EXPERIMENTS ON THE LOCALISATION OF THE SUBSTANCES IN PITUITARY EXTRACTS RESPONSIBLE FOR METAMORPHIC AND PIGMENTARY CHANGES IN AMPHIBIA.*

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1. Introduction.

THE close anatomical connection between the two portions of the pituitary body and the definite but dissimilar influences apparently exerted by the autacoids present in their extracts emphasise the need for eliminating contamination in the preparation of the latter. Recently the author found only one of several commercial products of the anterior lobe giving a positive reaction when administered in comparable dosage in experiments on metamorphosis of the axolotl larva of the Mexican salamander. The melanophore stimulant characteristically present in quantity in extracts of the posterior lobe was, however, found to be present in each preparation.

It seemed, therefore, that attention might be profitably directed to investigating the reason for these divergent results, with a view to throwing further light on the localisation and interaction (if any) of the active substances concerned with amphibian metamorphosis and pigmentary response.

In actual practice an appreciable interval necessarily elapses between the death of the animal and the removal of the pituitary body from the skull, the dissection of the gland,

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and the preparation of the extract. There is thus opportunity for diffusion, if such takes place, from one portion of the gland to another. If extracts of the anterior and posterior lobes could be prepared from glands after varying intervals, one would suppose that diffusion would be detected by testing each product for the amount of melanophore stimulant present by the response of the pigment cells of the frog, and comparing results with the ability of the extracts to cause the successful transformation of the axolotl. Two complete series of extracts, made at definite but increasing intervals, were obtained, (*a*) prepared under manufacturing conditions; (*b*) prepared in the laboratory, the one serving as check to the other and enabling any errors arising through differences in the method of preparation to be ascertained and allowed for in the comparisons. In each series the pituitary glands were dissected out from the skull (ox glands were used throughout) and dropped into ice. All intervals of exposure were reckoned from the time the whole glands were taken out of the ice, so that although the actual period between death and extraction was only approximately known, it was exactly the same for all members of the series. The time spent by the glands in ice was very small, and it was assumed that this treatment rendered diffusion impossible, but to test this, preparations were made from glands kept in ice for long intervals. On being taken from ice the glands were exposed to the same room temperature (15° C.) under identical conditions for the necessary interval before the separation of the two lobes, except in cases to be mentioned. The extracts were made as soon as the lobes had been dissected out and weighed, but further treatment depended upon which series of extracts they were used for, since the commercial method of extraction differed in some respects from that employed in the laboratory, although an acid medium for extraction was used in each case. However, 20 per cent. sterile extracts were obtained in each series and they were stored in hermetically sealed tubes.

2. Manufactured Preparations.

The commercial preparations, carried out under the author's directions in the laboratory of Messrs Oppenheimer, will

be considered first. The series consisted of the following extracts :—

(*A*) Anterior Lobe. — (1) Extract made directly glands taken from ice and dissected.
(2) Extract made from glands exposed for two hours.
(3) Extract made from glands exposed for four hours.
(4) Extract made from glands exposed for eight hours.
(5) Extract made from glands exposed for twenty-four hours.

(*B*) Pituitary Body. — (6) Extract made from whole glands direct from ice.

(*C*) Posterior Lobe. — (7) Extract made directly glands taken from ice and dissected.
(8) Extract made from glands after eight hours' exposure.
(9) Extract made from glands after twenty-four hours' exposure.

(*D*) Anterior Lobe.—(10) Extract made from glands directly they were taken from ice and dissected.
(11) Extract made direct from glands kept in ice two days before dissection.
(12) Extract made direct from glands kept in ice four days before dissection.

The extracts prepared direct from glands placed in ice for a short interval only (Nos. 1, 6, 7, and 10) were made as quickly as possible to reduce the interval between death of the animal and actual preparation to a minimum, and further to prevent any undue changes due to alteration in temperature. Similarly the rapid preparation of extracts Nos. 11 and 12 practically eliminated temperature effects.

In the metamorphic tests the dose given was 0.75 gr. per 0.5 c.c., a quantity found in previous work to be approximately the smallest amount of a similarly prepared product bringing about metamorphosis in the shortest time for a full sized animal at a temperature of 22° C. When administered under similar conditions greater amounts of active extracts do not shorten the period to any extent, but smaller doses prolong up to a certain point, when the extract becomes too weak to be effective.

The axolotls used for these tests, as in previous work, were kept in glass containers of about 2 litres capacity, fed

with raw beef, weighed, and measured once a week; at the same time the water was changed. They were placed in a room kept at a temperature 22° to 24° C. A dose of 0.5 c.c. of the original extract diluted to 10 per cent. with Ringer's solution, equivalent to 0.75 gr. fresh gland was injected tri-weekly, and the same intraperitoneal injection was given to the same animal throughout the experiment. Conditions, therefore, were as uniform as possible and remained so during the period of treatment.

The melanophore test elaborated by Hogben and Winton and used by the author in recent work was utilised for studying the pigmentary response. Suitable frogs of known weight, bleached by placing when dry in muslin covered containers in strong light on a white background, were injected with 0.5 c.c. of various strengths of a 10 per cent. extract, the 20 per cent. extract having been diluted with Ringer's solution, and thereby those concentrations just failing to darken the frog's skin, and just sufficient to darken it were found. These conditions correspond respectively to a reticulate and contracted phase of the melanophores when examined microscopically. In this manner the minimal and sub-minimal doses with reference to a standard weight (20 gms.) were determined for each extract in turn. The approximate estimation was followed by an accurate one, in which frogs of various weights were injected with the minimal or sub-minimal dose. The response varies with the weight of the animal and so this reduction gave a uniform basis for comparison and the mean—that amount of melanophore stimulant just giving a response in a frog of 20 gms. weight—could be determined.

The results of this first series of experiments upon manufactured preparations are summarised in Table I. The salient points as regards the effect upon metamorphosis are:—

(1) Only preparations of anterior lobe proved efficacious in inducing metamorphosis.

(2) The activity of the autacoid concerned with this response is not impaired by exposure up to four hours: storage of glands on ice for short periods does not affect the activity of extracts prepared from them: exposure beyond four hours increases the time taken for completion of metamorphosis with equivalent injections; and no change whatever occurred with extracts of glands exposed to room temperature for twenty-four hours or more.

Localisation of Substances in Pituitary Extracts

In these experiments upon axolotls it may be noted that the characteristic pigmentary change noticed by Smith and by Hogben were less pronounced in animals treated with posterior lobe extracts subjected to prolonged exposure.

TABLE I.

Extract.	Metamorphic Changes.	Threshold Dose for Melanophore Test for Frog of 20 gms. Body-weight.
		Mean value—
A. 1. Anterior lobe direct from ice.	Metamorphosis complete in 42 days. Loss in weight 40 per cent.	= 0·01 c.c.
2. Anterior lobe after 2 hours.	Metamorphosis complete in 45 days. Loss in weight 40 per cent.	= 0·002 „
3. Anterior lobe after 4 hours' exposure.	Metamorphosis complete in 38 days. Loss in weight 40 per cent.	= 0·00015 „
4. Anterior lobe after 8 hours' exposure.	Metamorphosis complete in 55 days. Loss in weight 15 per cent.	= 0·001 „
5. Anterior lobe after 24 hours' exposure.	Practically no change. Loss in weight 20 per cent.	= 0·015 „
B. 6. Pituitary gland direct from ice.	Tissue reduction, no metamorphosis, colour changes. Loss in weight 40 per cent.	= 0·00015 „
C. 7. Posterior lobe direct from ice.	Slight tissue reduction, no metamorphosis, marked colour changes. Loss in weight 35 per cent.	= 0·0000005 „
8. Posterior lobe after 8 hours' exposure.	No tissue reduction, no metamorphosis, colour changes. Loss in weight 40 per cent.	= 0·00005 „
9. Posterior lobe after 24 hours' exposure.	No tissue reduction, no metamorphosis, slight colour changes. Loss in weight 40 per cent.	= 0·0004 „
D. 10. Anterior lobe direct from ice.	Metamorphosis complete in 45 days. Loss in weight 40 per cent.	= 0·006 „
11. Anterior lobe 2 days in ice.	Metamorphosis complete in 41 days. Loss in weight 55 per cent.	= 0·006 „
12. Anterior lobe 4 days in ice.	Metamorphosis complete in 50 days. Loss in weight 40 per cent.	= 0·006 „

As regards the melanophore test the main conclusions to be drawn are:—

(1) Every extract contained at least a trace of melanophore stimulant, but this substance is invariably present in greater quantity in posterior than in anterior lobe preparations.

(2) Gradual increase of the melanophore stimulant occurs with exposure of the gland up to four hours in the case of anterior lobe extracts: this is followed by a decline, so that after twenty-four hours the amount present is less than with extracts immediately prepared.

(3) The melanophore activity of posterior lobe extracts, on the other hand, diminishes continuously with exposure of the gland the diminution becoming less rapid after eight hours.

3. Laboratory Preparations.

Experiments dealing with extracts prepared in the laboratory will now be described and compared with those already obtained using the commercial extracts.

In previous work extractions from the manufactured products were made with Ringer's solution, but this method gave most unsatisfactory results when used for the extraction of the principle from fresh glands. However, later experiment showed that very active products were obtained using suitable concentrations of acetic acid as the extracting medium. The following description details the method used. The glands were dissected immediately after death and placed in a receptacle surrounded by a freezing mixture, so that its temperature was well below freezing point, and taken to the laboratory. The lobes were immediately separated and the anterior weighed, cut into small portions, minced, and pounded in a mortar. The mass was next transferred to the required amount of warm 0.1 per cent. acetic acid to give a 20 per cent. extract. After boiling a few minutes the solution was filtered, and cooled, and the extract stored in sealed tubes. Preparations of the posterior lobe, and also glands after exposure, were subjected to the same treatment to obtain extracts. Preparations were made using Ringer's solution in place of the acetic acid, but the procedure was identical in every way.

The series consisted of the following preparations of the same strength (20 per cent.):—

(1) Extract of the anterior lobe of glands taken direct from ice.
(2) Extract of outer portion of anterior lobe of glands taken direct from ice.
(3) Extract of inner portion of anterior lobe of glands taken direct from ice.
(4) Extract of whole glands direct from ice.
(5) Extract of posterior lobe of glands direct from ice.
(6) Extract of anterior lobe of glands direct from ice and extracted with Ringer's solution.

(7) Extract of posterior lobe of glands direct from ice and extracted with Ringer's solution.
(8) Extract of anterior lobe of glands exposed for three and a half hours at 15° C.
(9) Extract of posterior lobe of glands exposed for three and a half hours at 15° C.
(10) Extract of outer portion of anterior lobe of glands exposed for four and a half hours at 15° C.
(11) Extract of inner portion of anterior lobe of glands exposed for four and a half hours at 15° C.
(12) Extract of anterior lobe of glands exposed for four and a half hours at 15° C. extracted with Ringer's solution.
(13) Extract of posterior lobe of glands exposed for four and a half hours at 15° C. extracted with Ringer's solution.
(14) Extract of anterior lobe of glands exposed for six and a half hours at 15° C.
(15) Extract of posterior lobe of glands exposed for six and a half hours at 15° C.

As before the times of exposure were reckoned from the time the glands were taken from the ice. They were only placed for a few minutes on ice, the time being the same in each case. No accurate determination could be made, but it was approximately equal to that experienced by glands used for the commercial extracts. The glands were all taken from ice together, and those not being used direct for extracts were exposed on clean white tiles placed together ensuring uniformity, the necessary number being taken for preparations at the required intervals.

The melanophore test was applied under the same conditions as before, and the comparison showed the results of each series to be identical, thereby confirming and supporting those deductions already made. A similar series of experiments testing the activity of the anterior principle was not considered necessary in view of this close agreement, as no new information would be gained, but the extracts direct from ice were tested to prove the efficacy of the method used for extracting the active anterior principle. That prepared from ice using acetic acid as the medium proved very active, that prepared using Ringer's was unsatisfactory, while the preparation from the inner portion was also very active and the outer apparently ineffective.

The following tests were made upon axolotls :—

(1) Anterior lobe direct from ice—first signs of change after 8th injection—sixteenth day; complete after 14 injections—thirty-one days; occasionally refused food, otherwise normal. Loss in weight 45 per cent. and steady; no alteration in length.

(2) Anterior lobe direct from ice. Ringer's extracting fluid. Slight reduction about twentieth day, but no change indicating activity of anterior lobe principle.

(3) Anterior lobe (inner portion) direct from ice—first signs of change at 7th injection—thirteenth day; complete after 13 injections, twenty-eight days. Normal in every way; no alteration in length. Loss in weight 45 per cent. and uniform. Throughout change advanced more rapidly than in any other animal treated with anterior lobe extracts whether commercial or laboratory preparations, and this was very apparent when the stages in the reduction of the dorsal fin were compared, so that the metabolic rate was much higher.

(4) Anterior lobe (outer portion) direct from ice—no sign of change at all after 14 injections—thirty-four days. Steady loss in weight of 40 per cent. Perfectly normal so that failure to metamorphose could not be due to abnormality of animal.

The first record shows that the laboratory method of extraction with 0.1 per cent. acetic acid yields a product having the same action as the commercial extract. Ringer's solution, judged by these experiments, is not a suitable medium for the extraction of the anterior lobe principle from the fresh gland. The object of the preparations of the inner and outer portions of the anterior lobe was to trace, if possible, the diffusion of the melanophore stimulant through the anterior lobe, and hence they were not made in sufficient quantities to permit extended investigations into the question of the location of the anterior metamorphic principle as these records demand. Histological preparations of the anterior lobe pituitary reveal two distinct regions, an inner and outer, and these regions were dissected and extracts prepared as described, and these results suggest the inner portion as the seat of origin. Smith has claimed to find a different growth rate when feeding the outer portion to animals and concludes that growth promoting principles are confined to this part. However, the observations recorded here are merely tentative, and further investigation on a larger scale is required before definite conclusions can be drawn.

The records of the experiments to find the amount of

melanophore stimulant in each preparation are tabulated in Table II.

The following summary enables an easier comparison with similar results detailed in Table I. and shows the general confirmation of previous conclusions together with further information :—

(1) Traces of the melanophore stimulant are always present in anterior lobe extracts in spite of expeditious preparation.

TABLE II.

Extract.	Threshold Dose for Melanophore Test for Frog of 20 gms. Body-weight.	Extract.	Threshold Dose for Melanophore Test for Frog of 20 gms. Body-weight.
	Mean value—		Mean value—
1. Anterior lobe of glands direct from ice.	= 0·01 c.c.	9. Posterior lobe of glands exposed 4½ hours at 15° C.	= 0·00008 c.c.
2. Outer portion of anterior lobe of glands direct from ice.	= 0·04 „	10. Outer portion of anterior lobe of glands exposed 4½ hours at 15° C.	= 0·00025 „
3. Inner portion of anterior lobe of glands direct from ice.	= 0·01 „	11. Inner portion of anterior lobe of glands exposed 4½ hours at 15° C.	= 0·0001 „
4. Whole glands direct from ice.	= 0·00035 „	12. Anterior lobe of glands exposed 4½ hours at 15° C. extracted with Ringer's solution.	= 0·0004 „
5. Posterior lobe of glands direct from ice.	= 0·00001 „	13. Posterior lobe of glands exposed 4½ hours at 15° C. extracted with Ringer's solution.	= 0·0003 „
6. Anterior lobe of glands direct from ice extracted with Ringer's solution.	= 0·01 „	14. Anterior lobe of glands exposed 6½ hours at 15° C.	= 0·0002 „
7. Posterior lobe of glands direct from ice extracted with Ringer's solution.	= 0·00001 „	15. Posterior lobe of glands exposed 6½ hours at 15° C.	= 0·0003 „
8. Anterior lobe of glands exposed 3½ hours at 15° C.	= 0·0002 „		

(2) The amount present increases with exposure in anterior lobe extracts, but decreases in posterior lobe extracts.

(3) Ringer's solution or dilute acetic acid are equally potent extractors of the melanophore principle, since the same amount was found to be present in each case; but only the latter can extract the anterior principle.

(4) The melanophore principle can always be detected, irrespective of the presence of other principles.

(5) There is a greater amount of melanophore stimulant present in the inner portion than the outer of the anterior lobe, but this difference decreases with exposure.

These observations throw some light on conflicting statements which have been made as to the localisation of the melanophore stimulant and the activity of pituitary extract in relation to metamorphic processes.

In a recent paper Smith (1923) has maintained that the melanophore stimulant is distributed throughout the entire hypophysis. This view conflicts with the earlier results of implantation experiments of Swingle (1921), and the careful quantitative comparison of extracts from different parts of the gland by Hogben and Winton (1922) and Hogben (1924). The present experiments reinforce the view that the melanophore stimulant is in life restricted to the posterior lobe; and Smith's results based on extracts of glands prepared several hours after slaughter would appear to be due to post-mortem diffusion.

As regards metamorphosis in contrast with Hogben's (1922) observations on the Mexican axolotl, Smith (1922) found that injection of anterior lobe extract prolonged the larval condition. The necessity of preparing the extract in acid medium would explain why Smith did not obtain metamorphic changes, but the retardation of metamorphosis recorded by Smith requires another explanation. In the author's experiments extracts of whole gland were inefficacious to produce metamorphic change, such as accompany administration of similarly prepared extracts of the anterior lobe alone. This suggests that substances present in the posterior lobe interfere with the reaction. I learn from Dr Hogben (unpublished) that he obtained inhibition of metamorphosis in thyroid fed axolotls by injection of posterior lobe extracts alone, and the author has also inhibited metamorphosis in axolotls injected with (1) extracts of thyroid, and (2) anterior lobe, in sufficient quantities to complete the change, by injections of posterior lobe extract. It would seem, therefore, that to prepare extracts of anterior lobe which will induce metamorphosis in Urodeles, it is necessary not only to extract the gland substance with dilute acid, but to separate the lobes and prepare the extract itself as quickly as possible after the death of the animal from which the gland is taken.

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Oppenheimer for co-operation and supply of products. The expenses of the research were defrayed by a grant from the Dixon Fund.

4. Summary.

1. Metamorphosis can, as originally stated by Hogben, be induced in the neotenous axolotl larva of the Mexican salamander by regular injection of fresh extracts of the anterior lobe of bovine pituitary; the failure of Smith to obtain this result in experiments upon the Colorado axolotl appears to be due to the necessity of performing the extraction in acid medium.

2. Such traces of melanophore stimulant as are found in the anterior lobe of the mammalian pituitary result from post-mortem diffusion.

3. The substances responsible for production of metamorphic changes and pigmentary response are not identical.

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