BIOLOGICAL AND CHEMICAL STUDIES OF EXTRACTS OF THE ANTERIOR LOBE PITUITARY

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I. INTRODUCTION.

The relation of the anterior pituitary to the metamorphosis of Amphibia has provided a means of securing more intimate knowledge of the endocrine activity of this portion of the pituitary gland. The effects of extirpation and implantation of the gland upon the development of the larval forms indicated its relationship to metamorphic change, but the preparation and suitable administration of extracts of the anterior lobe capable of influencing these changes widened the field of investigation. As a consequence quantitative and qualitative information has been acquired, permitting the distinction of its activity from that displayed by the thyroid in metamorphosis, and its correlation to some extent with other known activities of the pituitary itself and those of other endocrine organs. Furthermore the standardisation of the method of preparation and the adoption of uniform conditions and procedure for the specific response—the production or acceleration of metamorphosis in the Amphibia—have made possible the biological assay of extracts (Spaul, 1930). The successful application of this method of assay is, however, limited considerably by the range—determined by the minimum dose influencing metamorphosis and the maximum response of the animal—and the degree of accuracy—dependent upon animal and other variations—that can be obtained. Hence, to further the study of the characteristics of this activity, a search is needed for some efficient reliable test and means of assay giving equivalent indication of biological activity and, at the same time, responsive beyond the limits imposed by biological assay, in which case the presence and amount of the activator failing to express itself biologically could be detected.

So far the approach to the problem has been mainly biological, but its consideration from a chemical standpoint might conceivably provide some means of satisfying these conditions by a suitable correlation of chemical methods with biological activity.

In the experiments recorded here, extracts, prepared by the same method but under various conditions and submitted to varying treatment, have been tested biologically and chemically and, by comparing the results, the possibility of checking the biological activity chemically has been examined.

II. BIOLOGICAL TESTS.

The standardised method and procedure of extraction by decoction of the fresh tissues with the required amount of the extracting medium adopted in previous work (Spaul, 1928, 1930) was used for all extracts, and they were sterilised for the same length of time. Subsequent treatment varied, but finally each extract was subjected in turn to the biological and chemical tests. Ox pituitaries were used for the gland extracts.

The biological tests consisted of (*a*) the metamorphic test—the accelerated metamorphosis produced in tadpoles, and (*b*) the melanophore test—the capacity to darken "bleached" frogs.

The approximate response of many of the extracts in these tests was known already from previous investigations, but for uniformity and comparative purposes a complete series of estimates was obtained.

(*a*) *The metamorphic test.* Batches of ten tadpoles at the same stage of development (from the appearance of limb buds onwards) from groups of animals obtained from different sources, were placed in small glass dishes containing 100 c.c. of tap water in uniform environmental conditions at room temperature (18° C.), and given tri-weekly injections (0·1 c.c.) of an extract. The injections were continued until metamorphosis was complete, when the time required and the number of injections were noted. A modification of this test was introduced in some cases. The progress made from the same stage of development after 8 days and three injections was observed, and the advance at the end of this period, compared with uninjected controls and those given injections of 0·125 per cent. acetic acid extracts, gave sufficient indication of activity for the purposes of this work.

(*b*) *The melanophore test.* Frogs of known weight were paled by putting them in dry glass containers covered with gauze placed on a white background in bright light. Each extract was diluted with Ringer's solution until such concentrations were reached that paled frogs were just darkened, or just failed to darken, when given a 0·5 c.c. injection. The minimal and submminimal, and so the threshold doses in c.c. for a standard weight (20 gm.) of frog were then determined for each extract.

III. CHEMICAL TESTS.

In a preliminary study of active extracts of the anterior pituitary, a precipitate was obtained by the addition of aqueous *N*/20 iodine solution (6·35 gm. I_2 and 15 gm. KI per litre). The volume of this precipitate was measured in a graduated centrifuge tube, and the amount of iodine in the precipitate ascertained by decanting the clear supernatant liquid, dissolving the precipitate in water and titrating the free iodine with thiosulphate. Titration of the decanted liquid gave the amount of free iodine remaining, whilst the difference between the total amount of iodine found by the two titrations and the amount introduced gave the iodine combined chemically.

It was found that a solution of potassium iodide containing the same weight of iodine as the *N*/20 solution, but entirely in the form of potassium iodide, gave no precipitate.

Aqueous *N*/20 bromine, either with or without the addition of potassium bromide, gave whitish precipitates which could not be centrifuged to give convenient and reliable volume measurements.

In the test finally adopted 10 c.c. of the extract were placed in a graduated centrifuge tube (15 c.c. graduated) and 6 c.c. *N*/20 iodine solution added from a burette. After mixing, the solution was allowed to stand for 1 minute and was then centrifuged for 1½ minutes at a speed of approximately 300 revolutions per minute. The volume occupied by the iodine precipitate was read off, and the amount of iodine in the precipitate and in the supernatant liquid determined. Alternatively the liquid can be decanted, and the iodine dissolved out of the precipitate by shaking and centrifuging alternately several times with absolute alcohol. The precipitate is then white and entirely free from iodine. No significant relationship between the amount of iodine adsorbed and that combined chemically has yet been found. The adsorbed iodine in the precipitate is chemically more reactive than dissolved iodine or iodine crystals. It causes immediate and rapid evolution of nitrogen from a solution of sodium azide, while a solution of iodine or iodine crystals fails to do so. Feigl (1928) has applied the activation of the reaction between iodine and sodium azide as a test for sulphide ions, but the precipitates here examined do not contain sulphur sufficient to respond to any other chemical test.

Maximum precipitation from 10 c.c. of the extract is produced by the addition of less than 6 c.c. iodine, but below a certain point the volume of precipitate depends upon the volume of iodine added. Table I shows the relationship. The volumes read off in these chemical tests can be repeated for the same extract, but for extracts made under apparently identical conditions they are by no means constant. The differences found agree with the variability observed in the biological activity.

The dilution of extracts with Ringer's solution reduces the amount of the precipitate proportionately with the biological activity.

An iodine precipitate can also be obtained with acetic acid extracts of the posterior lobe, and here again the volume is dependent upon the volume of iodine solution added to 10 c.c. of extract below a certain value (see Table I).

Table I. *Relation of the volume of the precipitate to the volume of iodine.*

Anterior lobe, 0·125 % acid extract			Posterior lobe, 0·5 % acid extract		
Volume of extract in c.c.	Volume of *N*/20 I_2 sol. in c.c.	Volume of ppt. in c.c.	Volume of extract in c.c.	Volume of *N*/20 I_2 sol. in c.c.	Volume of ppt. in c.c.
10	1·0	—	10	1·0	—
10	1·5	0·5	10	1·5	1·5
10	2·0	0·7	10	2·0	2·4
10	3·0	0·8	10	3·0	2·7
10	4·0	0·8	10	4·0	2·7
10	6·0	0·8	10	5·0	2·7

IV. EXPERIMENTAL OBSERVATIONS.

The following record deals with the results of the application of these tests to extracts in the different experiments carried out.

(1) *Specificity.*

The specific nature of the effect of extracts of the anterior lobe upon metamorphosis has been demonstrated by the failure of similarly prepared tissue extracts to induce any change, whilst a comparison of its influence with the activity of the thyroid in metamorphosis has served to differentiate between the active factors (Spaul, 1928, 1930). Further no melanophore response is shown by these extracts.

In the chemical tests with 20 per cent. extracts of tissues in 0·125 per cent. acetic acid the colour of the precipitates obtained, when less than 3 c.c. of iodine solution were added to 10 c.c. of each extract, permitted their distinction from that of a 20 per cent. extract of the anterior lobe with the same quantity of iodine solution. The latter, being less adsorbent, gave a lighter buff-coloured precipitate. The addition of more than 3 c.c. completed precipitation when there was little difference in colour between the various tissue extracts, but the anterior lobe precipitate was still lighter. Liver extracts (20 per cent.) in Ringer's solution and acetic acid (0·125 and 0·5 per cent.) gave dark brown precipitates with 3 c.c. and more of the iodine solution. With thyroid a white precipitate was obtained with Ringer's extracts and a dark brown precipitate with acetic acid extracts.

(2) *Extraction.*

(*a*) *Extracting medium.*

Similarly prepared 20 per cent. extracts of the anterior lobe in Ringer's solution, 70 and 0·125 per cent. alcohol, 0·125 per cent. acetone and distilled water fail metamorphically, but all contain approximately the same quantity of melanophore stimulant (threshold dose = 0·0025 – 0·0009 c.c.) as the acetic acid extract (Spaul, 1928, 1930). However, only the 70 per cent. alcohol extract gave a precipitate (0·2 c.c. white) in the chemical test.

(*b*) *Strength of the acetic acid in extraction.* (Table II A.)

The metamorphic activity of extracts has been found to depend within certain limits upon the strength of the acid used for extraction whereas the melanophore response is not appreciably affected (Spaul, 1930). 0·5 per cent. acetic acid extracts are without harmful effect upon the axolotl during treatment, but tadpoles are very susceptible, and dilution with Ringer's solution was necessary for these tests. The acceleration produced by the 0·5 per cent. acetic acid extracts diluted by half approximated with that induced by 0·25 per cent. extracts diluted to the same extent. Further equal dilutions of these extracts gave similar accelerations. Hence there was little difference in the quantity of active substance present in these extracts. However, with extracts prepared with acetic acid of strength varying from 0·125 to

0·03125 per cent., a graded decrease in activity was observed. No definite change was noted with weaker acid extracts.

In the chemical tests the volumes of the precipitates obtained were more in accord with the biological activity than with the strength of the acid used for extraction. The 0·5 per cent. acetic acid extract gave a precipitate only slightly greater than that of the 0·25 per cent. acetic acid extract. The remainder, including the biologically inactive, showed a graded decrease and provided thereby a quantitative expression of the minimal value of metamorphic activity. In 0·5 and 0·125 per cent. acetic acid extracts of the posterior lobe having the same melanophore activity, the volume of the precipitates approximated with those of the corresponding anterior lobe extracts.

Table II A. *Comparison of results of the biological and chemical tests.*

Extract	Metamorphic test	Melanophore test (threshold value in c.c.)	Chemical test (volume of ppt. in c.c.)
(1) 20 % ext. of ant. lobe in 0·5 % acetic (average of 3 preparations)	3 to 4 injections of extract diluted by half with Ringer's. Completion in 6–8 days	0·00125	3·1
(2) 20 % ext. of ant. lobe in 0·25 % acetic (average of 2)	3 to 5 injections (ext. diluted by half). Completion in 6–9 days	0·001	2·6
(3) 20 % ext. of ant. lobe in 0·125 % acetic (average of 4)	3 to 6 injections. Completion in 7–12 days	0·0015	1·3
(4) 20 % ext. of ant. lobe in 0·05 % acetic (average of 2)	After 6 injections and 12 days 50–60 % with fore limbs	0·002	0·31
(5) 20 % ext. of ant. lobe in 0·01 % acetic	Similar progress to control animals. Control tadpoles: after 12 days fore limbs in 10–15 %	0·001	0·10
(6) 20 % ext. of outer region of ant. lobe in 0·125 % acetic	Approximately as active as 0·05 % acid ext.	0·0027	0·23
(7) 20 % ext. of middle region of ant. lobe in 0·125 % acetic	Approximately as active as 0·125 % acid ext.	0·0012	1·0
(8) 20 % ext. of inner region of ant. lobe in 0·125 % acetic	Slightly less active than ext. of middle region	0·00087	0·8
(9) 20 % ext. of post. lobe in 0·125 % acetic (average of 3)	—	0·0000008	1·4
(10) 20 % ext. of post. lobe in 0·5 % acetic	—	0·00000065	3·2

The threshold value in the melanophore test is inversely proportional to the amount of the factor present in an extract.

(*c*) *Time of extraction and concentration of extract.* (Table II B.)

In the process of extraction from the fresh gland the liquid is boiled from 10 to 15 minutes before filtering, but re-extraction of the residues has shown that all the active substance is not necessarily released in that time. In this experiment 20 per cent. extracts of the fresh gland in 0·5 and 0·125 per cent. acetic acid were prepared

after (1) 10 minutes', and (2) 40 minutes' extraction. The residues in the first case were boiled again for 30 minutes with the required amount of 0·5 and 0·125 per cent. acetic acid respectively to give 20 per cent. extracts. A similar series of 50 per cent. extracts were made. The results of the tests are compared in Table II B and show:

(i) *Melanophore stimulant.* Extraction is continuous but more rapid at first, the amount obtained increasing with the concentration of the extract and the period of extraction. With the exception of the higher concentration the amount extracted does not appear to depend to any extent upon the strength of the acid used for extraction. There is no definite evidence of the destruction of the principle during extraction for these periods.

Table II B. *Time of extraction and concentration of extract.*

Extract	Concentration (%)	Time of extraction (mins.)	Tests		
			Metamorphic	Melanophore (c.c.)	Chemical (c.c.)
(1) Ant. lobe in 0·125 % acetic ...	20	10	Active	0·0025	0·7
(2) Residues of (1) in 0·125 % acetic	20	30	< 1	0·0029	0·9
(3) Ant. lobe in 0·125 % acetic ...	20	40	> 1	0·00007	0·8
(4) Ant. lobe in 0·5 % acetic ...	20	10	⩾ 1	0·001	2·4
(5) Residues of (4) in 0·5 % acetic	20	30	< 4	0·0014	1·0
(6) Ant. lobe in 0·5 % acetic ...	20	40	> 4	0·00006	1·9
(7) Ant. lobe in 0·125 % acetic ...	50	10	> 1 < 4	0·0008	2·3
(8) Residues of (7) in 0·125 % acetic	50	30	< 7 > 2	0·00025	2·3
(9) Ant. lobe in 0·125 % acetic ...	50	40	⊱ 4	0·00002	2·3
(10) Ant. lobe in 0·5 % acetic ...	50	10	> 4	0·0001	2·5
(11) Residues of (10) in 0·5 % acetic	50	30	< 10 > 5	0·00006	2·2
(12) Ant. lobe in 0·5 % acetic ...	50	40	⊱ 6	0·000008	2·3
(13) Post. lobe in 0·125 % acetic ...	20	30	—	0·00000001	1·0
(14) Post. lobe in 0·5 % acetic ...	20	30	—	0·00000001	2·0

(ii) *Metamorphic factor.* The potency is greater in the more concentrated and stronger acid extracts for short period extraction, but prolonged extraction is not advantageous in all cases, whilst the activity of residue extracts varies. There appears to be a maximum value which is found in both 0·5 per cent. acetic acid extracts and 50 per cent. extracts. The exhaustion of the extracting medium, the destruction of the activator or the gradual accumulation of inhibitory, toxic, or other products as extraction proceeds beyond a certain point, determined among other things by time and concentration, are possible factors tending to reduce the potency. The higher mortality with 0·5 per cent. acetic acid extracts after 40 minutes' extraction and with all 50 per cent. extracts, together with the decreased activity of the 50 per cent. extract after 40 minutes in 0·5 per cent. acetic acid, support this possibility.

(iii) *Precipitates.* The volume of precipitate produced by the addition of iodine showed a tendency to decrease with prolonged extraction (except in 20 per cent.

extracts in 0·125 per cent. acetic acid). Extracts made by re-extraction of residues gave precipitates approximating in volume to that produced in the first extract, excepting the 20 per cent. extract in 0·5 per cent. acetic acid, where the residue extract gave a much smaller precipitate than the corresponding extract of fresh gland. There is a fundamental change in the nature of the precipitate from residue extracts, which is at present under investigation. They differ from the precipitates of fresh gland extracts in being almost completely soluble in alcohol.

(3) *Effect of boiling extracts.* (Table II c.)

The activity of extracts was diminished by boiling, but the destruction of the melanophore stimulant was more gradual than that of the metamorphic activity. After an abrupt fall in the quantity of the iodine precipitate obtained there is little change.

Table II c. *Effect of boiling.*

Extract	Time of boiling (hours)	Tests		
		Metamorphic	Melano-phore (c.c.)	Chemical (c.c.)
(1) 20 % ext. of ant. lobe in 0·125 % acetic	—	Active	0·001	1·6
	2	Less active	0·0017	1·1
	4	Slight	0·0037	0·8
	6	—	0·0075	0·75
(2) 20 % ext. of ant. lobe in 0·5 % acetic	—	Very active	—	3·4
	2	Active	—	1·9
	4	Less active	—	1·8
	6	—	—	1·7
(3) 20 % ext. of ant. lobe in 0·05 % acetic	—	Slight	—	0·30
	2	—	—	Trace
	4	—	—	—

(4) *Action of pepsin and trypsin.* (Table II d.)

The destructive action of the proteoclastic ferments upon metamorphically active extracts has been demonstrated (Spaul, 1930), but the rate at which it occurs has not been studied. Hence the activity of extracts after definite periods of digestion with either pepsin or trypsin was examined. Rapid inactivation was noted with trypsin, but pepsin is less destructive. Further, treatment with tryptic digests of the extracts caused a high mortality, with the exception of 0·05 per cent. acetic acid extract. The somewhat similar effects of these enzymes upon the melanophore stimulant agree with the results of Hogben and Winton (1922). There was a sharp decline in the amount of iodine precipitate following tryptic digestion but the loss was more gradual with pepsin.

Table II D. *Action of pepsin and trypsin.*

Experiment	Time of digestion (hours)	Tests		
		Metamorphic	Melanophore (c.c.)	Chemical (c.c.)
(1) 30 c.c. of 20 % ext. of ant. lobe in 0·125 % acetic, 16 c.c. 0·2 % HCl, 4 c.c. 0·1 % pepsin in 0·2 % HCl. Digested at 40° C.	—	Active	0·0014	0·4
	2	Slight	0·0019	0·2
	4	—	0·0025	0·1
	6	—	0·004	0·05
(2) 30 c.c. of 20 % ext. of ant. lobe in 0·5 % acetic, 16 c.c. 0·2 % HCl, 4 c.c. 0·1 % pepsin in 0·2 % HCl. Digested at 40° C.	—	Active	0·001	1·7
	2	Less active	0·0015	1·05
	4	Very slight	0·0021	1·0
(3) 75 c.c. of 20 % ext. of ant. lobe in 0·125 % acetic (neutralised with NaOH), 50 c.c. of 0·5 % Na_2CO_3, 0·25 gm. trypsin. Digested at 40° C. Each sample reacidified with acetic before testing	—	Very slight	0·07	0·2
	2	—	0·6	Trace
	4	—	—	—
(4) 75 c.c. of 20 % ext. of ant. lobe in 0·5 % acetic (neutralised with NaOH), 50 c.c. of 0·5 % Na_2CO_3, 0·25 gm. trypsin. Digested at 40° C. Each sample reacidified before testing	—	Very slight	0·01	1·3
	1	—	0·2	0·5
	2	—	—	0·4

Table II E. *Effect of boiling with* (1) *hydrochloric acid*, (2) *sodium hydroxide*, (3) *sodium carbonate.*

Experiment	Time of boiling (hours)	Tests		
		Metamorphic	Melanophore (c.c.)	Chemical (c.c.)
(1) Equal vols. of 0·125 % acetic ext. of ant. lobe and 0·5 % HCl	—	Active	0·0016	0·7
	2	—	0·005	0·3
	4	—	0·025	0·2
	6	—	0·056	0·15
(2) Equal vols. of 0·5 % acetic ext. of ant. lobe and 0·5 % HCl	—	Active	0·0018	1·2
	2	Slight	0·004	0·8
	4	—	0·015	0·6
	6	—	0·048	0·35
(3) Equal vols. of 0·125 % acetic ext. of ant. lobe and 0·5 % NaOH (samples acidified with a few drops of acetic)	—	Active	0·002	0·4
	2	—	0·015	0·05
	4	—	0·08	Trace
(4) Equal vols. of 0·5 % acetic ext. of ant. lobe and 0·5 % NaOH (samples acidified)	—	Active	—	1·1
	1	Very slight	—	0·95
	2	—	—	0·85
	3	—	—	0·75
(5) Equal vols. of 0·125 % acetic ext. of ant. lobe and 0·5 % Na_2CO_3 (samples acidified)	—	Trace	0·002	0·6
	1	—	0·009	0·2
	2	—	0·018	0·1
	4	—	0·028	0·08
(6) Equal vols. of 0·5 % acetic ext. of ant. lobe and 0·5 % Na_2CO_3 (samples acidified)	—	Slight	—	1·5
	1	—	—	0·17
	2	—	—	0·125
	4	—	—	0·10

(5) *Effect of boiling with (a) hydrochloric acid, (b) sodium hydroxide, (c) sodium carbonate.* (Table II E.)

In each case the biological activity (metamorphic and melanophore) and the amount of precipitate are reduced according to the time of boiling, but their respective reductions are not in the same ratio, nor identical with each reagent. Hence there is varying stability of the biological factors and the iodine precipitate in the presence of these reagents. The most marked effect followed boiling with sodium carbonate.

(6) *Effect of exposure at various temperatures upon pituitary glands.* (Table II F.)

A study of the purity of anterior lobe extracts raised the question of the diffusion and autolysis of pituitary principles. Investigations revealed post-mortem diffusion

Table II F. *Effect of exposure at various temperatures upon pituitary glands.*

Extract	Exposure and temperature	Tests		
		Meta-morphic	Melano-phore (c.c.)	Chemical (c.c.)
(1) 20 % ext. of whole pituitary in 0·125 % acetic	—	—	0·00012	1·4
(2) 20 % ext. of whole pituitary in 0·125 % acetic	3 hr. at 39° C.	—	0·0004	1·1
(3) 20 % ext. of whole pituitary in 0·125 % acetic	5½ hr. at 39° C.	—	0·006	0·5
(4) 20 % ext. of whole pituitary in 0·125 % acetic	3 hr. at 26° C.	—	0·00028	1·1
(5) 20 % ext. of whole pituitary in 0·125 % acetic	6 hr. at 26° C.	—	0·0022	1·0
(6) 20 % ext. of whole pituitary in 0·125 % acetic	6½ hr. at 26° C.	—	0·0025	0·9
(7) 20 % ext. of ant. lobe in 0·125 % acetic	3 hr. before dissection at 39° C.	Active	0·0004	1·5
(8) 20 % ext. of ant. lobe in 0·125 % acetic	5½ hr. before dissection at 39° C.	—	0·0009	1·1
(9) 20 % ext. of ant. lobe in 0·125 % acetic	6½ hr. before dissection at 39° C.	—	0·0025	0·9
(10) 20 % ext. of ant. lobe in 0·125 % acetic	3 hr. before dissection at 26° C.	Active	0·0005	1·1
(11) 20 % ext. of ant. lobe in 0·125 % acetic	6 hr. before dissection at 26° C.	—	0·0008	1·0
(12) 20 % ext. of ant. lobe in 0·125 % acetic	3 hr. after dissection at 39° C.	Very slight	0·004	1·0
(13) 20 % ext. of ant. lobe in 0·125 % acetic	5½ hr. after dissection at 39° C.	—	—	0·6
(14) 20 % ext. of ant. lobe in 0·125 % acetic	3 hr. after dissection at 26° C.	Slight	0·001	1·3
(15) 20 % ext. of ant. lobe in 0·125 % acetic	6 hr. after dissection at 26° C.	—	0·001	0·7

of active substances between the lobes and later autolysis dependent upon the temperature and the time of exposure (Spaul, 1925, 1927). To study these changes in relation to the chemical test, a series of extracts of the whole gland and the anterior lobe were prepared from complete glands exposed in an oven for various intervals at different temperatures. The biological tests confirmed previous work. In the chemical tests little variation was noted in the precipitates of the whole gland extracts at the lower temperature until after 6 hours' exposure, when they decreased. At the higher temperature this decline was apparent much earlier. The amounts of the precipitates of the anterior lobe extracts approximated at first at the lower temperature, but a decline followed. At the higher temperature a small increase was followed by an earlier reduction. In each case these reductions approximate roughly with the loss of biological activity due to autolysis observed in the extracts.

Reductions showing similar agreement, and hence providing some degree of confirmation, were found in the precipitates and biological activity of extracts prepared from anterior pituitaries dissected from the posterior lobes whilst still frozen before the exposure for definite intervals at different temperatures. In this manner diffusion between the lobes during exposure was eliminated, leaving only the effects of autolysis to be observed. The reductions, however, were more rapid, since the conditions of the experiment considerably accentuated the loss (Spaul, 1928), and so introduced factors detracting from the value of the comparison. As both lobes respond to the chemical test, these results provide no satisfactory evidence of diffusion, although the increase in the anterior lobe precipitates at the higher temperature suggest the possibility of more rapid diffusion from the posterior lobe than in the reverse direction.

(7) *Regional distribution of activity in the anterior lobe.* (Table II A.)

Apart from its influence upon metamorphosis the anterior pituitary is associated with growth stimulation. Smith and Smith (1923) found that extracts of the central region of the ox pituitary, identified histologically by the predominance of basophil cells, induced metamorphosis, whilst the outer region, mainly oxyphil, stimulated growth only. In later work, however, such definite distribution of the activity was not observed, as accelerated metamorphosis was obtained with extracts of the outer region, although the rate of change was much less than that induced by extracts of the inner portion (Spaul, 1925, 1930). To further the study of the distribution of the activity, the frozen glands used in this experiment were divided into three, roughly equal, regions instead of the outer and inner portions, of which the latter approximated to the central region, and acetic acid extracts of each prepared in the usual manner and tested biologically and chemically. Here the outer portion consisted of the peripheral part of the lobe; the inner bordered the cleft and the pars intermedia and the remainder of the lobe made up the middle portion. The central region is more or less axial in position, so that it was distributed between the three portions.

The melanophore stimulant present decreased from the inner to the outer region as in previous observations, and indicated the post-mortem diffusion between the lobes.

The metamorphic activity was greatest in the middle region, somewhat less in the inner and only slight in the outer; hence it would appear that the responsible factor for metamorphosis is more concentrated in the middle than at the periphery, but its localisation solely in the central region doubtful. Recent histological studies of the gland tend to confirm this distribution (Spaul and Howes, 1930).

The precipitates obtained in chemical tests closely agreed with the biological activity, the middle being the greatest, next the inner, and lastly the outer with only a small precipitate.

V. DISCUSSION.

A summary of these observations reveals additional knowledge, gained by the biological tests, of the characteristics of the hormonic activity of the pituitary—the importance of time and concentration in extraction in relation to activity, the rate of destruction by the digestive ferments, the effects of boiling and the varying influence of various reagents (dilute HCl, NaOH, Na_2CO_3) upon the melanophore and metamorphic factors.

On the other hand, the capacity of the chemical tests to supplement the biological tests and acquire further information needs closer examination before any estimate of its value can be given.

Some degree of correlation would appear to be indicated by the approximate parallelism of the series of results in the tests applied to fresh extracts, but this is not maintained, as considerable divergence is evident in those results obtained with extracts after subsequent treatment or with preparations for which some modifications of the standardised procedure were introduced.

However, the active substances are precipitated by the iodine solution, since, after the complete removal of the iodine from the precipitate of fresh anterior and posterior lobe extracts by repeated shaking and washing with absolute alcohol and drying the residue *in vacuo*, 20 per cent. extracts prepared by extracting the dried products with the required amount of 0·125 per cent. acetic acid give indications of biological activity when tested. Only a small number of tadpoles with elongated limb buds were available for the metamorphic test, but distinct signs of accelerated change followed treatment with the extract prepared from the anterior lobe precipitate. There was no apparent increase with the extract of the posterior lobe precipitate. An interesting feature of the experiment was the absence of any marked shrinkage of the animals, so usual in the accelerated metamorphosis induced by fresh gland extracts. The melanophore test gave positive results, the posterior lobe precipitate extract being very much greater, but in each case the response was less than that of the original fresh gland extracts (threshold values: anterior lobe, 0·03 c.c.; posterior lobe, 0·00006 c.c.). The precipitate extracts gave the same amount of precipitate in the chemical test as their respective fresh gland extracts. It is difficult to explain the discrepancy in the value of the biological activities of the fresh gland and the precipitate extracts at this stage, but possibly the precipitation of the active substances was incomplete in the first instance or the treatment preparatory to re-extraction changed or rendered inert some portion.

Apart from the biological tests, the precipitates obtained in the chemical test with the extracts of both lobes of the pituitary cannot be distinguished without further chemical examination. Further no accurate valuation of the metamorphic and melanophore activities in terms of the precipitate by quantitative comparison is possible, since allowance cannot be made for other hormonic activities of the pituitary that might be present and able to influence the chemical test.

Analysis of the precipitates obtained from extracts of each lobe indicates a larger phosphate content in the anterior lobe than the posterior lobe precipitate, and this affords a basis for distinction between them. Total phosphate phosphorus precipitated with iodine solution in 10 c.c. of 0·5 per cent. acetic acid extract: anterior lobe, 0·058 mg.; posterior lobe, 0·003 mg.

The precipitate of the chemical test appears to be a complex association of iodine and the active substances, and possibly also inactive substances. The stability of this complex is not equivalent to that of the active substances themselves, as proportional variations are not evident in the results of the tests upon the extracts after treatment with enzymes or other reagents or with extracts prepared by other means, so that any practical co-ordination of the biological and chemical tests is limited.

In fresh gland extracts of the anterior lobe prepared by the standard method with suitable concentrations of acetic acid, the precipitate serves as an indicator of the presence of the metamorphic factor and, within certain limits, an apparent quantitative relationship exists, which, if the value of the melanophore stimulant of the original extract is known, permits the use of the quantity of the precipitate obtained as an approximate measure of the metamorphic activity. Any such relationship in the case of the melanophore stimulant and the posterior lobe is obscure.

The importance of the chemical test, however, is the provision of a possible line of enquiry into the chemical relationships of the factors and a means of studying the chemical aspect of activity, as a result of the difference shown by analysis in the phosphate content of the two lobes of the pituitary.

In conclusion, two interesting applications of this test, which supplied information as to the suitability and effectiveness of the material considered, may be mentioned. The first concerned a study of commercial preparations of the anterior lobe. They included extracts and dry powders. Acid extracts (20 per cent. by fresh gland weight) were prepared of the latter. Their *p*H with one exception was found to vary between 4·8 and 5·2. In the tests two only showed any capacity to accelerate metamorphosis. They gave a small melanophore reaction, but others which failed in the metamorphic tests also showed the presence of small amounts. The majority, however, gave a large melanophore response. There was considerable dissimilarity and variation in the quantity of the iodine precipitates and also in the phosphate content of the precipitates, but only those extracts affecting the rate of change in the tadpole gave results comparable with those recorded here indicative of metamorphic activity.

In the other case the anterior lobes of various breeds of cattle were studied. The selected breeds—Aberdeen Angus, Shorthorn and Hereford—were living in different

parts of South America. The glands were extracted from the animals in the abattoirs and placed immediately in cold storage and each batch forwarded to England, where the standardised routine was followed in every detail in the preparation of 20 per cent. extracts in 0·125 per cent. acetic acid of each series of anterior and posterior lobes (Spaul, 1930). Approximately two months elapsed in each case between the death of the animals and, therefore, dissection of the glands from the skull and the preparation of the extracts. The metamorphic activity was estimated by comparing the times required for inducing complete metamorphosis in axolotls under uniform conditions by each of the anterior lobe extracts. Those given the Aberdeen Angus extract were transformed in 24 days with twelve injections, giving a strength nearly equivalent to that of the standard laboratory extract of the ox pituitary (Spaul, 1930). Those given the Hereford extract needed 36 to 40 days, whilst the others failed to make any advance. This difference may have been due either to the breed or to post-mortem diffusion during a prolonged interval between death and the freezing of the dissected glands or deterioration during storage. The melanophore tests upon the extracts of both lobes showed that irrespective of specific differences post mortem diffusion had occurred and in the case of the Shorthorn autolysis also. This condition made it impossible to assess any difference due to the breed. The chemical tests confirmed this, and the iodine precipitates and phosphate contents of the Aberdeen Angus and Hereford extracts compared with the laboratory extract were graded as expected.

These results are supported by the histological studies of Howes (1930), in which a striking similarity was noted in preparations of these glands and certain of those of a series of ox pituitaries which had been exposed for various temperatures.

VI. SUMMARY.

1. A buff-coloured precipitate is obtained by the addition of *N*/20 iodine solution to dilute acetic acid extracts of fresh anterior lobe substance. The volume of the precipitate depends upon the volume of the iodine solution added. Similar acetic acid extracts of tissues and extracts of the anterior lobes prepared with other extracting media do not give the same precipitate with iodine.

2. The precipitate indicates the presence and, within limits, gives an approximate measure of the metamorphic factor in fresh gland extracts of the anterior lobe.

3. Dilute acetic acid extracts of the precipitates after removal of the iodine by shaking with alcohol are biologically active and characterised by their phosphate content.

4. Similar extracts of the posterior lobe give an iodine precipitate but the phosphate content is much lower than that of the anterior lobe precipitate.

5. The iodine in these precipitates is adsorbed or loosely combined. The adsorbed iodine is chemically more reactive than dissolved iodine or iodine crystals giving rapid evolution of nitrogen from sodium azide compared with the failure of the others.

6. The biological activity of anterior lobe extracts varies with the time of extraction and the concentration of the extracts. There is a maximum for the time of extraction at any particular concentration and acid strength after which a loss of potency occurs. There is a continuous extraction—rapid at first—of the melanophore stimulant. The amount increases with the period of extraction and concentration, but is independent of the strength of the acid used in extraction.

7. The metamorphic factor is slowly destroyed by pepsin but rapidly by trypsin.

8. The biological activity is diminished by boiling, or by boiling with dilute hydrochloric acid, sodium hydroxide, or sodium carbonate.

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