ON THE ACTIVITY OF THE ANTERIOR LOBE PITUITARY

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(With One Text-figure.)

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1. INTRODUCTION.

The failure of anuran tadpoles with the buccal anlage of the hypophysis removed in the embryo stage to undergo metamorphosis (Adler (1914), Allen (1917), Smith (1916 b)), and the accelerated transformation of normal tadpoles to adult forms produced by implantation of the pituitary gland, together with the discovery that the pars anterior was the responsible portion of the gland (Allen (1920), Swingle (1923)), directed attention to the possible significance of the latter in metamorphic changes and the particular nature of its influence, in view of the established position of the thyroid in relation to this phenomenon. In earlier work no precocious change was found in anurans after pituitary feeding as in the case of thyroid (Gudernatsch, 1912), and later Huxley and Hogben (1922) failed to metamorphose axolotls with a diet of anterior lobe pituitary substance. However, Hogben (1923) induced these changes successfully in axolotls, both normal and thyroidectomised, by injections of commercial extracts of this portion of the gland, and so demonstrated the physiological activity of the gland as regards metamorphosis. The author (1924 b) confirmed this, using other preparations, and
also produced acceleration in tadpoles by the same method (1924 a), but failed to
get any such response with several other commercial preparations. This lack of
consistency, coupled with the inhibiting effect of the anterior lobe upon meta-
morphosis recorded by Smith, P. E. (1926), in the Colorado axolotl, made the actual
existence of any accelerating activity of the anterior lobe in metamorphosis appear
doubtful, since only commercial products had been tried and contamination might
well account for the effects observed. Accordingly these facts were submitted to
a more critical examination in an attempt to clarify the situation.

In the first place, an active extract was prepared from fresh ox glands by
extraction with suitable concentration of dilute acetic acid and its behaviour in
metamorphosis studied. Further, it was ascertained that post-mortem diffusion
occurs within the pituitary gland between the anterior and posterior lobes, together
with autolysis during that inevitable period that elapses before complete removal
from the skull of the animal, owing to its inaccessibility for immediate dissection,
and continues until the separation of the two lobes. The loss of potency thus
entailed is enhanced by an inhibiting influence exerted by the posterior lobe
principles, and a stage is eventually reached at which it is impossible to detect
any metamorphic activity in anterior lobe preparations thus contaminated. This
knowledge made it possible to determine the most suitable conditions for extraction
and for a standardised preparation of active extracts; at the same time it afforded
a basis of reconciliation of apparently divergent results (Spaul, 1925 a, 1927). Strictly
comparative studies of metamorphosis produced by these active extracts and of
thyroid extracts have shown distinct differences in physiological activity (both
qualitatively and quantitatively) and in relation to iodine (Spaul, 1928), so that
contamination with thyroid or iodine is not the cause of the activity of the pituitary
extracts. These facts provide definite evidence of the endocrine function of the
anterior pituitary in metamorphosis, and it remains to study the characteristics and
behaviour of this factor, both in relation to the problems of metamorphosis and to
other records of hormonic activity of the anterior lobe. The experiments described
here have been undertaken with that purpose.

2. PREPARATION OF EXTRACTS AND APPLICATION
OF ANIMAL TESTS.

Extracts. The preparation of active extracts of this portion of the gland in the
laboratory has, so far, only been successfully attempted by the method of decoction
with dilute acetic acid. After expeditious removal from the skull immediately
after death, the glands are placed in a refrigerator and transferred to the laboratory.
Here the lobes (whilst still frozen) are separated and the anterior portion weighed,
cut into small pieces, minced, pounded in a mortar and added to the required
amount of warm liquid to give an extract of the required concentration. After
boiling for approximately 10 minutes, the solution is filtered, poured into clean
tubes and sterilised in boiling water for about 20 minutes, when the tubes are
sealed. This procedure has been adopted in this work as the standard method of
extraction for all preparations, whatever the tissues or extracting media used, so
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as to maintain uniformity and facilitate the comparative studies necessary for the investigation of the characteristics of the activity of the anterior lobe. A 20 per cent. extract of the anterior lobe substance in 0.125 per cent. acetic acid prepared in this manner is taken as the standard extract.

Tests. The metamorphic response of axolots and tadpoles to the administrations of extracts has been used as an indicator of their physiological activity, and here again, with exceptions mentioned hereafter, the procedure is standardised (Spaul, 1925).

Axolotl test. With axolots (A. trigrinum—Old Mexican form) tri-weekly injections of 0.5 c.c. are given and continued until metamorphosis is complete, the animal being kept in the same quantity of water in a glass container at a temperature of 22°-24° C. until this stage is reached. The animals are fed with raw meat or worms once a week, when the water is changed and their weight and body length recorded. Treated in this manner in this uniform environment, successful transformation of fully developed axolots of either sex and of any age and size between 8 cm. and 24 cm. and 8 gm. and 110 gm. or more can take place, the animals being quite normal and continuing a natural active existence in land quarters after the change. Smaller specimens do not respond so readily and consistently; death may supervene before completion. The upper limit represents the largest specimens used so far for the test in the laboratory, but there is little doubt that still larger specimens would respond.

Treatment with 20 per cent. extracts of the anterior lobe in 0.125 per cent. acetic acid—the standard extract—is taken as the standard test. The interval before any signs of change appear, the time for completion and the number of injections, all increase with the size of the animal, varying with the standard extract.
from 7 injections for a weight of 9 gm. during 16 days, the first sign of change appearing on the 10th day, to 21 injections for 90 gm. weight during 50 days with the first appearance of change on the 23rd day (see Table I). The data are fairly consistent for any particular size, but the gradation is not uniform and only a general relationship existing between the size and the dose is apparent.

Table I. *Average records of animals within selected limits of weight showing the relation between (a) size and dosage, and (b) concentration of acid used for extraction and metamorphic activity of extract as indicated by the time required for completion.*

<table>
<thead>
<tr>
<th>Extract</th>
<th>No. of animals tested</th>
<th>Average no. of injections</th>
<th>First sign of change (average) Day</th>
<th>Average time for completion in days</th>
<th>Average % weight loss</th>
<th>Weight limits of animals in gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 % in 0.125 % acetic acid</td>
<td>5</td>
<td>9</td>
<td>10th</td>
<td>22</td>
<td>38</td>
<td>8-12</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>14</td>
<td>12th</td>
<td>25</td>
<td>40</td>
<td>15-20</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>14</td>
<td>16th</td>
<td>26</td>
<td>36</td>
<td>30-45</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>21</td>
<td>23rd</td>
<td>50</td>
<td>35</td>
<td>80-90</td>
</tr>
<tr>
<td>10 % in 0.125 % acetic acid</td>
<td>5</td>
<td>15</td>
<td>19th</td>
<td>33</td>
<td>40</td>
<td>8-12</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>18</td>
<td>21st</td>
<td>42</td>
<td>37</td>
<td>15-20</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>25</td>
<td>23rd</td>
<td>56</td>
<td>35</td>
<td>30-40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Extract</th>
<th>No. of animals tested</th>
<th>Average no. of injections</th>
<th>First sign of change (average) Day</th>
<th>Average time for completion in days</th>
<th>Average % weight loss</th>
<th>Weight limits of animals in gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 % in 0.5 % acetic acid</td>
<td>2</td>
<td>10</td>
<td>8th</td>
<td>20</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>8th</td>
<td>21</td>
<td>38</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11</td>
<td>9th</td>
<td>23</td>
<td>18</td>
<td>28-32</td>
</tr>
<tr>
<td>20 % in 0.05 % acetic acid</td>
<td>3</td>
<td>14</td>
<td>12th</td>
<td>26</td>
<td>25</td>
<td>10-15</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>19</td>
<td>21st</td>
<td>43</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>36</td>
<td>26th</td>
<td>*</td>
<td>6-6</td>
<td>30</td>
</tr>
</tbody>
</table>

*Not complete after 86 days.*

**Limitations of test.** In spite of standardisation, there are decided limitations to the accuracy that can be attained which reduce the efficiency of the test in quantitative measurement and the extent of its application to biological assay. Hitherto this test has been used as an indication of the physiological activity of extracts, and for comparative purposes the times necessary for complete transformation in animals of approximately the same size have served as rough estimates of the relative strengths of extracts, but its potential value in direct quantitative determinations has not been investigated.

**Animal variations.** In the first place, a high degree of accuracy is impossible owing to individual differences of the animals. Age, lineage, development, the stability of the endocrine system, previous environment and diet, are all factors affecting the animal's condition and contributing to individual variations. They can neither be determined nor completely controlled nor eliminated, and may make a difference of anything up to 3 days or more in the time for the completion of metamorphosis of animals of the same weight using the same extract.

**Variability of extracts.** The quantities used in the laboratory for the preparation of extracts are small and only 8 to 12 glands are required for each extract. Also
the amount of hormone present in the glands depends, among other things, upon the condition and age of the animals from which they are taken, and the varying interval between death and dissection of the gland from the skull. Hence variations in the strength of the extracts are to be expected in spite of standardised preparation, and animal tests confirm these expectations.

Selection of the axolots of the same age from the same stock for the test, and of pituitaries from animals of the same age and condition for the extract, would appreciably reduce these variations, but such arrangements are not always practicable.

Threshold dose and maximum response of animal. Apart from these fluctuations the threshold value of the dose (the smallest dose that can induce metamorphosis) and the maximum dose to which the animal will respond (that amount inducing the greatest rate of change) increase with the size of the animal, introducing thereby further complications in quantitative estimations of the activity of extracts (Table I). The time for completion when injections of 10 per cent. extracts or 20 per cent. extracts diluted by half with Ringer's solution are given tri-weekly to medium-sized animals (30 to 40 gm.) is approximately twice that required for 20 per cent. extracts, but with small animals (10 gm.) the period is less than double, and large animals, although showing premetamorphic signs, fail to complete and remain at an intermediate stage unless the dose is increased. Dilutions of the 20 per cent. extract below 10 per cent. can be found at which small animals fail to give a complete response. Hence a certain quantity or minimal concentration per dose, which increases with the size of the animal, is necessary before a response can be obtained. The presence of quantities below this value cannot be detected unless the concentration of the extract is increased to reach the minimal required by the particular size of the animal used for testing purposes. Again, increasing the size of the usual dose (0·5 c.c.) of 20 per cent. extracts or dosing with extracts of higher concentration does not hasten the assumption of the adult characters of small and medium sized specimens, and the same number of injections are required, but the period for completion is shortened in the larger specimens so that an upper limit exists to the stimulation possible, and quantities given in excess of a certain maximum, which increases with the size of the animal, are ineffective so far as metamorphosis is concerned, even if retained within the animal, and so cannot be measured. These limits reflect the difficulties to be countered in the application of the test with any degree of accuracy, but a tabulation of the records of administration under standard conditions of specified doses of a standard extract, prepared from suitably selected glands to axolots of different weights from the same stock, would serve as a basis for estimating the values of similarly tested extracts.

Rate of injection. Another important feature of these tests is the necessity for continued injection till completion. If the sequence is interrupted, the progress of metamorphosis is suspended until the resumption of injections, and if stopped, no further progress is made, and the animal remains at the particular stage reached, so far as its external appearance is concerned. No regeneration of the dorsal fin or gill stumps has been observed in such cases, but such animals seem quite normal.
If the time between each injection is increased, the time for completion is considerably increased, and a stage may be reached when the stimulus fails to induce the change, whereas daily injections reduce the time for completion, although the number of injections necessary agrees with that needed for metamorphosis with tri-weekly injections (10 injections in 13 days, first signs upon the 5th day for small animals—10 gm., loss in weight—35 per cent.), which suggests that the effect of each injection is short-lived and a gradual recovery follows.

Therefore the metamorphosis of the axolotl by the metamorphic factor of the anterior pituitary is possible by the repetition of a stimulus which must have a quantitative value between certain limits, dependent upon the size of the animal, and must be applied at sufficiently frequent intervals to maintain progress. Variations in the quantitative value of the stimulus and in the times between its successive applications within these limits determine the period for complete transformation.

Temperature. There is one other factor, the temperature, whose influence has been considered. The most favourable temperature is 22°-24° C. for these changes by the standard procedure, but as it is raised the animals become sluggish, refusing food, showing eventually signs of distress, particularly as metamorphosis advances, whilst there is no increase in the rate induced. Retardation is noted as the temperature is lowered, and below 10° or 12° C. it does not seem possible to induce these changes with the anterior pituitary. Similar retarding effects have been noted in thyroid administration, but conversion is possible at still lower temperatures, the minimum being about 7° C.

Tadpole tests. With tadpoles (R. temporaria) tri-weekly injections of 0.1 c.c. are given under uniform conditions and temperature, without food during the period of the experiments, to groups of animals from various sources but at the same stage of development (from the appearance of limb buds onwards) and the activity of the factor judged by the acceleration of the normal metamorphosis produced (Spaul, 1928). The tadpoles are kept in glass tanks with a plentiful supply of algae for a short period previous to the experiment. For the test ten specimens are placed in 100 c.c. of tap-water in a small glass container on a white background with plenty of light at room temperature (17°-18° C.). The water is changed after each injection. Several such batches of ten at the different stages of development are selected from the separate groups for each test and the average acceleration ascertained (3 stages from 2 groups—60 animals in all—is the least number used for any test). These measures reduce individual variations which are considerable. The injections, as with the axolotl, must be continued at short intervals till completion to maintain the maximum acceleration the particular dose can produce, and prevent a return to the normal rate. As before, the administration of the standard extract constitutes the standard test. The acceleration increases with age (stage of development) and depends upon the quantity given per dose which has minimum (approximately 0.1 c.c. of 5 per cent. extracts) and maximum (0.1 c.c. of 20 per cent. extracts approximately) values. Overdosing demonstrated the inability of the animal to store the active principle.

The tadpole test has the advantage of giving quicker results with smaller
quantities and detecting smaller concentrations, but individual variability is greater and the reliability of the response not so trustworthy, as the dose is large compared with the bulk of the animal, and although given to animals at the same stage of development they are not necessarily of the same size. Hence a larger error is introduced into estimations by this method which cannot with certainty be eliminated by the use of several specimens for the test.

3. CHARACTERISTICS OF THE METAMORPHIC ACTIVITY OF THE ANTERIOR LOBE EXTRACT.

The principle responsible for metamorphic activity has yet to be isolated and its chemical constitution and behaviour studied, but the results of extirpation and implantation of the pituitary gland and the injection of extracts in tadpoles and axolotls justify the presumption of its existence. Further, in the absence of satisfactory chemical information, the exploitation of these animal tests afford a means of gaining knowledge of its properties, whilst the application of suitable methods of biological assay within the limits of their effectiveness render fairly accurate quantitative estimates possible.

(a) Specificity.

Successful treatment with anterior pituitary extracts, as already explained, depends upon the injection of an acid extract of the gland, and although the injection of similarly prepared extracts of thyroid also produce metamorphosis, the responsible factor is not identical with the pituitary (Spaul, 1928), but since a number of compounds, including proteins, are capable of increasing the basal metabolism, it is imperative to test the specificity of the reaction. Portions of liver, lung, kidney, brain, spleen, muscle, and pancreas of an ox were placed in a refrigerator immediately after death and transferred to the laboratory, and 20 per cent. extracts of each tissue in 0.125 per cent. acetic acid prepared in exactly the same manner as the active pituitary extracts. Clear more or less colourless fluids were obtained. Each extract was tested by injection into axolotls kept under the conditions usual in work with the pituitary extracts, and the same procedure as regards feeding, weighing, measuring and changing water adopted. Tri-weekly injections (0.5 c.c.) of the same extract were given to the same animal throughout the experiment. A control was given injections of an anterior pituitary extract of the same strength and prepared at the same time. The latter changed in the usual way, being complete in 32 days after 14 injections. No metamorphic signs were noted in those given tissue extracts after 7 weeks' treatment and 20 injections. They appeared unchanged and quite normal, the weight approximately the same except in the case of the spleen and lung extracts where the loss in weight was 22 per cent. and 21 per cent. respectively, and the length was unaltered. It is evident therefore that these tissue extracts contain no factor able to simulate the effect upon metamorphosis produced by the pituitary. At the same time these results emphasise the specific nature of the activity of this metamorphic principle.
The melanophore stimulant invariably present in extracts of the anterior pituitary due to post-mortem diffusion was absent in these extracts since bleached frogs were not darkened by injections, which agrees with the work of Hogben and Winton (1922). Two animals died after the cessation of the injections, but there was no evidence suggestive of harmful effects of the extracts used. The remainder metamorphosed later at the usual rate when anterior pituitary extracts were given, so that whatever may have been the influence of the extracts, if any, upon the animals, there does not seem to be any effect so far as metamorphosis is concerned.

(b) Extraction.

Extracting medium. Attempts have been made to prepare active extracts of the anterior lobe with other media in place of the acid, using identical methods for extraction. Extracts prepared with Ringer's solution, distilled water, acetone solution (0.125 per cent.) and alcohol (0.125 per cent.), gave no indication of change in either axolotls or tadpoles when tested in the usual way, even after prolonged treatment. The animals appeared quite normal throughout, although a few fatalities occurred during treatment with Ringer's and distilled water extracts. Evidently the factor is either not present at all, or in such quantities that it cannot be detected by this means, or else present in an inactive condition. The latter possibility was explored by acidifying the Ringer's extract with dilute acetic acid, and after boiling and filtering the solution was tested as before, but there was no indication of the presence of the activating principle. The melanophore stimulant was present in all these extracts, the media used appearing approximately equally effective in its extraction. 0.125 per cent. solutions of HCl and H₂SO₄ were also used for extraction. The solutions obtained after filtration were coloured and cloudy. When tested by injection into axolotls and tadpoles the H₂SO₄ extract proved toxic even when diluted, and scarcely any survived 2 or 3 doses, but no ill effects marked the treatment with the HCl extract. Metamorphosis was not induced in this case, but there appeared a slight increase in size in some tadpoles, and a small increase in length in spite of weight reduction in one or two axolotls. As this increase was by no means general, and since no more satisfactory results were obtained by repetition, there was no justification for suggesting growth promotion by these extracts.

Strength of acid. So far approximately the same strength of acid has been used for extraction without any consideration of the possible dependence of the activity of the extract upon concentration of the acid in extraction. For this investigation 20 per cent. extracts of the anterior lobe were prepared in the usual manner under identical conditions with 0.5, 0.25, 0.125, 0.0625, 0.05 and 0.03125 per cent. solutions of acetic acid. The rate of change in axolotls induced by 0.5, 0.125 and 0.05 per cent. extracts, and the acceleration in tadpoles (R. temporaria) by 0.5, 0.25, 0.125, 0.0625 and 0.03125 per cent. were studied and compared. The axolotls were about the same size for each test and the tadpoles at the same stage of development, and the usual experimental conditions for each group of tests contained. Tri-weekly injections of 0.5 c.c. (axolotls) and 0.1 c.c. (tadpoles) were given.
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The time required for completion of metamorphosis in the axolotls pointed to the greater effectiveness of those extracts prepared with the higher concentration of acid. This effect was not very obvious in the case of the smaller axolotls (10 gm.), but with the increasing weight of the animals the period lengthened whatever the strength of the acid, that increase being least with the 0.5 per cent. whilst belated precocious signs of fin and gill reduction apparent with 0.05 per cent. were not followed by the completion of the change even with continued injections in the larger forms. The quantity of active principle per dose in the latter case, although able to initiate these processes, was evidently not sufficient to maintain the necessary concentration long enough to be effective and hence give the minimum stimulus required for the complete conversion of the animals, since with increasing size the threshold dose rises (see p. 53). The animals were normal throughout the experiment. Controls were given injections of the same strength of acid, but except for loss of weight, shrinkage and occasional signs of distress shown by specimens receiving 0.5 per cent. acetic acid, the concentrations used were not unfavourable to normal existence, although higher were injurious. There was some variation in the time for completion of individuals of about the same size with the same extract, but the failure of 0.05 per cent. acid extracts is sufficient indication, apart from the shorter intervals obtained with the higher concentrations, of the significance of the strength of the acid in relation to the potency of the extracts. The records of these experiments showed that with animals about 10 gm. weight the loss of weight during treatment was approximately 25 per cent. with 0.05 per cent. extracts and 40 per cent. with 0.5 per cent. extracts, with no increase in length except a slight increase in one case with the former extract. The first sign of change appeared after 8 days with 0.5 per cent. extracts, completion in about 20 days after 10 injections, but with 0.05 per cent. 26 days passed before any visible signs of change appeared and completion had not taken place after 56 days (see Table I). In one case an animal under 10 gm. changed in 19 days after 9 injections under treatment with this extract, the first signs being noted after 10 days, but this was exceptional and probably due to unusual susceptibility. With animals of 30 gm. and over, the data (averages of results) were: (a) 0.5 per cent. extracts—loss of weight 18 per cent., length unaltered, first signs 9th day, 11 injections in 23 days for completion; (b) 0.05 per cent. extracts—loss in weight 6.6 per cent., first sign 26th day, reduction continuing slowly but remained at intermediate stage after 86 days and 36 injections. Change was always obtained with 0.125 per cent. acid extracts, but the interval for completion was greater than 0.5 per cent. acid extracts, more injections were required and the first signs appeared a day or so later.

These findings were confirmed in the tadpole tests in which the greatest acceleration occurred in those specimens receiving extracts prepared with the strongest acid, those given the lowest displaying little if any progress beyond the normal controls. The range of variability was high, but the individual variations of tadpoles themselves is high, and, as on previous occasions, these fluctuations were to a great extent eliminated by the comparison of the results obtained from
tests upon animals at different stages of development from the first appearance of
the limb buds to the stage with limb divisions, repeating with animals from various
sources. Harmful effects were produced by 0.5 per cent. extracts, since all speci-
mens succumbed after 2 or 3 injections, otherwise the mortality was remarkably
low and behaviour normal throughout. No unusual alteration in size beyond the
characteristic slight shrinkage during accelerated metamorphosis produced by the
anterior pituitary (Spaul, 1928) was noted. The acceleration induced by 0.5 per
cent. extracts was no greater than 0.25 per cent. extracts, but there was a graded
decrease with succeeding reductions in the concentration of the acid used for
extraction: 3 to 4 injections of 0.25 per cent. extract during 6 to 8 days, depending
on the stage of development, were enough to complete transformation to the
adult, whilst 4 to 6 injections during 7 to 10 days were required with the 0.125 per
cent. extracts. After 6 injections of the 0.0625 per cent. extract many had fore
limbs, but the difference in the rate induced here and that shown with 0.125 per
cent. extract was much greater than between the rates observed with 0.125 per cent.
and 0.25 per cent. extracts. This fact, and the similar response obtained with
0.5 per cent. and 0.25 per cent. extracts, suggests that a concentration somewhere
between 0.25 and 0.125 per cent. evokes the maximum response of these tadpoles
which excess quantities cannot extend. Those given 0.03125 per cent. extracts
showed only a small increase in limb development compared with controls after
6 injections. No acceleration, but greater shrinkage was observed in the controls
given the various strengths of acid. They appeared normal, but there were no
survivors after the first injection of 0.5 per cent. acid, and a few after 2 or 3 in-
jections of 0.25 per cent. acid.

Concentration of extracts. The strength of a dose depends upon the concentra-
tion of the preparation itself, apart from the strength of the acid used in extraction.
This is indicated in the record given elsewhere (Spaul, 1928) of the small but
regular increase in the acceleration of metamorphosis of tadpoles corresponding
to the increased concentration of the extracts (20, 30, 40 and 50 per cent.),
obtained by injection under the usual experimental conditions. These extracts
were prepared in exactly the same manner and 0.125 per cent. acetic acid was used
for extraction in each case. The test with axolotls was not so satisfactory as it was
confined to the administration of the 50 per cent. extracts to small specimens,
owing to the small number available. The rate induced was about the same as
that produced by a 20 per cent. extract and also a 20 per cent. extract in 0.5 per
cent. acid. Eleven injections were given in 23 days, the first signs of change being
on the 11th day, and the loss in weight 20 per cent. The close approximation of
these results is not due, in the case of the small axolotl, to the equal amount of the
active factor in each dose, as the test with tadpoles and larger axolotls show, but
to the fact that each dose contains at least sufficient to obtain a maximum response
of the animals, surplus quantities being ineffective.

To sum up, the use of neutral media and inorganic acids for direct extraction
of the metamorphic principle from fresh gland by decoction is not produc-
tion of active extracts, dilute acetic acid being necessary. The amount of the principle
so extracted is governed by the strength of the extract prepared and the concentration of the acid within limits prescribed by this method of preparation and the animal tests. The question of extraction is by no means exhausted and variations of the present method or other processes may be devised, providing more efficient extraction, as our knowledge of the principle is extended. Meanwhile the uniformity introduced by the adoption of this method for all extractions is essential for standardisation and comparative work. Investigations upon the length of time required for the complete extraction of the principle show that extracts of the residue obtained after the first extraction are active, causing acceleration in tadpoles, but they are not so potent and vary in strength, and hence the time allowed for boiling was arbitrarily fixed for the purpose of standardisation.

(c) Action of proteoclastic enzymes.

Feeding fresh gland substance of the anterior lobe to axolotls and tadpoles is without effect upon metamorphosis, which may be explained by the inability of the digestive system to absorb quantities sufficient for the maintenance of the threshold concentration of the active factor, but it is likely, in view of its rapid destruction by autolysis after death, to have been destroyed by the digestive ferments, although the alteration in the alimentary system and consequent loss of digestive power during the latter stages of metamorphosis must not be disregarded in consideration of these explanations. Negative results were, in fact, obtained with extracts after the action of either pepsin or trypsin.

Action of pepsin. The following mixtures were placed in separate beakers in an oven at 40 per cent. for 8 hours and then boiled and filtered: (a) 32 c.c. of fresh 20 per cent. anterior lobe extract in 0.125 per cent. acetic acid, 20 c.c. of 0.1 N HCl and 4 c.c. of 1 per cent. pepsin in 0.1 N HCl; (b) 32 c.c. of 20 per cent. extract and 20 c.c. of 0.1 N HCl; (c) 32 c.c. of 20 per cent. extract diluted with Ringer's to same volume as (a). The solutions were tested by injecting (0.1 c.c.) tadpoles tri-weekly in the usual manner. No acceleration was produced by (a), a slight acceleration in (b), and the activity of (c) did not appear to be impaired by the treatment.

Action of trypsin. A quantity of 20 per cent. extract was neutralised with sodium carbonate and the following mixtures were placed in an oven at 40° C. for 8 hours, then boiled and filtered: (a) 30 c.c. of neutral extract and 20 c.c. of 0.5 per cent. Na₂CO₃ solution and 10 c.c. of trypsin; (b) 30 c.c. of neutral extract and 20 c.c. of 0.5 per cent. Na₂CO₃ solutions; (c) 30 c.c. of neutral extract diluted with Ringer's to the same volume as (a); and (d) 30 c.c. of active extract diluted to the same volume with Ringer's as (a). Each filtrate was halved, and one portion reacidified with acetic acid until its pH was the same as the original extract. The solutions were next tested by the application of the tadpole test, and only the reacidified extracts which had not been subjected to tryptic action and (d), the active extract, showed signs of acceleration, but, from a comparison of the effects produced, a slight adverse effect of neutralisation seemed apparent.
There were many deaths due to toxic influences among the tadpoles given pepsinised and trypsinised extracts. It appears, therefore, that the principle is inactivated by both pepsin and trypsin, whilst its activity is diminished by dilute hydrochloric acid. In neutral solutions it is inactive, but activity is more or less restored upon the addition of acetic acid. Axolotls given pepsinised or trypsinised extract showed no signs of change, and toxic effects were noted in a few cases.

(d) Hydrogen-ion concentration and activity.

The activity of certain hormones is known to be dependent upon the $pH$ and, as the metamorphic principle has been shown to be quiescent in neutral solution, it seemed that an enquiry in this direction might be profitable. Accordingly the same quantities of sodium acetate were added to 20 per cent extracts in 0·5, 0·125 and 0·05 per cent. acetic acid and, their $pH$ having been determined by the colorimetric method, the time taken by axolotls to change under identical conditions with the same treatment observed. Small animals were used for these experiments. The results obtained are in Table II. Variations in the time required for complete change with animals of about the same size and extracts of the same strength, and the maximum and minimum values of the effective quantity per dose have already been explained, but they do not affect the main conclusions to be drawn even when an allowance is made for them. It can be seen that with the increase of the $pH$ the appearance of the first sign of change is delayed and the period for completion extended, and these effects become more marked as the concentration of acid used for the extracts decreases, the increase in the $pH$ produced by the same quantity of acetate in 0·05 per cent. acid extracts being much greater than in 0·5 per cent. acid extracts. Eventually as the value of the $pH$ approaches 6, metamorphosis does not proceed to completion. Also a comparison of the records of extracts having the same $pH$ gives further evidence of the greater quantities of active factor present in extracts prepared with the higher concentration of acid and undetected when administered to small animals. Similar results are obtained when other salts are used to reduce the $pH$. The hydrogen-ion concentration therefore plays a part in determining the activity of the metamorphic principle. This fact is important, since it provides further means of judging the quality and activity of extracts.

Fresh gland extracts prepared with Ringer's solution or distilled water are neutral, yet saline commercial extracts of the anterior pituitary are of acid reaction and some metamorphically active. A number of 20 per cent. extracts in Ringer's solution ($pH = 7$) of manufactured products (powder and tabloid form) were tested and showed a $pH$ range of 5·7 to 6·5, and among those below $pH = 6$ one or two accelerated tadpole metamorphosis. It is usual in the preparation of these products to treat the glands with acetone, and as this almost invariably contains acetic acid as an impurity, an acid reaction is not surprising. Samples of acetone tested ranged from $pH = 5$ to $pH = 5·7$, whilst the fresh gland extracts in 0·125 per cent. solutions of acetone and alcohol were distinctly acid. Hence it seems very probable that an acid contamination due to the manufacturing processes sufficient to give the
extract a pH below 6 was responsible for the production of the active Ringer's extracts, which would explain the previously reported cases of successful metamorphosis with saline commercial extracts. 0.125 per cent. acetic acid extracts of these products were also tested, and the pH ranged between 5 and 5.7, whilst three, including those whose saline extracts responded, influenced metamorphosis. The negative responses of the remainder and of those saline extracts whose pH is below 6 need no explanation, since such failures have already been analysed and factors discovered (autolysis and post-mortem diffusion), apart from manufacturing methods, tending to reduce potency (Spaul, 1925 a, 1927). The hydrogen-ion concentration plays no significant part in the loss of potency of the metamorphic factor due to exposure and temperature, since the determinations of the pH of acid extracts of the anterior and posterior lobes separated before and after exposure for various periods at different temperatures approximately agree with that of fresh extracts (pH — 5). Nor does the question arise with regard to the tissue extracts, as the pH ranged between 5.1 and 5.3. The various strengths of acetic acid used for extraction were prepared by the dilution of pure glacial acetic acid with distilled water, but the pH of each extract has been found to vary, which is not surprising in view of the fluctuations in activity of similar extracts revealed in the animal tests. The pH of the more concentrated 0.125 per cent. acid extracts appears to rise with the concentration (40 per cent—5.35; 50 per cent—5.45).

Table II. Records of the pH of extracts of the anterior pituitary and the time required for the completion of metamorphosis induced by them in axolotls. (Average of 3 tests.)

<table>
<thead>
<tr>
<th>Strength of acetic acid used for extraction %</th>
<th>Amount of sodium acetate per 100 c.c. in gm.</th>
<th>pH</th>
<th>No. of injections</th>
<th>First signs of change in days</th>
<th>Time for completion in days</th>
<th>Weight in gm.</th>
<th>% loss in weight</th>
<th>Length in cm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>—</td>
<td>4.7</td>
<td>10</td>
<td>8th</td>
<td>21</td>
<td>1.0</td>
<td>40</td>
<td>11.0</td>
</tr>
<tr>
<td>0.5</td>
<td>1.6</td>
<td>5.0</td>
<td>11</td>
<td>12th</td>
<td>24</td>
<td>0.9</td>
<td>39</td>
<td>10.0</td>
</tr>
<tr>
<td>0.5</td>
<td>3.2</td>
<td>5.3</td>
<td>12</td>
<td>13th</td>
<td>26</td>
<td>0.8</td>
<td>42</td>
<td>9.0</td>
</tr>
<tr>
<td>0.125</td>
<td>—</td>
<td>5.0</td>
<td>9</td>
<td>10th</td>
<td>20</td>
<td>0.9</td>
<td>22</td>
<td>10.0</td>
</tr>
<tr>
<td>0.125</td>
<td>1.6</td>
<td>5.35</td>
<td>11</td>
<td>12th</td>
<td>26</td>
<td>1.0</td>
<td>40</td>
<td>10.5</td>
</tr>
<tr>
<td>0.125</td>
<td>3.2</td>
<td>5.7</td>
<td>16</td>
<td>13th</td>
<td>35</td>
<td>0.6</td>
<td>25</td>
<td>7.5</td>
</tr>
<tr>
<td>0.05</td>
<td>—</td>
<td>5.2</td>
<td>10</td>
<td>11th</td>
<td>21</td>
<td>0.8</td>
<td>25</td>
<td>10.0</td>
</tr>
<tr>
<td>0.5</td>
<td>—</td>
<td>4.8</td>
<td>10</td>
<td>9th</td>
<td>22</td>
<td>1.5</td>
<td>38</td>
<td>11.5</td>
</tr>
<tr>
<td>0.5</td>
<td>2.0</td>
<td>5.25</td>
<td>13</td>
<td>10th</td>
<td>28</td>
<td>1.0</td>
<td>40</td>
<td>10.5</td>
</tr>
<tr>
<td>0.5</td>
<td>5.0</td>
<td>5.6</td>
<td>20</td>
<td>20th</td>
<td>46</td>
<td>1.2</td>
<td>36</td>
<td>11.0</td>
</tr>
<tr>
<td>0.125</td>
<td>—</td>
<td>4.9</td>
<td>12</td>
<td>12th</td>
<td>26</td>
<td>0.9</td>
<td>47</td>
<td>10.0</td>
</tr>
<tr>
<td>0.125</td>
<td>2.0</td>
<td>5.6</td>
<td>21</td>
<td>21st</td>
<td>48</td>
<td>1.0</td>
<td>30</td>
<td>11.0</td>
</tr>
<tr>
<td>0.125</td>
<td>5.0</td>
<td>5.95</td>
<td>25</td>
<td>30th</td>
<td>*</td>
<td>1.0</td>
<td>33 1/2</td>
<td>11.5</td>
</tr>
<tr>
<td>0.05</td>
<td>—</td>
<td>5.1</td>
<td>13</td>
<td>14th</td>
<td>28</td>
<td>1.3</td>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td>0.05</td>
<td>2.0</td>
<td>6.2</td>
<td>25</td>
<td>32nd slight</td>
<td>†</td>
<td>1.0</td>
<td>33 1/2</td>
<td>11.0</td>
</tr>
<tr>
<td>0.05</td>
<td>5.0</td>
<td>6.9</td>
<td>25</td>
<td>33rd slight</td>
<td>†</td>
<td>1.2</td>
<td>40</td>
<td>11.5</td>
</tr>
</tbody>
</table>

* Not complete after 56 days.  † No further progress after 56 days.
Finally, in view of the reduced activity of the factor as the pH of the extract is increased, some such similar alteration within the animal might conceivably account, in part at least, for the apparent short and rapid effect of each injection, and the ineffectiveness of surplus quantities given even if retained, since the animal would attempt to counteract the acidity as soon as possible. On the other hand, if rapid elimination of the factor occurs, the influence of each dose would be to a great extent governed by the period of retention within the animal.

(e) Comparison of the effects of the thyroid and the anterior pituitary upon metamorphosis.

At this stage a comparison of the activities of these two principles can be profitably made. A series of comparative studies of the accelerated metamorphosis of frog tadpoles have already been carried out (Spaul, 1928), and it is interesting to ascertain whether these investigations having extended the field for comparison, will allow further distinctions to be made.

The first point to be considered is the activity of 20 per cent. thyroid extracts prepared under the same conditions and by the same methods employed for pituitary extracts using Ringer's solution, distilled water, and various strengths of acetic acid (0.25, 0.125, 0.0625 and 0.03125 per cent.). The tadpole test was applied to each extract and a control was given 20 per cent. extract of anterior pituitary in 0.125 per cent. acetic acid. Three injections (0.1 c.c.) were given in the first week, and as the increased rate of change produced appeared about the same in each case, the injections were stopped. Those given Ringer's and distilled water extracts continued at the same rate and rapidly completed metamorphosis, whereas 3 or 4 days passed before the first of the remainder was complete. Evidently the dose of each given was at least sufficient to induce the maximum acceleration in the specimens for that concentration, since the control given anterior pituitary advanced at the same rate, but the excess given per dose was greater in the case of the Ringer's and distilled water extracts, for as surplus quantities of thyroid are stored, the rate could be maintained, due possibly to the chemical stability of the thyroid activator compared with the instability of other gland extracts. The reserve was smaller with acid extracts, and so the time for completion was extended. Hence the Ringer's and distilled water extracts were more potent. Similar results were obtained by placing tadpoles in solutions of the various extracts (1 c.c. of each extract in 75 c.c. of tap-water) without injections. No food was given and the solutions were changed 3 times in 8 days. Rapid transformations to the adults were observed in the Ringer's and distilled water extract solutions, the remainder changing less quickly. Several fatalities occurred among those given these two extracts by injection, otherwise there were few deaths. Shrinkage and reduction in size characteristic of hyperthyroidism were noticeable features of treatment with these two extracts, but were not so marked in the remainder. The presence of acetic acid therefore reduces rather than increases the activity in metamorphosis of extracts of the thyroid. Precipitation would explain this loss, since Harrington...
A relationship between the activity of this principle and the pH similar to that observed in the case of the pituitary extracts would hardly be expected from this result and, in fact, no sign indicative of any definite relation was found in tests upon tadpoles with the acid extracts to which various quantities of sodium acetate had been added as in the series of experiments with the pituitary extracts. The accelerations induced were approximately the same as those of the original extracts.

A comparison of the response of axolotls to Ringer’s and acid extracts of thyroid (tri-weekly injections under the usual conditions) gives further confirmation of the greater activity of the Ringer’s extract, and additional evidence of the storage of the active factor and subsequent use as required for metamorphic purposes. The initial period before any signs of change appeared and the time for completion varied with the dose given and the weight of the animal. There is a maximum, increasing with size, beyond which a greater response with increased dosing cannot be obtained, and a certain minimum concentration of hormone is necessary before any change is possible, and this must be maintained for completion. With acid extracts the indication of change was late (17th day with small animals (10 gm.), and 30 to 40 days with animals of 50 gm. and more) compared with Ringer’s extract (8th day for small and 15th for large) (see Table III). The change was then rapid except with large animals given acid extracts, when the threshold concentration for complete change is not always maintained and the animal remains in an intermediate condition, which effectively illustrates the greater potency of Ringer’s extracts. The time required for completion with Ringer’s extracts is 14 days for small animals and 22 to 30 days for larger sized specimens, but 30 days or more are necessary for small animals with acid extracts. It would seem from these periods that the axolotl is more susceptible to thyroid than anterior pituitary treatment so far as the rate of metamorphosis is concerned, but when the concentration of the doses of each given are just sufficient for the maximum response in specimens of the same weight, the rates induced approximate, for the extra stimulus arising from surplus thyroid given is eliminated. Daily injections of maximum doses of the anterior pituitary induce about the same rate as large tri-weekly injections of thyroid in Ringer’s since a stimulus—a maximum—corresponding to that of the thyroid surplus is maintained (see p. 53).

The failure of excess quantities to give an extra stimulus was further demonstrated by giving injections (0.5 c.c.) tri-weekly, containing 0.1 gr., 0.05 gr. and 0.025 gr. of thyroid in Ringer’s solution, one to each of 3 groups of animals of about the same weight. The times for completion were not in the same proportion to the dosage or the number of injections (5 injections in 8 days, 7 in 10, and 8 in 11). Also the retention of thyroid given was shown by giving injections containing 0.1 gr. at longer intervals. The animals changed in approximately the same time until the interval between the injections was such that the stimulus or metabolic level could not be maintained owing to the exhaustion of the dose. Whether all the thyroid given was retained and for how long or what proportion
Table III. *Examples illustrating: (1) inhibiting influence of posterior pituitary upon metamorphosis induced in axolotls by either anterior pituitary or thyroid; (2) the greater rate of metamorphosis induced by Ringer's extracts compared with acetic acid extracts of thyroid.*

<table>
<thead>
<tr>
<th>Extracts given</th>
<th>No. of injections</th>
<th>First sign of change</th>
<th>Time for completion</th>
<th>Weight</th>
<th>% loss in weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% ant. pit. and Ringer's solution</td>
<td>12 ant. pit.; 7 Ringer's</td>
<td>10th</td>
<td>26</td>
<td>10</td>
<td>35</td>
</tr>
<tr>
<td>20% ant. pit. and tissue ext.</td>
<td>12 ant. pit.; 8 tissue</td>
<td>11th</td>
<td>27</td>
<td>12</td>
<td>33</td>
</tr>
<tr>
<td>20% ant. pit. and 20% Ringer's post. pit.</td>
<td>12 ant. pit.; 8 post. pit.</td>
<td>7th</td>
<td>Not complete after 28; small reduction only</td>
<td>14</td>
<td>35</td>
</tr>
<tr>
<td>20% ant. pit. and 20% acetic post. pit.</td>
<td>12 ant. pit.; 10 post. pit.</td>
<td>8th</td>
<td>Not complete after 28</td>
<td>13</td>
<td>46</td>
</tr>
<tr>
<td>5% ant. pit. and 20% acetic post. pit.</td>
<td>13 ant. pit.; 9 post. pit.</td>
<td>14th</td>
<td>Not complete after 35</td>
<td>9</td>
<td>66</td>
</tr>
<tr>
<td>10% ant. pit. and 20% acetic post. pit.</td>
<td>13 ant. pit.; 13 post. pit.</td>
<td>8th</td>
<td>Not complete after 35</td>
<td>17</td>
<td>41</td>
</tr>
<tr>
<td>20% ant. pit. and 10% Ringer's post. pit.</td>
<td>14 ant. pit.; 12 post. pit.</td>
<td>8th</td>
<td>Nearly complete after 32</td>
<td>13</td>
<td>54</td>
</tr>
<tr>
<td>20% ant. pit. and 5% Ringer's post. pit.</td>
<td>14 ant. pit.; 13 post. pit.</td>
<td>10th</td>
<td>31</td>
<td>14</td>
<td>43</td>
</tr>
<tr>
<td>20% ant. pit. and 20% acetic post. pit.</td>
<td>36 ant. pit.; 27 post. pit.</td>
<td>16th</td>
<td>Not complete after 82</td>
<td>87</td>
<td>51</td>
</tr>
<tr>
<td>20% acetic thyroid</td>
<td>13</td>
<td>17th</td>
<td>30</td>
<td>8</td>
<td>31</td>
</tr>
<tr>
<td>20% acetic thyroid</td>
<td>16</td>
<td>42nd</td>
<td>53</td>
<td>20</td>
<td>37</td>
</tr>
<tr>
<td>Ringer's thyroid</td>
<td>9</td>
<td>15th</td>
<td>22</td>
<td>21</td>
<td>30</td>
</tr>
<tr>
<td>20% acetic thyroid and Ringer's solution</td>
<td>15 thyroid; 22 Ringer's</td>
<td>42nd</td>
<td>53</td>
<td>39</td>
<td>39</td>
</tr>
<tr>
<td>20% acetic thyroid and 20% tissue ext.</td>
<td>16 thyroid; 24 tissue</td>
<td>42nd</td>
<td>55</td>
<td>49</td>
<td>35</td>
</tr>
<tr>
<td>20% acetic thyroid and 20% acetic post. pit.</td>
<td>19 thyroid; 25 post. pit.</td>
<td>10th</td>
<td>69</td>
<td>50</td>
<td>44</td>
</tr>
<tr>
<td>Ringer's thyroid and 20% Ringer's post. pit.</td>
<td>9 thyroid; 10 post. pit.</td>
<td>9th</td>
<td>28</td>
<td>15</td>
<td>40</td>
</tr>
<tr>
<td>Ringer's thyroid and 10% Ringer's post. pit.</td>
<td>9 thyroid; 12 post. pit.</td>
<td>9th</td>
<td>24</td>
<td>15</td>
<td>37</td>
</tr>
<tr>
<td>Ringer's thyroid and 20% Ringer's post. pit.</td>
<td>9 thyroid; 12 post. pit.</td>
<td>10th</td>
<td>Not complete after 20</td>
<td>10</td>
<td>15</td>
</tr>
</tbody>
</table>
was utilised it is impossible to say, but it is apparent the greater part is and can be used, for a time at least, and actually used, but discrepancies in the comparisons between the period for completion and the dose suggest that a small portion perhaps may be lost or non-effective. At low temperatures the thyroid can be used up in antagonising cold (Huxley, unpublished).

Hence with thyroid the rate of acceleration or the time for completion is determined by the quantity given. It may be administered irregularly in large or small doses, an amount above the concentration for maximum response from the animal being retained and used later if needed, and quantities below a minimum value accumulate until that concentration necessary for response is reached. Also extraction with Ringer’s or distilled water is more effective than dilute acetic acid, whilst the activity of such extracts does not appear to be a function of the pH. On the contrary, the effect upon metamorphosis of the anterior pituitary depends upon the repetition of a stimulus, varying between definite limits, at short regular intervals by the injection of extracts, prepared with suitable strengths of dilute acetic acid, whose activity is limited by the hydrogen-ion concentration. The size of the axolotl and the temperature exert further modifying effects, nevertheless these results provide qualitative and quantitative confirmation and extension of those already obtained from the comparison of the effects of the two principles upon the acceleration of metamorphosis in tadpoles.

4. METAMORPHOSIS AND THE POSTERIOR LOBE OF THE PITUITARY.

In an account of the effect of the posterior lobe extracts upon normal and accelerated metamorphosis in tadpoles and metamorphosis in axolotls, it was concluded that no metamorphic principle was contained in this portion of the pituitary, but evidence was brought forward suggesting the possibility of an antagonistic influence (Spaul, 1924b, 1925c). The question as to the direct or indirect nature of this influence, however, was not decided. It had been found previously that extracts of the whole pituitary gland failed to shorten the larval period, but this may be explained by the inevitable smaller concentration of the principle. A similar dilution would partially account for the reduced activity of extracts of the anterior lobe prepared after exposure, in spite of autolysis and post-mortem diffusion. On the other hand, the retarded growth observed in some mammals following the administration of posterior lobe, and in the more definite retardation in tadpoles after implantation of this lobe from other tadpoles (Allen, 1920) and in axolotls after feeding with posterior lobe (Uhlenhuth, 1921, 1922), strengthened the view that a direct effect rather than an indirect, such as dilution, was responsible for the inhibition recorded of metamorphic processes. The demonstration of such action is important, for, apart from the question of contamination of extracts of the anterior lobe, it indicates interrelationships between members of the endocrine system. Hence an investigation was undertaken, in which the action of 20 per cent. extracts of the posterior lobe in Ringer’s and 0.125 per cent. acetic acid, made by the usual method, was compared with the action of (a) Ringer’s, and (b) similar tissue extracts upon the metamorphic influence of thyroid and anterior pituitary
in axolotls under standard conditions. Tri-weekly injections (0.5 c.c.) of the thyroid (Ringer's and 0.125 per cent. acetic acid) and anterior pituitary (0.125 per cent. acetic acid) were given and alternated with the 0.5 c.c. injections of the posterior lobe or tissue extract or Ringer's. Controls were given tri-weekly injections of thyroid or anterior pituitary. The effect of varying the strength of the dose was also studied. The comparison with Ringer's made it possible to estimate any dilution effect due to the presence of the extra fluid injected, and the tissue extract enabled any effect due to the protein content of the injected substances to be observed.

**Experimental results.** Animals receiving posterior pituitary, together with either thyroid or anterior pituitary, showed signs of change earlier than controls given only thyroid or anterior pituitary, and appeared to transform at a greater rate for a while, the dorsal fin and gills reducing rapidly, but later the rate slackened, and eventually the controls completed their change first. These effects depended upon the quantities of each given, but in all the premature reduction of the dorsal fin and slight gill absorption were observed (Table III).

**Thyroid and posterior pituitary.** In small animals the effect was small with thyroid and weak doses of the posterior pituitary, for apparently the surplus thyroid was sufficient to overcome any action of the posterior lobe. As the strength of the posterior pituitary dose or the size of the animal increased, less thyroid would be available, with a resultant prolonged period for completion and earlier initial signs of change. Finally a stage would be reached when the animal failed to change and remained at an intermediate stage, but generally in these cases few survived many weeks the metabolic strain imposed by this treatment. Reducing the thyroid dose would have similar effects, and eventually the amount available would be less than the threshold value, and no definite metamorphic signs would be noted at all.

In these experiments the injections have been continued till completion or stable conditions were reached, but if only the number of injections required by the control for completion were given and the post pituitary injections continued as before, the period for completion was extended or, more usually, further change ceased. Similar inhibitions are obtained if a few large doses or a single thyroid meal are given with continuous injections of the posterior lobe extract (Huxley, Hogben, unpublished). The increased effects obtained when using the acetic acid thyroid extracts compared with Ringer's extract emphasised their weakness, whereas the extracts of the posterior pituitary appeared equally effective.

For injections of Ringer's or the tissue extract instead of the posterior pituitary in addition to thyroid only a limited number of animals were available, but there was not one instance of premature signs of transformation. The change proceeded normally as in the controls, the first indication being somewhat delayed and the period longer, but the difference was too slight and varied to justify any inference of definite interference with the thyroid activity.

**Anterior pituitary and posterior pituitary.** In the case of the anterior pituitary the effects observed were not so marked in the smaller animals, as they completed a few days later, but the larger either changed much later or failed to complete,
the effects increasing as the quantity of posterior lobe per dose increased. These facts agree with the earlier contentions of the significance of the concentration of the active principle for successful conversion and also the increase with size of the animal of the minimal effective concentration. In those given Ringer’s and tissue extracts with the anterior pituitary, no fin reduction occurred before the expected time, nor was the time for completion unduly extended.

The time elapsing after the thyroid or anterior pituitary dose before the posterior pituitary injection is given, whether 15 minutes, 2 hours after or the next day, does not appear to appreciably influence the final result, for inhibitory effects are always noted.

Earlier work upon tadpoles (Spaul, 1925 c) was extended, and the influence of the posterior lobe extracts compared with the effect of Ringer’s and tissue extracts upon normal and accelerating metamorphosis, and the results obtained agreed entirely with the work upon axolotls.

To sum up, these experiments present definite evidence of an inhibitory influence upon metamorphosis through direct action of some factor present in the posterior lobe, for neither the extra fluid injected nor its protein content appears to be directly responsible. How this is achieved, whether by direct influence upon the tissues, metabolism, or the endocrine organs themselves, cannot yet be stated.

The early tissue reduction and the delay, and in many cases failure, of transformation would imply the encouragement of the destruction of tissues in the early phases and partial or complete hindrance of the constructive phase or reorganisation for the land habitat, including later tissue resorption.

The evidence so far available does not help to fix the responsibility of inhibition upon any particular autacoid or factor of the posterior pituitary. It is true the melanophore stimulant has been the means of detecting the presence of the posterior lobe and that both Ringer’s and acetic extracts have shown antagonism, but it does not follow necessarily that the responsible factor is identical with the melanophore stimulant. Until these observations have been extended and correlated with details of the activities of the other autacoids, no statement as to the identity of the factor is permissible.

Due emphasis has already been placed upon the detrimental influence of the posterior lobe in anterior lobe extracts, and these results serve to stress still more the neutralisation of the principle’s activity and the need for minimum contamination.

Moreover, it seems probable that the reduction of the activity of anterior lobe extracts with increased pH, and as the concentration of the acid used for extraction becomes weaker, might be accentuated by the inhibiting factor present retaining its effective strength.
5. ACTIVITY OF DIFFERENT REGIONS OF THE ANTERIOR LOBE

In a recent record tentative observations were made upon the location of the metamorphic principle in the anterior lobe consequent upon the injections of extracts prepared from the inner and outer portions of the anterior lobe (Spaul, 1925). Fuller investigations have now been made and a discussion of the problem is possible.

From histological studies the existence of more than one kind of cell is evident. Clear and granular cells are present, the latter being divisible into oxyphil and basiphil according to their readiness to stain with acid or basic dyes. Their relationship, if such exists, has yet to be settled. They may be distinct or represent different functional stages or activity of the same cell. These cells are distributed more or less irregularly, but a recognisable central area in the ox contains most basiphil cells. Smith, P. E. and Smith, I. P. (1923) found that injections of the central area induced rapid metamorphosis in tadpoles, whilst the outer portion containing most oxyphils promoted growth without metamorphosis. This suggests the presence of two active principles, one promoting growth and the other metamorphosis. The histological observations of Reiss upon the position of the Golgi body in the cells are suggestive of two kinds or different phases of secretion in the gland. Apart from evidence of secretion from the cells, a colloid material has been described, which, unlike that found in the thyroid, contains no iodine, is insoluble in water, alcohol and ether, but dissolves in acids. It is contained usually in vesicles surrounded by cells, sometimes diffuse, and abundant after thyroidectomy. Fatty granules are present in most cells and are said to increase with age.

The separation of Smith's basiphil area with any certainty is doubtful, but in the writer's previous work extracts were made of the outer and inner regions of the anterior lobe, the latter including this area so far as could be ascertained. These preparations were made primarily to demonstrate the post-mortem diffusion of the melanophore stimulant through the anterior lobe, and only limited observations were possible upon metamorphosis. Only the inner region was found responsive. More extensive observations have since been made, using 20 per cent. extracts in 0.125 per cent. acetic acid, the division of the anterior lobe into inner and outer regions being kept as nearly the same as practicable. Successful transformation of axolotls, together with failures to complete, have been obtained with extracts of either region, but in no single case was the change more rapid than that produced by extracts of the whole anterior lobe. There was no great difference with small animals, but, as the weight increased, it was observed that the period necessary for change was generally longer in those given extracts of the outer region. This was more pronounced when 10 per cent. extracts of these regions were used, whilst the majority of failures received extracts of the outer region. Tadpole tests gave similar results. The response of the inner region was considerably influenced by the inhibition of the diffused posterior lobe which is greater here than in the outer region. It would appear therefore from these results that, although there is not definite localisation or concentration of the metamorphic principle in a particular
6. OTHER EFFECTS OF ADMINISTRATION OF THE ANTERIOR LOBE.

(a) Influence upon growth.

Since the discovery of the association of acromegaly and gigantism with the enlargement of the anterior lobe, attempts have been made to produce experimentally increased body growth by the administration or implantation of anterior lobe substance. The results have by no means been uniform or conclusive. Some success has attended transplantation in mammals, whilst Goetsch (1916) records a stimulating effect upon growth and sexual development and activity in young rats and a retarding influence of the posterior lobe. Schafer (1912) found that the addition of small amounts of pituitary substance to the food of young rats causes increased growth rate after 3 months, although no change was noted during the first 6 weeks. Later Evans and Long (1921) described a positive effect upon the growth by the injections of extracts of the anterior lobe, but no effect by feeding, and repression of sexual development. Prolonged feeding with the whole pituitary gland had no effect according to C. S. Smith (1923), but Dott (1923), feeding large amounts of anterior lobe, obtained acceleration of growth in kittens and puppies. Brailsford Robertson (1916) concluded that alcoholic extracts of the anterior lobe contain a growth-promoting substance—tethelin—and found its effect upon mice depended on age. Wulzen (1914) and others, however, observed the reverse effects in chicks following the addition of the anterior lobe substance to the food. This conflict of evidence does not help in the interpretation of the action of anterior lobe or its method of functioning, and it is impossible to formulate general principles as to the relation between growth and the anterior lobe on account of the lack of uniformity in experimental conditions, the age and stage of development of the animals treated, the substance given, whether fresh gland extract or manufactured product, and the quantity, frequency and nature of the administration, apart from the knowledge of other influential factors, including the reaction of other members of the endocrine system in the presence of excessive activity of one gland. The purity of the substance administered is another important factor which does not appear to have received adequate consideration, and contamination with the posterior lobe or its factors likely to exhibit inhibitory tendencies may explain some at least of these discordant results. However the amphibia provide more convincing evidence, and as the removal of this portion of the gland retards growth it is generally accepted that the anterior lobe furnishes some growth-promoting substance particularly favourable to bony and connective tissues.

Administration of the anterior lobe to the amphibia reveals new and interesting features of its activity. P. E. Smith (1916) noted an increased growth-rate in normal and hypophysectomised tadpoles by feeding, and Allen (1920) similar effects in
tadpoles of *R. pipiens* (normal, hypophysectomised, thyroidectomised) after grafting anterior lobe of adult frogs, but retardation with posterior lobe. Swingle (1922), and also Smith and Smith (1922 b), accelerated metamorphosis of tadpoles by grafting the adult frog's anterior lobe. Uhlenhuth (1923) produced gigantism in transformed axolotls (*A. opacum, A. tigrinum*) by feeding anterior lobe, and Huxley and Hogben (1922), noticing increased growth in axolotls after feeding anterior lobe, concluded that it possessed growth-promoting properties. Later Hogben (1923) induced the metamorphosis of normal and thyroidectomised axolotls by the injection of commercial extracts of the anterior lobe. The subsequent confirmatory investigations of the author and the reduction of the larval period in tadpoles by the same means have served to introduce this series of studies upon the activity of the anterior lobe in relation to metamorphosis. P. E. Smith (1926) has observed the reverse effect upon injection into Colorado axolotls, but with knowledge of the conditions tending to destroy the effectiveness of anterior lobe extracts, these results may be taken to indicate the contaminating influence of the posterior lobe principles (Spaul, 1925 a, 1927). Hoskins and Hoskins (1920) obtained precocious metamorphosis in tadpoles by feeding anterior lobe preparations containing iodine, but Smith and Cheney (1921) have shown that the latter was the activator. The writer has fed other anterior lobe preparations with no results upon metamorphosis. The independence of this pituitary metamorphic factor of iodine and its separate identity from the thyroid principle have been demonstrated (Spaul, 1925 b, 1928). Also Simpson and Hunter (1910) found no iodine in fresh bovine or sheep pituitaries even after removal of the thyroid.

Therefore a twofold expression of the activity of the anterior lobe is possible in the amphibia. Growth at an increased rate follows prolonged feeding of sufficient quantities of the fresh substance without effecting metamorphosis and a size greater than the normal may be attained. Metamorphosis is either accelerated or produced in certain amphibians not normally undergoing such changes in that environment by the injection of suitable extracts in sufficient quantities, whilst in some cases growth or metamorphosis in normal animals may be influenced by implantation. This suggests the existence of two factors, already indicated by the histological evidence, but before discussing this aspect of the problem the ability of the extracts producing metamorphosis to promote growth must be considered and the relation between metamorphosis and growth defined.

**Experimental observations. Weight and length during metamorphosis.** The records of the weights and measurements of the axolotls metamorphosing under the influence of the anterior pituitary show no increase in weight or length. In every instance an initial loss of weight during the first few days of treatment was followed by a slower decline until completion, the total loss varying between 35 and 30 per cent., though extremes of 45 and 25 per cent. were noted. This loss bore no apparent relationship to dosage or other factors. Further, no change in length was noted, except a slight decrease at times as the tail fin was absorbed. Approximately the same loss in weight has been noted in axolotls metamorphosed by thyroid treatment. With tadpoles, only records of measurements have been taken during the accelera-
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of metamorphosis, and they show a small decrease in size depending upon the
f, and hence the dose. However, this reduction is not so considerable as that
noted with thyroid (Spaul, 1928). During normal and induced metamorphosis,
and especially in the latter stages, animals take little or no food, owing to the change
in the digestive tract associated with this process, so that a loss is not surprising.
Hence it appears that either growth and metamorphosis cannot be stimulated simul-
taneously or that extracts producing metamorphosis are not able to initiate an
increase in size.

Experiments on frogs. Experiments were performed to test the capacity of these
extracts to influence growth. Small frogs (1.5–2.5 gm.) were placed in glass con-
tainers (10 in each) each having earth covering the bottom, a water trough and
gauze covers. The conditions were kept uniform at room temperature. The speci-
mens were weighed and measured (body and hind limb) and tri-weekly injections
of 0.4 c.c. of a 20 per cent. extract of the anterior lobe in 0.125 per cent. acetic acid
given. Entirely satisfactory and consistent results were not obtained owing to
the difficulty of maintaining the most suitable experimental conditions for captivity
for any length of time. In the first series during the early summer at 17° C. the
animals were fed with worms and a decided increase in body length (15 per cent.)
was noticed within 14 days compared with a slight increase (5 per cent.) in some
controls, but there was a decline in weight in each case. After this period deaths
occurred among both injected and control specimens, and became more frequent,
so the experiment was stopped after 21 days when the numbers were reduced by
half. Increased growth in length was still progressing at this stage. As feeding
might introduce an unknown quantity not easily determined, the experiments were
repeated in the late autumn with small frogs under the same conditions. No food
was given and the effect of other 20 per cent. extracts of the anterior lobe (Ringer's;
alcohol; 0.5 and 0.03125 per cent. acetic acid; 0.125 per cent. HCl; neutralised
extract; trypsinised; pepsinised; 0.125 per cent. acetic acid of outer and inner
regions), whose metamorphic effect had already been studied, was examined
together with the uninjected controls and others given Ringer's and tissue extracts.
Only 0.2 c.c. injections were given and the temperature was lower (14° C.). There
were fewer fatalities and the experiment continued 28 days. Another similar series
started in the summer at 18° C. with 5 in each container lasted 16 days. Increase
in body length (3.5 per cent.) and in the length of limb (10 per cent.) in excess of
that observed in controls was obtained in those given extracts of the outer region
of the anterior lobe, and to a much less extent in some given extract of the inner
region (0.5 and 2.5 per cent.) of the lobe and the whole lobe itself (0.6 and 3.0 per
cent.). There was an increase in weight at first, but a decrease followed even in
controls. Some specimens given Ringer's extract showed a slight increase in
length, but no other extracts gave any indication of stimulated growth. In spite
of inconsistency and variation and the desirability of some reliable standard test,
it would seem that there is some factor present in acid, and Ringer's extracts of
the anterior lobe, able to promote growth in size and that it predominates in the
outer region. The effects are most marked and persistent when the specimens are
regularly well fed, and no doubt the failure to make satisfactory feeding arrangements in these experiments would account for many fatalities and the falling of the growth-rate induced. This recalls the histological evidence of the different cells in the anterior lobe and the possible existence of two principles or two phases of activity of the one, and also the greater ability of the outer region, to increase the rate of growth recorded by P. E. and I. P. Smith (1923).

**Other results.** Apart from these experiments other examples of growth have been obtained.

(i) A few large transformed axolotls were given tri-weekly injections (0.5 c.c.) of the anterior lobe extract again after some weeks of normal existence following metamorphosis, and a record taken of their weights and lengths. They were kept in a constant environment, separate from controls, and fed three times a week with worms or raw meat. After 50 days an increase in length, averaging 6.7 per cent. of the total length compared with 2.8 per cent. of the controls, was found. The weights fluctuated and only a slight advance occurred. Uhlenhuth (1923), feeding anterior lobe to *Amblystoma* for many weeks obtained considerable growth in length beyond the known maximum and also increased weight.

(ii) Some axolotls given tri-weekly injections of 10 per cent. extracts of the outer region of the anterior lobe in either Ringer’s or acetic acid, and showing no signs of metamorphosis, increased in length as treatment continued: 1.5 per cent. in 28 days and 12 injections; 4 per cent. in 36 days and 14 injections; 11.5 per cent. in 38 days and 16 injections (Ringer’s extracts) are examples. There was a loss in weight, although the last regained its weight and was eventually heavier than at the beginning of the experiment.

(iii) Another case of increased length was found in a medium sized axolotl failing to complete metamorphosis with injections of 20 per cent. extracts of the anterior pituitary in 0.05 per cent. acetic acid. After the initial period, during which a late reduction of fin and gills but no change in length occurred, the injections ceased, to be resumed 4 weeks later for a period of 66 days. 26 injections were given, and there was an increase in length of 6.5 per cent. but a loss of weight. Controls for these axolotls showed no change in length and slight loss of weight. All these animals were fed once a week.

The evidence from tadpoles has little critical value, as no measurements were taken of those failing to accelerate metamorphosis and no food was given. However, a distinct tendency was observable of those given either Ringer’s or acid extracts of the outer region of the lobe to become larger than controls, with no increase in the rate of metamorphosis. Such increases were not noted with extracts of the inner region, but there was a small increase noticeable sometimes with Ringer’s extracts of the whole lobe.

**Conclusions.** These records, though a somewhat odd and incomplete assortment, strengthen the previous deductions upon the stimulating effects of extracts of the anterior lobe, particularly the outer region, upon growth. The extracts of the outer region seem more potent, but the inhibitory activity of diffused posterior factors as in metamorphosis may prevent the expression of such stimulation by
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extracts of the inner region, since the administration of the lobe retards growth (Hen, 1920).

Acid extracts of the anterior lobe are able, then, to influence metamorphosis, provided the concentration permits effective stimulus, and growth in size, but not apparently at the same time, as might be expected a priori from our knowledge of the weight loss normally accompanying metamorphosis. Ringer's extracts can influence growth. Limits defined by concentration, frequency of dosage, the size of the animal, and the temperature in standard environmental conditions, exist to the period of metamorphosis, and in the later stages of the process, especially, little food is taken. For effects upon growth, treatment must be prolonged, and the response not very definite for a time, gradually increases aided by continuous and plentiful feeding. Exact determinations of factors influencing the rate of growth have yet to be made, before quantitative comparison with the rate of metamorphosis is possible, but it would seem the details given of the effect upon growth and metamorphosis of extracts of the whole lobe and the inner and outer regions, taken in conjunction with the histological evidence and Smith's results, favour separate factors or principles for each effect, or, if one agent only is concerned, these distinct effects are the result of different stages or phases of its activity determined by both its concentration and chemical environment or combination. Metamorphosis itself, though actually a growth process, is more than an increase in size or rate of processes already in progress, since it involves a general reorganisation and reconstruction, the differentiation of some structures, the reduction and absorption of others, which alone points to the particularity of the stimulus required to induce these changes.

(b) Relation to gonads.

Another phase of the activity of the anterior lobe is shown in its relation to the gonads and secondary sex characters. Their activity and state of development are considerably modified in mammals by hypo- or hyperpituitarism or by the removal, injury, or disease of the gland. Evans and Long (1921), injecting aqueous or saline extracts of bovine anterior lobe, found delayed and eventual suppression of the oestrus as well as a body growth in contrast to the stimulated sex development noted by Goetsch (1916) and also Marinus (1919). More recently fuller investigations upon the pituitary-ovary mechanism of the mammal have been carried out (Smith and Engle, 1927; Parkes, 1928). Sexual precocity has been observed in birds in some cases after feeding anterior lobe. No observations appear to have been made upon this relationship in the amphibia.

(c) Interrelationships of thyroid and anterior pituitary.

Lastly there is the question of the relation between the anterior pituitary and the thyroid and whether, in view of the presence of a metamorphic principle in each, they can function vicariously. The removal of the pituitary results in atrophy arrested development of the thyroid, whilst the pituitary becomes altered and enlarged after thyroidectomy, and tadpoles deprived of either gland cannot meta-
morphose and limb growth is equally retarded (Allen, 1925), so that some relationship exists. Greenwood (1925) found no altered growth-rate in axolots after removal of the pituitary when the thyroid was developed, and Huxley (unpublished) kept thyroidless and pituitaryless axolots for more than a year with no change in growth-rate. Recently Uhlenhuth and Schwartzbach (1927), in studying the changes in the thyroid of the larvae of _A. tigrinum_ during metamorphosis induced by injection of extracts of Armour's anterior lobe powder (thereby confirming the work of Hogben and the author upon the metamorphic activity of the anterior pituitary), found that the gland passes through a whole cycle of functional changes characteristic of thyroids in larvae during normal metamorphosis. He concluded that the anterior lobe does not enforce metamorphosis by direct action but indirectly by stimulating the thyroid to functional activity. Further, he showed by examination at various periods that thyroids of specimens metamorphosing in solutions of Bayer's iodothyreine were unchanged, and concluded that thyroid substance induces metamorphic changes directly by its action on the tissues without the intermediation of the animal's own thyroid, and therefore behaves entirely differently from what he believed the anterior lobe substance did. No examination of the pituitary was made similar to that of the thyroid in the previous experiment. These conclusions agree with the views upon the ineffectiveness of the axolotl's thyroid expressed by the author (1925), following experiments showing the necessity of dosing with thyroid before the axolotl could utilise iodine in solution and also the experimental evidence, both quantitative and qualitative, as well as in relation to iodine, distinguishing the metamorphic activity of the thyroid and anterior pituitary, but the production of metamorphosis by the anterior pituitary through the thyroid is difficult to reconcile with the results obtained in animals from which the glands have been removed. Further observations made by Uhlenhuth concerned the changes in the thyroid of axolotls—swellings of some follicles and disintegration of others and diminution in numbers—when given a dose of iodine. Inorganic iodine has no effect upon metamorphosis in axolots, but these changes are not surprising considering the sensitiveness of thyroid tissues to iodine as such, in contrast to the tissues in general. Absorption to some extent took place in Uhlenhuth's experiments, but the ineffective condition of the thyroid prevented the successful elaboration of active hormone. A possible defect in the releasing mechanism of the thyroid itself would explain its failure to co-operate with the active thyroid substance given to effect metamorphosis, so that during treatment the gland remained unchanged. The sensibility to iodine and the power to use it and elaborate some active agent are retained in tadpoles not only by the thyroid but to a less extent by the tissues themselves, as illustrated by the conversion to adults of specimens without the thyroid and pituitary by iodine. Consideration of the response of specimens without either or both glands to suitable stimulation with thyroid or pituitary would show that the metamorphic factors function independently. Hypophysectomised tadpoles metamorphose when given thyroid (Allen, 1920, Swingle, 1923 a), and injection of anterior pituitary extracts change thyroid-ectomised axolotls (Hogben, 1923).
Experimental evidence. The following investigations upon axolotls from which either or both glands were removed provide confirmatory evidence. The glands were removed from the axolotls whilst under an anaesthetic (urethane). The thyroid was approached from a mid-ventral cutaneous incision in front of the opercular fold and each half removed from its position by the inferior jugular vein. The incision was closed by one or two stitches. The pituitary was exposed with the aid of a dental drill, fitted with a small rose burr, by piercing the parasphenoid immediately below the gland, which was previously located as a white spot in the roof of the mouth beneath the thin membrane and translucent cartilage. The same technique was employed by Hogben. After loosening the gland with a fine cataract knife it was sucked up a pipette and the hole recovered with dental cement. In removing both glands from an animal it was found that the two operations could be performed in succession, the extirpation of the pituitary first, with a quick recovery and no harmful consequences. A permanent palor was assumed by the black variety of axolotl a few hours after the removal of the pituitary, similar to that recorded by Hogben in hypophysectomised axolotls and frogs, and by P. E. Smith (1916a, b), Allen and Atwell in tadpoles without glands. The animals were able to feed a short time after the operation, and remained under normal conditions and temperature for several weeks to ensure complete recovery. For the experiments the usual conditions and temperature (22°C.) were maintained and tri-weekly injections (0.5 c.c.) given and weights and measurements taken. Thyroidectomised animals given 0.125 per cent. acetic acid extracts of the anterior pituitary transformed in approximately the same time as the controls given the same extract; showing the first signs of change on the same day, and about the same loss of weight with no change in length. Thyroidectomised animals given either acid or Ringer’s extract of thyroid appeared to change at the same rate as the controls. The acid extract, as shown, is not so potent, so that the first signs of change in this case were not visible for some time, and the time required for completion was longer. Hypophysectomised animals given anterior lobe extracts required about the same time and number of injections for transformation as similarly treated controls and thyroidectomised animals of about the same weight with the commencement of the change signified on the same day, similar percentage loss of weight, and only a very slight decrease in length. Animals with both thyroid and pituitary removed respond in like manner to anterior pituitary treatment. The pale hypophysectomised forms returned to normal coloration shortly after the injections of the anterior pituitary owing to the presence of the melanophore stimulant in the extract.

Unfortunately only a limited number of animals (20) were available for these experiments, but the results were consistent and present a means of examining the behaviour of the thyroid and anterior pituitary of the axolotl during induced metamorphosis. The similarity in the times required for the assumption of adult characters by both normal and thyroidectomised animals when given thyroid and also anterior pituitary is significant, for it would appear that the thyroid of the axolotl, in spite of the known physiological activity of its contents shown by the accelerated anuran metamorphosis produced by transplantation (Swingle, 1923a),
plays no active rôle in the changes induced, as no augmentation of the activity of the injected thyroid is apparent, and the anterior pituitary is seemingly able to function independently of the thyroid, as originally shown by Hogben. These observations upon the axolotl’s thyroid are in keeping with the previous conclusions based upon the comparison of anuran and urodele metamorphosis (Swingle, 1923a), experiments showing the effective utilisation of iodine by axolotls only when given thyroid (Spaul, 1925b), and histological studies of the thyroid during metamorphosis by thyroid (Uhlenhuth and Schwartzbach, 1927), and reflect the peculiarity of its behaviour—the retention of the thyroid hormone during larval life. Various explanations of this condition have been put forward, such as a defective releasing mechanism or factor controlling the secretion, lack of factors involving the sensitisation of the tissues to the thyroid hormone, or insufficiency of hormone. It can be seen from the experiments described here that the tissues of the animals respond to thyroid treatment if sufficient is given to maintain the necessary threshold concentration. The amount of hormone present, therefore, in the animal’s thyroid would probably be too small to make any appreciable difference in the times required for metamorphosis by normal and thyroidectomised animals. On the other hand, its activity may be controlled or suppressed by some internal influence or factor either preventing its escape from the gland or neutralising its effect upon the organism when free, so that additions are necessary to overcome these resistances. From Uhlenhuth’s observations upon the cycle of functional changes in the thyroid during metamorphosis by anterior pituitary injections, and its unchanged condition during thyroid treatment, it would appear that the elaboration of the hormone was incomplete and required some stimulus for completion, or the removal of such influences as impeded functional activity. This may have been the direct or indirect influence of the anterior pituitary. Similar changes may have occurred when the gland was transplanted into anuran larvae with the resultant acceleration. This implies (a) interrelationship of the glands, and (b) metamorphosis by thyroid activity through pituitary stimulation, although the axolotl’s pituitary does not appear able to do this of its own accord. The former has already had experimental demonstration, but the latter is difficult to reconcile with the metamorphosis of the thyroidectomised animals by the anterior pituitary and the distinct qualitative and quantitative differences noted in comparative studies of accelerated metamorphosis of normal tadpoles by treatment with thyroid and anterior pituitary. Incomplete removal of the glands or regeneration, the existence of accessory portions, or the presence of a small quantity of hormone in the tissues, since the glands were removed from fully developed animals, are possibilities which may be brought forward to account for the transformation of thyroidectomised forms by anterior pituitary stimulation. It was only possible to examine two of these animals immediately after the change, but no definite signs of thyroid tissue were found. However, these experiments themselves do not justify any conclusion upon vicarious functioning, but they gain support from evidence derived from other sources. In those experiments in which the anterior pituitary injections were given to axolotls in iodine solution, no increase in the rate of change was noted,
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whereas, if the changes proceeding were due to the activity in the tissues of the stimulated and circulating hormone of the stimulated thyroid, there does not appear to be any reason for the failure to utilise iodine as in the case of those given thyroid in iodine solutions, even with doses producing a slower rate of change. Again, although the histological studies show functional changes of the gland, there is no indication of the amount of active hormone formed, or whether the hormone was released even and able to effect the tissues. It is difficult to estimate the amount of hormone in the gland, but from transplantation work, although able to metamorphose some tadpoles, it is incapable of affecting an axolotl in the same way, and, in view of the negative response to iodine, no other source of increase is apparent. In the absence of a releasing factor there is no secretory activity. Hence, although an interrelationship exists between these glands, the extent to which they are interdependent is doubtful. The evidence certainly favours the ability of the anterior lobe to produce metamorphosis directly and independently, and the effect upon the thyroid is probably a subsidiary effect of this same stimulation.

The approximate times for metamorphosis noted in normal and hypophysectomised animals and those with both glands removed when given anterior pituitary injections, suggest that the change was due solely to the injected principle with practically no intervention or contribution of the animal's own gland. This may signify a low level of functional activity of the pituitary gland of the normal axolotl, as regards metamorphosis, similar to that characteristic of its thyroid. This condition of the gland would explain the state of the thyroid, in view of their interrelationship, and also the unaltered growth-rate of hypophysectomised axolotls noted by Greenwood (1925).

As to any effects due to the posterior lobe of the pituitary of the animal, it is impossible to base any statement upon these records. It seems therefore that the axolotl depends entirely upon the direct action of these principles added from outside sources to initiate and sustain the processes involved to achieve complete metamorphosis, which activity its own endocrine system seems little able to supplement.

The influence of thyroid injections upon hypophysectomised forms was only studied in one case owing to the limited supply. Death supervened before completion, but the first signs of change appeared at the same time as in the controls and its progress gave indications of agreeing with the other results.

The information provided by these experiments is by no means adequate, and confirmation and a more extensive quantitative search supported by histological examination are needed before the exact relation between these glands and metamorphosis is elucidated with any certainty.

No useful information was gained by giving both thyroid and pituitary in alternative injections. Many died during treatment, but those surviving showed no undue acceleration beyond that already noted with either anterior pituitary or thyroid.
7. DISCUSSION.

The data given above demonstrate the existence of an active substance of the anterior lobe of the pituitary which influences amphibian metamorphosis. The principle itself has yet to be isolated and its chemical composition and properties investigated, but utilising the specific response—the production or acceleration of metamorphosis in the amphibia—it has been possible to identify its activity in extracts of this portion of the gland and study some of its characteristics, particularly those features serving to distinguish it from other active metamorphic agents, and those reflecting some light upon its relationship to the other known activities of the pituitary itself or other members of the endocrine system. The knowledge gained, at the moment, is small but significant, dependent upon the biological approach, and defined by the extent to which its activity can be recognised by this response. An exploration of the range of this reaction revealed limits of concentration and variations with size and age suitable as a basis for biological assay, but the individual differences of animals and other variables, which could neither be eliminated nor successfully controlled, introduce a degree of uncertainty in the determination of these limits and an inconsistency in the time required for these changes incompatible with standardisation or a higher order of exactitude in assessment. However, with conditions arranged as uniformly as possible, and the adoption of a standard method of extraction, consistent results were obtained by comparative methods. From these approximate estimates of the strength of extracts could be made which were sufficiently accurate to afford a satisfactory and reliable means of interpreting the various phases and aspects of the factor's activity, and greatly extended the field of study of its characters and facilitated the comparison with other agents. Acid extraction of fresh glands with suitable concentrations of dilute acetic acid appeared to be the most appropriate method, giving extracts whose activity was high enough to stimulate these animals. The activity, within limits, increased with the strength of acid used for extraction; the pH of the extract also affects it. Solutions of strong acids (HCl, H2SO4) used for extraction failed, and it is probable that the heavy precipitates obtained with them removed the active substance. It appears that the factor is stable in the presence of weak acids but sensitive to alkalies. (Saline extracts have been used with success in a few cases, but the method of preparation was different from that used here, and other species of Rana were used for the test; as the response varies considerably with the species, it is difficult to compare these from the results obtained.) The active extract can be boiled for a short time at least without appreciably reduced activity, and can be stored for some months unaffected. Its destruction by autolysis has already been shown, and the negative response to metamorphosis by feeding the fresh gland or preparations, even in large quantities, is no doubt due in part to the action of the digestive ferments which have been shown above to render extracts inactive after some hours. These properties point to the hormonal character of the active substance. Brailsford Robertson (1916) describes the presence of a growth-promoting autacoid in alcoholic extracts, but confirmation of this is lacking.
I. Smith (1920) finds a growth autacoid in the residue after extraction with water and boiling alcohol. Evans and Long (1921) used yet another method of preparation to obtain extracts promoting growth and inhibiting ovulation. Other workers have also prepared active products, but their method of extraction and the test applied were different and gave no hint as to their metamorphic value. Clearly reciprocal testing and closer analysis and experimentation in extraction are needed before any common identity is established between the different active products and a clear conception of the active substance or substances of the anterior lobe is gained.

The manner in which the anterior lobe is able by its activity to produce metamorphic effects is by no means established, and the problem is complicated by the fact that, apart from the influence of the thyroid upon these changes, a definite functional correlation has been shown to exist between these structures. The two activators are distinct, but the question is, whether the action of the pituitary is direct, or indirect through the stimulation of the thyroid. The problem has already been discussed in relation to the axolotl, but consideration and analysis of amphibian metamorphosis generally and the modifications suffered in the presence of excess or entire absence of the glands may provide yet other viewpoints for a more intimate understanding of the rôle of these glands in this phenomenon.

Metamorphosis of tadpoles is inhibited by the removal of either or both glands, whilst, in the absence of the hypophysis, the thyroid remains rudimentary. The possibility of inducing metamorphosis in hypophysectomised tadpoles by thyroid was shown by Allen (1920), but P. E. Smith (1920) was unable to complete the changes in similar tadpoles by thyroid or thyroxin-iodine feeding. The transplantation of the anterior lobe into hypophysectomised tadpoles having rudimentary and functionless thyroids produced metamorphosis, according to Allen (1920), whereas transplantation into thyroidectomised larvae had no effect, since no thyroid mechanism was present for stimulation. These results, like the histological studies of Uhlenhuth, show the dependence of the thyroid upon the pituitary for normal development and differentiation and their essential co-operation in normal metamorphosis. On the other hand, the failure with the thyroidectomised anurans compared with the successful treatment by injection of the anterior pituitary of the thyroidless axolots requires examination. This involves a consideration of the larval phase and the sequence of developmental changes in anuran and urodele metamorphosis.

The acquirement of limbs and the absorption of the tail, together with the closure of gill clefts and the shedding of the larval skin, are associated with metamorphosis in anuran tadpoles. The larval urodele has fully developed limbs, tail and external gills. Of these the tail is retained and external gills are absorbed in metamorphosis. The shedding of the larval skin, the protrusion of the eyes, alterations in the nature of the integument externally, and reduction of the alimentary system and ossification to a varying degree internally, are changes occurring in each group, but the gonads, and to some extent the lungs, appear to be independent of metamorphosis. Hence distinct variations exist in the larval phase and in the sequence of changes from the aquatic to the terrestrial form characteristic of these
groups, and changes, such as development of limbs, definitely associated with metamorphic changes in the one case, are apparently unconnected with it in the other. The rate of change is influenced by favourable or adverse environmental conditions (food supply, temperature, etc.) or experimentally by the addition or the removal of the thyroid or pituitary. The neotenous condition of the anuran tadpole following removal of either gland approaches but is not equal to the condition found in the axolotl, for the growth of limbs is retarded, and although the gonads continue to develop and the germ cells mature, the ducts do not appear. Normally, metamorphosis of the tadpoles is inevitable and an accelerating stimulus may be given at almost any pre-metamorphic stage in both sexes. The susceptibility increases with age, but the stimulus given can be too great at the younger stages for survival till completion. The axolotl does not transform unless it receives a suitable stimulus which may be applied to the fully developed mature animals of any size and age irrespective of sex. The acceleration of tadpoles is reduced to normal if the stimulus ceases, and the change may be checked or fixed at any stage in the axolotl. These facts serve to illustrate still further the difference between these groups. The tadpole and the axolotl represent somewhat extreme phases of the larval condition which is correlated with the fundamental difference between the thyroids of the larvae of these groups—the liberation of the hormone throughout larval life in the anuran and its retention in the urodele, with release only at metamorphosis in those forms normally transforming.

It can now be seen, returning to the thyroidless tadpoles and axolotls given anterior pituitary, that the thyroid was removed at an earlier (and different) stage of the larval life of the tadpole than in the case of the axolotl, the latter only, for instance, having fully developed limbs, and it is possible that the thyroid is concerned with early phases of development for which the axolotl's thyroid was sufficient and had been successfully reached when the gland was removed, but so far as the tadpoles were concerned such stages could not take place without the presence of thyroid tissue. The activating influence of the transplanted anterior pituitary upon the rudimentary thyroid in the hypophysectomised tadpole enabled this stage to be completed, but probably the later stages of metamorphosis, as in the case of the thyroidless axolotl, could take place under the stimulus of the anterior pituitary alone. This interpretation gains support from Hoskins' (1922) work upon the relation of thyroid to certain stages of metamorphosis, and Helff's (1926) studies dealing with the sequence of metamorphic change. The thyroid anlage was removed by Hoskins and grafted into tails of frog larvae, and when the legs were well developed the tails were cut off. These thyroidless animals completed metamorphosis, so that the thyroid was not directly necessary for the later stages of the change. Helff found that some of the structural and physiological changes occurring during metamorphosis are not due to the direct action of the thyroid hormone, though it must be considered the primary activating agent. He concludes that the thyroid supplies the initial impulse for differentiation and is but a link, yet a vital and essential link, in the chain of reactions aroused and culminating in transformation. In a similar indirect manner, by intermediate steps, the anterior
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The anterior lobe pituitary may also produce definite change. It is conceivable, therefore, that the thyroid and anterior pituitary, as such, are not necessarily required to function in any special manner to produce metamorphosis, but by the exercise of their characteristic activity they create and maintain metabolic conditions or metabolic levels within the organism providing stimuli which initiate series of interdependent reactions producing those changes which end finally in metamorphosis. Hence the ability of the thyroid and pituitary glands of animals other than the amphibia to initiate these same changes is not surprising, and renders superfluous the postulation of a definite metamorphic factor, although this is a useful and helpful conception. The interrelationship of these glands is possibly the results of indirect stimulation and intermediate reaction. Metamorphic variations in tadpoles and axolotls when given either thyroid or anterior pituitary (quantitative differences, greater rate of absorption of gut and tail, and differential tissue response with thyroid) may reflect the greater stimulus received by particular series of intermediate reactions most susceptible to the activity of one or the other of these glands. In normal metamorphosis it would seem that both the thyroid and the pituitary are active, the combined stimulus bringing metamorphosis, for if either acted alone the change would not proceed, as seen after extirpation. Increasing either stimulus produces acceleration by increasing the rate of those processes primarily susceptible to the particular stimulus given, and the others receive impulses by virtue of their direct or indirect dependence upon or their relation to these processes in the general sequence of metamorphosis. The production of metamorphosis in animals from which either or both glands are removed would depend upon (a) whether the strength of the stimulus given was enough to initiate those reactions not directly susceptible to its effect, and for which the stimulus is lacking, but influenced by virtue of their relation as part of the whole to those effected, or (b) whether the stimulus was applied at such stage in the development of the organism as a whole, that indirect stimulation alone was possible and adequate. If the stimulus through experimental, environmental, specific, or other causes, is insufficient or fails to reach the required standard conditions at any phase the progress of the changes becomes slower and larval life extended, and below certain values metamorphosis is not completed and a neotenous condition supervenes. Upon suitable application of either stimulus again to these forms changes proceed with eventual metamorphosis except in certain forms with no capacity to respond to any further stimulation. As to influences tending to reduce or counteract these stimulating influences, apart from the actual extirpation of the gland and the records of environmental effects, the inhibiting influence of the posterior pituitary upon metamorphosis has, among others, been indicated. Its metabolic effect may slacken those reactions which are in progress, or break the sequence of intermediate steps in a series and so prevent metamorphosis, its effectiveness depending upon the quantity and the stage at which its influence is introduced, or modify in some degree a particular sequence.

The possibility of such an influence is important as it reveals the potentialities of a mechanism having probably some evolutionary significance. The experimental
evidence suggests that the conditions of neoteny and metamorphosis are largely determined by the interrelationship of the different glands, and the conditions of endocrine balance attained in larval and adult life and in different species appear to vary considerably. It may well be, therefore, that some such co-ordination of the members of the endocrine system and variations in their relationship, impelled by, or correlated with, environmental factors, to a greater or lesser degree, have been operative within the organism and concerned in some way in addition to other factors in changes in evolutionary time contributing to particular characters, adaptive rather than morphological, and so giving another outlook upon the problems of amphidian phylogeny. Watson (1926) observed in his studies of palaeozoic amphibia the steady evolution with time of certain structures (dentition, skeletal peculiarities, shoulder girdle) through the Embolomeri, Rachitomi, and Stereospondyli, although the mode of life of these animals changed from a primarily aquatic through a terrestrial to a secondary aquatic habit. Similar evolutionary trends to those exhibited in these Labyrinthodonts occur in other orders, and it is now known that the living Perennibranchiate Urodeles are descendants of terrestrial forms. It would seem justifiable, from evidence supplied by the line of enquiry pursued in this paper, to assume that some readjustment of the endocrine balance or the balance between endocrines and tissues would in part be responsible for these changes in the mode of life and the secondary acquirement of an aquatic existence. Among the Urodela, the perennibranchiates represent an extreme condition, for, whereas there is practically no real larval period in some salamanders (e.g. *S. atra* is viviparous), the larval life of newts is prolonged and axolotls reach sexual maturity in the larval state and only assume adult characters under favourable environmental conditions or suitable experimental stimulation, members of this group fail to metamorphose under any conditions. Here a state is reached in the endocrine relationship when, instead of a continuous or partial, but nevertheless eventual, predominance of accelerating factors during metamorphosis, inhibitory influences gain and retain supremacy at some stage before final metamorphosis and establish a permanent relation and stability in the endocrine mechanism as far as metamorphosis is concerned; hence the characteristic condition—the persistence of larval characters throughout life. How this condition arose, whether through a reduction of the accelerating agents themselves, following glandular atrophy or other causes such as defective releasing mechanisms preventing secretion or simply increased activity of the inhibitors, or whether other factors altering the sensitivity of the tissues were responsible, it is not possible to say with certainty, but it is of interest to compare the thyroids of these animals. The size and weight of the thyroid in relation to those of the body are not necessarily criteria of activity, and this is illustrated by the perennibranchiates, for the thyroid of *Siren* appears to be abnormally large, greater even than that of a normally metamorphosing species (Huxley and Hogben, 1922), *Necturus* has a well-developed physiologically active thyroid (Swingle, 1921), whilst in *Proteus* it is very small, consisting of only a few vesicles and apparently it is almost entirely absent in *Typhlomolge* (Uhlenhuth, 1926). It does not follow that the considerable reduction observed in the two latter species
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is entirely associated with the failure to metamorphose, since this may have occurred consequently in relation to other factors concerned with the mode of existence peculiar to these animals. A similar study of the pituitaries of these animals might provide interesting evidence in connection with the endocrine relations. The complete reversal of the endocrine relationship which seems to be indicated in the perennibranchiates would suggest an earlier return phylogenetically to the aquatic habitat, and it would be reasonable to expect the exhibition of primitive features in such animals. Morphological examination does, in fact, show that in some respects the structure of the skull and vertebral column of the majority of the neotenous genera is more primitive than the terrestrial and rapidly metamorphosing forms. However, it is probable that this return did not occur at the same stage or at the same time in the ancestral history of the various genera, and it is conceivable that any readjustment of the endocrine balance would not be effected with the same rapidity in each case, nor would the extent of the responsibility or relation of such adjustments to this return be identical, which would account for the perennibranchiates not being a natural group.

As regards growth, it would appear that the stimulating influence of the activity of the anterior lobe in this case is not identical with that effective in metamorphosis, and the exact relationship is not yet clear. The extent to which any growth effect is due to direct action or to co-operation with or dependence upon other factors such as environment, nutrition, age and stage of development, and the endocrine system, has to be ascertained and analysed before complete comprehension of the actual influence of the anterior pituitary is gained.

The field of enquiry has been confined to the amphibia and the examination within these limits has been by no means exhaustive, yet the information yielded sheds some light of distinct critical value upon the activity of the anterior lobe pituitary which, when considered in relation to the knowledge supplied by previous work, makes it possible to generalise with some justification upon the functions of this portion of the gland. It would seem that its activity is vital and essential to developmental processes by virtue of its capacity to stimulate and maintain directly favourable metabolic conditions for certain of the complex series of changes involved, and influence indirectly others dependent upon them. Little is yet known of the intricacies of these processes, so that it is difficult to analyse and define their susceptibility, and any effects produced can only be gauged by a study of outward aspects in the final condition or, where possible, intermediate states according with the stimulus given.

In the amphibia it has been shown that metamorphosis is definitely dependent upon its activity, whilst growth in size in vertebrates does appear to be controlled, in some way less clearly defined, by the anterior lobe. These effects are limited by certain conditions and at times need the co-operation of other factors. Further, in certain cases the development and normal function of the gonads is influenced by its activity.

In its stimulating influence upon metabolic processes and the activities of the body tissues the anterior lobe resembles the thyroid, but there is evidence that their
selective action and the range of susceptibility of the different processes to their influence is not identical.

In conclusion, it would appear that the anterior lobe has a regulative and apparently directive effect within the organism and hence an important rôle in the internal environment—one, possibly, having some evolutionary significance.

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8. SUMMARY.

1. The standardisation of the method of preparation of extracts and the adoption of a uniform procedure for the estimation of their ability to induce metamorphosis in axolotls or accelerate the transformation of tadpoles to adults permit a biological assay of the metamorphic activity of extracts of the anterior lobe.

2. The metamorphic activity of the anterior lobe is specific, since similar tissue extracts fail to excite a like response and qualitative and quantitative characteristics distinguish it from the activity of the thyroid in metamorphosis.

3. Active preparations are not produced when neutral media or inorganic acids are used for extraction from fresh gland by decoction.

4. The potency of extracts depends upon the strength of the acetic acid used for extraction, the concentration of the extract and the hydrogen-ion concentration.

5. The threshold value of the dose inducing metamorphosis in axolotls by injection and the amount inducing the maximum rate of change increase with the size of the animal. Injections must be continued at regular and suitably short intervals till the completion of the change to maintain the maximum response to any particular dose.

6. The activity of extracts is destroyed by pepsin and trypsin and diminished by dilute hydrochloric acid. Neutralised extracts are inactive.

7. Ringer's and distilled water extracts of thyroid are more active than similar extracts in dilute acetic acid. There is no relation between the activity of these extracts and the hydrogen-ion concentration.

8. There is evidence of an inhibiting influence of the posterior lobe upon metamorphosis.

9. The distribution of the factor responsible for the metamorphic activity of the anterior lobe is not uniform throughout the lobe, less being present at the border than within. No definite localisation is apparent.

10. The anterior lobe influences metamorphosis and growth in size, but the same influence does not appear to operate in the production of each of these effects. The extent to which the growth stimulus is dependent upon and determined by other factors has yet to be determined.
The thyroid and pituitary are interdependent, but both seemingly are able to induce metamorphosis in the axolotl independently, and in the absence of the other. In the tadpole independent action in stimulating metamorphosis appears to be possible only in the later stages, co-operation being necessary in development and the earlier stages.

The thyroid and pituitary of the adult axolotl appear to be at a low level of functional activity, and metamorphosis is dependent upon the stimulus of glands from external sources when suitably administered, since the rate of change induced is approximately the same in these animals, whether normal, thyroidless, pituitary-less or with both thyroid and pituitary removed.

The relationship between the endocrine balance and metamorphosis in the amphibia and its evolutionary significance is discussed.

The anterior lobe appears to have a regulative and directive effect within the organism which, as a factor in the internal environment, may have some evolutionary significance.

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