered at increasing intervals after mating. Differences of this sort were found among litters of all ages but the data available are too meagre for a comparison to provide conclusive results, and there is reason to doubt how far a comparison of this sort would be valid even with more data because of lack of knowledge about the rate of cleavage of ova at different stages; but there is no real suggestion here that, as regards the stage of development, the range among ova tends to become more spread out among older litters within the range of ages studied. Considering only litters containing at least two cleaved ova, two of seven sows killed at 48 hr had only 2-cell ova; eleven of twenty-seven sows killed 72 hr after heat and one of eight killed at 96 hr had all 4-cell ova. Two of five sows killed at 144 hr had all morulae or blastocysts. The greatest spread was shown within one litter from a sow killed 120 hr after mating. This litter contained one 3-cell, one 4-cell, three 6-cell, two 8-cell, seven 12-cell ova and one morula. Morphological examinations of the embryos before and after fixation and staining yielded no clear evidence of abnormality in any of these ova.

**Table 6**

**Numbers of Cytologically Normal and Degenerate Unfertilized Ova Recovered from Unmated* Sows Killed at Varying Intervals After Onset of Heat**

<table>
<thead>
<tr>
<th>Ova</th>
<th>Intervals: onset of heat to autopsy (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 to 72</td>
</tr>
<tr>
<td>Normal</td>
<td>14</td>
</tr>
<tr>
<td>Degenerate</td>
<td></td>
</tr>
<tr>
<td>1-cell</td>
<td>9</td>
</tr>
<tr>
<td>Fragmented</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>26</td>
</tr>
<tr>
<td>Ova</td>
<td>3</td>
</tr>
<tr>
<td>Sows</td>
<td></td>
</tr>
</tbody>
</table>

* Includes sows mated to vasectomized boars.

**Numbers of Spermatozoa on Recovered Ova**

The numbers of spermatozoa reaching the ova were studied in serial sections of ova in egg-plugs recovered from four sows killed at varying intervals up to 5½ hr after mating and in whole-mounts of aceto-carmine stained ova. Spermatozoa in these preparations tend to lie in two focal planes, one above and one below the ovum (Pl. 1, Fig. 11).

No spermatozoa were found in either the egg-plug or on the ova in sows killed 10, 70 or 90 min after mating. Spermatozoa were found in the egg-plug of one sow killed 5½ hr after mating; no spermatozoa were found in contact with the ova. Table 7 shows the numbers of penetrated ova, pronucleate ova and cleaved ova distributed according to the number of spermatozoa present in the zona pellucida. The data refer only to ova recovered from sows mated naturally and examined as stained whole-mounts. It is shown that of seventeen penetrated ova none had more than fifty spermatozoa and only three had more than ten spermatozoa. In contrast to the few spermatozoa generally found on
penetrated ova, 152 of 195 cleaved ova had ten or more spermatozoa, more than half (109) had more than fifty spermatozoa and forty-nine (25%) were estimated to have more than 200 spermatozoa. Pronucleate ova tend to show more variation as regards the numbers of spermatozoa present, thirteen of a total of 138 had none but twenty-one had more than 200. The numbers of spermatozoa on ova with more than two pronuclei are apparently not different from the numbers on ova with only two pronuclei. The above data suggests that very few spermatozoa are in the vicinity of the ovum at the time of fertilization and that the number increases with time. Examination of the data as

\[ \text{Table 7} \]

**PENETRATED, PRONUCLEATE AND CLEAVED OVA FROM FORTY-FIVE MATED SOWS DISTRIBUTED ACCORDING TO THE NUMBERS OF SPERMATOZOA ON THE ZONA PELLUCIDA**

<table>
<thead>
<tr>
<th>Class</th>
<th>Numbers of spermatozoa on ova</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Penetrated</td>
<td>n</td>
</tr>
<tr>
<td>Pronucleate</td>
<td>1</td>
</tr>
<tr>
<td>(two nuclei)</td>
<td>13</td>
</tr>
<tr>
<td>Pronucleate</td>
<td>0</td>
</tr>
<tr>
<td>(three nuclei)</td>
<td>12</td>
</tr>
<tr>
<td>Cleaved</td>
<td>n = number of ova in class.</td>
</tr>
</tbody>
</table>

\[ \text{Table 8} \]

**PRONUCLEATE OVA FROM SOWS MATED AT VARYING INTERVALS AFTER THE ONSET OF HEAT, DISTRIBUTED AMONG FIVE CLASSES ACCORDING TO THE NUMBER OF SPERMATOZOA ON THE ZONA PELLUCIDA**

<table>
<thead>
<tr>
<th>Sperm numbers in class</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ova</td>
</tr>
<tr>
<td>0</td>
<td>39</td>
</tr>
<tr>
<td>1 to 10</td>
<td>14</td>
</tr>
<tr>
<td>11 to 50</td>
<td>63</td>
</tr>
<tr>
<td>51 to 200</td>
<td></td>
</tr>
<tr>
<td>&gt;200</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>157</td>
</tr>
</tbody>
</table>

regards the numbers of spermatozoa on ova recovered from sows killed at varying intervals up to 24 hr after mating provides little support for this view but the issue is confused by the effect of variations in the interval between mating and onset of heat. Sows mated early in heat were killed after a greater interval than sows mated late in heat; this was necessary to ensure that ovulation would have occurred at the time of slaughter. The effects of time of mating relative to onset of heat and of the related interval between mating and slaughter are most likely to affect the findings for pronucleate ova, since nearly all this material was collected as part of an experiment to examine the effects of time.
of mating on fertilization. Examination of these data (Table 8) shows in fact that of the twenty-one pronucleate ova recorded as having more than 200 spermatozoa present, twenty were recovered after delayed matings, i.e. at matings made 32 hr or more after the onset of heat.

In view of the suggestion that the rate of cleavage increases with increasing sperm numbers, the numbers of spermatozoa on 4-cell ova recovered at 48 hr have been compared with the numbers on 4-cell ova recovered at 72 hr after mating. This is the largest homogeneous sample of data available for comparison of this sort. The results (Table 9) show that the ova recovered earlier tend to have fewer spermatozoa than those recovered later; this is contrary to what might be expected if larger sperm numbers caused precocious cleavage.

**Table 9**

<table>
<thead>
<tr>
<th>Interval: mating to recovery (hr)</th>
<th>Number of spermatozoa on zona pellucida</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1 to 10</td>
</tr>
<tr>
<td>48</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>72</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

**DISCUSSION**

**FERTILIZATION**

A survey of previously published work shows that until 1955 no real account was available of the changes that occur in fertilized pig ova. Corner & Amsbaugh (1917) say that they examined fertilized ova before the stage of pronucleus formation but gave no information at all about their findings. Heuser & Streeter (1929) and Green & Winters (1946) were concerned only with the later stages of embryonic development and their papers contain virtually no information about the cytological changes associated with fertilization. Recently, Pitkjanen (1955) and Thibault (1959) have both published accounts based on the examination of sectioned ova and Spalding, Berry & Moffat (1955) have described the maturation changes shown by pig ova examined in stained whole-mounts.

The account given here of the morphological changes that follow penetration and fertilization agrees well with previous findings but some points of difference deserve mention. Both Pitkjanen and Thibault claimed to be able to distinguish the male from the female pronucleus. In both cases, the initial distinction is the presence of the sperm tail in the immediate vicinity of the male pronucleus. Using this feature as a marker, they both found that the female pronucleus shows characteristic concentrations of chromatin on the nuclear membrane at the face opposed to the male pronucleus. Because precise figures are given by neither author, it is impossible to know how frequently it is possible to make the distinction in this way, but Thibault suggests that it is possible in nearly every case although he implies that the sperm tail is seen more easily in some
preparations than in others. Whatever the difficulties about identifying the male pronucleus by the sperm tail, there is agreement here with Pitkjanen and Thibault that the two pronuclei of normal ova differ morphologically as regards the disposition of the chromatin and in this investigation, in the few cases where it was possible to identify with certainty the male pronucleus, the findings were in complete agreement with the two earlier observers that the female pronucleus shows characteristic chromatin concentrations which are absent from the male pronucleus. The observations here also agree with Thibault’s findings that the nucleoli of the male pronucleus are larger and fewer than those of the female pronucleus, but one must conclude that the nature of the evidence so far on the morphological differences between the two pronuclei is such that the only safe criterion is the presence of a sperm tail clearly associated with one (the male) pronucleus. It seems clearly desirable to continue to examine critically the evidence that one can separate the two pronuclei by other morphological criteria.

The distinction between the male and female pronuclei presents a problem of particular urgency when dealing with ova with more than two pronuclei. Pitkjanen’s findings enabled her to class all ova with more than two pronuclei as polyspermic. The findings on this point in this investigation have been presented earlier, elsewhere (Hancock, 1959). It was shown then that an increase in the frequency of ova with more than two pronuclei was associated with delayed mating. No evidence of retention of the second polar body was found in any ovum and it was concluded that the presence of more than two pronuclei was probably evidence of polyspermic fertilization.

Thibault found evidence of retention of the second polar body in eleven of fifty-three ova from six sows killed more than 37 hr after mating or insemination. He does not say precisely what were the criteria used to distinguish polyspermic ova from those said to show retention of the second polar body; but he says that when retention occurs it can easily be recognized because remains of the second maturation spindle can be found in the region of the most peripheral pronucleus. Presumably, this evidence was taken into consideration with other evidence as regards the presence or absence of spermatozoa and nuclear morphology. Because the observations made here were not made on permanent preparations, it is impossible to re-examine them in the light of Thibault’s observation, but it is of interest that in one photomicrograph, of an ovum classified as polyspermic (Pl. 1, Fig. 9) two of the three nuclei show traces of features that are now believed to be characteristic of female pronuclei. Dr Thibault has seen this photograph and has expressed the opinion that it is an example of a polygynic ovum, but even if one accepts this interpretation of the findings the revision does not modify the conclusions drawn earlier about the likely consequences for the embryo of delayed mating.

Pitkjanen’s account contains little information on the timing of the events in fertilization. The findings here that fertilization takes place about 6 hr after mating are virtually the same as Thibault’s, although one cannot see any need to invoke, as Thibault does, the phenomenon of ‘capacitation’ to explain the delay. The evidence here suggests that the delay may be the time needed for the spermatozoon to reach the ovum. It is true that spermatozoa reach the Fallopian
tube much earlier than 6 hr after mating but there is no evidence that they reach the site of fertilization in much less than 5 hr. In this connection, it is puzzling to note that Thibault in considering the probable time of fertilization in the pig apparently did not find it necessary to examine ova from pigs killed earlier than about 5 hr after mating; one concludes that he had access to other data not referred to in his paper. It is difficult to know what weight to attach to Thibault’s statement that penetration and pronucleus formation up to the stage of syngamy occupy about 1 hr. It is perhaps important that Thibault’s findings and those recorded here, that syngamy takes place about 6 to 8 hr after mating, were all made on sows where mating was delayed; hence, in both cases the observations were made on ova differing greatly in age from newly fertilized ova from sows mated at the normal time. Since the behaviour of ageing ova at fertilization has been shown to differ so strikingly from that of ova of normal age, it seems unwise to draw conclusions about normal ova from observations made on ageing ones.

**CLEAVAGE**

It has been shown that cleavage may occur in unfertilized ova and this probably explains the isolated findings of cleaved ova from sows killed within a few hours of mating. The weight of evidence here is that the first cleavage takes place not earlier than 20 hr after mating; this agrees well with Thibault’s estimate. An examination of the distribution of 2-cell and 4-cell ova from sows killed at varying intervals after mating suggests that the second cleavage does not normally occur until at least 12 hr later than this. The findings here of two apparently normal 4-cell ova from one sow killed only 24 hr after mating and Green & Winter’s (1946) finding of two 3-cell and two 4-cell ova from a sow killed 25 hr after mating are obviously exceptional.

Although there are difficulties about comparing the present findings with those recorded by earlier workers, the evidence is that here subsequent development is slower than has previously been found to be the case.

In this investigation, no ovum recovered within 72 hr of a single mating or insemination had developed beyond the 8-cell stage and the evidence here is that this stage is not usually reached until 96 hr after mating, but Heuser & Streeter (1929) found 12-cell embryos at 72 hr and Green & Winters (1946) recorded the finding of morulae at this time. Blastocysts were recovered by Heuser & Streeter 114 hr after the sows had been mated.

Heuser & Streeter also recorded the finding of three morulae from a sow killed 47 hr after mating and their text-figure, showing graphically the number of ova in each cleavage class plotted against time after mating, is somewhat misleading in that these morulae are omitted, without direct comment, but apparently because the number of cells per morula could not be counted. Heuser & Streeter clearly never regarded their modest data on the cleavage stages of the pig ova as more than an approximate statement, but later workers (Pincus, 1936), in the absence of more reliable data, have tended to credit them on this point with an authority that they never claimed.

An outstanding feature of the data on cleavage rate is the way in which the results are affected by the time of mating or insemination. For example, 48 hr
after insemination, 68% of cleaved ova from sows inseminated on Day 0 are at the 2-cell stage and only 29% at the 4-cell stage; these proportions are virtually reversed for sows inseminated on Day 1 as might be expected if the first and second cleavages occur with an interval of 24 hr. (Ovulation occurs on the second day of heat so that the ova from sows mated on Day 0 are unlikely to be fertilized until 24 hr later.) In view of these observations, it is surprising to find how little comment this factor has received in accounts of some investigations that set out to deal with the rate of cleavage of pig ova. Some workers have acknowledged the importance of time of mating but have failed to say specifically what their own mating procedure was. Green & Winters (1946) say that 'the oestrous cycles of sows were charted and the females were bred as near to heat as possible'. More information is needed to know exactly what was done, but it seems reasonably certain that these sows were mated not less than 15 hr after the onset of heat. It is not said whether or not the boars had free access to the sows or were handmated. Pomeroy's (1955) material was from sows mated twice but he does not give the precise interval between recovery of ova and mating.

Heuser & Streeter (1929) were less informative. In their experiments, sows were mated and killed in circumstances that probably prevented any critical supervision of the timing of the operations.

More recently, Pitkjanen (1955) has published data that are presented as evidence that cleavage rate is more rapid in pigs mated twice than in pigs mated once. Pitkjanen's data have been recalculated so that they can be compared with other data (Table 10). Unfortunately, the original data on the numbers of ova from sows in the two experimental groups give only the percentages of ova in each cleavage class and these percentages do not total 100, so that in recalculating them it is impossible to account for some ova in each experimental group. These difficulties aside, the only evidence of any difference in cleavage rate is the difference in the frequencies of ova developed beyond the

**Table 10**

**NUMBERS AND PERCENTAGES OF OVA AT DIFFERENT STAGES OF CLEAVAGE AMONG CLEAVED OVA FROM SOWS MATED ONCE AND FROM SOWS MATED TWICE**

<table>
<thead>
<tr>
<th>Cleavage stage</th>
<th>Single matings</th>
<th>Double matings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>1-cell</td>
<td>205</td>
<td>29-4</td>
</tr>
<tr>
<td>2-cell</td>
<td>62</td>
<td>64-8</td>
</tr>
<tr>
<td>3- to 4-cell</td>
<td>131</td>
<td>3-3</td>
</tr>
<tr>
<td>5-cell</td>
<td>7</td>
<td>1-0</td>
</tr>
<tr>
<td>6- to 10-cell</td>
<td>2</td>
<td>9-0</td>
</tr>
<tr>
<td>Total ova</td>
<td>407</td>
<td>283</td>
</tr>
<tr>
<td>Cleaved ova</td>
<td>202</td>
<td></td>
</tr>
</tbody>
</table>

* Data recalculated from Pitkjanen (1955).
Figures in parentheses refer to original totals (see text).
4-cell stage and in twice-mated sows more than half of these are only at the 5-cell stage. The frequency of ova at this stage (10-2% of cleaved ova from twice-mated pigs) is much higher than that recorded here (1-0% at 48 hr and 2-0% at 72 hr after mating) but the percentages for this class given here refer to ova examined after fixation and staining. The difference between the experimental groups was said to hold for ova recovered 72 and 96 hr after mating, but only the data for the 48-hr group are presented. The usefulness of this data is limited by the fact that not all unfertilized ova may be cleaved by this time and among the uncleaved group it is impossible to distinguish between fertilized and unfertilized ova. The differences in the proportion of cleaved ova between the two groups could well be due to either differences in frequency of fertilization or differences in cleavage rate. If the data are intended to show differences in cleavage rate, then they are less well suited to this purpose than data (which are not given) obtained from sows killed later, at times when differences due to fertilization failures would be evident.

These criticisms aside, it is clear that some ova obtained by Pitkjanen from sows killed 48 hr after mating were at a more advanced stage of cleavage than anything observed at the same stage in these investigations where single matings or inseminations have been used throughout. However, differences between sows in such characteristics as duration of oestrus and time of ovulation might well account for these differences; differences in rate of cleavage are said to occur between different strains of rabbit (Castle & Gregory, 1925).

One of the features of the data on cleavage of pig ova as measured in stained preparations of whole ova is the very clear evidence of the occurrence of asynchronous cleavage. Three-cell, 5-cell and 7-cell ova are clearly normal stages which could arise respectively by cleavage of a single blastomere of a 2-cell ovum, of a single blastomere of a 4-cell ovum and of three of the four blastomeres of a 4-cell ovum, although Heuser & Streeter, after a careful analysis of the relative sizes of the different blastomeres, give a different interpretation of the origin of these ova.

ABNORMAL AND UNFERTILIZED OVA

Anomalies at fertilization in the pig are apparently to be associated mainly with disturbances occurring within originally normal ova; the total incidence of ova considered to be intrinsically abnormal was low in this investigation. The apparently premature cleavage observed among ova from delayed matings may be related to the phenomenon of ‘immediate cleavage’ described by Braden & Austin (1954a) in the mouse. They found that hot-shock treatment of mouse ova 8 to 12 hr after ovulation was followed by cleavage 4 to 5 hr later. They suggested that this cleavage was the result of completion of the second meiotic division in an ovum where the spindle had previously migrated to the centre of the ovum. They later (1954b) suggested that the finding of two nuclei in a single blastomere in prematurely cleaved hot-shocked ova might be due to penetration by a spermatozoon, the second nucleus being a male pronucleus formed from the penetrating spermatozoon.

The findings recorded here of the changes observed with time in fresh unfixed unfertilized ova are in good agreement with previous observations by Dziuk
There appears to be no previous detailed study of the cytological changes that take place in unfertilized pig ova, although Bischoff (1844), Spalding, Berry & Moffat (1955) and Pitkjanen (1955) refer briefly to the degenerative changes of the pig ovum.

Previous descriptions exist of the cytological changes that occur spontaneously in vivo in unfertilized ova of the mouse (Charlton, 1917), rat (Mann, 1924, Austin, 1949), opossum (Smith, 1925), rabbit (Pincus, 1930), hamster (Austin, 1956), ewe (Thibault, 1949) and ferret (Chang, 1957). The findings here suggest that changes resembling those that occur in fertilized ova are more common in the pig than in other species, with the exception of the hamster and possibly the ferret, but it is important to note that all 'cleaved' ova recovered from unmated sows have been found to be cytologically different from normal cleaved ova.

Multinucleate cells were found here in cleaved ova only from sows that had been in contact with spermatozoa. Thibault also recorded these only from inseminated sows (although he does not comment on the finding from this point of view) and it seems likely that these are abnormal fertilized ova; this interpretation can be applied to similar ova (believed to be unfertilized) found in the mouse (Charlton, 1917) and opossum (Smith, 1925), since it is clear that the material used in those investigations included ova from females in contact with males; but this interpretation will not hold for Mann's observations on rat ova because her animals had been isolated from the male since before parturition. One must agree with Thibault that the precise origin of these abnormal ova is obscure. Budding of nuclei described by Charlton in the mouse was not found in pig ova known to be unfertilized; a few nuclei with similar features were observed in otherwise normal ova from mated pigs.

These findings on the changes that occur in unfertilized ova are of particular interest. Estimates of the proportions of fertilized ova from inseminated pigs are frequently used to measure the success of inseminations in experimental investigations. The usual criterion of fertilization is cleavage, but from Dziuk's (1960) observations and from those reported here it is clear that cleavage of unfertilized ova occurs with increasing frequency with time after ovulation and is likely to be a considerable source of error among estimates of fertilization based on cleavage of ova recovered more than 72 hr after the onset of heat. Errors of this sort may explain the observation that the fertility of certain experimental inseminations is apparently higher when judged on the frequency of cleavage among ova recovered 3 to 4 days after insemination than when judged on the numbers of foctuses recovered 3 to 4 weeks later. (Dziuk, 1959, Self, 1959). It is reasonable to conclude that where cleavage is to be used as evidence of fertilization, the most suitable time for the recovery of ova is about 72 hr after the onset of heat for sows mated on the first or second day of heat.

If it is impracticable to kill pigs at this time and one has to choose between recovery of ova at 96 hr or later (when there is a real possibility of classifying unfertilized ova as fertilized) and earlier recovery (with the risk that some fertilized ova will not yet be cleaved), then it seems advisable to prefer the risk of false negative information and to choose the earlier time. Where facilities exist for examining stained preparations of whole ova, the false negatives can be
virtually eliminated by routine examination by this method and this enables one
to detect cleaved ova that are unfertilized. Although, therefore, the recom-
mandation here is that examination should be made 48 or 72 hr after the onset
of heat, it is clear that there is a need for a critical examination of the value
of estimates of fertilization based on the microscopical features of pig ova.
However, there are difficulties about designing an experiment to examine the
problem critically. The most obvious approach would be to compare estimates
made on coded samples of ova from mated sows and from unmated sows. The
great difficulty here is that cleaved ova recovered more than 48 hr after mating
usually show large numbers of spermatozoa, which are detectable at the low
magnification used for examining ova for cleavage, and uncleaved ova recovered
at the same time generally show few or no spermatozoa. This means that the
probability that an ovum is fertilized can be judged in these circumstances
from the presence or absence of spermatozoa and this reduces the value of
information about the value of cleavage as a criterion of fertilization when it
must be used to distinguish fertilized ova from unfertilized ova in circumstances
where fertilized ova can be expected to have very few or no spermatozoa as, for
example, among ova from sows inseminated with small numbers of sperma-
tozoa.

For these reasons, it is now standard practice in this laboratory to code all
material so that decisions about the occurrence of cleavage are made in circum-
stances that make it difficult for the observer's decision to be biased by having
other information about the probability of fertilization. It is probable that,
without these precautions, estimates of fertilization based on cleavage of ova
are susceptible to considerable errors.

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EXPLANATION OF PLATES

Unless otherwise stated, all are phase-contrast photomicrographs of aceto-carmine stained whole-
mounts. (Times given refer to time of recovery of ova.)

Fig. 1. Paraffin section of ovum in dispersing cumulus-cell mass (egg-plug). First polar body and
chromosomes on second maturation spindle at periphery (1 o'clock). Note vacuolation of cytoplasm
and unvacuolated cortex at periphery. 10 min post coitum. Haematoxylin and eosin. ×125.
Fig. 2. Second maturation spindle at periphery of unfertilized ovum. Note cortical unvacuolated layer
and vacuolated cytoplasm below. 48 hr post coitum. ×600.
Fig. 3. Pronucleate ovum showing first and second polar bodies. Polar body on right shows attached
spindle remnant (arrowed). 8 hr post coitum. ×450.
Fig. 4. Spermatozoon in zona pellucida of penetrated (see below) ovum. 8 hr post coitum. ×450.
Fig. 5. Penetrating spermatozoon in vitellus. Same preparation as Fig. 3. ×450.
Fig. 6. Nuclear apparatus of penetrated ovum shown in Figs. 4 and 5. Presumptive female pronucleus
lies to right of second polar body at 9 o'clock position (arrowed). ×450.
Fig. 7. Conjugating pronuclei showing chromatin clumps at face of presumptive female pronucleus
(right). Note larger nucleolus of presumptive male pronucleus. 8 hr post coitum. Ester-wax section.
Weigert's iron haematoxylin. ×550.
Fig. 8. Conjugating pronuclei showing coarse chromatin clumps (arrowed) on face of right (?) female
pronucleus. See text. 48 hr post coitum. ×460.
Fig. 9. Three conjugating pronuclei, with evidence of chromatin clumps on nuclear membrane of two
smaller pronuclei (arrowed). 6 hr post coitum. ×460.
Fig. 10. Pronucleate ovum showing two centrosomes (arrowed) near lower (male) pronucleus. Note
large nucleolus of lower pronucleus (arrowed). (The nuclear membrane of the lower pronucleus is not
in focus.) 8 hr post coitum. ×425.
Fig. 11. Spermatozoa in zona pellucida of 4-cell ovum. 96 hr post coitum. ×460.
Fig. 12. Unfertilized ovum from inseminated sow showing separation (spontaneous activation) of diads into two groups. 72 hr post oestrum. \( \times 460 \).

Fig. 13. Unfertilized ovum from sterile mated sow showing scattering of chromosomes in cytoplasm. 96 hr post oestrum. \( \times 600 \).

Fig. 14. Unfertilized ovum from sterile mated sow showing single (diploid ?) pronucleus. Refractile material is uncleared yolk. 120 hr post coitum. \( \times 460 \).

Fig. 15. Unfertilized ovum from sterile mated sow showing two (haploid ?) pronuclei. Whole-mount. 120 hr post oestrum. \( \times 460 \).

Fig. 16. Late morula showing mitotic figures. Direct light. 144 hr post coitum. \( \times 330 \).

Fig. 17. 4-cell ovum showing bodies on nuclear membrane resembling sex chromatin. 72 hr after insemination. \( \times 270 \).

Fig. 18. Ovum showing blastomere with two nuclei (arrowed). 72 hr after insemination. \( \times 400 \).

(Facing p. 329)
NUMBERS OF SPERMATOZOA ON OVA

One of the advantages of the method used here to examine fixed and stained whole ova is that it is easy to count spermatozoa present on the zona pellucida of the ovum. No precise estimate has been made of the errors likely to affect counts but it has been shown (Hancock, 1959) that the method is sufficiently sensitive to detect differences between numbers of spermatozoa on ova from mated and from inseminated sows, and more recently it has been shown (Hancock & Hovell, 1961) that differences between ova from sows inseminated with different numbers of spermatozoa can also be detected. There is thus reason to believe that counts of spermatozoa on whole fixed and stained ova give a more or less accurate estimate, at least for comparative purposes, of the numbers of spermatozoa reaching the ova and that the method can be used for this purpose in the same way as counts made on whole unfixed ova (Adams, 1956) or on formalin-fixed unstained ova (Hancock, 1957). In view of results of extensive use of the method, it is difficult to understand Thibault’s statement that one of the disadvantages of the method used here is that it does not permit sperm counts to be made; this is very clearly not the case. One might reasonably expect that counts made in this way would be more accurate than counts made on sectioned ova where there is an obvious risk that spermatozoa may appear in more than one section, but in fact a comparison of the results obtained here with whole ova with the results obtained by Pitkjanen and by Thibault on sectioned ova agree very well. Very few spermatozoa are present in the vicinity of the ovum at the time of fertilization. Thibault found not more than six spermatozoa on fertilized ova recovered up to 7 hr after mating; here, of seventeen penetrated ova recovered before the stage of pronucleus formation, fourteen had less than ten spermatozoa. The numbers of spermatozoa increase with the increasing interval between mating and recovery. The finding that numbers of spermatozoa are greater on ova at more advanced stages of cleavage than on earlier stages is regarded by Pitkjanen as evidence that cleavage is more rapid in ova with more spermatozoa. This conclusion is, of course, quite unjustified on evidence consisting of ova recovered at different times from different sows. A within-sow analysis would not be free from the same defect because of the possibility that ova are shed at different intervals; here again, the issue might be confounded by the strong possibility that ova at later cleavage stages have more spermatozoa because they have been in contact with them for a longer time. The results given here of counts of the number of spermatozoa on ova recovered at the same cleavage stage, at different intervals after mating, do not support her view. Pitkjanen’s argument that increased sperm numbers are associated with increased cleavage rate was used to explain in part the observation that double mating increases the rate of cleavage. In this connection, she says that the majority of ova from the once-mated group contained less than 100 spermatozoa and the majority of ova from twice-mated sows contained more than this number. This statement as it stands is not very convincing. One must conclude that the interesting interpretations offered by Pitkjanen, which cannot be rejected out of hand, need to be substantiated by critical experimentation.

To return to the observation that very few spermatozoa are present at the time of fertilization; sperm numbers on the ovum increase with time after
fertilization presumably because either spermatozoa reach the ovum in increasing numbers or because, with time, there is an increased likelihood that if they reach the ovum they will remain in contact with it. The first explanation seems the simpler and therefore more likely, and it remains to be seen if the increased rate of contact is due to upward movement of the spermatozoa or to downward movement of the ovum. In view of the evidence about the rate of tubal journey of the pig ovum (Anderson, 1927) and the distribution of spermatozoa in the Fallopian tube (du Mesnil du Buisson & Dauzier, 1955), it seems reasonable to conclude that the increase in sperm numbers occurs as the ovum migrates into a medium containing an increasing concentration of spermatozoa. (It is worth noting here that Anderson’s estimate of the time taken for the ovum to traverse the different sections of the Fallopian tube needs to be revised, because of her erroneous assumption that the tubal journey takes 72 hr. Pomeroy (1955) has shown that the actual time is less than 48 hr.) Although there is no clear evidence here that polyspermic fertilization is associated with increased sperm numbers at the time of fertilization, it could well be that with delayed mating the ovum meets a high concentration of spermatozoa at its first contact because it is already in the lower part of the tube when mating takes place. In these circumstances, the time available for the operation of a block to polyspermy (which in the pig is apparently associated with a ‘zona reaction’) will be shorter than in conditions of normal mating where successive spermatozoa probably reach the ovum at greater intervals of time; it is equally possible that there is an increase with the age of the egg of the time needed for completion of the block to polyspermy. Both factors may be important as causes of polyspermic fertilization in delayed matings.

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