CCLXXV. THE ENDOCRINE FACTORS CONCERNED IN THE CONTROL OF THE OVARIAN CYCLE.

II. *RANA TEMPORARIA* AS TEST AMIMAL.

III. THE ACTION OF ANTERIOR LOBE PITUITARY EXTRACTS ON THE OVARY.

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II. *RANA TEMPORARIA* AS TEST ANIMAL.

It is now generally believed that the nature and extent of the periodic changes occurring in the ovary are controlled by the anterior lobe of the pituitary. The study of this relationship has been seriously handicapped, however, because the gonad-stimulating properties of extracts and other preparations can only be adequately studied in the hypophysectomised animal. Since it is practically impossible to remove the anterior lobe of the mammal alone or even carry out total hypophysectomy with ease, progress is beset by serious obstacles. In a previous paper [Bellerby, 1933] it was pointed out that this difficulty can be overcome by making use of certain amphibia, and attention was drawn to the suitability of the African Clawed Toad (*Xenopus laevis*) for use in the study of the ovary-pituitary relationship particularly when used for the assay of a gonad-stimulating substance. Before the practical possibilities of *Xenopus* were ascertained experiments were carried out to determine the suitability of the common English frog (*Rana temporaria*) for the same purpose.

As regards the removal of the pituitary *Rana* possesses the same main advantages as *X. laevis* [Hogben, 1923].

(*a*) It can be easily and rapidly hypophysectomised without injury.

(*b*) One or both lobes of the pituitary can be removed with precision.

(*c*) Completeness of removal can be checked by the characteristic colour response of the animal.

The present investigation shows that *Rana* is much less satisfactory than *Xenopus* for use in the study of the gonad-stimulating properties of extracts. It can nevertheless be used with advantage to confirm conclusions derived from experiments on *Xenopus* and will be made use of in the present studies for that purpose.

Experimental.

After the female has spawned in the spring the resultant atrophic ovary undergoes rapid development throughout the year until it is practically fully developed again by the autumn. A period of slow growth then occurs until the following March or April. The ovary has finally increased about ten times in weight and consists almost exclusively of black ova.

At spawning time the ova are first liberated *en masse* into the body cavity (ovulation *sensu stricto*). Then as a result of muscular movements of unknown origin they enter the oviducts which become enormously distended, leaving the ovary yellowish in colour and a mere vestige of its former size. The ova are finally expelled from the oviducts through the cloaca (oviposition) as a result of some stimulus the nature of which is at present unknown. The process takes place in three distinct stages, and some time may elapse between ovulation and oviposition.

In the present series of investigations suspensions of anterior lobe tissue were employed. The sole reason for this is that experiments were carried out before the work on *Xenopus* was undertaken. It was not until some progress had been made with the latter that it was found that extracts yielded consistent results.

Suspensions were prepared as follows. The glands were dissected out within 4 hours of the death of the animal and were ground to a fine paste with sand. The mass was agitated with twice its weight of normal saline for about 10 minutes and centrifuged. The fine suspension was then decanted off the sand and residual tissue and injected without delay. All injections were made into the dorsal lymph sac.

The great advantage of *X. laevis* as a test animal is that eggs are ejected externally within 24 hours as a result of a single injection of an active preparation. This dispenses with the necessity of killing the animal. An attempt was therefore made to procure a similar reaction in *Rana*. Two series of experiments were carried out.

In the first, frogs were injected with a volume of suspension equivalent to 2 g. of original tissue and were killed 3 days later. Oviposition only occurred in 2 cases, one frog ejecting three ova on the 2nd day, the other two ova on the 3rd day. The result therefore was quite abnormal because in the normal frog several hundreds of ova are shed at one time.

In Table I are given details of the *post mortem* results. It was somewhat surprising to find that although definite oviposition had not occurred marked

Table I.

Weights in g.

Injected			Controls		
Body weight	Weight of ovary	Weight of oviducts	Body weight	Weight of ovary	Weight of oviducts
51·50	0·850	27·75	37·75	7·02	8·50
50·25	0·605	24·25	36·25	3·56	12·00
46·52*	0·425	14·02	33·50	5·55	7·75
44·75	0·415	14·72	28·70	3·57	8·01
42·62	0·495	17·95	28·45	3·74	7·15
41·00*	0·552	17·50	28·25	5·25	5·75
40·05	0·650	17·25	28·02	3·32	7·76
39·82*	0·575	13·25	24·55	4·52	7·25
39·61	0·625	13·27	23·15	3·25	5·48
39·25	0·425	14·02	23·10	3·09	6·32
38·87	0·385	15·05	20·55	2·57	5·50
34·35	0·425	9·61	20·15	3·74	3·85

* Oviposition occurred.

changes had taken place in the injected animals. All exhibited the typical changes found during the immediate pre-spawning period; that is to say the ova had been released from the ovary and had passed into the oviducts, whilst in the controls the ovaries remained intact and the oviducts empty.

The second series of frogs was injected with a volume of extract equivalent to 1 g. of fresh tissue, the animals being killed 7 days later. The results confirmed those of the first series. Out of 16 frogs, only 3 shed their eggs into the water, the total number not exceeding half a dozen. On *post mortem*, it was found that ovulation had occurred, however, in 12 animals.

Further experiments, though useless from the practical point of view, were carried out to see whether typical oviposition could be brought about by (*a*) further injections of anterior lobe substance, (*b*) subsequent injection of the oxytocic principle of the posterior lobe of the pituitary, (*c*) by placing injected animals in contact with males. All these were without result. It thus became clear that normal oviposition in *Rana* could not be taken to indicate activity of a preparation. It would be necessary to kill animals to confirm the result of an experiment. As the macroscopic appearance alone of the ovaries and oviducts in injected animals was sufficiently definite to indicate a positive result experiments were carried out to determine the earliest time that a reaction could be obtained. In Table II are shown the results of an experiment embracing 4 series of 8 frogs killed at different times after injection.

Table II.

Series	Time killed after injection hours	No. of frogs ovulating	Remarks
A	24	0	—
B	36	3	A few ova in body cavity. Ovary full
C	48	7	Body cavity full of ova. Ovary vestigial
D	72	7	Oviducts full. Several ova in body cavity. Ovary vestigial

The results show that a well-defined positive result can be obtained within 72 hours. If, however, this is taken to be liberation of ova from the ovary into the body cavity and not into the oviducts the reaction time can be reduced to 48 hours.

Several estimations of the gonad-stimulating power of anterior lobe preparations were carried out using this reaction.

Ovulation could be produced in 50 % of animals with a volume of suspension corresponding to as little as 0·25 g. of original tissue. It was also found that injection of acid extracts of the gland prepared by the writer's method [Bellerby, 1929] was effective.

The above experiments were carried out in the months of January and February. Subsequent investigation demonstrated a further disadvantage of *Rana*. The animal cannot be used in March or early April as it normally spawns during that time and ovulation can only be induced with a single injection from about the end of August to February. Experiments showed that during the rest of the year it was necessary to administer a series of injections to produce ovulation, the number of which varied according to the time of the year. As this phase of the work has been thoroughly investigated by Spaul [private communication] no further details are given here.

Apart from the indication of the drawbacks of *Rana* as an experimental animal two main points have emerged from the present enquiry. Firstly the results suggest that the process of ovulation and oviposition in *Rana* is more complicated than in *Xenopus*. It appears that some factor other than secretion of the anterior lobe of the pituitary is involved. In *Xenopus* the whole process of ovulation and oviposition can be attributed to anterior lobe activity. Secondly, *Rana* has one definite advantage over *Xenopus*. Because the ovary remains

undeveloped for several months it can be used to determine the effect of an extract in producing development of ova as distinct from one which induces their liberation from the fully developed ovary during that time. In *Xenopus* the ovary remains fully mature throughout the year when the toad is kept under optimum conditions. It cannot be used in consequence to demonstrate the former effect.

SUMMARY.

1. Ovulation without oviposition can be induced by injection of saline suspensions of anterior lobe pituitary tissue in the common frog *Rana temporaria*.

2. Oviposition only occurs in few injected animals and is never complete as in the normal animal.

3. Ovulation induced by single injections cannot be obtained during the whole year.

III. THE ACTION OF ANTERIOR LOBE PITUITARY EXTRACTS ON THE OVARY.

In a previous paper [Bellerby, 1933] and the foregoing part of the present paper it was shown that *Xenopus laevis* and *Rana temporaria* have certain advantages in work directed to extend knowledge of the chemical co-ordination between the ovary and the anterior lobe of the pituitary. In this paper are given details of the first of a series of observations made on the number and nature of the endocrine factors which are definitely concerned in the control of the ovarian cycle using *X. laevis* as the experimental animal. Before proceeding to the main issue discussed, it is necessary to refer to a matter which was not dealt with in the first paper of this series, namely the relation of external temperature to the ovulation process induced in *Xenopus* by extracts. Formerly, all experiments had been carried out at room temperature, but it transpired later that the temperature could be raised with advantage to the experimental procedure.

Relation of ovulation to external temperature.

Temperature variation might influence ovulation in two ways.

(*a*) It might increase or decrease the time elapsing between injection and ovulation.

(*b*) It might render the animal more or less sensitive to the effect of the extract so that different amounts would be required to produce the same effect.

Table I gives the results of a series of seven experiments regarding this point. Groups of 20 toads were divided into two batches of 10 each which were kept at different temperatures. They were injected with less than the quantity of acid extract required to produce ovulation in 100 % of the animals and received a threshold amount which under ordinary conditions would produce a positive response in about 60 % of animals. Toads were allowed to remain at their respective temperatures for four or five days before injection. As laboratory facilities did not permit of each batch being kept at a stipulated temperature, the difference for the two series was obtained by keeping one batch in a cold underground room and the other in a room heated with an electric fire. Both series were kept on a white background and under similar conditions of illumination.

It is clear that temperature has a marked influence in reducing the time taken for ovulation. In some hundreds of experiments which have been carried

Table I.

Temperature ° C.	Temperature difference ° C.	Number of toads ovulating				
		0–10 hrs.	10–24 hrs.	24–36 hrs.	36–48 hrs.	Total
31·5	15·5	7	1	0	0	8
16·0		0	4	3	0	7
27·0	11·0	6	0	0	0	6
16·0		0	0	5	3	8
24·0	10·0	4	0	0	0	4
14·0		0	1	5	0	6
26·5	12·3	7	0	0	0	7
14·2		0	3	3	0	6
25·2	10·7	3	4	0	0	7
14·5		0	4	4	0	8
26·2	7·7	4	2	0	0	6
18·5		0	7	0	0	7
23·5	7·0	5	2	0	0	7
16·5		0	2	2	0	4

out at room temperature (14–18°), ovulation under 12 hours only occurred once, the average time of occurrence being 18 hours.

In the animals kept at the high temperature (23–31°) ovulation took place in the majority of cases within 10 hours and in no case was it delayed beyond 48 hours. The average time was reduced to about 9 hours, all ova in some cases being shed within 7 hours of injection.

The results are summarised in Table II. It will be noted that a high temperature has no recognisable effect on the sensitivity of the response, practically the same number of toads ovulating at both temperatures. Temperature can therefore be disregarded in determining activity of extracts. At the same time the fact that a high temperature quickens the reaction increases the usefulness of *Xenopus* for purposes of assay.

Table II.

Temperature ° C.	No. of toads injected	No. ovulating				
		0–10 hrs.	10–24 hrs.	24–36 hrs.	36–48 hrs	Total
23·5–31·5	70	36	9	0	0	45
14·0–18·5	70	0	21	22	3	46

Relation of percentage response to amount of active substance injected.

A previous enquiry showed how it was possible to ensure a 100 % response with a sufficient dosage. In perfecting any method of assay based on the principle of the minimum dose, it is of the utmost importance to explore the range of dosage between quantities just sufficient to give a 100 % result and just too little to give any response at all. This point has been fully investigated in a series of experiments with acid extracts, the results being summarised in the graph shown in Fig. 1. Extracts were prepared as described in Part I of this series.

Roughly speaking a 50 % response requires about 4 times the absolute minimum dose and the least quantity required to evoke a 100 % response is 4 times the dosage required to produce any result at all. The reader may judge the reliability of this estimate by the very satisfactory consistency in the way the points arrange themselves in relation to a logarithmic curve. However, some attention was given to the reliability of each estimate in the mid region of the curve. In the neighbour-

hood of a 70 % response, an estimate based on 4 series of 10 toads is seen from the data in Table III to be subject to a maximum variation of 5 % when the whole is considered as a group of 40 animals. Each of the points in the graphs in Figs. 1 and 2 is based on 40 toads.

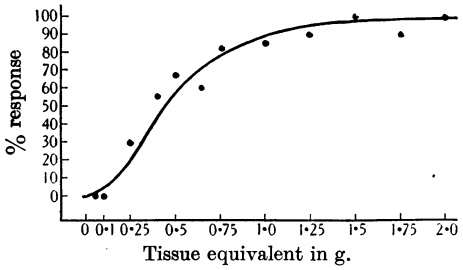

Fig. 1.

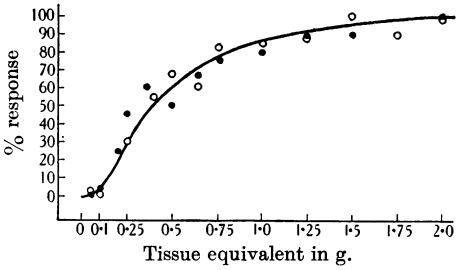

Fig. 2. o = Acid extract. • = Alkaline extract.

Table III.

No. of batch	No. injected	No. ovulating	Percentage response: 10 toads	20 toads	40 toads
A	10	3	30	65	70
B	10	10	100		
C	10	8	80	75	
D	10	7	70		

The action of alkaline extracts of anterior lobe.

The chief point which must be considered in any explanation of the mechanism by which the anterior lobe of the pituitary controls ovarian periodicity is whether one or more hormones are concerned. Evans and Long [1921] showed that injection of mature female rats with saline suspensions or extracts prepared with NaOH was followed by cessation of the oestrous cycle as a result of intensive luteinisation of the ovary. In the immature rat no effect was produced. Later Smith and Engle [1927] and Zondek and Ascheim [1927] found that injection of suspensions of anterior lobe tissue in the immature rat was followed by intensive follicular development, rupture of follicles and precocious oestrus. In the mature rat superovulation and superfoetation could be obtained [Engle, 1927]. At first the problem seemed a simple one. Basing his views mainly on the fact that injection of NaOH extracts in any dilution was ineffective in causing follicular growth and oestrus in the immature rat the writer suggested that two distinct substances were involved [Bellerby, 1928]. One was stable to alkali and on injection caused development of luteal cells; the other was stable to acid and induced growth and rupture of follicules. A similar view was expressed later by Evans and Simpson [1928] and Wiesner and Crew [1930]. However, further work [Bellerby, 1929] showed this view to be unsound. Making use of the rabbit, an animal in which no periodic changes take place in the ovary, it was found that injection of acid extracts of anterior lobe was followed by luteinisation as well as follicular growth and rupture. In fact, the injection of any anterior lobe preparation resulted in both luteinisation and follicular growth occurring collaterally to a corresponding extent.

Recently Fervold *et al.* [1931] and Claus [1931] claimed to have actually separated two substances from anterior lobe tissue with the specific physiological actions stated above. Subsequently, however, Van Dyke and Wallen-Lawrence

[1933] could not obtain evidence for the separation of the active substance into two fractions with solely follicle-stimulating or luteinising actions. Apart from this work no further reference is made to the numerous papers published on the "hormones of the anterior lobe of pituitary." The justification for this reticence is that in the majority of cases work on the existence of two or more substances in the anterior lobe transpires on further reading to be based on experiments in which no anterior lobe substance was employed. The large bulk of literature ostensibly dealing with the biochemical aspects of the anterior lobe is actually devoted to a detailed study of a certain novel but irrelevant attribute of the kidney. At the present time, therefore, no definite evidence is available to demonstrate the existence of two hormones directly influencing the ovary in the anterior lobe of the pituitary itself.

As stated in a previous paper, preliminary experiments showed that alkaline extracts were apparently just as effective as acid extracts in producing ovulation in *Xenopus*.

Extracts were prepared from ox pituitaries as follows.

The anterior lobes were dissected out within 4 hours of the death of the animal, weighed and ground to a fine paste with sand. The mixture was then extracted with 1½ times its weight of *N*/10 NaOH for 24 hours at a temperature of 0°.

After first allowing the solid mass to thaw out it was carefully neutralised with 50 % acetic acid, phenol red being used as indicator. The mixture was then centrifuged and the red turbid extract decanted off. Injections were made without any delay into the dorsal lymph sac, strict attention being given to the points raised in the first paper.

In Fig. 2 is shown a curve plotted from experiments in which injection of alkaline extracts was carried out under the same conditions governing the results obtained with the acid extracts. Points obtained from the latter series are also included for purposes of comparison. Emphasis must be laid on the fact that ten out of twelve points on the alkaline curve were derived from experiments in which the tissue-equivalent dose given was the same as in the acid series. It will be seen that there is close similarity in the configuration of the two curves. No difference therefore is apparent in the effect of alkaline and acid extracts in producing ovulation in *Xenopus*.

Conclusions.

The work which has been done in the past seems to show that the ovary-pituitary relationship is common to all the land vertebrates, a fact which need evoke no surprise when we consider the widespread distribution of substances such as adrenaline (Cannon), secretin (Bayliss and Starling) and of the oxytocic and melanophore principles of the pituitary (Hogben and de Beer). In mammals the relationship is complicated by the highly complex specialisation of the follicle before and after ovulation. In amphibia this specialisation is lacking. For this reason the effect of pituitary extracts upon *Xenopus* is a valuable source of supplementary evidence when interpreting the data derived from the study of mammals alone. In the latter the dual action of anterior lobe extracts which evoke follicular growth and ovulation on the one hand and luteinisation on the other might be interpreted in two ways. One is that there are two substances respectively extracted more readily in acid or alkaline media. The other is that one and the same substance stimulates two processes which may be antagonistic. The assumption implied in the last remark would explain why on some occasions extracts have been found to evoke the first type of response and on other occasions the second.

A point of pivotal importance in the discussion is that both these processes, namely follicular development and ovulation on the one hand, and luteinisation on the other, affect different stages in the development of cells derived from the same primordial elements of the gonad. The action of extracts upon the ovary in *Xenopus* involves the response of cells derived from primordial elements with developmental potentialities of a simple order.

At this stage, although the data derived from the study of *Xenopus* do not permit us to make a confident decision in favour of either one or the other of the two hypotheses stated above, they do permit us to narrow the issues involved in the assumption of two separate substances. That in some experiments on mammals alkaline extracts have given luteinisation alone whilst other extracts have only evoked follicular development might be explained by those who invoke two separate substances in either of two ways. The first is that a luteinising substance which is more readily extracted in alkaline medium completely neutralises the action of another substance extracted both by alkaline and acid media, thus preventing the latter substance from evoking follicular development. The second is that such a luteinising substance does not directly neutralise the alternate component of an alkaline extract but stimulates the formation of cells with an antagonistic action so that the seat of the inhibition ultimately rests in the ovary itself.

In other words, the process of luteinisation may inhibit the response of immature follicles to the action of the follicular component. The fact that acid and alkaline extracts are equally effective in acting upon ovarian cells which cannot undergo a luteinising process definitely disposes of the first alternative. Thus we have now to decide in favour of the second alternative or of the hypothesis that only one substance is involved in the response of the ovary to anterior pituitary extracts. The latter is the more economical hypothesis and there are at present no conclusive objections to it. An underlying assumption in the previous remarks is that alkaline extracts which have been found by some workers to induce conspicuous luteinisation also contain the substance which promotes cell division of the primordial ovarian cells, culminating in the discharge of the ovum. If we prefer to accept the dual hypothesis this assumption is not only justified by the experiments on *Xenopus*. It is also implicit in the testimony derived from mammalian studies since the presence of numerous corpora lutea presumably involves the previous development of follicles.

In conclusion it will not be out of place to point out the relation of the present work on *X. laevis* and *R. temporaria* to similar studies on amphibia. The results obtained are entirely in accord with investigations on the induction of ovulation by means of pituitary transplants [Wolfe, 1929; Morgan and Sondheim, 1932; Noble and Richards, 1930; 1932]. They also confirm the work of Kehl [1930], Adams [1931] and Buyse and Burns [1931] who have induced ovulation by means of injection of extracts of mammalian pituitary substance. Thus no support is given to the contention that ovulation can only be induced in amphibia by homeo-transplants or extracts of glands from amphibia themselves [Houssay *et al.*, 1929]. The above investigations coupled with those carried out on mammalia clearly show that an ovary-stimulating substance in anterior lobe tissue has a wide distribution throughout the whole vertebrate series.

SUMMARY.

1. Alkaline extracts of anterior lobe pituitary injected in equivalent dosage throughout the whole range of effective concentration are as effective as acid extracts in producing ovulation in *Xenopus laevis*.

2. By keeping toads at a high temperature (23–31°) the time elapsing between injection and ovulation can be reduced from an average of 18 to 9 hours, thereby increasing the value of the animal for test purposes.

3. Temperature variation was not found to have any perceptible effect on the sensitivity of the response of ovulation to injection of extract.

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