THE EFFECTS OF DELAYED INSEMINATION ON FERTILIZATION AND EARLY CLEAVAGE IN THE PIG*

R. H. F. HUNTER†

*A.R.C. Unit of Reproductive Physiology and Biochemistry,
University of Cambridge*

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Summary. Eggs were examined from a control group of gilts (Group 1) which were inseminated at least 6 hr before ovulation, and from five other groups (Groups 2 to 6) in which the postovulatory age of the eggs was estimated to be 4, 8, 12, 16 or 20 hr at the time of sperm penetration. Penetrated eggs were obtained from twelve animals in each group.

The conception rate fell from 100% in the control group to 66.7% in Group 6, whereas the incidence of unilateral fertilization increased from 8.3% in the controls to 33% in Group 6. Within the seventy-two animals with some penetrated eggs, the mean percentage of unfertilized eggs increased from 1.3% (Group 2) to 23.9% (Group 6), but unfertilized eggs occurred principally as a result of unilateral fertilization.

No decrease in the mean proportion of fertilized eggs developing normally was found until the eggs were 12 hr old at penetration (Group 4), when the figure fell to 73.6%. A further decrease occurred in Groups 5 and 6, means of 55.4% and 66.9% respectively, being obtained. The mean percentage of polyspermic eggs increased from 1.3% in Group 2 to 15.4% in Group 5, and the proportion of eggs showing varying degrees of fragmentation also increased from 3.8% in the control group to 29.2% in Group 5.

The results indicate that a very high proportion of eggs can be fertilized up to 8 hr after ovulation and undergo apparently normal development at least until the 4- to 8-cell stage.

INTRODUCTION

The fertilizable life of mammalian eggs is generally considered to be quite short, and in several species lowered fertility frequently occurs when mating is delayed until after the time of ovulation. The early studies of Hammond & Walton (1934) in the ferret, and Hammond (1934) in the rabbit, attributed this loss of fertility to an increase in the proportion of eggs failing to undergo

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† Present address: Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts, U.S.A.

fertilization, but increased embryonic mortality may also result from fertilization of aged eggs (for guinea-pig, Blandau & Young, 1939; rat, Blandau & Jordan, 1941; Braden, 1959; rabbit, Chang, 1952).

A number of studies in the pig have indicated that matings or inseminations made late in oestrus tend to result in lowered fertility, as measured by both the proportion of eggs fertilized (Hancock & Hovell, 1962) and embryonic survival rates (Burger, 1952). It is, therefore, important to make an accurate estimate of the viable life of the ovulated egg, especially as ageing eggs are more likely to be encountered under conditions of artificial insemination than natural mating. Moreover, ageing of one or both of the gametes could be a contributory factor to the high incidence of embryonic mortality which has been reported for this species (for review see Hanly, 1961).

An essential preliminary to a study of the viable life of the egg would be an accurate method of pre-determining the time of ovulation. This is possible by means of a single injection of human chorionic gonadotrophin (HCG) given

TABLE 1
THE ESTIMATED AGE OF EGGS AT THE TIME OF SPERM PENETRATION IN SIX GROUPS OF PIGS INSEMINATED AT VARYING INTERVALS AFTER INJECTION OF HCG

<i>Group</i>	<i>Interval from HCG injection to insemination (hr)</i>	<i>Estimated age of eggs at sperm penetration (hr)</i>	<i>No. of animals yielding some penetrated eggs</i>
1 (controls)	<36	<4	12
2	40	4	12
3	44	8	12
4	48	12	12
5	52	16	12
6	56	20	12

during pro-oestrus (Dziuk, Polge & Rowson, 1964). In the experiment reported below, induced ovulation has been used as a means of studying the fertilizable life of eggs, and the effects of delayed insemination on fertilization and early cleavage.

MATERIALS AND METHODS

Design of experiment

For the purposes of the experiment, 42 hr was taken as the time after HCG injection when completed ovulation would have occurred, and 6 hr as the interval that would elapse between insemination of an oestrous animal and penetration of the eggs.

This time schedule was based on the following observations. A preliminary study (Hunter, 1965) indicated that in Large White × Essex gilts, ovulation occurred between 41 and 42 hr after an HCG injection given during pro-oestrus. The results of a number of other studies indicated that 6 hr would elapse between insemination of an oestrous animal and penetration of the eggs. Rodolfo (1934), Burger (1952) and Ito, Kudo & Niwa (1959) reported finding

spermatozoa in the oviducts within an hour of mating, and du Buisson & Dauzier (1955) located spermatozoa in the ampullary region of the oviduct within 2 hr of inseminating animals that were in mid-oestrus, but Thibault (1959) did not observe any penetrated eggs until 5½ hr after mating. The earliest evidence of sperm penetration recorded by Hancock (1961) was 6 hr after mating, and similar figures have been given by Baker & Dziuk (1964) and Polge (unpublished).

Inseminations were timed so that eggs should be penetrated at specific intervals after ovulation. Eggs were examined from six groups of animals: a control group in which the animals were inseminated at least 6 hr before ovulation, and five other groups in which the postovulatory age of the eggs was estimated to be 4, 8, 12, 16 or 20 hr at the time of sperm penetration (Table 1 and Text-fig. 1).

Penetrated eggs were obtained from twelve animals in each group, six animals being killed 14 to 20 hr after insemination so that eggs could be recovered at the pronucleate stage, and six, 40 to 72 hr after insemination so that uterine eggs would be recovered at the 4- to 8-cell stage. Sufficient animals were treated

TEXT-FIG. 1. Experimental procedure for obtaining sperm penetration of eggs 4 hr after ovulation in the pig.

until at least one penetrated egg was recovered from each of twelve animals in any particular group.

Animals

Large White x Essex gilts, weighing between 200 and 275 lb and approximately 6 to 8 months of age, were purchased from a number of local breeders. They were penned and fed as groups of five to ten, and tested for oestrus twice daily in the presence of a vasectomized boar, the latter being prevented from mounting. Animals with oestrous cycles within the range of 20 to 22 days were selected for treatment.

Induction of ovulation

Ovulation was induced by an intramuscular injection of 500 i.u. HCG (Lutormone, Burroughs Wellcome) in 5 ml sterile saline given during late pro-oestrus. This stage was indicated by vulval swelling and colour, as well as by reference to the previous oestrous cycle. Designating the first day of oestrus as Day 0, all injections were made on Day 19 or 20 of the cycle, and it was confirmed that animals would not stand to the boar at this time.

Collection and insemination

All inseminations were made with fresh, undiluted semen collected from boars of known fertility. The frequency of collection from individual boars rarely exceeded three times a week, and semen samples were examined routinely for the motility and density of spermatozoa using a heated stage microscope.

Insemination was performed intracervically using a spiral rubber catheter (Melrose & O'Hagan, 1961) and a collapsible polythene bottle (150 ml), from which the semen was expelled by gentle manual pressure. The volume of semen used varied from 80 to 120 ml, insemination taking place within 30 min of collection. Only animals that were in oestrus at the scheduled time were inseminated.

Procedure at autopsy

After stunning and bleeding, but before scalding or evisceration, the reproductive tract was removed and returned to the laboratory within 20 min in a dry vacuum flask. Observations were then made on the gross morphology of the ovaries, and the number and size of the corpora lutea and follicles recorded.

Egg recovery

The oviducts and uterine horns were dissected free from their ligaments, separated just caudal to the utero-tubal junction, and flushed with 0.9% saline into polythene petri dishes. Eggs were located under a stereoscopic microscope ($\times 12.5$) and individually transferred to a covered watch glass (5 cm diameter) containing saline. They were then examined for the presence of spermatozoa in the zona pellucida and for evidence of cleavage or fragmentation ($\times 100$).

Treatment of eggs

Whole mounts were prepared, immersed in fresh acetic alcohol (25% acetic acid by volume) and left approximately 24 hr for fixation and clearing. After staining with 1% natural orcein in 45% acetic acid, detailed cytological examination of the eggs was made by phase-contrast microscopy. The nuclear morphology of both cleaved and uncleaved eggs, the number of nuclei and the number of polar bodies were recorded. The presence of any other chromatin that could have arisen from either abnormal development or polyspermy was noted. Eggs were then classified as: (a) fertilized and developing normally, (b) fertilized but developing abnormally, or (c) unfertilized.

Criteria of fertilization

Single-cell eggs were considered fertilized if they contained the presumptive male and female pronuclei, even though the stage of syngamy may not have been reached or, alternatively, if they showed metaphase, anaphase or telophase of the first mitotic division. The extruded second polar body could be seen on such eggs, and in most cases the first polar body could also be detected.

Cleaved eggs were considered fertilized if each blastomere contained a single rounded nucleus with a complete nuclear membrane and one or more nucleoli. Chromatin strands or masses were also normally visible in such nuclei, but these criteria had to be modified if a mitotic division was taking place. The

nuclear findings were interpreted only after a careful consideration of the general morphology of the egg.

Eggs were classified as unfertilized on the basis of the morphological features described by Hancock (1961). Abnormal forms of fertilization are considered below.

RESULTS

Oestrous behaviour

Four animals did not come into oestrus after the HCG injection, and one animal in Group 5 was no longer in oestrus at the time scheduled for insemination.

The interval from HCG injection to the onset of oestrus in the eighty-seven inseminated animals varied from less than 12 hr to more than 24 hr. Consequently, the point during oestrus at which an animal was inseminated was not known precisely. Thirteen of the animals (14.9%) were in oestrus for less than 24 hr.

TABLE 2

THE CONCEPTION RATE (PERCENTAGE OF ANIMALS WITH PENETRATED EGGS) AND THE FREQUENCY OF UNILATERAL FERTILIZATION IN PIGS INSEMINATED AT VARYING INTERVALS AFTER INJECTION OF HCG

<i>Interval from HCG injection to insemination (hr)</i>	<i>No. of animals inseminated</i>	<i>No. with penetrated eggs</i>	<i>Conception rate (%)</i>	<i>Incidence of unilateral fertilization (%)</i>
< 36	12	12	100	8.3
40	12	12	100	0
44	13	12	92.3	0
48	14	12	85.7	8.3
52	18	12	66.7	25.0
56	18	12	66.7	33.0

Conception rate

The proportion of inseminated animals with one or more penetrated eggs decreased with the delay of insemination (Table 2). A conception rate of 100% was obtained in both Groups 1 and 2, but it then declined progressively to 67% in Groups 5 and 6. Fifteen of the animals inseminated late were completely infertile.

Recovery of penetrated eggs from one side of the tract only also increased with the delay of insemination, reaching figures of 25 and 33% in Groups 5 and 6, respectively (Table 2). The overall incidence of unilateral fertilization was 12.5%.

Ovulation rate

Within the eighty-seven inseminated animals a total of 1209 corpora lutea was counted at autopsy, giving a mean ovulation rate of 13.9, with a range from 10 to 19. All ovaries were of normal macroscopic appearance. Injection of HCG seemed to be a very efficient method of inducing ovulation, since the

diameter of unovulated follicles rarely exceeded 4 to 5 mm, and such follicles were very few in number.

Egg recovery

A total of 1135 eggs was recovered, representing an overall recovery rate of 93.9%. Within the seventy-two animals yielding some penetrated eggs, 937 or 94.3% of the eggs were recovered (Table 3). All the eggs were recovered in thirty-five (48.6%) of these animals. The oviducts were never flushed for eggs later than 33 hr after the estimated time of ovulation, and in many instances considerably earlier. On the other hand, recovery of uterine eggs was never attempted earlier than 46½ hr after the estimated time of ovulation. The mean recovery rate for tubal eggs was 97.1%, as compared with 91.5% for eggs recovered from the uterus. The majority of the eggs had therefore left the oviducts 46½ hr after ovulation.

TABLE 3
THE NUMBER OF EGGS SHED AND RECOVERED FROM PIGS
INSEMINATED AT VARYING INTERVALS AFTER INJECTION
OF HCG

<i>Interval from HCG injection to insemination (hr)</i>	<i>Total No. of corpora lutea</i>	<i>Total No. of eggs recovered</i>	<i>% of eggs recovered</i>
<36	163	148	90.8
40	161	154	95.7
44	176	167	94.9
48	167	161	96.4
52	162	150	92.6
56	165	157	95.2
Total	994	937	94.3

Fertilization

Approximately 150 eggs were examined from each insemination group, and a summary of the results on fertilization appears in Table 4. The mean percentage of unfertilized eggs increased from 1.3% in Group 2 to 23.9% in Group 6. Unfertilized eggs occurred principally as a result of unilateral fertilization; with the exception of eggs resembling primary oocytes, ten of a total of seventy-five unfertilized eggs were recovered that were not coincident with the occurrence of unilateral fertilization. Eight of these ten unfertilized eggs were recovered from the oviducts of two animals in Group 6, in both of which some fertilized eggs were present, but only at the early pronucleate stage. The other two eggs, however, were recovered in the presence of normal 4-cell eggs. Many spermatozoa were attached to the zona pellucida in both of these eggs but none had penetrated into the vitellus; first polar bodies were present, but the chromosomes of the second maturation spindle were scattered in the cytoplasm.

Seven eggs were recovered that were considered either immature or atretic. The nucleus in each of these eggs was located peripherally, and was similar to

the large dictyate nucleus of primary oocytes, there being no indication of any resumption of meiotic activity. Spermatozoa were present in the cytoplasm of five of these eggs, although the sperm heads were not detectably swollen. The remaining two eggs, which were both from an animal in which the other eggs were at the early pronucleate stage, had not been penetrated.

TABLE 4

THE NUMBER OF EGGS EXAMINED, AND THE PERCENTAGES OF NORMAL AND ABNORMAL FERTILIZED EGGS AND OF UNFERTILIZED EGGS FROM PIGS INSEMINATED AT VARYING INTERVALS AFTER INJECTION OF HCG

Interval from HCG injection to insemination (hr)	No. of eggs stained and examined	Eggs fertilized and developing normally (%)	Eggs fertilized but developing abnormally (%)	Eggs not fertilized (%)
<36	141	90.8 ± 4.5*	3.5	5.7
40	152	92.1 ± 2.7	6.6	1.3
44	166	94.6 ± 2.3	3.6	1.8
48	155	70.3 ± 7.8	25.2	4.5
52	149	48.3 ± 8.4	38.9	12.8
56	155	50.9 ± 7.5	25.2	23.9

Eggs were examined by phase-contrast microscopy after staining with 1% natural orcein in 45% acetic acid.

* Mean ± S.E.

TABLE 5

THE PERCENTAGES OF NORMAL AND ABNORMAL FERTILIZED EGGS FROM PIGS INSEMINATED AT VARYING INTERVALS AFTER INJECTION OF HCG

Interval from HCG injection to insemination (hr)	% of fertilized eggs developing normally			% of fertilized eggs developing abnormally
	From oviducts	From uterus	Total	
<36	93.5	98.6	96.2	3.8
40	91.1	95.8	93.3	6.7
44	97.7	94.7	96.3	3.7
48	67.7	78.7	73.6	26.4
52	71.6	33.9	55.4	44.6
56	80.0	57.3	66.9	33.1

Egg development

There was no decrease in the mean percentage of eggs developing normally until the eggs were 12 hr old at penetration (Group 4), when the figure fell to 73.6% (Table 5). A further decrease occurred in Groups 5 and 6, means of 55.4 and 66.9%, respectively, being obtained. The mean percentage of fertilized eggs developing abnormally increased with the age of eggs at penetration. Thus, in the controls, only 3.8% of the eggs were developing abnormally, whereas the figure increased to 44.6% in Group 5.

Table 5 also presents a breakdown of the percentage of normally developing

eggs recovered from the oviducts and from the uterus. In both Groups 5 and 6, there was a conspicuously lower percentage of normally developing uterine eggs as compared with the figure for tubal eggs, suggesting that in these older eggs irregularities of development increased with time.

TABLE 6
THE MEAN PERCENTAGES OF THREE CLASSES OF ABNORMAL EGGS
FROM PIGS INSEMINATED AT VARYING INTERVALS AFTER INJECTION
OF HCG

Interval from HCG injection to insemination (hr)	% of eggs			
	Polyspermic	With one anucleate fragment	Fragmenting	Total
<36	0.0	1.5	2.3	3.8
40	1.3	2.0	3.4	6.7
44	1.2	1.2	1.2	3.6
48	6.8	9.5	10.1	26.4
52	15.4	6.1	23.1	44.6
56	11.1	3.4	18.6	33.1

Abnormalities of development

The abnormally developing eggs were classified into three groups: (a) polyspermic and/or digynic eggs, (b) eggs with an anucleate fragment noticeably smaller than the nucleated blastomeres, and (c) fragmenting eggs.

The relative percentages of these abnormalities appear in Table 6.

Polyspermy. An egg was judged to be polyspermic if: (1) in the pronucleate state, it had an extruded second polar body and three or more pronuclei (Pl. 1, Figs. 1 and 2); (2) following cleavage, it had an extruded second polar body and an accessory nucleus (pronucleus) in any one blastomere (Pl. 1, Fig. 3); and (3) before or after cleavage, accessory sperm heads, either unswollen or beginning to swell, could be detected within the cytoplasm.

EXPLANATION OF PLATE 1

Abnormalities observed in fertilized pig eggs after delayed insemination. All figures are phase-contrast photomicrographs of fixed and stained preparations.

FIG. 1. A polyspermic pronucleate egg showing an accessory male pronucleus and an unswollen sperm head (arrowed) in the cytoplasm. First and second polar bodies cannot be distinguished at this focal plane. $\times 375$.

FIG. 2. A trispermic pronucleate egg containing four pronuclei, one of which is out of focus. Note the appearance of the cytoplasm and the absence of vacuoles in the cortex. Neither polar body is visible in this photograph. $\times 375$.

FIG. 3. A dispermic egg with second polar body (arrowed). The '2-cell' condition may have arisen from fragmentation of a pronucleate egg, leaving the fusing pronuclei in the upper fragment and an accessory male pronucleus in the lower fragment. Note the appearance of the peripheral cytoplasm. $\times 300$.

FIG. 4. A 3-cell egg recovered from the uterus. Note the apposing position of the two nuclei in the upper blastomere. No polar bodies could be detected on this egg. $\times 375$.

FIG. 5. A 2-cell egg with an anucleate fragment. The cytoplasm is of normal appearance. $\times 300$.

FIG. 6. A fragmenting egg recovered from the oviduct. Note the recent mitotic division (arrowed). $\times 300$.

PLATE 1

PLATE 2

FIGS. 7 and 8. Normal 5-day embryos at early egg cylinder stage.

FIG. 9. Degenerated 5-day embryo from DDK females $\times$ KK males mating.

FIG. 10. Degenerated 5-day embryo from DDK females $\times$ NC males cross. Only a single embryonic cell remains.

FIG. 11. Implantation site with dead and degenerated embryo (7 days after copulation) from DDK females $\times$ KK males mating.

FIG. 12. Normal 8-day embryo from DDK females $\times$ NC males cross.

The mean percentage of polyspermic eggs increased from 1.3% in Group 2 to 15.4% in Group 5. Furthermore, in both Groups 4 and 5, an appreciably higher percentage of polyspermic eggs was recovered from the uterus than from the oviducts (Table 7).

On three occasions, the presence of more than one sperm tail or midpiece in the cytoplasm of pronucleate eggs confirmed the diagnosis of polyspermy. Sperm tails were also observed in eleven other polyspermic eggs but were attached to, or in association with, sperm heads, not pronuclei.

TABLE 7
THE PERCENTAGES OF POLYSPERMIC EGGS FROM THE
OVIDUCTS AND UTERI OF PIGS INSEMINATED AT
VARYING INTERVALS AFTER INJECTION OF HCG

<i>Interval from HCG injection to insemination (hr)</i>	<i>Polyspermic tubal eggs (%)</i>	<i>Polyspermic uterine eggs (%)</i>	<i>Total (%)</i>
<36	0.0	0.0	0.0
40	1.3	1.4	1.3
44	1.1	1.3	1.2
48	2.9	10.0	6.8
52	6.8	26.8	15.4
56	14.0	8.8	11.1

Forty-four of the 918 eggs that were stained and examined were classified as polyspermic. Twenty of these were pronucleate eggs: there were nine 2-cell eggs, thirteen 3- to 4-cell eggs and two were at later stages of development.

Of these forty-four eggs, fourteen contained unswollen or only slightly swollen sperm heads, twenty-two had accessory pronuclei, and eight had both sperm heads and accessory pronuclei. Sperm heads were found in twelve uterine eggs, but in only two tubal eggs, both of which had cleaved. On the other hand, accessory pronuclei were found in thirteen tubal eggs and in nine

EXPLANATION OF PLATE 2

Abnormalities observed in penetrated pig eggs after delayed insemination. The figures are phase-contrast photomicrographs of fixed and stained preparations.

FIG. 7. Fragmentation of a pronucleate egg. The male pronucleus is visible in the lower fragment, while the female pronucleus is out of focus in the main body of the egg. $\times 300$.

FIG. 8. Fragmentation of a pronucleate egg, with the 'fusing' male and female pronuclei in one of the fragments. Note the appearance of the cytoplasm and the large size of the second polar body (arrowed). $\times 300$.

FIG. 9. A fragmenting, pronucleate egg. The male pronucleus and adjacent sperm tail are in one fragment and the early female pronucleus in the other. The spindle remnant (arrowed) lies between the female pronucleus and the large cytoplasmic second polar body. $\times 300$.

FIG. 10. Fragmentation of the second polar body observed in a pronucleate egg. This egg possessed a morphologically normal first polar body. $\times 760$.

FIG. 11. A fertilized 2-cell egg with chromatin arranged at early metaphase in one of the blastomeres. Note the degenerate appearance of the peripheral cytoplasm and the absence of vacuoles. $\times 300$.

FIG. 12. A nuclear lobe or bud (arrowed) found in a fertilized 2-cell egg. $\times 475$.

uterine eggs. Eight uterine eggs contained both accessory sperm heads and pronuclei.

The number of accessory sperm or male pronuclei seen was variable. There were 24 eggs with one, ten eggs with two, one egg with three and nine eggs with four or more.

Of the twenty eggs containing two or more extra spermatozoa, sixteen were recovered from the uterus but only four from the oviducts.

Digyny. Only one egg was classified as digynic; this was at anaphase of the first cleavage, with a second spindle at a similar stage. If it is assumed that the egg had been ovulated as a secondary oocyte, then the second polar body must have been retained and undergone syngamy with an accessory spermatozoon. Four other eggs may have been digynic; these were all 3-cell eggs recovered from the uterus with two nuclei or pronuclei in one of the blastomeres (Pl. 1, Fig. 4). No polar bodies could be detected on any of these eggs. Although

TABLE 8
THE PERCENTAGES OF FRAGMENTING EGGS AMONG
EGGS RECOVERED FROM THE OVIDUCTS AND UTERI
OF PIGS INSEMINATED AT VARYING INTERVALS AFTER
INJECTION OF HCG

<i>Interval from HCG injection to insemination (hr)</i>	<i>Fragmenting tubal eggs</i>	<i>Fragmenting uterine eggs</i>	<i>Total</i>
<36	3.3	1.4	2.3
40	3.8	2.8	3.4
44	0.0	2.6	1.2
48	16.1	5.0	10.1
52	16.2	32.2	23.1
56	6.0	28.0	18.6

failure of cytoplasmic division at the second cleavage could have occurred, in each instance the two nuclei in the one blastomere were apposed to each other as though about to fuse, as was the case in some 2-cell eggs that were classified as polyspermic.

Fragmenting eggs. The proportion of eggs with a small anucleate fragment, i.e. one cytoplasmic fragment in which no chromatin material whatsoever could be detected (Pl. 1, Fig. 5), increased with delayed insemination (Table 6). The percentage of fragmenting eggs with two or more anucleate fragments (Pl. 1, Fig. 6) increased to 23.1% in Group 5 as compared with 2.3% in the controls. Although a higher incidence of fragmenting eggs was recovered from the oviducts than uterus in Group 4, in Groups 5 and 6 this situation was completely reversed (Table 8).

Fragmenting eggs recovered from the oviducts in these animals appeared to have undergone a form of 'cleavage' while still in the pronucleate stage. Thus, in four eggs from two animals in Group 5 in which the egg was composed of two uneven fragments, the male pronucleus and adjacent sperm tail were found in one fragment and the female pronucleus in the other (Pl. 2, Fig. 7).

A further tubal egg from Group 5 had 'cleaved' into two fragments of approxi-

mately equal size, but one fragment contained the fusing pronuclei and adjacent sperm tail while the other was anucleate (Pl. 2, Fig. 8). First and second polar bodies were identified on all these eggs and they were classified as fertilized but fragmenting.

Appearance of second polar body

In a number of fertilized eggs recovered from Groups 5 and 6, the second polar body presented an unusual appearance. Thirteen eggs from four animals were observed in which the second polar body had been formed as a large cytoplasmic fragment, and within this the chromatin was arranged in the form of a nucleus or pronucleus. In seven of these eggs, a spindle remnant was observed in association with the cytoplasmic fragment (Pl. 2, Fig. 9), and in one such egg the cytoplasm of the second polar body had been penetrated by a spermatozoon. The first polar body could be identified in nine of these thirteen eggs. On the basis of criteria outlined above, three of these eggs were classified as normally developing, whereas the remaining ten were classified as fertilized but fragmenting. A further four eggs were recovered from two animals in Group 6, each of which had fragmented second polar bodies as well as a single first polar body (Pl. 2, Fig. 10). These were pronucleate eggs recovered from the oviducts, and all were classified as developing normally.

Appearance of cytoplasm and nuclei

The cytoplasm of fertilized eggs was of an unusual appearance in a number of instances. In 2-cell eggs, there was often a loss of structure in the cytoplasm around the periphery of the blastomeres, this being quite prominent in eggs from Groups 5 and 6 (Pl. 2, Fig. 11). The nuclei of several cleaved, fertilized eggs in these groups also presented an unusual appearance, some being rather angular in outline, while others possessed nuclear lobes or buds (Pl. 2, Fig. 12).

Cleavage rate

A number of eggs recovered from the uterus showed retarded development. Thus in Group 5, six pronucleate, eight 2-cell and nine 3-cell eggs were flushed from the uteri of five animals. Three of the 2-cell eggs and one of the 3-cell eggs looked morphologically normal, having second polar bodies still attached. However, the remaining eggs showed either fragmentation or polyspermy.

Six pronucleate, eight 2-cell and eleven 3-cell eggs were recovered from the uteri of five animals in Group 6. Eight of these twenty-five eggs appeared morphologically normal. Two were pronucleate eggs, two were 2-cell eggs, and four were 3-cell eggs. Five of the eight eggs still had second polar bodies. Apart from these eggs, and a small number of tubal eggs which appeared to be in advance of the normal rate of cleavage ($<3\%$), eggs recovered within any one animal were at very similar stages of development.

DISCUSSION

The results obtained in this study show that a very high proportion of pig eggs can be fertilized up to 8 hr after ovulation and undergo normal development at least until the early uterine stage. However, fertility decreased very considerably

thereafter due to: (1) a fall in conception rate; (2) an increase in the incidence of unilateral fertilization; and (3) an increase in the proportion of abnormally developing fertilized eggs. Together, these sources of loss of fertility following delayed insemination were of considerable importance, and indicate that insemination should be performed not later than 2 hr after ovulation in order to obtain the maximum number of normally fertilized eggs. Furthermore, a consideration of the number of unfertilized eggs recovered from all inseminated animals confirms the observation of Hancock & Hovell (1962) that failure of fertilization is likely to be a greater source of infertility in animals inseminated late in oestrus than is the formation of defective zygotes.

The fall in conception rate with the delay of insemination may be due to deficiencies in sperm transport, as in Groups 5 and 6 the animals would certainly be nearing the end of oestrus at insemination. Similarly, the chances of unilateral fertilization appear to increase with the delay of insemination, and the results obtained suggest an inverse correlation between the conception rate and incidence of unilateral fertilization. There was no indication that unilateral fertilization resulted from any anatomical abnormality of the reproductive tract. Hancock (1962) has also recorded the occurrence of unilateral fertilization in the pig and Pitkjanen (1961), using uterine fistulae, has shown that semen is sometimes recoverable only from one horn of the uterus, a finding reported previously by Kvasnitsky (1959).

It is important to note that within the seventy-two animals, the overall incidence of unilateral fertilization was 12.5%, and if a comparable figure were to be obtained under field conditions, this would represent an appreciable decrease in fecundity. An incidence of 8 to 10 % of unilateral fertilization has previously been found in this laboratory after routine inseminations on the morning of the second day of oestrus, and a similar figure was reported by Hunter (1964).

That the incidence of polyspermy increased with delayed insemination is in agreement with the findings of Pitkjanen (1955), Hancock (1959) and Thibault (1959), although in these earlier studies the incidence of polyspermy could not be related to the postovulatory age of the egg. Nevertheless, the figures of 10 and 11% polyspermy, recorded by Pitkjanen and Thibault, respectively, are in close agreement with the mean figure of 11.1% for Groups 4, 5 and 6. There is, also, evidence from a number of laboratory species that the incidence of polyspermy may be greatly increased by delayed mating, this having been shown in the rat (Austin & Braden, 1953; Odor & Blandau, 1956; Braden, 1958), rabbit (Austin & Braden, 1953) and hamster (Yanagimachi & Chang, 1961).

Because no difficulty was experienced in distinguishing between first and second polar bodies, location of an extruded second polar body in trinucleate eggs became a major criterion for confirming any diagnosis of polyspermy. On the other hand, both Pitkjanen (1955) and Thibault (1959), working with sectioned eggs, claimed to be able to distinguish between male and female pronuclei on morphological grounds, and, furthermore, were able to observe the sperm tail or midpiece in association with the male pronucleus. On this basis, Pitkjanen classified all eggs with more than two pronuclei as polyspermic. However, the frequency with which sperm tails could be detected in the cyto-

plasm was not disclosed by either Pitkjanen or Thibault. The results of the present experiment, in agreement with Hancock (1959, 1961), indicate that the sperm tail is only occasionally seen in whole mounts of fixed pronucleate eggs.

The finding of sperm heads in the cytoplasm of cleaving eggs, in some instances together with accessory pronuclei, is suggestive of recent penetration of such eggs. The increased incidence of polyspermy in uterine eggs compared with tubal eggs in Groups 4 and 5 would also seem to point to the same conclusion. The proportion of sperm heads that would be able to form pronuclei can only be left to speculation, as must the fate of eggs subject to this abnormality. Pitkjanen (1955) and Hunter (1966) have also recorded the presence of supplementary spermatozoa in cleaving pig eggs, and Pitkjanen suggests that such spermatozoa penetrate during the course of segmentation.

In contrast with the findings of Thibault (1959) who observed 21% of digynic eggs in pigs mated later than 36 hr after the onset of oestrus, this abnormality was almost completely absent from the present study. Apart from five eggs described above, there was no evidence whatsoever that retention of the second polar body had occurred after delayed insemination, a conclusion also reached by Hancock (1961). However, digynic eggs have been found in a number of laboratory species following delayed mating or insemination (hamster: Chang & Fernandez-Cano, 1958; Yanagimachi & Chang, 1961; mouse: Marston & Chang, 1964).

No previous investigator has noted the formation of second polar bodies of unusual size and morphology following delayed insemination in the pig, but these have been observed in the mouse under such conditions (Marston & Chang, 1964). Abnormal second polar bodies may result from a delayed and disorganized meiotic division, in which case retarded extrusion or retention of the second polar body could be regarded as different degrees of the same abnormality. Furthermore, if the cytoplasm of the second polar body was large enough to appear as a 'blastomere,' this observation could then correspond with the 'immediate cleavage' found in some laboratory species and discussed by Austin (1961) and Marston & Chang (1964). As already noted, the chromatin of these abnormal second polar bodies tends to form a nucleus or pronucleus which could be confused with the expected nuclear configuration of a normal blastomere.

'Immediate cleavage' has been suggested to result from migration of the second maturation spindle towards the centre of the egg, so the size of the two fragments produced in this condition will depend on the location of the spindle relative to the vitelline membrane when the egg becomes penetrated by a spermatozoon. Hancock (1961) found a number of 2-cell eggs in the pig with two nuclei in one blastomere and one in the other, and considered that the apparently premature cleavage shown by such eggs from delayed matings might be related to 'immediate cleavage'.

The finding of fragmented second polar bodies after delayed insemination has not been reported for the pig before. In the present study, fragmented second polar bodies were found on eggs of normal appearance undergoing syngamy. Marston & Chang (1964), working with the mouse, observed two second polar bodies only on eggs that they classified as androgenetic. The

cytoplasm of such eggs contained one large pronucleus together with a sperm tail, and it was therefore considered that completion of the second maturation division had resulted in the extrusion of two second polar bodies, thus leaving no chromatin to form a female pronucleus.

The ovulation of eggs resembling primary oocytes has been reported previously in the pig, both after induced ovulation (Spalding, Berry & Moffit, 1955; Hunter, 1964, 1966; Polge & Dziuk, 1965) and spontaneous ovulation (Hancock, 1961). Although Hancock found three such eggs in a total of 1677 eggs (0.2%), none has been recorded under conditions of spontaneous ovulation in this laboratory. However, seven in a total of 918 primary oocytes (0.8%) were recovered in the present study, and this increase is possibly a reflection of the HCG treatment, causing such eggs to be released from follicles that would not normally have ovulated at that time. Apart from these instances, there was no reason to suspect that eggs induced to ovulate by means of HCG treatment differed physiologically from those ovulated spontaneously.

Hancock (1961) suggested that pig eggs fertilized as a result of delayed insemination tend to have accelerated rates of cleavage. It is important to note, though, that Hancock did not know the time of ovulation and, further, considered that the second mitotic division does not normally occur until 12 hr after the first, whereas findings in this laboratory indicate that it occurs considerably earlier and that the 4-cell stage is relatively long (≥ 20 hr). Contrary to Hancock's findings, retarded development of eggs was a much more common observation in the present experiment than premature cleavage. The presence of pronucleate 2-cell and 3-cell eggs in the uterus was presumably an abnormal condition associated with fertilization of old eggs. In the hamster, Chang & Fernandez-Cano (1958) found some evidence of delayed cleavage of otherwise morphologically normal eggs after delayed mating, and, in the mouse, Marston & Chang (1964) found that embryonic development up to 80 hr after delayed insemination was distinctly inferior to that following mating or insemination at the time of ovulation.

Apart from the exceptions described above, the fact that eggs recovered within individual animals were all at very similar stages of development would seem to be a reflection of both the efficiency of HCG treatment in causing synchronous ovulation and the efficiency of insemination. It has been shown that the eggs are shed within a period of 1 hr following induced ovulation (Hunter, 1965). The other major factor which would influence the spread in developmental stages is the time it takes for all eggs to become penetrated. Because a large number of spermatozoa were used for each insemination ($> 10 \times 10^9$), it would seem that this period had been kept to a minimum.

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