CXX. THE COMPOSITION OF THE TISSUES OF ALBINO RATS TREATED WITH ALKALINE ANTERIOR PITUITARY EXTRACTS.

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As Evans and Long have shown [1921], a bovine anterior pituitary extract, prepared and administered in the proper manner, induces a pronounced increase of weight in rats. This was confirmed by Hewitt [1929], Johnson and Everett [1929], and Gordon and Handelsmann [1930]. The phenomenon has been reproduced in dogs by Putnam and Benedict [1928], Reichert [1929], Cushing and Teel [1929], and in pigeons by Riddle [1931]. The negative results of experiments which have been published by some authors [Larson *et al.* 1929, Korenchevsky 1930] may be attributed either to unsuitable preparation of extracts (addition of antiseptics, heating, *etc.*) or to the incorrect choice of experimental objects. (With regard to rats, for instance, the growth curve must have reached a plateau in order that the phenomenon may be demonstrated at all.) According to Evans and Long [1922], the induced increase in weight is due partly to the formation of fat, partly to the growth of organs and bone. The majority of later writers have agreed in finding an increase in the weight of certain parenchymatous organs and in the dimensions of the skeleton, changes which have been interpreted as acromegaly by some investigators. There are no analyses, however, of the composition of the tissue of animals treated with such extracts, and the organ weights and bone measurements published cannot be statistically treated, so for the present it must suffice to state that potent pituitary extracts induce a statistically significant increase of weight in suitable experimental objects. On the other hand the past few years have seen the appearance of a number of reports indicating that this weight increase is not permanently established. Hewitt [1929] was, we believe, the first to make the observation that discontinuation of the injections brings about an immediate and rapid falling off in weight. In the same year Downs and Geiling [1929] announced that experimental giant mice deprived of water fall so much in weight in the course of 24 to 48 hours that they approach the controls, and Lee, Teel and Gagnon [1929] similarly maintain that experimental giant rats lose much more in weight during the first 22 hours after death than the controls. These observations seem to indicate that the increase of weight is exclusively or principally due to the retention of water, which is

excreted as soon as the injections are stopped, and they have resulted in doubt whether any growth hormone at all occurs in the extracts employed.

In our laboratory we have long been familiar with the fact that discontinuation of extract injections involves immediate and marked loss of weight (Fig. 1). In our experience (confirmed quite recently by Evans and Simpson [1931]) this loss, however, amounts to only approximately 35 % of the weight increase that has been obtained. If this is true, it is extremely improbable that the induced gigantism should be due to water retention alone. Since there are no known investigations into the composition of the tissues of anterior pituitary-treated animals, we report below on the result of a series of dry-matter and total-N determinations on albino rats treated with extract, with parallel controls.

Details and technique of experiments.

The material used was 8 albino rats for the tests (6 ♂ and 2 ♀) and 7 albino rats for control (6 ♂ and 1 ♀). Unfortunately, at the time it was not possible to secure couples of the same litter. The significance of this will be referred to later. When the experiments were started (3/2/31), the average weight of the experimental animals was 75 g. and of the controls 78 g. The food was the standard diet of Gudjonsson [1930], consisting of powdered skim milk 30 %, ground rice 40 %, yeast 15 %, and cocoa butter with liver oil 15 % (liver oil 0.3 %). Food and water were given *ad lib.* and the quantity of water consumed was measured daily.

The extract employed was prepared according to Evans with certain modifications. The pituitary glands were removed as quickly as possible after the slaughtering of the cows (less than half an hour after killing) and put on ice at once. Experience had shown that this latter point is of vital importance to the obtaining of potent extracts. The Seitz-filtered extract is a slightly yellowish, sometimes reddish, somewhat cloudy fluid of p_H about 6.7 and containing about 1.4 % dry-matter (N 0.2 %). It produces colour changes in axolotls and frogs and thus undoubtedly contains posterior lobe (*intermedia*) hormones. Titration of the melanophore-expanding principle on *Rana temporaria* showed that 1 cc. of extract corresponded to 0.13 cc. of pituitrin, 0.13 cc. pitressin and 6.6 cc. of pitocin (Parke Davis).

Dose: For the first three months each rat was given 0.5 cc. of extract daily, injected intraperitoneally, corresponding to about 0.1 g. of fresh anterior lobe. Later on 1 cc. was given daily.

The rats were killed (with coal-gas) over the period 13/10–31/10. One rat was killed every day, experimental animal and control animal being killed at intervals of 24 hours. Immediately after death the animal was weighed, then placed in the ice-box for 24 hours—which very much simplifies the subsequent mincing. After 24 hours the animal was weighed again. The average loss of weight during chilling was 0.5 g. (max. 3 g.), an error which of course can easily be eliminated by using a correction in every case. The mincing process consisted in grinding the whole rat (stomach, guts and skin) a sufficient number of times (average 10) in an ordinary sausage machine. As a rule it was possible to complete the mincing without the least loss of dry matter.

If we call the weight prior to mincing b , that of the instruments used (mincing machine, spatula, forceps, scissors, *etc.*) c , and that of minced meat + instruments after mincing d , the loss of weight through evaporation during mincing is $b + c - d$, and the weight of the meat $d - c$. The loss by evaporation was on an average 3 to 5 g. (max. 15 g.). This error, of course, can also be eliminated by correction.

The mince thus obtained was an extremely homogeneous, red mass, although some fragments of hair were distinguishable.

The following samples were taken of this mixture as quickly as possible: (1) 4 samples of about 0.5 g. for N analyses, (2) 2 of 1 g. for dry matter, (3) 4 (reserve samples) of 1 g. All samples were weighed to an accuracy of 0.1 mg.¹, and of course the samples were taken from various parts of the mince.

¹ In reality such extreme accuracy is superfluous for the present purpose.

The N determinations were made according to Kjeldahl's method. The samples were weighed on dried, cleaned fragments of object-glass, which were immediately thrown into the incineration-tube so that waste of mince through transfer was entirely avoided.

The real N % (X) was calculated from the N % found (f) by means of the following correction:

$$X = \frac{f \cdot e}{b} \cdot \frac{b}{a} = \frac{f \cdot e}{a}$$

where a and b are the weight of the rat before and after chilling, e the weight of the total mince (e/b correction for evaporation when mincing and b/a correction for evaporation when chilling).

The dry matter tests were performed in weighing-tubes. All samples were dried for 4 days at about 90°. When this was completed, small irregular fluctuations were observed of < 0.001 g. With the quantities employed (1 g.) these involve an error of < 0.1 %, which may be regarded as insignificant for present purposes.

Fig. 1.

Fig. 2.

- Weight curve of 10 Wistar rats (4 ♂ and 6 ♀) injected with anterior pituitary extract for 7 months.
 - - - - - Controls. 10 Wistar rats (4 ♂ and 6 ♀).
 ÷ means injections stopped. + means injections started.

Fig. 2. Weight curves of rats used for the analyses.

- Injected rats. - - - - - Controls.

Results.

The average weights of experimental animals and controls are given graphically on Fig. 2. It will be seen that the curves almost completely coincide in the first two months, which argues that the spontaneous growth energy has been the same for both treated and untreated animals, and makes it improbable that the subsequent dissociation of the curves is caused by accidents. In this connection we may refer to Fig. 1, which illustrates the effect of the extract upon a series of Wistar rats consisting of couples of the same litter. On the whole, extracts prepared in the manner described have in our laboratory without exception given a distinct difference in the average weight of experimental and control animals in favour of the former. It will be noticed that the curves do not deviate until at about 200 g., which agrees with the experience

of other laboratories. The reason is not known. If rats of this weight are employed it is possible to determine the activity of an extract in about 14 days.

The weight increase attained at the end of the experiments (13/10/31) amounts to about 21.5 %. At this time the average weight of the experimental animals was steadily rising, whereas the weight-curve of the controls ran parallel with the abscissa. If the extract dose and experimental period are varied there is no doubt that much greater weight differences can be obtained than in the present series. It is possible that the vitamin content of the food plays an important rôle in this respect; this is a question that will be dealt with in a future publication. It will merely be pointed out here that the diet employed contains sufficient, but not a surplus of, vitamins. As the killing of the rats was spread over one month, it will be most correct to calculate their mean weight from weighings just after death; the weight difference was then 23.4 % (experimental animals: 355 g., controls: 288 g.).

Dry-matter determinations. The average dry matter percentage for the extract-treated rats (M_E) proved to be 36.5 ± 0.7 , and for the controls (M_C) 38.6 ± 1.68 . It will be observed that the individual variations in dry matter content are much greater for controls than for experimental animals. The great error in M_C has the effect that no importance is to be attached to the difference $M_C - M_E = 2.1 \pm 1.82$. This difficulty, of course, may be solved by increasing the number of controls. Fortunately, by means of Chanutin's investigations [1930], we are enabled to form a reliable idea as to the variability of the dry matter content in a random sample of rats comprising a sufficient number. In a series of 39 albino rats of both sexes Chanutin found an average dry matter content of 38.4 % (max. 45.7 and min. 32.8) and in another series of 45 similar animals 38.6 % (max. 42.2, min. 36.4), *i.e.* for both series a mean of 38.5. Unfortunately, the author does not give the mean error for this figure. For safety's sake we will therefore take it that the greatest deviation observed by him (7.2) corresponds with twice the standard deviation. The mean error of the mean (38.5) is then $7.2/2 \sqrt{84} = 0.39$. There can scarcely be any doubt as to the justification for employing this author's experiments as a standard, *i.e.* substituting M_C ($= 38.6 \pm 1.68$) with 38.5 ± 0.39 , because in the first place these two figures are practically identical, and in the second place the greatest deviation in our control material amounts to 5.5, or $< 2\mu$ (2×3.6). The difference between the dry-matter contents of controls and experimental animals will then be:

$$(38.5 - 36.5) \pm \sqrt{0.39^2 + 0.7^2} = 2.0 \pm 0.8.$$

As the difference is $>$ twice the mean error, it is probable that it is reliable, but by no means certain.

The total N determinations showed that the average N % in the experimental animals was 3.29 ± 0.49 and in the controls 3.16 ± 0.085 . The difference is 0.13 ± 0.098 , in other words insignificant from a statistical point of view, although of course this does not preclude the possibility that the test animals

nevertheless contain more N. The question calls for supplementary investigation.

With the collected data for dry matter and total N as a basis we are in a position to form an idea as to the nature of the growth that is induced by the extract employed. At the beginning of the experiments (3/2/1931) both experimental animals and controls weigh almost the same (Fig. 2). At the end (13/10–30/10/1931) the experimental animals on an average weigh 355 g. and the controls 288 g. with dry matter contents of 36.5% and 38.6 % respectively. Thus the increase of weight is 67 g., the increase of dry matter 18.4 g. and the percentage of dry matter in the increase 27.5. In normal rats weighing > 70 g. the percentage of dry matter is practically independent of the weight [*cf.* for instance Donaldson, 1915, p. 300]. Thus when a rat (> 70 g.) increases its weight by 67 g., it will, regardless of its momentary weight, increase its dry matter content by $\frac{67 \times 38.6}{100} = 26$ g. Appraised in this manner we have thus to do with $\frac{18.4 \times 100}{26} =$ about 71 % normal growth for our experimental animals. The N % in the experimental animals was 3.29 and in the controls 3.16. As no importance may be attached to this difference (see above), we will take it that experimental animals and controls contain an average of 3.23 % N. As the experimental animals weigh 67 g. more, they contain a N-surplus of $\frac{3.23(355 - 288)}{100} = 2.16$ g., which corresponds with 13.5 g. of protein.

It will be seen that the animals treated with extract not only add less dry matter than normal animals, but that their dry matter addition is of abnormal composition, having a much higher N content.

If the increase of weight induced by the anterior pituitary extract were exclusively due to retention of water, we should have found a dry matter content (M_E) in the experimental animals of $\frac{38.5 \times 288}{355} = 31.3$ % with a mean error (see above) of about ± 0.7 . If the dry matter percentage of the controls (M_C) is put at 38.5 ± 0.39 (see above) the difference $M_C - M_E$ will be

$$(38.5 - 31.3) \pm \sqrt{0.39^2 + 0.7^2} = 7.2 \pm 0.8.$$

The difference actually found being 2.0 ± 0.8 , it is out of the question that the induced weight increase is due exclusively to water. The same can be proved by the aid of the N analyses. If the weight difference were exclusively due to water, the N % in the experimental animals would fall to $\frac{3.16 \times 288}{355} = 2.56$. The difference between the average percentage in experimental animals and controls would then be:

$$(3.16 - 2.56) \pm \sqrt{0.085^2 + 0.049^2} = 0.6 \pm 0.098.$$

The difference between this figure and the difference found (0.13 ± 0.098) is 0.47 ± 0.14 , *i.e.* statistically significant.

As was observed in the introduction, discontinuation of the injections causes an immediate loss of weight which amounts to about 35 % of the

difference attained. As the experimental animals have a higher water content than the controls, the obvious assumption is that it is this store of water which the animals get rid of when the treatment is discontinued. Therefore it will be of interest to calculate how much the animals can lose in weight by ridding themselves of the surplus quantity of liquid. The necessary data may be obtained from the curve in Fig. 1. It will be seen that on 22/12 the injected rats weigh 340 g. whereas the controls weigh 256 g. Discontinuation of the injections brings about a loss of weight of 32 g. If the water content of experimental animals is put at 63.5 % and of controls at 61.5 %, we obtain

$$\frac{\left(340 \times \frac{63.5}{100} - X\right) 100}{340 - X} = 61.5.$$

where X signifies the loss of weight through yielding up the surplus quantity of water. From the equation X is found to be about 18 g. Consequently, only about 60 % of the loss of weight actually found is due to elimination of the surplus water.

SUMMARY.

In recent years various writers have published reports indicating that the increase of weight induced by bovine alkaline anterior pituitary extracts is principally the result of the retention of water. For the purpose of elucidating this problem a series of dry matter and total N analyses has been performed on extract-treated albino rats with corresponding controls. In the extract-treated animals there was found an average dry matter content of 36.5 %, in the controls 38.6 %. The difference is > twice the mean error. The average total N % in experimental animals and controls was 3.29 and 3.16 respectively. The difference is statistically insignificant. The figures show conclusively that the induced increase of weight is not exclusively due to retention of water; on the contrary, only a small part of the weight difference obtained seems to be a result of an abnormal accumulation of water. If the injections are stopped, the weight falls immediately, it is true, but the loss amounts to only about 35 % of the weight difference recorded. Only about 60 % of this loss of weight is explained by the excretion of surplus water. The nature of the growth induced by the extract employed may be characterised by the following figures. When an injected and a normal rat increase their weight by 100 g., these 100 g. will be of the following composition.

Injected rat: 72.5 g. water, 27.5 g. dry matter (consisting of 73 % protein and 27 % ash and fat).

Normal rat: 61.4 g. water, 38.6 g. dry matter (consisting of 52 % protein and 48 % ash and fat).

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