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

I. *XENOPUS LAEVIS* AS A TEST ANIMAL.

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EXPERIMENTAL studies upon the relation of the pituitary gland to the ovarian cycle in mammals involve three principal difficulties:

- (i) that of removing the pituitary itself without injury to the animal;
- (ii) the practical impossibility of removing the anterior lobe without removing or damaging the posterior lobe;
- (iii) the necessity of killing the test animal, in most cases, to confirm whether injected extracts or transplants have produced ovulation or other changes in the ovaries.

None of these objections arises, however, in the case of certain amphibia. In a recent series of papers by Hogben [1930], Hogben, Charles and Slome [1931] and Zwarenstein and Shapiro [1933] reporting work carried out on the African Clawed Toad (*Xenopus laevis*) it has been shown:

- (i) that ovarian atrophy follows removal of the anterior lobe alone;
- (ii) that injection of acid extracts prepared by the method elsewhere described by the writer [Bellerby, 1929] will induce the extrusion of eggs during the period of the year when the animal does not breed either in its natural surroundings or in the laboratory;
- (iii) that the characteristic colour response of the animal serves as an infallible indication as to the completeness of removal of either one or both lobes of the pituitary.

The facts that hypophysectomy involving one or both lobes can be carried out in a few minutes with a negligible mortality and a high degree of accuracy, and that the extrusion of the amphibian egg is a sufficiently obvious and unequivocal phenomenon justify the attempt to explore the use of *Xenopus laevis* as a test animal for assay purposes in investigations directed to extend knowledge concerning the chemical properties of anterior lobe hormones and related substances and improve existing methods of extraction, etc.

Before ovulation could be regarded as adequate for practical purposes several points which had not been dealt with by the workers already quoted required thorough investigation. These were in particular the reliability of the test, its time relations, and its sensitivity.

Reliability of Xenopus laevis.

The question of reliability demands special attention, because it is well known that a considerable percentage of failures is usually recorded in mammalian experiments. Firstly, there is no danger of error arising in the case of

Xenopus through spontaneous ovulation. *Xenopus* does not ovulate even during the breeding season, when kept under laboratory conditions (Hogben, Zwarenstein). In any case ova extruded after pituitary injection possess no mucilaginous envelope, so characteristic of the amphibian egg when ovulation occurs in the normal way. If the animals are well fed and kept in a warm, well-lighted laboratory the ovaries remain in a healthy condition with black pigmented ova (Hogben) indefinitely. On the other hand unfavourable conditions of captivity (darkness in particular and apparently a temperature lower than that of the animal in its natural surroundings) lead to a retrogression of the ovaries after 4 or 5 months (Zwarenstein and Shapiro). In these circumstances no ripe ova are present in the ovaries, and the animals are, in consequence, unsuitable for experimental work, when results are demanded from a single injection of extract.

The first experiments undertaken by the writer were carried out on animals which had been kept for some months in the laboratory, in a cold underground room, and many failures to produce ovulation with single injections were recorded. Table I shows such an experiment. The acid extracts used in this and all subsequent experiments were prepared in the following way. The glands were collected at the abattoir and dissected out within 4 hours of death. The anterior lobes were weighed and ground with sand in a mortar. The paste was extracted with 1.5 times its weight (*i.e.* original tissue) of 1 % acetic acid for 24 hours at room temperature. The mixture was then centrifuged and the clear extract decanted from the residue and carefully neutralised with 50 % NaOH, using phenol red as indicator, so that the p_H of the fluid was eventually about 7.4. On removing the precipitate by centrifuging a second time, the extract was ready for immediate use. Injection was made into the dorsal lymph sac to leave no doubt concerning the transport of the active substance in the extract to the ovaries *via* the circulation. The final number of cc. of extract obtained was usually greater than g. of tissue taken. In all cases therefore sufficient volume of extract was injected to ensure that the equivalent of a definite amount of tissue was administered, *i.e.* 0.25 g., 0.5 g. or some simple multiple of these amounts.

Table I.

Dosage*	No. of toads used	No. ovulating	Mean no. of eggs shed
0.5	8	0	0
1.0	8	4	17
2.0	8	2	19

* Equivalent g. of original tissue.

In the experiments recorded in Table I, it will be seen that a large number of animals failed to give the expected response of ovulation. Furthermore, a better response was given by the batch which received the intermediate dose and not the maximum dose. These results suggested that some factor or factors, in addition to the potency of the extract was involved. This was probably the condition of the ovaries.

Two series of experiments were carried out to ascertain the cause of failure. In the first 24 toads were selected and divided into 2 batches of 12 each. One batch (A) had all ovulated previously after injection. The other batch (B) consisted of animals which had not done so. All toads received a dose of extract equivalent to 2 g. of fresh gland. In series (B) 2 out of 12 gave a positive response. In series (A) 8 out of 12 gave a positive response. Complete results are given in Table II.

Table II.

No. of eggs shed	
Batch A	Batch B
263	0
8	11
0	0
303	0
10	0
479	12
0	0
540	0
0	0
38	0
0	0
91	0
Total no. of toads ovulating = 8	Total no. of toads ovulating = 2

In the second experiment, recorded in Table III, the non-ovulating animals were killed to ascertain the reason for failure to give the response. It will be seen that 5 animals out of 11 failed to respond to an injection of 2 cc. of extract equivalent to 2 g. of tissue. When killed and examined these animals alone were found to have atrophic ovaries.

Table III.

No. of toad	No. of eggs shed	Condition of ovary when killed
A	0	Ovary atrophic
B	0	" "
C	294	" normal
D	34	" "
E	0	" atrophic
F	392	" normal
G	466	" "
H	64	" "
I	236	" "
J	0	" atrophic
K	0	" "

From these experiments it became evident that the condition of the ovaries was a deciding factor if perfect reliability was to be guaranteed, and that it was necessary to pay special attention to this point. Subsequently to these experiments all toads to be used were removed to a warm, well-lighted room, and, by only using animals that had been tested for a positive response, it was found possible to approach 100 % reliability in subsequent experiments.

A further issue of considerable importance in safeguarding a high standard of reliability for experiments involving the injection of extracts concerns the relation of degree of absorption of the injected extract to its volume and salt concentration. In Table IV an experiment which only yielded a positive response in 5 out of 12 animals is recorded. The dosage was, in this case, equivalent to 1.0 g. of fresh gland substance in 4.4 cc. of fluid. It was evident from the greatly distended lymph sac that the fluid was not absorbed in the majority of the animals. This was tested by an incision of the skin from which the fluid squirted out under pressure. Even after 3 days the 7 animals which failed to ovulate showed that the extract was still largely unabsorbed (Table IV). That failure to ovulate was not due to atrophic ovaries was ascertained by autopsy.

These experiments show that it is very important to use as small a volume of fluid as is practicable and to avoid methods of preparing extracts which will

Table IV.

No. of toad	No. of eggs shed	Whether extract absorbed	Condition of ovaries of toads killed
P	17	+	—
Q	0	0	Normal
R	82	+	—
S	87	+	—
T	0	0	Normal
U	0	0	"
V	0	0	"
W	264	+	—
X	0	0	Normal
Y	0	0	"
Z	0	0	"
A 1	37	+	—

ultimately yield a fluid containing a high salt concentration, which will, in consequence, differ largely in osmotic concentration from the lymph and body fluids.

Time relations of the response to single injections.

The chief advantage of *Xenopus* as test animal for work involving assay of pituitary extracts is that extrusion of eggs follows rapidly after introduction of active preparations into the lymph sac. It generally begins about 12 hours and lasts till about 24 hours after injection. The results of a number of experiments, in which the time relations were noted carefully, are given as a frequency distribution in Table V. In these experiments, as in others, toads were kept in

Table V. *Time relation of ovulation following injection.*

Hours	0-12	12-24	24-36	36-48	48-60	60-72	Total no. of cases
Eggs first extruded	1	49	14	4	3	0	71
Completion of ovulation	0	18	17	9	3	0	47

separate containers and were examined for the presence of eggs at 12 hourly periods after injection. The eggs found at the end of each period were removed and counted. This was a necessary precaution in determining number of eggs shed, since it was observed that the toads frequently ate them, if they were left in containers for more than 24 hours.

Sensitivity of the response.

Either of two criteria might be selected as a basis for comparing the amounts of gonad-stimulating substance in extracts. One is the number of eggs laid. The other is whether any eggs are laid or not. The first, however, is not a very reliable index of the dosage, for a great variation exists in the number of eggs shed per toad, even when receiving the same amount of extract. For instance, in one experiment embracing 10 toads the number of eggs shed varied per individual from 3 to 1539. In Table VI will be seen results obtained on 76 animals. The figures form a frequency distribution with unequal class intervals.

Table VI. *Frequency distribution of eggs laid.*

0-25	25-75	75-175	175-375	375-725	725-1575
20	15	16	11	10	4

The second criterion gives results, however, which are consistent with the view that response varies with the potency of the extract injected. In the series of experiments recorded in Table VII 3 different series of toads received the

Table VII.

Dosage	No. of toads used	No. of toads ovulating	% of toads ovulating	Extremes of eggs laid	Mean no. of eggs shed
0.25	8	0	0	0	0
0.50	21	3	14	53-85	63
1.00	22	5	22	33-285	228
1.50	20	11	55	7-494	112
2.00	16	11	68	15-1062	206
2.50	10	10	100	60-423	247

same volume of extract but corresponding to different amounts of fresh tissue. The 3 series are combined and the mean number of eggs and range for each batch are given together with the percentage positive response.

It will be seen that there is no relation between number of eggs shed and dosage, but that there is a distinct relationship between percentage response and the latter. The data contained in Table VII enable a provisional definition of a unit for use in comparative work to be made. For instance, if the minimum amount of extract is chosen which will produce a response in 50 % of 10 toads, of an average weight of 35 g., this amount of extract would contain 1 toad unit. That is to say 1 kg. of fresh tissue would contain 750 units.

The chief point of practical interest which emerges from these experiments is that there is a much more constant relation between dosage and response in *Xenopus* than the writer has been able to establish using mammals as test animals. Further, the same set of animals may be used repeatedly for assay work. This is advantageous, not merely because it diminishes expense, but also,

Table VIII.

No. of toad	No. of eggs shed after successive injections			
	First	Second	Third	Fourth
36 H	423	403	156	294
36 S	169	87	15	392
36 W	110	264	0	236
36 A	131	17	1062	765

because it ensures that the ovaries are in good condition. In Table VIII is recorded an experiment in which four successive injections were made on the same batch of animals, at fortnightly intervals.

CONCLUSIONS.

It would be premature to give full details of the chemical work which has been carried out using *Xenopus laevis* as a test animal. Three facts which have emerged from the present enquiry, at this stage, throw light on the property of anterior lobe extracts. The first two are merely practical. If glands are dissected immediately after killing, the anterior lobes can be kept at a temperature about freezing point for several weeks without affecting greatly the potency of extracts subsequently made from them. A second fact of some interest, in view of the very large quantity of glandular material which is necessary to evoke ovarian changes both in *Xenopus* and in the mammal, is that a subsequent extraction

of the solid matter which is separated after centrifuging, for the first time, yields an extract which may have a potency equal to a third of that of the original extract. A third point is of more general interest. In the case of the rabbit, acid extracts prepared by the writer's method and alkaline extracts prepared in the way prescribed by Evans and Long [1921; 1922] both induce ovulation and both induce luteinisation on injection. Experiments on *Xenopus*, made with alkaline extracts, show that these are almost as effective as acid extracts in producing ovulation.

SUMMARY.

1. Acid extracts of ox anterior pituitary, when injected in sufficient quantity into females of *Xenopus laevis* with healthy ovaries, uniformly produce extrusion of eggs.
2. Ovulation usually occurs within 18 hours and is rarely delayed beyond 48 hours.
3. The same animals may be used successively for the assay of extracts.
4. There is a more constant relation between dosage and response than is obtained when the mammal is used as test material.

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