THE EFFECT OF PITUITRIN INJECTION ON THE WATER BALANCE OF *BUFO REGULARIS* REUSS.

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(With Three Text-figures)

I. INTRODUCTION

In 1921, Brunn showed that injection of neurohypophysial extracts causes a temporary increase in the body water content of Amphibia kept in water. Since then the existence of this water-balance effect has been confirmed by numerous workers, but its mechanism is by no means fully understood. The increase in body water following pituitrin injection might be due either to an increased rate of water uptake through the skin, or to a decreased rate of elimination of water through the kidneys, or to a combination of the two. Each type of effect has been claimed to have been demonstrated by various workers, but many of the experiments are open to criticism. It is convenient to consider separately the evidence for the existence of the two mechanisms.

(1) *Increased water uptake*

Brunn (1921) concluded that the water-balance effect was extra-renal in origin. He performed experiments on frogs in which the cloaca was ligatured. When kept in water pituitrin-injected animals were found to increase in weight more rapidly than uninjected controls, thus reflecting an increased rate of water uptake. After periods of 3, 6 and 8 hr. the ligature was untied, and the volume of urine that had been produced was measured. He found the same values for pituitrin injected as for normal animals, and therefore concluded that there was no renal effect. This latter conclusion is hardly justified, since normal urine production cannot be expected to occur with the cloaca blocked for long periods. Biasotti (1923), Steggerda (1931) and Rey (1935), in similar experiments, also demonstrated an increased rate of water uptake.

Novelli (1936), in experiments on toads, also claimed to have demonstrated an increased rate of water uptake in skin pouches with intact circulation. The pouches of pituitrin-injected animals were found to contain more fluid than those of uninjected controls. This finding, however, does not satisfactorily demonstrate an increased rate of water absorbtion; it might equally well result from a decrease in the rate of elimination of water from the pouches of the injected animals.

(2) *Decreased urine flow*

As mentioned above, Brunn's experiments do not warrant his conclusion that there is no decrease in urine production after pituitrin injection. Oehme (1919), and Burgess, Harvey & Marshall (1933) also failed to find any anti-diuretic effect.

On the other hand, Houssay & Potick (1929), Rey (1935), Adolph (1936) and Pasqualini (1938), in experiments similar to Brunn's, found a decreased rate of urine production following pituitrin injection. Howes (1940), using a dye-elimination method not involving cloacal ligature, also found an anti-diuretic effect. Howes points out that the water-balance effect in intact animals cannot be the result only of an increased rate of water uptake with no renal effect. The kidney is not normally functioning at its maximal rate, and therefore if renal physiology were unaffected the increase in water absorbtion would be compensated by an increase in urine production, and no gain in weight would occur.

There is thus considerable evidence in favour of the existence of both a renal and an extra-renal effect of neurohypophysial extracts. Unfortunately, however, most of the experiments involved the functional elimination of the kidneys, and are therefore open to the objection that the physiological condition of the animals was so abnormal as to render difficult the evaluation of the results obtained. Further experiments in which both the renal and the extra-renal effects can be studied simultaneously in a normal animal are therefore desirable. A method of cannulating the cloaca of toads has been devised, and this makes it possible to measure simultaneously both the uptake of water and the urine production. This technique has therefore been used to study the water-balance effect of mammalian posterior pituitary extract.

II. MATERIAL AND METHODS

The species used was *Bufo regularis* Reuss. The animals ranged in weight from 42 to 133 g. All experiments were carried out at 26° C. The pituitrin used was Parke Davis Pituitary (posterior lobe) Extract. The cannula consists of a piece of glass tubing with a slight bulb at one end, divided by a constriction from the main part of the tube (Fig. 1*a*). The bulbed end of the cannula is inserted into the animal's
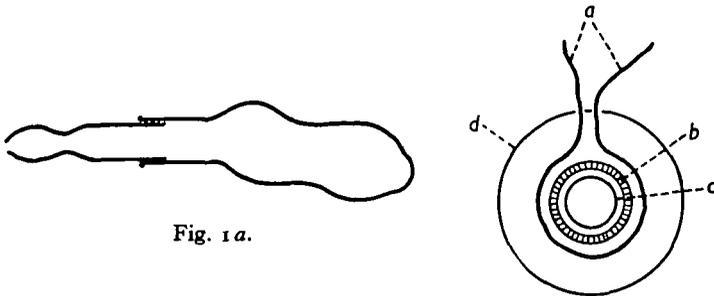

Fig. 1*a*.

Fig. 1*b*.

Fig. 1. (*a*) Cannula with balloon attached. (*b*) Diagrammatic cross-section of hindend of toad, showing mode of attachment of cannula. *a*, loose ends of thread ready for tying; *b*, cloacal wall; *c*, cannula; *d*, skin of toad.

cloaca and held in place by a cotton thread passing through the skin on the dorsal surface just above the anus, round the cloaca and out again through the skin close to where it entered (Fig. 1*b*). The single stitch necessary to place the thread in this position is made with a fine curved surgical needle. The two ends of the thread are

then tied tightly in such a way that the thread firmly holds the constriction in the cannula in position in the cloaca. A toy balloon is attached to the end of the cannula, and in this the urine is collected. In preliminary trials it was found that the animals showed no ill effects as a result of this treatment.

The toads were kept without food, but with access to water, for a few days before an experiment. This allowed the gut to be emptied and thus prevented complication due to the elimination of faeces during an experiment. The procedure was as follows. The toad was placed in a covered dish of water, kept in a thermostatically controlled water-bath, for 1 hr. The depth of water was such as to cover the greater part of the toad's body, but allowed it to rest with its head above water. This period allowed the animal to adjust to the experimental temperature and reach its equilibrium water content. The animal was then removed from the water, the cannula inserted under light ether narcosis, and the animal allowed to recover. It was then dried carefully, weighed and returned to the water-bath. At approximately hourly intervals the animal was removed, dried and weighed, the increase in weight giving the water uptake during the previous period. The balloon was then removed from the cannula, emptied, replaced and the animal weighed again. The amount of urine voided each hour, as well as the total water uptake, was thus known. After five such hourly weighings pituitrin was injected into the crural lymph sac and the experiment continued. As will be seen from the results quoted later, the injection was followed by a weight increase which reached its maximum in 3 or 4 hr., and thereafter recovery started. In the first experiments weighings were continued until recovery was complete, and the original weight had been regained, but later this was considered unnecessary, and the experiments were stopped as soon as the weight began to decrease.

In the control series the procedure was as before, except that the injection made after the first 5 hr. consisted of mammalian Ringer-Locke solution. Controls and pituitrin-injection experiments were run alternately, with the experimental and control animals as far as possible paired for size, sex and general condition. In each case the volume of Ringer injected into the controls was the same as that of the pituitrin used for the corresponding experimental animal.

III. RESULTS

(1) *General*

Fig. 2a shows a typical control experiment. The weight of the animal remains more or less constant with only minor oscillations. The rate of water uptake is very high in the first hour; this was frequently, but not invariably, the case, and is probably due to incomplete recovery from the anaesthetic. It was observed that the ether caused sufficient peripheral vasodilation for there to be visible flushing of the skin. If recovery from this effect were not complete when the experiment was started a high initial water uptake might well result. For this reason it was decided to discard the first hour's readings in all calculations. Apart from this initial high value there is a tendency, more marked in some experiments than in others, for the rate of water

uptake to show a gradual decline during an experiment. The large oscillations in urine production are probably not due to real variations of such magnitude in the rate of urine formation, since the toad does not always empty its bladder just before being weighed. The urine collected in the balloon at each weighing is therefore not necessarily equal to the urine produced in the previous hour. However, over a period
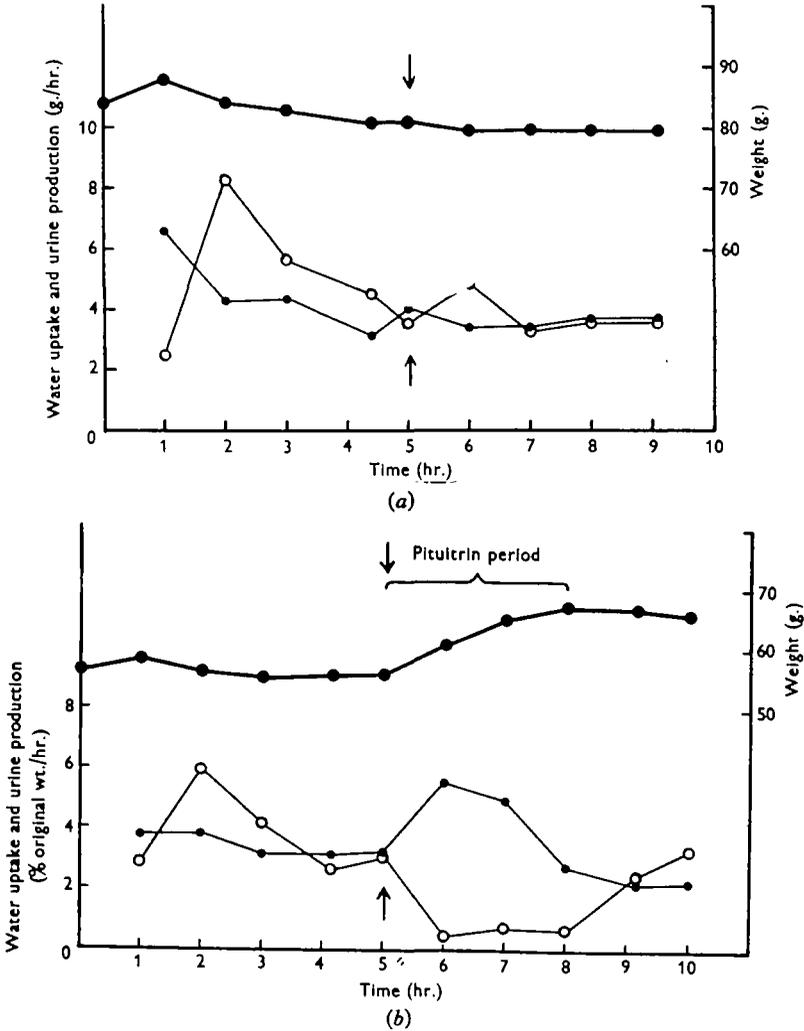

Fig. 2. (a) Typical control experiment. (b) Typical pituitrin injection experiment. Large filled circles, weight of toad (right-hand scale); small filled circles, rate of water uptake in previous period; small open circles, rate of urine production in previous period. The arrows indicate the times at which the injections were made.

of several hours the irregularities due to this factor will tend to even themselves out, and a reasonable measure of the rate of urine production may be obtained.

Fig. 2b shows the results of a typical pituitrin-injection experiment. The weight remains more or less constant throughout the first period, but after the injection of

pituitrin it increases markedly, reaching a maximum after 3 hr., and then begins to decrease. The urine production shows a sharp fall after injection, and later a rise which ultimately brings the animal's weight back to normal. In the pre-injection period the water uptake shows the usual slight fall, but injection is followed by an increase, after which the value gradually returns to slightly less than its original level.

It was decided to treat the results as follows. The 'pituitrin period' is taken as the period from the time of injection until the weight is at a maximum. Recovery is taken as having started as soon as the animal's weight starts to decrease. The rate of water uptake per hour is calculated for the whole of the pituitrin period, and then expressed as a percentage of the rate of uptake during the pre-injection period, exclusive of the first hour. The urine production is treated in the same way. These values will be referred to as the relative water uptake and relative urine production. In the control experiments the rates of water uptake and urine production in the post-injection period are similarly expressed as percentages of the corresponding rates in the pre-injection period, excluding the first hour. In any control experiment the post-injection period is taken as being the same length of time as the pituitrin period for the corresponding experimental animal.

(2) The effect of pituitrin on water uptake

In the first experiments the dosage of pituitrin was not controlled, 5 i.u. (0·5 ml.) were injected, irrespective of the weight of the toad. It was soon found that the results were very variable. Although after pituitrin injection there was always a weight increase and a diminished urine flow, the rate of water uptake sometimes increased and sometimes showed the slight decrease which was usual in the controls The variability seemed to be a function of dosage, and therefore in subsequent experiments the amount of pituitrin injected was varied so that the dosage covered a range of 1·9–15·3 i.u. per 100 g. body weight. Table 1 shows the relative rates of water uptake for all the pituitrin dosages used, together with the values found in the control series. Fig. 3 shows the same results graphically. Although the variability

Table 1. *Relative rates of water uptake of pituitrin-injected toads, and of saline-injected controls*

Low dosage		High dosage		Controls
Dosage (i.u./100 g.)	Relative water uptake	Dosage (i.u./100 g.)	Relative water uptake	Relative water uptake
1·9	88	8·5	145	76
2·1	88	8·8	134	74
3·4	171	8·9	117	83
3·7	94	10·0	123	80
4·5	98	11·8	210	86
5·5	116	12·0	145	96
6·3	56	13·5	161	101
6·8	100	15·3	115	106
7·5	76			92
8·2	68			
Mean	95·50 ± 9·93		143·75 ± 10·99	88·2 ± 3·74

is considerable, it is clear that water uptake is consistently increased by pituitrin only when the dosage is high, while low dosages are often without effect. The experiments were therefore divided into two groups, one with dosages of up to 8·4 i.u./100 g., the other 8·5–15·3 i.u./100 g. In each category the mean relative rate of water uptake was calculated and compared with the value found for the controls. With a dosage less than 8·5 i.u./100 g. the rate is 95·5 ± 9·93, while for the controls the value is 88·2 ± 3·74. These two values are not significantly different. The pituitrin-injected animals include one which gave an extraordinarily large effect, 171%. If this value is neglected then the mean relative water uptake for dosages of less than 8·5 i.u./100 g. becomes 87·11 ± 6·04, which is very close to the value found for the controls. We can therefore conclude that, apart from the one exceptional animal which had a very high sensitivity to pituitrin,* dosages of pituitrin less than 8·5 i.u./100 g. have no effect on the rate of water uptake.
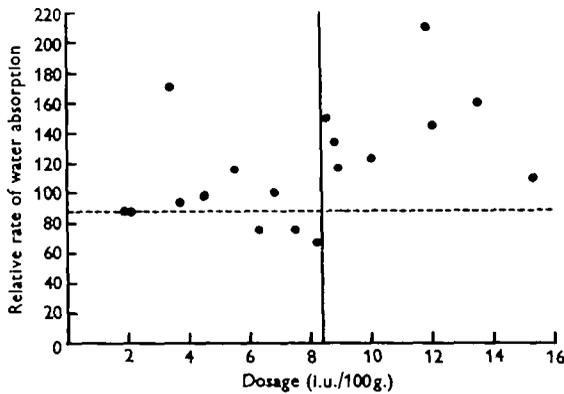

Fig. 3. Relation of rate of water uptake to pituitrin dosage. The relative water uptake is the rate of water uptake during the pituitrin period expressed as a percentage of the rate in the pre-injection period. The broken line represents the value for the controls, and the vertical line represents the point at which the division into low and high dosages was made.

For dosages of 8·5 i.u./100 g. and over, the mean relative rate of water uptake is 143·75 ± 10·99. This differs significantly from the control value. A dosage of 8·5 i.u./100 g. or over therefore causes a significant increase in the rate of water uptake.

(3) The effect of pituitrin on urine formation

Table 2 shows the relative rates of urine production at low and at high dosages of pituitrin, together with the values found for the controls. The mean values for the urine production at the two dosages are 26·20 ± 3·17 and 26·25 ± 4·78 respectively, while the control value is 79·2 ± 5·46. The rates at the two dosages do not differ significantly from each other, but both are significantly less than the control value. Pituitrin injection at all the dosages used thus has an anti-diuretic effect.

* Moulting in Amphibia is controlled by the posterior pituitary. This animal may have been about to moult and may therefore have had an unusually high concentration of pituitary hormones in its blood.

Table 2. *Relative rates of urine production of pituitrin-injected toads, and of saline-injected controls*

Low dosage		High dosage		Controls
Dosage (i.u./100 g.)	Relative urine production	Dosage (i.u./100 g.)	Relative urine production	Relative urine production
1·9	13	8·5	26	68
2·1	25	8·8	17	71
3·4	48	8·9	21	56
3·7	25	10·0	8	70
4·5	37	11·8	44	111
5·5	19	12·0	44	78
6·3	25	13·5	15	92
6·8	23	15·3	35	91
7·5	29			76
8·2	18			
Mean	26·20 ± 3·17		26·25 ± 4·78	79·2 ± 5·46

(4) *The distribution of the excess water*

During the course of the experiments it was noticed that in animals which had taken up excess water after injection the lymph sacs were extremely full of fluid. It therefore seemed desirable to find out whether most of the excess water was retained in the lymph spaces, or whether it was more or less uniformly distributed throughout the tissues. For this purpose toads were placed in the water dish for an hour to equilibrate. They were then removed, injected with 5 i.u. of pituitrin, weighed and replaced in the water-bath for a further period of 2 hr. They were then dried and weighed again to find the percentage weight increase. In the eleven determinations made the mean weight increase after injection was 16·55%. The animals were then killed rapidly with ether, and certain tissues removed for water content determinations. The tissues used were skin, muscle, gut and liver. The whole skin was not used, but only the part from the 'waist' down. The muscles were the two gastrocnemii. The gut was removed by two cuts, one through the oesophagus just above the stomach, the other through the distal end of the rectum. The stomach was slit open and blotted dry on both surfaces, while the rest of the gut was emptied of its contents by squeezing under the blunt edge of a scalpel blade. The tissues were weighed as quickly as possible, after blotting off the surface fluid, and then dried at just over 100° C. for 3 days and reweighed after cooling. In a control series the normal water content of the tissues was determined in the same way after the animal had remained in the water-bath for the usual hour's equilibration period.

The results are given in Table 3. In the case of skin and liver the differences between controls and injected animals are not significant, in the case of gut the significance is doubtful, while for the muscles the difference is significant. The magnitude of the effect, however, is very small, less than 3%, while the mean increase in body weight of the animals from which the tissues had been taken was 16·55%. Thus, although the increase in body weight is considerable, the degree of

tissue hydration is very slight. An attempt was made to assess the degree of dilution of the blood, by making erythrocyte counts before injection and just before killing. The first sample can be satisfactorily withdrawn from the mandibular artery without subsequent bleeding. It was found, however, that it was impossible to obtain a satisfactory second sample. The waterlogged state of the lymph spaces made it impossible to withdraw a sample uncontaminated with lymph, and the blood counts obtained were therefore artificially low. In spite of this extra dilution the values obtained showed a mean percentage dilution which was approximately equal to the percentage increase in water content of the whole animals. The real degree of dilution of the blood, as a result of the increased water uptake following injection, must therefore have been considerably less. Since the excess water is thus not accounted for either in the blood or other tissues it is clear that most of it is retained in the lymph spaces. This implies a type of internal osmotic regulation, but the mechanism by which it is achieved has not yet been studied.

Table 3. *Percentage water contents of tissues of pituitrin-injected and of normal toads*

(Each value represents the mean of ten determinations in the control series, and eleven in the pituitrin-injected animals.)

	Injected	Controls
Skin	76·6 ± 0·48	76·1 ± 0·50
Muscles	80·8 ± 0·56	78·5 ± 0·53
Gut	81·7 ± 0·50	79·9 ± 0·79
Liver	64·7 ± 2·80	67·3 ± 2·12

IV. DISCUSSION

The experiments described above have shown that in *Bufo regularis* the water-balance effect is the result both of an increased uptake and a diminished output of water. Using mammalian posterior pituitary extract the latter effect is seen to be produced at lower dosages than the former. This could be interpreted as a difference in thresholds for response to a single substance, or equally well as indicating that the two types of response are elicited by distinct substances, present in widely different concentrations in the extract used. Heller (1945) assumes that a single substance is responsible for the two types of effect, while Houssay (1949) assumes that two are involved. No critical evidence exists to decide the question. Previous work on the active principle responsible for producing the water-balance effect is difficult to evaluate, since the effect has generally been treated as a unit, without differentiation into renal and dermal components. There is agreement that pitocin produces a greater effect than pitressin (Heller, 1930*b*; Steggerda & Essex, 1934; Oldham, 1936; Boyd & Brown, 1938). This has usually been interpreted as indicating that the oxytocic fraction either contains or is itself the factor causing the amphibian water-balance effect. This is an unjustifiable conclusion. The workers mentioned above, although they found pitocin the more effective, did not find pitressin devoid of activity: moreover Heller & Smith (1948) obtained from crab's eye-stalks an extract devoid of oxytocic power which produces a water-balance effect in *Rana*

temporaria. It is not possible to reach any conclusion until the effects of the oxytocic and the pressor fractions have been tested, using a method which differentiates between the renal and the dermal components of the water-balance effect.

The mechanisms of the two components of the water-balance effect are not understood. Houssay (1949) implies that the anti-diuretic effect is the result of increased tubular resorbtion, just as it is in mammals, but little evidence is adduced to support this view. Posterior pituitary extracts have been shown to cause anti-diuresis in reptiles by constriction of the glomerular arterioles (Burgess *et al.* 1933), and also to cause constriction of the glomerular vessels in the frog's kidney (Richards & Smith, 1924; Adolph, 1936; Pasqualini, 1938). It therefore seems more probable that the anti-diuretic effect in Amphibia results from a reduction in glomerular filtration, rather than from an increase in tubular resorbtion.

The increased rate of water uptake after pituitrin injection may result either directly from changes in the skin permeability, or indirectly as a consequence of dilation of the skin capillaries. In favour of the latter view are Howes's (1940) experiments, showing that the water-balance effect is absent in tadpoles of *Bufo bufo bufo*, and appears gradually, *pari passu* with the development of the skin capillaries. It should, however, be noted that Howes did not differentiate between the renal and dermal components of the water-balance effect, and makes no mention of the relation of the magnitude of the effect to the stage of development of the kidney.

The results obtained on the distribution of the excess water indicate that most of it is retained in the lymph spaces, and that the tissues are protected against excessive hydration. Heller (1930a), working with *Rana esculenta*, obtained similar results, the degree of hydration of the tissues being somewhat higher than I have found in *Bufo regularis*. Although the excess water load is, of course, abnormal, the regulative mechanism controlling its distribution may be of considerable significance in normal conditions. The body water in Anura can vary very widely. When the animal is in contact with water the lymph spaces are filled, and when it leaves water it thus has a considerable reserve which can be allowed to evaporate with little effect on the tissues. For this water to be kept available for evaporation it is essential that it should not simply be eliminated by the kidneys. This implies some sort of internal osmotic regulation which, presumably, involves the integrated control of the blood pressure, the activity of the lymph hearts and the capillary permeability. Further work is necessary before this can be regarded as more than a hypothesis, but if it is correct then the adaptive significance of the peculiar organization of the anuran lymphatic system becomes clear. Its function is to maintain a 'reserve tank', in which water can be stored temporarily and allowed to evaporate gradually when the animal is on land, without greatly influencing the water content of the tissues of the body.

V. SUMMARY

1. A method of cannulating the cloaca of Anura is described.

2. Using this method it is found that in *Bufo regularis* the increase in body water which follows pituitrin injection is the result of an increased rate of water uptake, together with a diminished urine flow.

3. The excess water is mostly retained in the lymph spaces, while the water content of the tissues of the body increases very little.

4. The bearing of these results on the problem of the identification of the substance or substances which elicit the amphibian water balance effect, and the biological significance of the organization of the anuran lymphatic system are discussed.

A brief account of this work has already been published (Ewer, 1949). In this account there is an error, the figures given for the water contents of gut and liver have been transposed.

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