THE RESPONSE OF THE WATER-REGULATING MECHANISM OF DEVELOPMENTAL STAGES OF THE COMMON TOAD, *BUFO BUFO BUFO* (L.), TO TREATMENT WITH EXTRACTS OF THE POSTERIOR LOBE OF THE PITUITARY BODY

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(With Two Text-figures)

Investigations made in recent years have demonstrated a connexion between the posterior lobe of the pituitary gland and water regulation in Amphibia. Injection of extracts causes a retention of water in the body of frogs (Brunn, 1921; Heller, 1930; Steggerda, 1931; Collin & Drouet, 1932; Steggerda & Essex, 1934; Oldham, 1936; Boyd & Brown, 1938; Boyd & Whyte, 1939), toads (Steggerda, 1931; Novelli, 1933), Amblystoma (Bělehrádek & Huxley, 1927), *Necturus maculatus* (Steggerda, 1937), and some reptiles (Boyd & Dingwall, 1939). In mammals the antidiuretic effect of the posterior lobe is now well established. On the other hand, freshwater fish show no such effect even when given doses of pituitrin up to 2·0 I.U. per 10 g. body weight. Nevertheless, the fish pituitary contains a substance causing a typical water retention in frogs (Boyd & Dingwall, 1939). It appears therefore that while the principle concerned is elaborated in the fish pituitary, either the appropriate effector is not developed or an alternative system which deals with the greater part of water regulation, is present. Thus, on the basis of the data available, it may be said that the regulation of the body water by agents of the posterior pituitary is characteristic of terrestrial but not of primarily aquatic vertebrates.

The Salientia during development undergo a transition from an aquatic to a terrestrial mode of life and it seemed a matter of some interest to determine whether Amphibia exhibited the adult response from an early stage or whether this was acquired during metamorphosis. In the latter case, interesting information would be obtained on the adjustments undergone by individual animals passing from an aquatic to a terrestrial habitat and on the larger problem of the mechanisms involved in the invasion of land by vertebrates. For this purpose, the effect on body water of extracts of the posterior pituitary has been investigated on a series of developmental stages of the common toad.

MATERIAL AND METHODS

Recently hatched tadpoles of *Bufo bufo bufo*, having well-developed ventral suckers, were collected on 3 May and placed in large bowls containing tapwater and plants of *Elodea canadensis*. They were fed each morning and evening on powdered wheat germ and the water was changed twice weekly. A piece of liver or beef was suspended in each bowl for about 6 hr. daily from 15 May onwards. Tadpoles in which one or both forelimbs had emerged were transferred to glass jars containing a little water and were fed on *Drosophila* spp.

The toads continued to develop normally and individuals were taken from stock and used in experiments between 30 May and 25 August, when the last had metamorphosed. Observations were made on all stages from the appearance of the hindlimb bud until immediately after metamorphosis.

Tadpoles of the common toad survive treatment involving injection and considerable handling much better than do those of *Rana t. temporaria*. Their skin is tougher and precise quantities can be injected into this species more readily since the body wall is flaccid and the coelomic fluid does not gush out when the skin is pierced. On the other hand, the consistent succession of external changes associated with metamorphosis in the frog (Etkin, 1932) is not found in the toad. For instance, the emergence of the last forelimb is frequently delayed until the tail has nearly been absorbed, and again, the loss of the horny jaws and final development of the toad-like mouth may take place at any time during this process. For this investigation, a developmental schedule, closely correlated with time except in the case of certain of the three-limbed forms, was established by neglecting the finer changes and referring only to limb development and tail absorption (Table I, left-hand column). An extra stage was added as it was observed that after tail absorption was complete, the toad remained a deep greenish brown (*dark toad*) for about 30 hr. before suddenly assuming the adult colour (*yellow toad*).

Two types of experiment were made to test the response of the developmental stages of the toad to posterior lobe extracts. First, adult 20 g. *Bufo b. bufo* were injected with extracts and the effect on body water was followed by weighing the animals immediately before injection and afterwards at intervals for 8 hr. The animals had access to water. The adults behaved similarly to other Salientia, showing an increase up to about 40 % of their initial weight 2–3 hr. after treatment, followed by a return to normal. Since the animals were given no food, this weight change has been taken as a measure of the effect on body water. A similar procedure was carried out with metamorphosing toads and is described below.

Second, in an attempt to determine how far this effect was due to direct action of the extract on the kidney, tadpoles were stained with phenol red and the rate of elimination of this dye compared in untreated and treated animals.

The results of the two sets of experiments are correlated in the discussion.

EXPERIMENTS ON WEIGHT CHANGE

The weighing of tadpoles at regular intervals with any degree of accuracy presented some difficulty, especially as the earlier stages (*hindlimbs two- or three-jointed*) lost up to 30 % of their initial weight as the result of carrying out this process five times over a 6 hr. period. As, however, no alternative presented itself, experimental animals were controlled by others which were weighed at the same times, but which received no other treatment. The procedure was as follows: each animal was dried on a soft linen cloth, transferred to a tared specimen tube containing 5 ml. of water, rapidly weighed on an Oertling aperiodic reflecting balance and restored, together with the water, to its bowl. In the case of terrestrial forms the animals were weighed, after drying, in a dry weighing bottle. The great majority of the animals survived such treatment and later passed normally through metamorphosis.

The Burroughs Wellcome preparation of the posterior lobe, "Infundin", was used for most (88/127) of the pituitary injections, but Parke Davis "Pitressin" (22/127) and "Pitocin" (17/127) were used in confirmatory experiments. Preliminary tests having shown that tadpoles supported the injection of volumes up to 2 % of their body weight without ascertainable injury, the following doses were used: "Infundin", 2 I.U.; "Pitocin", 2 oxytocic I.U.; "Pitressin", 4 pressor I.U.; in every case per 10 g. animal. These doses produced comparable responses, but the pitocin was more active with postmetamorphic stages. This agrees with the findings of Steggerda & Essex (1934) and Boyd & Brown (1938). The extracts were not diluted. Injections were made with a Burroughs Wellcome "Agla" micrometer syringe fitted with a fine needle, which enabled quantities as small as 1·5 mg. to be injected with accuracy. The syringe was clamped to a stand and the animal manipulated so that the tip of the needle entered at the posterior end of the body, low on the side, and passed a short distance obliquely forward just within the body wall, avoiding injury to the viscera. The injection was then made into the body cavity. The room and water temperatures were between 17 and 20° C. in all experiments.

The experimental procedure was standardized as follows: eighteen numbered tongue glasses, each containing 40 ml. of tapwater for the aquatic stages, or 10 ml. for terrestrial animals so that the latter were not drowned, were set out in three groups of six. These groups were to receive animals, (1) injected with posterior lobe, (2) controls injected with the same volume of 0·5 % NaCl in London tapwater, and (3) controls weighed only. Sets of three tadpoles of similar size and metamorphic stage were selected from stock, weighed individually, the time being noted, injected if destined for groups (1) or (2) and put singly into tongue glasses, one animal to each of the three different groups. This was done six times so that each tongue glass received one animal. After the lapse of 1½ or 2 hr., the tadpoles were weighed again and so on at equal intervals during the 6 or 8 hr. following the initial weighing. The groups were made up of tadpoles of different developmental stages when these could be obtained simultaneously from stock. In this way, tadpoles treated with posterior lobe were controlled against saline-injected and "weighed only" animals,

and against others at different stages of metamorphosis. Animals of groups (2) and (3) lost similar percentages of their weight in the same period so that this effect can be attributed to handling and to the progressive evacuation of the gut, rather than to the injections. Stock tadpoles were observed to feed continuously during the day, especially in the early stages, but no food was supplied during the experiments.

Table I. *Showing the response to injection of extracts of the posterior lobe of the pituitary, as measured by change in weight, of developmental stages of the toad. The animals were given access to water*

Stage of metamorphosis	No. of animals injected with posterior pituitary extract	No. of animals showing gain in weight after injection	No. of animals showing loss of weight after injection
Hindlimbs three-jointed and earlier stages	63	—	63
Anal canal piece resorbed	6	—	6
Three-limbed	12	2 (both with medium tails)	10
Four-limbed:			
Long tails (> 10 mm. long)	5	—	5
Medium tails (5–9 mm. long)	5	2 (< 4 %)	3
Short tails (< 5 mm. long)	8	4 (< 5 %)	4
Knob tail	8	7 (< 10 %)	1
Dark toads	5	5 (< 10 %)	—
Yellow toads	15	15 (> 10 %)	—

The results of these investigations are summarized in Table I and typical curves of individuals are shown in Fig. 1. It will be seen from the table that posterior lobe injections have no effect on the body water of the developing toad before tail absorption begins. On the other hand, when the tail is almost completely absorbed (*knob-tail*), the majority of the animals show an increase, although not pronounced, in water content, whereas later, when the tail has diminished to a small tubercle, a response is invariable and of greater magnitude. Thus as development proceeds, a gradual increase of sensitivity to posterior lobe extracts is apparent, both in the frequency with which water retention is induced, and in degree. The full response is not obtained until the skin ceases to be a dark greenish brown (*dark toads*) and assumes the adult colour (*yellow toads*). The period when the positive reaction gradually develops appears to coincide with the progress of tail absorption and to begin when this process has been under way for some time, i.e. in *medium* (5–9 *mm. long*) *tailed* and *short* (< 5 *mm. long*) *tailed* stages. Perfect correlation between the physiological reaction and external form was not obtained, nor is it to be expected since it is unlikely that the processes are causally connected. Where a response was obtained, this reached its maximum between 1½ and 3 hr. after injection, and the effect subsided within 6–8 hr., as had been found to be the case in adult toads. The lack of response of the earlier stages cannot, therefore, be attributed to the shortness of the experiments.

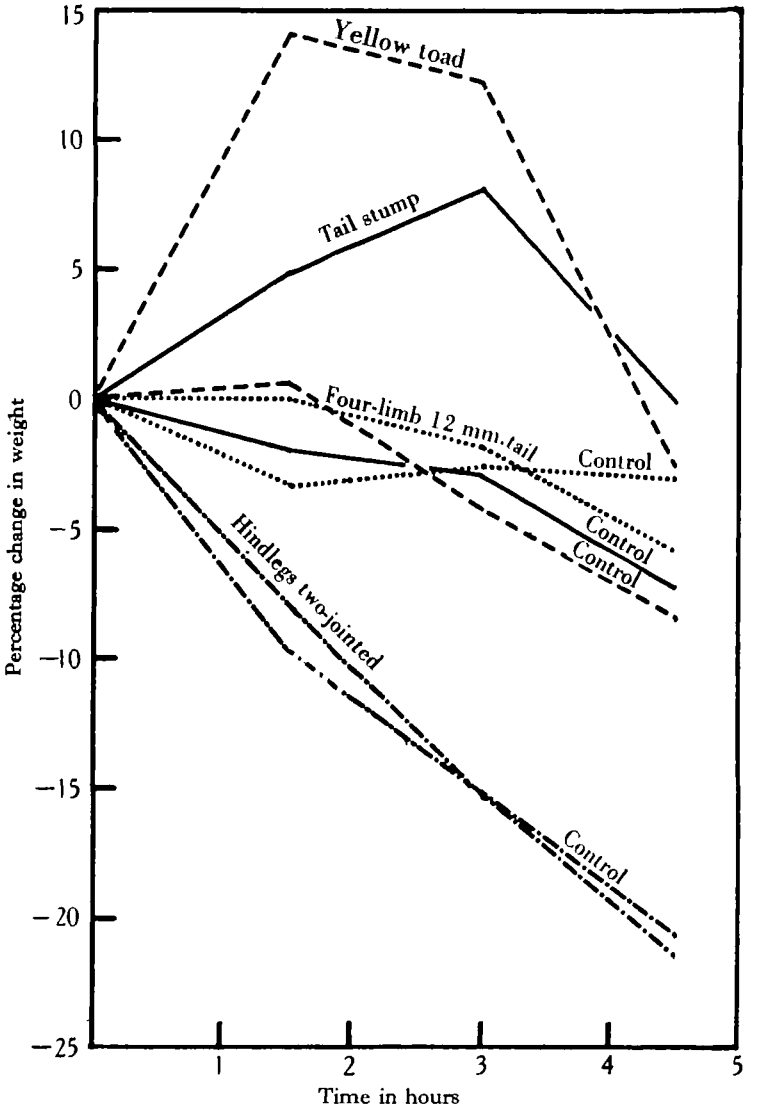

Fig. 1. Percentage weight change of representative animals of four stages in the development of the toad following injection with "Infundin" (2·0 I.U. per 10 g. body weight) or with 0·5 % saline (Controls) and allowed access to water. The same type of line is used both for the "Infundin"-treated animal and its control.

EXPERIMENTS WITH PHENOL RED

The changes in weight following injection with pituitary extracts give no information as to the nature of the mechanism involved in causing the increased water content of the body. This response in Amphibia may possibly be due to two factors, (*a*) an increased water uptake through the skin, and (*b*) a possible mechanism preventing loss of water through the kidneys. Experiments were undertaken to elucidate this problem.

When tadpoles are immersed in an 0·1 % solution of phenol red for from 12 to 15 hr., they become deeply stained with the dye. When returned to clean water, they gradually give out the dye, at first rapidly and later more slowly until at the end of about 120 hr. it is completely eliminated from their bodies. The animals show no ill effects from this treatment.

The amount of dye given off by the tadpoles in any period could be estimated by comparing the depth of colour of the water in which they had been kept with that of a series of dilutions of the original solution of phenol red. The colour in all cases was brought to that beyond the acid range of the dye by the addition of a few drops of acetic acid. This inversion of the normal procedure used in previous studies on renal activity was necessitated by the cloudiness caused in the water by the addition of alkali. Standard dilutions of the dye and test solutions were compared in Hehner tubes which gave columns about 350 mm. long with 50 ml. water, the tubes being illuminated from below. Many dilutions were made so that the test solution never differed from one standard by more than 30 %.

Preliminary tests were made with ten animals which were immersed in 200 ml. of 0·1 % phenol red (B.D.H. indicator) for 15 hr. and then washed in running water for 15 min. They were then divided into groups of five and each group was placed in 50 ml. of tap water. At intervals this water was poured off through a coarse sieve and collected, and the animals were given a further 50 ml. of clean water.

It was found that in similar groups the amount of phenol red lost by one group might be as much as twice that lost by the other during the first hour, but that during longer periods the maximum loss never exceeded 1·4 times the minimum. The discrepancies were greater in younger than in later forms because dye was retained within the opercular cavity. The method is thus not wholly satisfactory but was considered suitable for preliminary experiments.

Since trial experiments showed that immersion of animals in water containing extracts of posterior pituitary induced changes of weight similar to those following injection, it was decided to use immersion rather than injection as the method of administration to tadpoles in the phenol red experiments. Thus excessive handling of the dyed animals was avoided.

To test the influence of posterior lobe extracts, animals were treated as described above. Two groups of similar tadpoles were placed in clean tapwater after immersion in phenol red solution overnight, followed by washing. At the end of the first hour or so, one group was transferred to a similar measured quantity of tap water to which posterior lobe extract (1·0 I.U. per 10 ml.) had been added. After about 4 hr., this solution was replaced by clean water, the water in the control group being changed as usual. Clean water was again substituted at longer intervals up to about 60 hr., and the concentration of phenol red in the discarded water estimated both before and after treatment with extract. It was found necessary to halve the quantity of water for terrestrial forms and to place a sheet of gauze about 2 mm. above the surface of the liquid so that they remained immersed but could breathe air.

Such experiments were made on the following developmental stages: (1) up to *three-jointed hindlimbs*, five experiments on groups of five animals; (2) *anal canal piece*

resorbed, three on groups of five; (3) *long tails*, three on groups of five; (4) *knob-tails*, three on groups of five; and (5) *yellow toads*, four on groups of four; with a similar number of controls in each case. It was necessary to omit the *dark toad* stage, as its duration was usually less than that of one of the experiments. This period was not lengthened artificially by conducting the experiments at lower temperatures, since the skin of frogs has a very high temperature coefficient of permeability (Overton, 1904; Hevesy *et al.* 1935) and the water loss of untreated frogs is reduced to one-sixth of the normal at 4° C. (Boyd & Whyte, 1938).

The results of three experiments are shown in Fig. 2. Of the stages investigated, only the *yellow toads* showed a pronounced suppression of phenol red elimination when treated with posterior lobe extract. In none of the four groups was this suppression quite complete, although invariably marked compared with the controls, partly no doubt owing to the time elapsing before the extract penetrated into the animals. There was never any compensatory extra loss of phenol red in the succeeding period, both controls and treated animals losing about the same amount of dye. If it be assumed that the degree of retention of the dye is a measure of the suppression of the secretion of urine by the animal, since the weighing experiments show that water uptake is certainly not reduced, then it may be concluded that the effector mechanism for this action of posterior lobe extracts comes into play only when metamorphosis is quite complete. This is in agreement with the finding that, following injection of extracts, *yellow toads* exhibit a 50 % greater rise in weight than do the other sensitive forms and suggests that while the response of earlier stages is at least predominantly due to increased water uptake (cf. behaviour of *knob-tails* in Figs. 1 and 2), that of the metamorphosed animal is due both to increased transfer of water through the skin and to diuresis inhibition.

DISCUSSION

The fact emerges from the experiments described above that in the duration of the response induced by injections of posterior pituitary extract, the newly metamorphosed *Bufo b. bufo* behaves like all other Salientia that have been investigated. Where the behaviour of two or more species has been compared simultaneously, it has been shown that while the time relationships remain the same (Boyd & Brown, 1938), the amplitude of the weight changes varies from species to species. The more terrestrial toads *B. arenarum* (Novelli, 1933) and *B. americanus* (Steggerda, 1937) show a greater increase in weight than do frogs. There does, then, appear to be some relationship between the degree of response and the danger of desiccation. *Bufo b. bufo* shows a percentage increase in weight which falls within the range found in previous work.

It thus seems justified to compare the behaviour of the English toad with that of the various different species of frog used by other workers.

The exact mechanism of the response is not yet solved in spite of the study that has been devoted to it. Bělehrádek & Huxley (1927) suggest that the effect of posterior pituitary extracts on water regulation in amphibia involves (1) an action on the water intake, i.e. on the permeability of the skin, (2) an action on the powers

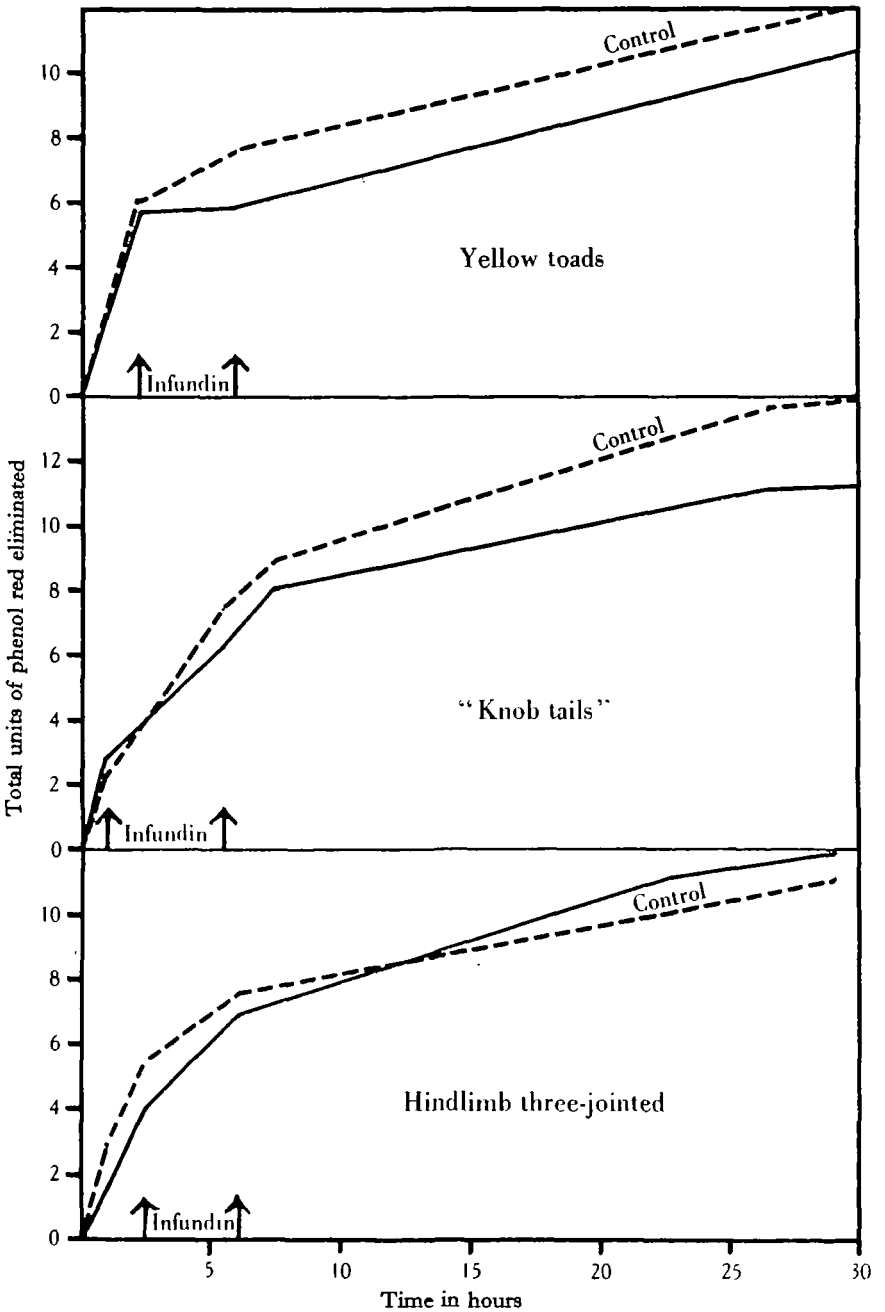

Fig. 2. Elimination of phenol red by groups of toads at different developmental stages. Each point represents the total amount of phenol red eliminated by a group from the beginning of the experiment. The experimental animals, represented by unbroken lines, were placed in dilute infundin (1 I.U. per 10 ml. water) for the period indicated by the arrows. The control animals were simultaneously placed in water.

of imbibition of the tissues, and (3) an action on water excretion, i.e. on kidney activity. The brief response of the water-regulating mechanism to a single injection of Infundin is scarcely comparable to the action, which may be due to different agencies, of posterior lobe extracts in retarding metamorphosis (Spaul, 1925) nor are the profound metabolic disturbances associated with this process considered relevant. If the weighing experiments are a measure solely of the increased water uptake of the skin, and the kidneys are unaffected, the frog must be almost unique in normally working its kidneys so near the upper limit of their capacity that they are unable to cope with the extra water inflow. Normally, *Rana pipiens* excretes about one-third of its weight of water per day (Adolph, 1933), but Boyd & Whyte (1939) found that frogs could take in and lose up to 20 % of their weight in 3 hr. The accumulation of water in a frog injected with pituitrin is at the rate of about 160 % of its weight per day, i.e. the rate of water uptake is apparently increased to five times the normal if Adolph's low figure be taken. Frogs used by Richards (1938) had a normal rate of urine formation of 2·5 ml./hr., although it could be calculated from the glomerular number and the maximum rate of filtration through a single glomerulus that the kidneys might form glomerular filtrates at rates up to 16 ml./hr., over 6 times as fast. Again, evidence from injection experiments with posterior pituitary shows that the kidneys can deal with a great deal more water than Adolph's figure suggests, since after the peak increase in weight, animals so treated do in fact lose more than 20 % of their weight as water in about 3 hr. Increased water uptake by the skin cannot then be the sole cause of the rise in weight. Nevertheless Novelli (1936), by experiments with skin pouches in which the circulation was kept intact, showed that it is an important factor.

The powers of imbibition of the tissues are affected by injections of "Pitressin" into the intact animal and the hydration of the muscles is considerably increased (Steggerda, 1931) and Rey (1935) showed that there was an increased hydration of the blood as well as of the tissues but that also a decreased kidney output was responsible for the gain in weight. In recent years the importance of this last factor has been shown to be greater than was at first believed. Rey (1935) and Boyd & Whyte (1939) found injected water to be retained after treatment with pituitrin and concluded that extracts of the posterior pituitary contained a substance which rendered the kidneys unresponsive to increased hydration of the blood.

Thus all three factors, namely, increased permeability of the skin, imbibition of water by the tissues and a failure of the kidney to respond to changes in the internal medium brought about by these, are concerned in the response of adult Salientia to injections of pituitrin. It may be concluded that the same factors are at work but in differing degrees, in the response of the developing toad. Since, however, until the later stages these animals are solely aquatic, water exchange in both directions through the alimentary canal may also be important. We know that during metamorphosis the urodele skin undergoes profound morphological changes (Wilder, 1925; Reis, 1930), so it may be concluded that the skin of the toad undergoes a similar alteration in structure, although we have no data on the nature of these changes nor at what stage they take place. It is likely that the tissues can imbibe

water as in the adult. From the results of the weighing and phenol-red experiments described above, the conclusion may tentatively be drawn that the early response of the developing toad from the *four-limb, medium tail* stage onwards is due to the skin becoming sensitive to the influence of the posterior pituitary. Phenol red injected into the blood stream of frogs is eliminated partly by glomerular filtration but mainly by secretion from the tubules (Richards & Walker, 1930; Smith, 1937; Richards, 1938), so that these experiments merely show that pituitrin had some effect on the kidney without giving any information as to whether that effect is due to a suppression of glomerular filtration or to an increased resorption of water. Either or both of these factors would reduce the elimination of phenol red by suppressing the secretion of urine. Actually, according to Adolph (1936), "Infundin" reduces glomerular blood flow and this is correlated in degree with the rate of urine formation. Blount's (1936) evidence also indicates that the glomerulus is affected. The "pressor" action of posterior pituitary extracts does not appear to influence water retention by the kidneys.

The data presented imply that suppression of urine production can only be brought about by pituitrin at the very end of metamorphosis but the experiments allowed of observations of only a general nature. If the number of nephrons progressively increases from about the *hind-limb-bud* stage onwards as in the frog (Gray, 1930), more refined experimentation may make it possible to show that this response gradually develops as more nephrons appear.

The factors involved in the action of extracts of the posterior pituitary on the body water of urodeles have not yet been investigated. Steggerda (1937) showed that *Necturus maculatus* increased slightly in weight after injection with pituitrin and Bělehrádek & Huxley (1927) produced evidence that metamorphosed and larval axolotls both gave a limited response, but the time relations in the latters' experiments were different from those of later work and the larvae failed to survive the treatment. More work is needed before one can decide whether changes in the mechanism of water regulation, similar to those described above for *Bufo bufo bufo*, occur during metamorphosis in urodeles and whether the response to posterior lobe extracts is characteristic of primarily terrestrial vertebrates or not.

SUMMARY

1. The effects of extracts of the posterior lobe of the pituitary on the body water of the developmental stages of the toad, *Bufo bufo bufo* (L.) have been investigated by injecting extracts and weighing the animals at short intervals afterwards.

2. No response was shown until marked absorption of the tail had occurred. From this stage onwards it became progressively established and gradually increased in magnitude. The adult Salientian type of response was only elicited at the very end of metamorphosis.

3. By staining tadpoles with phenol red and measuring the rate of elimination of this dye, it has been shown that the action of the extracts is not antidiuretic except in completely metamorphosed animals.

4. It is provisionally concluded that the extracts have a double effect, first to increase the rate of water uptake and second, to diminish water loss. During the life-history of the toad, the first response is established when tail absorption is progressing rapidly, i.e. at a comparatively early stage, whereas the latter only comes into play at the very end of metamorphosis.

5. Data at present available suggest that an influence of the posterior pituitary on body water is characteristic of terrestrial but not of primarily aquatic vertebrates.

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