

TIME RELATIONSHIPS IN FROG OVULATION¹

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In recent tests with excised ovaries of *Rana pipiens* (Wright, '45), it was found that ovulation in vitro during March usually begins within 8 to 10 hours after the experiment is started and terminates within 24 to 28 hours. In December, ovulation does not commence until 12 to 14 hours but ceases in 28 hours. The progress of ovulation between these limits follows a sigmoid pattern, the greatest response being achieved between the 14th and 18th hours of the test in March, and between the 18th and 22nd hours in December. In obtaining these data, bits of ovary from normal adult frogs were placed in pituitary solutions and left undisturbed for the full 28 hours. It seemed of considerable importance to ascertain more information concerning the time during this period when pituitary factors acted upon the ovarian tissue, and whether the presence of hypophyseal principles was essential throughout the experiment to effect maximal ovulation. Consequently a procedure was devised to permit observation of the responses of pieces of ovary following contact with pituitary suspensions for limited periods of time.

MATERIALS AND METHODS

Large (60 to 100 gm) sexually mature female *Rana pipiens* were used throughout the work as the source of hypophyseal and ovarian tissue, and were obtained from hibernation in small batches by collectors in Wisconsin. After arrival in the laboratory, the frogs were stored at 1° to 4°C. in covered pans

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containing a small amount of water. It was reported earlier (Wright, '45) that only ovaries obtained from frogs weighing 70 gm or more could be used successfully in vitro. Animals were obtained for these earlier experiments from Vermont, and it is possible that a strain difference in frogs collected for the present tests in Wisconsin could have been responsible for the fact that ovaries of smaller females were readily stimulated in vitro. Left ovaries were used exclusively for the sake of uniformity, and one gonad supplied more than ample material for all tests begun on any one day. All experiments were conducted at room temperature (22° to 23°C.).

Since some variation in potency of individual pituitary glands has been noted previously, concentrated stock solutions of fresh pituitary glands were used routinely. Stock solutions consisted of 15 to 20 triturated anterior lobes taken up in a small volume of Ringer's solution. Dilutions for individual experiments were made readily from these concentrated preparations, which were kept frozen to prevent deterioration while not in use.

EXPERIMENTAL PROCEDURE AND RESULTS

Experiments described here were divided into two groups. The first series of tests was performed during March and April, and at that time a study was made of the ovulatory responses of ovarian fragments after exposure to dilute pituitary solutions for a varying number of hours. These experiments were repeated in December using both dilute and a more concentrated pituitary solution, making possible thereby comparison not only of the effectiveness of two different potencies of pituitary solution, but also of the responses achieved during winter and spring.

The procedure used in March and April was as follows. Excised ovaries were cut into small fragments with 15 to 25 eggs, and these fragments were suspended by cotton thread in vials each containing the equivalent of one-sixteenth anterior lobe in 10 cm³ buffered Ringer's solution (0.65% NaCl) buffered with NaH₂PO₄-NaHCO₃. The vials were then tightly

stoppered and set aside, in groups of 6 vials, for periods of time ranging from two and one-half to 8 hours. At the end of the experimental period of exposure to pituitary solution, the ovarian fragments were removed, rinsed thoroughly in fresh Ringer's solution, and placed again in vials containing Ringer's fluid. Ovulation began in many of the ovarian fragments soon after they were changed to Ringer's solution, in fact, in several instances some ovulation had occurred before the pieces were removed from pituitary solution. Final percentages of ovulation were recorded 28 hours after the start of the experiment. Tabulations of percentages were made readily since ovulated eggs (free of the ovary and resting on the bottoms of the vials) as well as those remaining in the ovarian stroma could be counted accurately. One set of 6 vials served as controls in each experiment in that ovarian fragments were placed in pituitary solution at the beginning of the test and left undisturbed for the full 28 hours.

When these tests were repeated in December, fragments of ovary were exposed to the equivalent of one-fourth anterior lobe in 10 cm³ Ringer's solution for periods of time ranging from 4 to 12 hours, and to the equivalent of one-sixteenth anterior lobe for 8 hours. Subsequently, fragments of ovary were changed as described above to Ringer's solution where ovulation ensued. Two sets of controls were included in each of these experiments, i.e., vials in which fragments were exposed to one-fourth and one-sixteenth anterior lobe respectively for the full 28 hours.

From the results (tables 1 and 2) it is apparent that the percentage of ovulation bears a direct relationship to the length of time ovarian fragments were kept in pituitary solution. In the March and April experiments, two and one-half to three hours' contact with pituitary factors seems to be close to the threshold for stimulation of ovulation. Beyond this point, the longer the exposure to pituitary factors, the greater the percentage of ovulation obtained. But even 8 hours' stimulation (at which time ovulation could be expected to begin) did not effect so great a percentage of ovulation as that which

TABLE 1

Results of in vitro experiments in which fragments of normal ovary were exposed to the equivalent of one-sixteenth anterior pituitary gland in 10 cm³ buffered Ringer's solution for the number of hours shown. Following contact with pituitary solution, the fragments were washed thoroughly in Ringer's fluid and placed in vials containing fresh Ringer's solution. Each value represents an average percentage obtained in 6 similar vials 28 hours after the start of the experiment

DATE	PERCENTAGES OF OVULATION OBTAINED AFTER EXPOSURE TO PITUITARY SUSPENSIONS FOR							28 hrs. control
	2½ hrs.	3 hrs.	4 hrs.	5 hrs.	6 hrs.	7 hrs.	8 hrs.	
3-16	..	1.0	13.1	26.4	21.6	..	55.2 ¹	77.5
3-17	0.0	0.0	0.0	3.8	5.1	30.0	28.8	57.8
3-18	0.0	0.0	11.6	15.6	17.7	15.3	45.9	50.9
3-23	1.5	2.9	28.8	52.9	80.3	85.8 ¹	86.2 ¹	98.7
3-24	0.0	0.0	0.0	17.2	21.6	24.6	51.7 ¹	97.0
3-30	1.7	4.7	14.3	11.1	29.5	39.4	50.9	90.5
4-14	0.0	0.0	0.0	5.6	25.4	49.4	46.5	53.0
4-29	0.0	0.0	4.9	8.9	27.3	86.5	88.7 ¹	98.4
Av.	0.5	1.1	9.1	17.7	28.6	47.3	56.8	78.0

¹ Ovulation had begun in these vials before fragments were removed from pituitary solution.

TABLE 2

Results of in vitro experiments conducted as in table 1, except that two different potencies of pituitary solution were used

DATE	PERCENTAGES OF OVULATION OBTAINED AFTER EXPOSURE TO THE EQUIVALENT OF							28 hrs. control
	¼ pit. gland for						½ pit. gland for	
	4 hrs.	6 hrs.	8 hrs.	10 hrs.	12 hrs.	28 hrs. control	8 hrs.	
12-2	0.0	0.0	1.2	19.6	0.0	58.1
12-7	51.9 ¹	73.2 ¹	73.1 ¹	72.7	79.2 ¹	87.8
12-9	2.8	14.3	32.9 ¹	27.9 ¹	..	67.1	13.6	71.2
12-14	0.0	6.8	38.2 ¹	28.4 ¹	..	69.6	25.3 ¹	89.0
12-16	0.0	0.0	2.5	22.9 ¹	..	26.7	0.0	73.1
12-21	0.0	4.5	19.4 ¹	25.1	54.4 ¹	65.4	0.0	58.0

¹ Ovulation had begun in these vials before fragments were removed from pituitary solution.

followed continued exposure for the full 28 hours. In December, when ovarian tissue was less sensitive to ovulatory factors, 4 hours' contact with the equivalent of one-fourth anterior lobe approximates the ovulatory threshold, and 10 to 12 hours results in ovulatory responses comparable to those achieved in the 28-hour controls. In the majority of instances, 8 hours' exposure in December to one-sixteenth of an anterior lobe was insufficient to cause ovulation, although two ovaries were exceptionally sensitive and responded well. It should be noted in connection with the two controls used in these experiments that the more dilute pituitary solution (one-sixteenth), with only one exception, effected greater percentages of ovulation than the more concentrated. The explanation for this occurrence is not clear, but it has been observed previously (Wright, '45) that concentrated pituitary solutions are less effective in causing ovulation *in vitro* than dilute ones, i.e., one-sixteenth or one-thirty-second in 10 cm³ Ringer's solution.

It is interesting to note that the length of time fragments were exposed to pituitary solution also determined the approximate time when ovulation began. Percentages of ovulation varied directly with the time of exposure, but onset of ovulation bore within limits an inverse relationship to exposure time. It will be noted (table 1, March and April experiments) that ovulation had begun in several vials before 8 hours had elapsed. But as the time of exposure to pituitary factors is decreased, the time required for ovulation to commence is correspondingly lengthened. Data obtained in the experiments of March 23 (table 3) demonstrate this principle particularly well, and are typical of all tests performed during the spring months. In December, in spite of a somewhat lower ovarian sensitivity, the times for initiation of ovulation following exposure to pituitary solution compare favorably with data obtained in March. In the majority of these winter experiments (table 2) ovulation had started by 8 hours, but the average time when ovulation began after exposure for 6 hours was 12

hours as opposed to an average of 10 hours under the same conditions in March.

The observation that ovarian fragments after contact with pituitary solution for 8 to 10 hours fail (with one exception) to release as many ova as control fragments exposed for 28 hours indicates that pituitary factors exert an important influence even after many follicles have started to ovulate. This statement stands to be made more tenable if it could be shown that pituitary solutions still possessed ovulatory capacity after standing at room temperature for 8 or 10 hours. Consequently, vials were filled with pituitary solution (the equivalent of one-

TABLE 3

*Times when ovulation began in the experiment of March 23 (table 1).
These values are typical of all March experiments*

EXPOSURE TIME	START OF OVULATION
<i>hrs.</i>	<i>hrs.</i>
2½	28
3	27⅔
4	12½
5	9
6	8½
7	7
8	6½
28	6½

sixteenth anterior lobe in 10 cm³ Ringer's per vial) at intervals during the day, and ovarian fragments were placed in the vials after 4 to 12 hours had elapsed. Timing was arranged so that fragments of one ovary were used, being added to all vials at approximately the same moment. Percentages (table 4) obtained 24 hours later showed that 8 hours' standing at room temperature seemed to increase, and even 12 hours did not appreciably reduce, the ability of the solution to induce ovulation.

It is interesting to note, in this connection also, that pituitary solutions in which ovarian fragments had been kept for 8 hours were quite capable of effecting ovulation in a second

set of ovarian bits. In two experiments (March) the average ovulation in those fragments which had been exposed for 8 hours to fresh pituitary solution (one-sixteenth in 10 cm³ Ringer's solution) was 57.8%, and 36.6% response was achieved in the second set of ovarian fragments added to the pituitary solution after the first was removed. As many as 5 sets of fragments from different females have ovulated in Ringer's fluid at room temperature after successive exposures to the same pituitary solution at 4°C. (Wright, '45).

TABLE 4

Results of in vitro experiments in which pituitary solutions (equivalent of one-sixteenth anterior lobe in 10 cm³ Ringer's) were allowed to stand at room temperature for the number of hours shown before ovarian fragments were added

DATE	PERCENTAGES OF OVULATION AFTER PITUITARY SOLUTIONS HAD STOOD AT ROOM TEMPERATURE FOR			
	0 hrs.	4 hrs.	8 hrs.	12 hrs.
3-23	43.0	41.4	52.2	27.8
3-25	46.7	50.0	55.7	40.8
3-30	61.5	61.7	81.7	69.3
3-31	59.9	61.7	79.3	65.3
Av.	52.8	53.7	67.3	50.8

DISCUSSION

Although knowledge concerning many phases of reproductive physiology has advanced considerably in recent years, the biochemical processes involved in ovulation in the frog, or, for that matter, in any vertebrate, are still entirely unknown. Histological and cytological changes, including rupture of follicles and extrusion of ova, have been studied in considerable detail for the frog (Brandt, 1877; Rugh, '35), but the biochemical reactions responsible for these anatomical manifestations are as yet undiscovered. It seems almost certain that one or more factors of the anterior pituitary gland serves as the sole instigators of ovulation, and that regulation of the process is entirely hormonal. Since ovulation occurs

normally *in vitro*, it is apparent that constituents of the blood and nervous connections are not essential. Fractionation of amphibian hypophyses has not been carried out, up to the present, at least, but use of fractionated extracts of mammalian pituitary glands has revealed several important points concerning the ovulatory process in the frog (Wright and Hisaw, '46). Pure preparations of the follicle-stimulating hormone (FSH) did not cause ovulation *in vitro* or in hypophysectomized animals, but were effective in the intact frog. FSH did maintain and increase the sensitivity of ovarian tissue both *in vitro* and in the hypophysectomized frog so that greater percentages of ovulation than normal were obtained when ovulatory factors were added. Combinations of FSH and the luteinizing hormone (LH) brought about ovulation *in vitro* and in both normal and hypophysectomized frogs. Since the sensitizing effect of FSH and the ovulatory action of combinations of FSH and LH can be duplicated by proper administration of extracts of frog anterior pituitary glands, it is assumed that both FSH and LH normally take part in ovulation in the frog as in other vertebrates.

An enzymatic theory has been proposed (Rugh, '35) to account for the rupture of the follicular wall in the frog, and a proteolytic enzyme has been discovered in the frog egg (Hartog, '04). According to this theory maturing oocytes (or possibly the follicular cells themselves) are stimulated by pituitary factors to produce an enzyme which, in turn, digests an opening in the follicle. The exact means whereby the egg then escapes is not known, but it is thought that contractions of smooth muscle cells in the follicular wall aid in the process. The enzymatic theory is substantiated in part by the observation that pepsin activated by HCl causes follicular rupture in a very short time when added to Ringer's solution in which a normal ovary has been placed.

Just how the action of FSH and LH fits into the chain of events leading to the production of a proteolytic enzyme is not understood, but data presented above will be helpful in understanding the steps involved. It is possible that FSH is

responsible for production of the enzyme and LH for its activation. Very likely one or both factors take part in energy transfers which must occur during and prior to ovulation. The findings of this paper show, among other things, that all follicles do not have an equal threshold for stimulation of ovulation. During March and April, the natural breeding season of this species, it was shown that some follicles require only two and one-half to three hours' stimulation by pituitary factors to initiate ovulation, while fully one-fourth to one-third (or more) follicles require more than 8 hours. At a time when some follicles have ovulated, then, others have not received sufficient stimulus to complete the process, even if given 28 hours.

These time studies have served to show further that ovulation is not an all-or-none reaction, at least of the usual type. A brief exposure to pituitary factors results in a small percentage of ovulation which does not occur until 20 hours or more after fragments are removed to Ringer's solution (table 3). These same follicles would have begun to ovulate much earlier if exposure had been lengthened, as shown by the fact that other fragments of the same ovary exposed for 7 hours released many more ova, and ovulation began at the time fragments were removed to Ringer's solution.

In the December experiments described above, opportunity is presented for comparison of the effectiveness of exposure to two different potencies of pituitary solution for the same length of time. The results suggest that increasing the potency of the pituitary solution while the time remains constant has the same effect as increasing the time while the concentration remains unchanged. This relationship applies only within limits, however, since fragments exposed to the equivalent of one-sixteenth anterior lobe for 28 hours ovulated more eggs than identical fragments which remained in solutions of one-fourth pituitary gland for the same length of time. As mentioned above, no explanation for these results is immediately apparent, although it is a common observation in work with endocrines that overdoses are less effective than smaller

amounts. One might have expected that shorter exposures to this concentrated solution would not show the inhibitory behavior seen in the 28-hour controls, but the data do not indicate removal of this inhibition. It is possible that reduction in inhibitory effect would have appeared if the exposure time had been carried beyond 12 hours. Experiments to test this possibility are in progress.

SUMMARY

It has been shown that the amount of ovulation achieved in vitro bears, within limits, a direct relationship to the length of time ovarian tissue was exposed to pituitary factors. An exposure time of two and one-half to three hours to the equivalent of one-sixteenth triturated anterior lobe in 10 cm³ Ringer's solution was the approximate threshold for stimulation of ovulation in ovarian fragments during March and early April. Four hours' stimulation with one-fourth anterior lobe in 10 cm³ was the apparent threshold in December. Greater percentages of ovulation were obtained as the exposure time was lengthened, but 8 to 10 hours' contact, when ovulation ordinarily commenced, did not effect so great a response as continued exposure for 28 hours. The length of time required for initiation of ovulation varied inversely with the length of exposure to pituitary factors.

Experiments in which fragments were exposed to two potencies of pituitary glands for 8 hours showed that the more concentrated effected greater percentages of ovulation. If the exposure time was increased to 28 hours, however, the weaker solution was more effective.

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