CCXLII. QUANTITATIVE STUDY OF THE THYROTROPIC ACTIVITY OF ANTERIOR PITUITARY EXTRACTS.

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THE earliest experiments on hypophysectomy showed that atrophic changes take place in the thyroid after removal of the pituitary gland [Cushing, 1912; Dott, 1923]. This relationship is now well authenticated [Houssay *et al.*, 1931, for the dog; Smith, 1930, and Anderson and Collip, 1933, for the rat; White, 1933, for the rabbit]. Hypophysectomy also abolishes the power of the thyroid to undergo compensatory hypertrophy [Houssay *et al.*, 1932; Smith, 1930]. Replacement therapy has been successful in overcoming the effect of hypophysectomy. Thus Smith [1930] was able to restore the atrophied thyroid of the hypophysectomised rat by the implantation of anterior lobe tissue, while Anderson and Collip [1933] prevented atrophy by starting treatment immediately after hypophysectomy. Similar results have been obtained by Freud *et al.* [1933] for the rat, and Houssay *et al.* [1932] for the dog.

Normal animals have naturally been more used for studying the thyrotropic effect of anterior lobe extracts. As early as 1908, Rénon and Delille [1908, 1, 2] administered pituitary extracts to rabbits and observed changes which they considered as indicative of thyroid hyperactivity. Successful experiments on rabbits have also recently been reported by Baumann and Marine [1932]. It is now evident that the thyroids of different species vary greatly in their power to respond to the thyrotropic substance. The guinea-pig is particularly sensitive, and, as Freud *et al.* [1933] remark, has become the classical animal for the study of thyroid hyperplasia. The reaction in this species was obtained by Loeb and Bassett [1929; 1930] and Aron [1929], and afterwards by a large number of workers, including Janssen and Loeser [1931], Loeser [1932], Junkmann and Schoeller [1932], Anderson and Collip [1933], Castillo [1932], Greep [1933], and Friedgood [1934]. Marine and Rosen [1934] found that thyroid transplants can be stimulated as well as normal glands. Characteristic histological changes are produced in the guinea-pig thyroid. In the early stages of stimulation the epithelium undergoes hyperplasia, and becomes very high columnar, with large rounded nuclei. At the same time the colloid in the follicles becomes vacuolated and gradually decreases in amount. The gland may increase greatly in weight and macroscopically has a much more fleshy appearance than is normal. At the same time a rise occurs in the basal metabolism and symptoms of exophthalmos, presumably indicative of increased thyroxine output, may subsequently appear [Friedgood, 1934; Anderson and Collip, 1934].

In the rat however Loeb and Bassett [1929], Aron [1930, 2; 1932, 2], Schockaert [1932], and Anderson [1933] all failed to produce abnormal hyperplasia of the

thyroid of the rat by injection of anterior lobe extracts. Junkmann and Schoeller [1932] found the rat thyroid to be far less sensitive than that of the guinea-pig, while Houssay [1932], though obtaining a slight effect, states that a dose which will cause a response in a 10 kg. dog or in a guinea-pig is not adequate to affect a rat. Anderson and Collip [1933] however produced an effect in the rat and an increased metabolic rate with a purified preparation. These authors [1934] have now published a full account of their work on normal and hypophysectomised rats. Freud *et al.* [1933] obtained a slight effect on the rat.

The explanation of the comparative insensitivity of the thyroid of the rat to anterior lobe extracts seems to lie in the fact that the thyroid of this species is normally hyperactive. In this connection Junkmann and Schoeller's observation that the rat pituitary contains much more of the thyrotropic substance than that of the guinea-pig may be recalled. As Collip [1934] says, "it may follow as a general rule that the sensitiveness or responsiveness of an animal to injected hormone varies inversely with the hormone content or production of the animal's own gland," though this generalisation does not hold for the gonadotropic activity of the rat and rabbit pituitary as described by Hill [1934].

Various workers have shown that similar extracts of organs other than the anterior pituitary body do not affect the thyroid [*cf.* Aron, 1930, 2], and that no symptoms of hyperthyroidism are produced by administration of pituitary extracts to thyroidectomised animals [Verzár and Wahl, 1931]. It may thus be regarded as established without doubt that the anterior pituitary body elaborates a specific substance which stimulates the thyroid gland and which is presumably concerned with the regulation of thyroid activity in the normal animal.

In spite of the large amount of work which has been done on the thyrotropic activity of anterior pituitary extracts, remarkably little attention has been paid to the quantitative measurement of such activity, and the present paper is devoted to this aspect of the subject.

I. *Test animal and criterion of response.*

The work summarised above makes it evident that the guinea-pig is the obvious test animal for thyrotropic preparations. Possible criteria of response fall into two categories: (*a*) changes in the thyroid itself and (*b*) metabolic and other systemic changes depending on the increased production of thyroxine. The systemic changes, in addition to being extremely difficult to put on a quantitative basis, except perhaps the basal metabolic rate, suffer from the disadvantage that they involve two variables: (*a*) variation in the degree of thyroid stimulation produced by a given amount of anterior lobe extract and (*b*) variation in the secondary effect produced by a given amount of thyroxine. In this particular, of course, use of the B.M.R. or any other general effect is equivalent to the use of the vaginal or uterine reaction in assessing the gonadotropic activity of anterior lobe extract. Chief among workers using the B.M.R. is Collip, who defines a unit as "the minimum amount administered daily in two injections, which will cause a rise of 20 % in the metabolism of the hypophysectomised rat by the fourth day" [1934].

Direct examination of the stimulated gland is more satisfactory in some ways, and the changes in the size and histological appearance of the gland have often been used as test objects. Aron [1930, 1; 1932, 1], Loeser [1932] and Severinghaus [1933] give detailed descriptions of the histological criteria of stimulation, while Heyl [1933] considers the precautions necessary in defining stimulation histologically. Junkmann and Schoeller [1932] define a unit as the

amount required to produce a definite histological reaction in one out of two animals. The work of Castillo and Magdalena [1931] seems to throw some doubt on the validity of considering slight histological hyperplasia as a sign of extraneous stimulation, a view shared by Collip [1934]. Up to the present there appears to have been no systematic examination of the weight changes in the stimulated thyroid—*a priori*, an easy method of assay—and we have tentatively selected this criterion as a basis for the quantitative assay of thyrotropic preparations.

II. *Technique.*

Size of animal. Immature female guinea-pigs as near as possible to 200 g. body weight were used. The size and variability of the thyroid at this body weight are considered in the next section. All animals used were from the inbred strain maintained at the Institute's Farm Laboratories, the availability of which has made possible the work described in this paper.

Extract. One extract only (T.17.B) was used for the work described in Section III on the variables affecting the response of the thyroid. This was a dry friable powder obtained by precipitating with alcohol and ether a neutralised alkaline extract of acetone-desiccated ox pituitaries. The powder which represented about 10 % of the original desiccated gland or about 2 % of the fresh gland was readily soluble in dilute sodium hydroxide and stayed in solution when neutralised for injection. All injections were subcutaneous.

Details of other extracts used are given in the relevant places.

Weighing and histology. The thyroids, which can be dissected out very cleanly in the guinea-pig, were removed, fixed in Bouin's fluid and weighed at the 70 % alcohol stage in the up-grading. Certain of the glands were sectioned at 7μ and stained in the ordinary way.

III. *Examination of variables in the assay of thyrotropic activity on the immature guinea-pig.*

This section is concerned with an examination of the initial variability of the thyroid of the immature guinea-pig, and the variability in response to anterior lobe extract, together with a consideration of the most efficient system of injection *etc.* All the experiments described in this section were carried out with a single preparation, T.17.B, which under the conditions of preservation showed no signs of deterioration.

(a) *Initial size of the thyroid.* Since it would be extremely difficult to secure groups of young guinea-pigs of exactly the same body weight, and since even then variability in size of the thyroid might be expected, we have investigated the size and variability of the thyroid of normal guinea-pigs over a range of body weight more than covering all the animals used in these experiments. Thirty-eight normal animals with body weights of 150–300 g. were examined. The weights of the thyroids, plotted against body weight, are shown in Fig. 1. As will be seen, the mean thyroid weight at 200 g. body weight is 31 mg. The positions of these points obviously indicate a linear relation over the range of body weight used. Dealt with as a correlation table, these figures show a correlation coefficient of 0.808. The regression line calculated for these points has the formula $y = 0.159x - 1.02$, in other words the thyroids increase in weight almost exactly 1 mg. for each 6 g. increase in body weight. Individual variation is slight. Actually, even including the increase due to increasing body weight, the average thyroid weight for these 38 animals, 34.6 mg., only has a standard error of ± 1.3 . It may be concluded therefore that both the size changes due

to increasing body weight, and also the individual variation in thyroid weight, in animals of 150–300 g. body weight, are insignificant compared with changes of the magnitude of those described below. Even so however we have made every effort to obtain animals of about 200 g. at the beginning of the experiment, and where the experiment was of sufficiently long duration to allow of appreciable body growth, we have taken into consideration the normal growth which would have taken place in the thyroids. The subsequent growth of the normal guinea-pig thyroid, as well as that of the adrenals and reproductive organs of both sexes will be dealt with elsewhere.

Fig. 1. Weight of the thyroids of the guinea-pig of 150–300 g. body weight.

Table I. *Effect of duration of injection.*

Daily amount given mg.	Days injected	Body weight when killed g.	Thyroid weight mg.	Normal thyroid weight for the same body weight mg.	Increase in weight of thyroids above normal mg.
2	3	214	45.2	33.0	12.2
"	5	211	53.8	32.5	21.3
"	8	207	60.2	31.9	28.3
"	10	248	62.8	38.4	24.4
"	14	208	60.0	32.0	28.0
5	2	198	47.0	30.5	16.5
"	5	187	58.0	28.7	29.3
"	6	170	61.2	26.0	35.2
"	10	188	78.2	28.9	49.3
"	15	220	81.0	34.0	47.0
"	20	254	84.2	39.4	44.8

Five animals per group, except in the first 5-day group, which had ten, and second 5-day group which only had four.

(b) *Effect of duration of injection.* Our first experiment was designed to show what was the most suitable period of injection for securing a good reaction without unduly prolonging the time of injection. Five groups of animals were therefore injected with 2 mg. daily of the preparation, groups being killed after 3, 5, 8, 10 and 14 days of injection. The results are shown in Table I. Measured either by the gross weight of the thyroids, or by their increase above normal for the body weight of the group, the maximum reaction is reached at 8 days

and further daily injection does not increase the response. A further six groups were given 5 mg. daily of the same preparation for 2, 5, 6, 10, 15 and 20 days; each group being killed on the day following the last injection. As with the smaller dose, the maximum thyroid weight is reached quite soon, in this case at 10 days, and further injection of the same daily amount hardly increases the response. On the other hand, there is no sign of any failure to maintain the development induced by 10 days of injection. So far as these results go, they do not suggest that insensitivity and failure to maintain early hypertrophy appear in the course of prolonged injection, as described by Collip and Anderson [1934]. It is possible, of course, that 20 days is inadequate for the development of what they term an "anti-hormone" phenomenon, but according to Anderson and Collip [1934] the increased B.M.R. begins to fall after 7 days' injection.

Fig. 2. Effect of duration of injection of anterior lobe extract on the weight of the thyroid.

— Gross weight of thyroids.
 - - - Increase above normal for the corresponding body weight.

The maximum response with 2 mg. daily, reached at 8 days, was a thyroid weight of 60 mg., about double the normal for the body weight. With 5 mg. daily the maximum was about 80 mg., reached at 10 days, and the curve was higher from the beginning. The maximum development attained with 5 mg. daily seems to come about 2 days later than that with the smaller dose. In both these series most of the growth took place during the first 5 days, and this time was obviously adequate for producing a good reaction. As there is little to be gained by continuing beyond 5 days, we have adopted this as a standard period of injection.

(c) *Time of killing after last injection.* It was unlikely that there would be much delay in the response to treatment, so the animals described above were killed on the day after the last injection. It seemed desirable however to investigate this point and at the same time to extend the observations to ascertain whether the hypertrophied gland underwent regression and, if so, at what rate. Groups of animals were given 10 mg. Extr. T. 17. B daily for 5 days, an amount known to produce marked hypertrophy of the thyroid. The groups were killed at 1, 5, 10 and 20 days after the last injection. Results are shown in Table II, and Fig. 3. Since the body weight increased appreciably in animals killed in the later stages, a more accurate value for the extent of the regression is obtained by considering the excess thyroid weight above normal for the size of the animal. On this criterion, the weight of the hypertrophied thyroid drops rapidly in the first few days after the cessation of injection until about half the excess weight is lost. Subsequent regression is slow so that 20 days after the last injection

the thyroid is still slightly above its proper weight. The thyroid presumably regains its normal relation to body weight partly as a result of its own regression and partly as a result of body growth. These findings make it clear that to measure the maximum hypertrophy of the thyroids the animals should be killed very soon after the last injection, and as a routine we have killed on the following day.

Table II. *Involution of the hypertrophied thyroid after the cessation of injection of anterior lobe extract.*

Time killed after last injection days	Body weight when killed g.	Thyroid weight mg.	Normal thyroid weight for same body weight mg.	Increase in thyroid weight above normal mg.
1	217	82.6	33.6	49.0
5	242	62.4	37.5	24.9
10	264	57.8	41.0	16.8
20	289	51.8	44.9	6.9

Ten animals in the first group, five in the others.

Fig. 3. *Involution of the hypertrophied thyroid after the cessation of injection of anterior lobe extract.*

— Gross weight of thyroids.
 - - - - Excess above normal for the corresponding body weight.

(d) *Effect of various doses over a constant period.* After determining the growth and variability of the normal thyroid, a suitable duration of treatment and the time of killing after the last injection, it was necessary to consider the response in relation to dosage, so that a standardisation curve could be made. In the first experiment a wide range of dosage was used. Five groups of five animals each were injected with 4, 10, 20, 30 and 40 mg. of Extr. T. 17. B daily for 5 days. The results (Table VII and Fig. 6) showed that 10 mg. daily caused enlargement to 82.6 mg. and that further increase of dosage had little effect. The group receiving 40 mg. daily actually had slightly smaller thyroids than the one receiving 30 mg., but this may have been due in part to their having a slightly lower body weight. In view of these results it was clear that hypertrophy above 80 mg. weight was no use for assay work but that the steepness of the response/dose curve during rapid enlargement of the thyroid offered good chances of obtaining a curve suitable for assay work.

Attention was next turned to extending the experiments on hypertrophy up to 80 mg. Groups of ten guinea-pigs were injected with 2 and 7 mg. daily, and the previous experiments were duplicated so as to bring the groups at 4 and 10 mg. daily up to ten animals. The final results for this dose/response curve are shown in Table III and Fig. 4. The control figure was obtained from

a group of ten uninjected animals of comparable body weight. The curve given by these results is of a well-recognised type and can obviously be used as the basis of a method of assay. The statistical aspect of the use of this curve for this purpose is discussed in Section IV.

Table III. *Relation between dose given and weight of thyroid produced.*

Amount given daily mg.	Body weight when killed g.	Thyroid weight mg.
0	208	31.8
2	211	53.8
4	210	64.2
7	202	79.9
10	217	82.6

Ten animals per group.

Fig. 4. Relation between dose of anterior lobe extract given and weight of thyroids.

(e) *Effect of a constant total dose over various periods.* From the data discussed above it is possible to obtain information (summarised in Table IV) of the effect

Table IV. *Effect of duration of administration of a constant total dose.*

Total amount given mg.	Amount daily mg.	Days given	Thyroid weight mg.
10	2	5	53.8
	5	2	47.0
20	2	10	62.8
	4	5	64.2
50	5	10	78.2
	10	5	82.6

Five or ten animals per group.

of varying the time over which a constant total dose is given. It is evident that where the period of injection is not extended unduly, the influence is surprisingly slight: thus 10 mg. Extr. T.17.B give much the same effect whether given as 2 mg. for 5 days or as 5 mg. for 2 days, while 5 mg. for 10 days give a very similar result to 10 mg. for 5 days. It is evident from Fig. 2 however that where injection is carried on beyond 10 days the efficiency of a constant total dose will fall. Thus 2 mg. for 20 days would presumably not give a thyroid weight much above 60 mg., whereas from Fig. 4, 8 mg. for 5 days would give a weight of nearly 80 mg.

(f) *Control experiments.* A control experiment on the effect of injecting foreign material was made by giving a similarly prepared extract of rat testis in comparable amounts for 5 days. As will be seen from Table V there was no

Table V. *Control experiments.*

Substance given	Total amount	Body weight g.	Thyroid weight mg.	Ovary weight mg.	Uterus weight mg.
Alkaline extract rat testis	From 5 g.	202	27.4	—	—
Urine of pregnancy extract	50 rabbit units	193	29.4	82.6	136.0
Oestriol	1.2 mg.	190	26.6	55.7	316.0
Anterior lobe extract to ovariectomised animals	From 5 g.	207	83.2	—	104.0
Anterior lobe extract	25 mg. T.17.B	186	60.0	29.0	101.4
Anterior lobe extract to ovariectomised animals	25 mg. T.17.B	192	51.2	—	101.0
Anterior lobe extract to ovariectomised animals receiving oestriol	25 mg. T.17.B + 1.2 mg. oestriol	186	40.4	—	328.4

Five animals per group.

result on the thyroids. Other control experiments were necessitated by Biale-Laprida's [1933] conclusion that oestrin has an inhibiting action on the thyroid. Since the thyrotropic extract used by us contained the gonadotropic principles, which might be responsible for the secretion of oestrin by the immature ovary, it seemed necessary to ascertain what effect, if any, this complication might have on the response of the thyroid to the thyrotropic substance. In the first experiment, oestriol (trihydroxyoestrin) was given to a group of animals. As shown in Table V, this treatment caused a marked reaction in the uterus and the vulva became patent. The average thyroid weight, however, was only a little over 1 mg. below the normal for the body weight. This experiment, carried out with pure crystalline oestriol, fails to give any confirmation to Biale-Laprida's statement that a comparatively small dose of folliculin will halve the weight of the immature thyroid. In the second experiment, 5 mg. daily for 5 days of a urine of pregnancy extract were injected into each of a group of animals. This dosage, in rabbit ovulation units, contained about the same gonadotropic activity as the largest of the doses of T.17.B given. The ovarian response was very strong and there was appreciable uterine reaction, indicating secretion of oestrin. The thyroid weight was entirely normal.

These experiments however did not necessarily preclude the possibility of the presence of oestrin inhibiting thyroid hypertrophy as suggested by Aron and Benoit [1932]. In other experiments, therefore, alkaline extracts of two samples of the same batch of dried gland, made in the same way, were injected in similar amounts into (a) normal immature guinea-pigs, (b) ovariectomised immature guinea-pigs. The average thyroid weights for the two groups happened to be identical (83.2 mg.) and there is no doubt that the thyrotropic activity observed in the two groups was very similar, and that any stimulation of oestrin production in the first group had not materially affected the thyroid response.

Finally, 5 mg. daily of T.17.B were injected into groups of (a) normal immature guinea-pigs, (b) ovariectomised animals and (c) ovariectomised animals simultaneously injected with 0.24 mg. oestriol daily. The results (Table V) show that large doses of oestrin tend to inhibit thyroid hypertrophy. The thyroids of

the normal animals receiving the anterior lobe extract however were appreciably heavier than those of the ovariectomised animals, and it seems certain that the amount of oestrin produced by the ovary under the influence of pituitary extracts such as these can have had no inhibiting effect on the thyroid development. In actual fact the amount of oestrin produced in most of the test animals (as judged by the size of the uterus and by the failure of the vulva to become patent) seems to have been remarkably small, a conclusion in keeping with Hill's [1934] observation of the low activity of ox pituitary tissue on immature animals.

IV. *Standardisation curve for thyrotropic substance and definition of a unit.*

Prof. Gaddum pointed out to us that the curve shown in Fig. 4 is of the type which placed on a logarithmic co-ordinate becomes a straight line, and that in such a form it is more suitable for use in assay work. Conversion to a logarithmic scale necessitates omitting the point for the control animal (dosage 0). The other four points of Fig. 4 are shown on a logarithmic scale in Fig. 5, from which it is evident that they fall on a good approximation to a straight line. The line is so obvious that there seemed little to be gained by calculating its regression formula.

It must be remembered that assay curves of this kind change both in position and in slope, and that if tests are made only infrequently it may be necessary to give two doses of a standard and two doses of the unknown extract for each determination. At present we have no information as to the probable changes in the curve from time to time. The whole of the work described in this paper was carried out over a comparatively short space of time, the standardisation curve being made simultaneously. The groups of ten animals used for the standardisation curve are actually composed of groups of five done at different times (3-4 weeks apart), and in only one was there appreciable difference in the results obtained from the paired groups of five animals. This means little since the time range was short, but as a basis for comparing the results described elsewhere in the paper we have considered the standardisation curve shown in Fig. 5 as adequate. It is evident however that steps would have to be taken to verify the slope and position of this curve before it could be used in future work.

At present we are concerned therefore only with the inherent error due to individual variation in response. It is possible to consider this by the usual methods.

Each of the four points is the mean figure for ten animals; and σ is 9.0, 7.5, 15.9 and 11.3 for the 2, 4, 7 and 10 mg. daily means respectively. The high figure for the third point was mainly due to a very eccentric figure for one animal, so that there is no indication here of a regular increase in variability as the response increases. The coefficient of variation ($\sigma/\text{mean} \times 100$) is much less for the last group than for the first, so that the hypertrophied thyroid shows a lower percentage variation than the normal, though actual variability may be slightly greater. From this it seems reasonable to conclude that the hypertrophy caused by a given dose of anterior lobe extract tends to be a regular amount, and is not proportional to the original weight of the thyroid—a useful fact in view of the inevitable variation in the initial size of the gland.

In view of the above it seems justifiable to arrive at a σ for the 40 observations combined, which would give a σ for the thyroid response over the range of dosage and under the existing conditions. The figure obtained is 10.8, so that the standard error of the response would be ± 4.8 , ± 3.4 and ± 2.4 respectively, using groups of 5, 10 and 20 animals. Turning again to the line in Fig. 5, it can

be found graphically that where u is the weight of the thyroid in mg. and x is the log of the daily dose of anterior lobe extract in mg., $du/dx = 47/1$. With a group of five animals therefore the error ± 4.8 in the determination of the mean will represent $\pm 4.8/47 = 0.102$ as the error in the determination of the log of the dose given. This is equivalent to an error of about 25 % in the determination of the dose, and also therefore in the determination of the unit as shown on the top co-ordinate in Fig. 5. For groups of ten animals, the error is reduced to a little above 15 %; with groups of twenty animals, the corresponding figure is near 10 %.

It is clearly not worth doubling the cost and labour of the tests to reduce the error from 15 to 10 %, but groups of ten seem to be desirable. In practice we have generally been unable to use larger groups than five, which implies an error of ± 25 % in the results described below.

Adoption of a unit. Pending an international agreement as to a unit of thyrotropic activity, it is necessary to have some provisional definition for comparative work in the laboratory, and the size changes considered above seem to offer a suitable basis. It is evident that for purposes of practical convenience the unit should fall somewhere in the middle of the line shown in Fig. 5. The weights of the thyroids in the immature guinea-pig of the size we have used average very near 30 mg.; double this weight falls well into the most serviceable part of the dose/response curve. It seems sound therefore to name 60 mg. as the critical weight required, and to adopt as unit: the thyrotropic activity contained in an amount of extract, which, given daily for 5 days, will cause the thyroids of the immature guinea-pig to attain a weight of 60 mg. To cause such a response requires 3.0 mg. daily of the extract used for the experiments recorded in Fig. 5. The dosage shown on the bottom co-ordinate can be converted into units as shown on the top co-ordinate in the ordinary way. The unit so defined is obviously a large amount considered in terms of original pituitary tissue. Cow pituitary, for instance, contains only about 6 units per g. fresh tissue (or about 30 units per g. desiccated tissue). The adoption of a system of assay entailing a large unit has certain disadvantages, notably the larger consumption of material in assay work and the apparent decrease in the activity of extracts. There are however compensating advantages, particularly the fact that the diagnostic reaction is far removed from the range of normal variation.

V. Quantitative observations on the preparation of thyrotropic extracts.

Advantage has been taken of the quantitative work described above to examine the efficiency of some of the processes commonly used in the preparation of thyrotropic extracts.

(a) *Acetone desiccation.* The initial acetone desiccation of the fresh gland is so useful a process and so widely used that it is important to know whether it entails loss of thyrotropic activity. 120 g. fresh ox anterior lobe were divided

Fig. 5. Standardisation curve for thyrotropic hormone.

into two portions. Part (a) was extracted overnight with 60 ml. *N*/10 NaOH, neutralised, centrifuged and the whole of the supernatant fluid injected into five animals over 5 days. Part (b) was desiccated in 500+500 ml. of acetone, dried and extracted overnight with 60 ml. *N*/10 NaOH+48 ml. water, this being the calculated amount of water removed in the desiccation. The extraction was completed and injections made as with the first preparation. The thyroids of the animals receiving the fresh tissue extract averaged 90 mg. in weight, those of the other group 101 mg. The second group of animals were of rather high body weight (average 240 g.) compared with the others used in these experiments, and this probably accounts for the very high thyroid weight obtained, but it is obvious that the acetone-desiccated gland retains its full thyrotropic potency.

(b) *Alkaline extraction.* Extraction of the acetone-desiccated tissue with dilute alkali, precipitation of the neutralised extract with alcohol and drying of the precipitate produces a clean friable powder which contains the active substances of the anterior pituitary in a much reduced bulk and forms a good basis for further purification. It seems important therefore to know how thoroughly the active substances are removed from the dried gland by this method. 100 g. acetone-desiccated ox anterior lobe were extracted with 1000 ml. *N*/20 NaOH overnight, with vigorous stirring at the beginning and at the end. The mixture was then neutralised and centrifuged. The supernatant fluid was precipitated with 5 vols. of 96 % alcohol and 1 vol. of ether and the precipitate dried and weighed. The residue was re-extracted with 500 ml. *N*/20 NaOH in the same way. The residue from the second extraction was finally re-extracted with 500 ml. saline. The results of the assays are shown in Table VI, and suggest

Table VI. *Number of alkali extracts.*

No. of extract	Nature of extract	Total extract obtained g.	Thyroid weight produced by 7 mg. daily mg.	Weight of unit mg.	Total units in extract (approx.)
T.19.B1	1000 ml. <i>N</i> /20 NaOH on 100 g. desiccated ox anterior lobe	10.05	71.0	4	2500
T.19.B2	500 ml. <i>N</i> /20 NaOH on residue of above	3.21	51.4	11	290
T.19.B3	500 ml. saline on residue of 19.B2	0.50	81.0	2½	200

7 mg. daily of each fraction given for 5 days to groups of five animals.

that so far as thyrotropic activity is concerned, though the second extraction may be worth while, the third is not. This relation, of course, might not hold in successive extractions of horse pituitary, in which the total soluble matter is much greater. The comparatively high activity of the small amount of material obtained by the third extraction is probably due to the removal of less inert matter by the saline than by the neutralised alkali. We have not yet assayed these successive fractions for gonadotropic activity, and such assay may, of course, modify the conclusion about the optimum number of extracts where maximum all-round activity is desired.

It may be noted here that the amount of dry extract given by the above method is of the order of 10-13 % of the original dried tissue.

(c) *Precipitation by alcohol.* Unless an extract is to be used immediately, it is highly desirable to reduce it to a dry powder. In the case of the alcohol-insoluble principles of the anterior pituitary, precipitation by alcohol is the

usual method for doing this, and it is generally assumed that the process is efficient. As we are not aware that there have been any quantitative experiments in relation to the thyrotropic activity, an experiment was performed in which the effect of injecting the neutralised alkaline extract was compared with the effect of giving the alcohol-ether precipitate from a comparable amount of extract. The average thyroid weights of the two groups of animals were less than 4 mg. different on a strong response, and precipitation by four volumes of alcohol and one of ether therefore seems a safe procedure.

(d) *Solubility in pyridine.* 50 % aqueous pyridine has been shown to be a most useful solvent for the gonadotropic substances [Fevold *et al.*, 1931]. Greep's conclusion [1933] that the thyroid hypertrophy is produced either by the luteinising principle or by another substance associated with it during preparation, shows that the thyrotropic substance, of whose separate identity there can be little doubt, is probably soluble in aqueous pyridine. Tests were made on three residues after aqueous pyridine extraction of dried ox anterior lobe. In the first preparation only one pyridine extraction was made. Assay of the residue showed that about 4 units of the thyrotropic substance remained. In the second experiment, 20 g. dried gland were extracted four times with 500 ml. lots of aqueous pyridine. A residue of 15.7 g. was obtained; tests on this showed that only about 2 units per g. remained. In the other experiment, 100 g. dried gland were extracted twice with 2.5 l. of 50 % aqueous pyridine. The residue (T.14.A), when dried, weighed 45 g. and contained a little over 2 units per g. As opposed to this, over 500 units were recovered from the pyridine extract in spite of the heavy loss which may have occurred during evaporation. These experiments make it clear that pyridine extraction either removes or destroys the thyrotropic activity. The latter alternative is disposed of by the next experiment.

The evaporation of large amounts of pyridine extract is extremely troublesome, and it seemed that it might be much more convenient not to make the pyridine extraction on the large bulk of desiccated gland but to reserve it until the bulk had been reduced by preliminary alkaline extraction and alcohol precipitation. In practice, this procedure proved very satisfactory. 6.5 g. alcohol precipitate from the alkali extract of 50 g. dried gland, known to contain about 1500 units of thyrotropic activity (5 mg. daily = 64.2 mg. thyroids = 1.15 units), were extracted three times with a total of 200 ml. aqueous pyridine. The residue (T.18.C), washed and dried, weighed 2.9 g., and contained the barest trace, if any, of thyrotropic activity; 3 mg. daily for 5 days gave an average thyroid weight of 33.6 mg. The pyridine extract (T.18.D) was precipitated with alcohol and ether, and the precipitate, washed and dried, weighed 2.8 g. 3 mg. daily produced an average thyroid weight of 69.6 mg., an effect equivalent to 1.6 units. The unit weight of this fraction was therefore 1.9 mg., *i.e.* the total activity of this pyridine-soluble fraction was a little over 1450 units. This experiment seems to show conclusively that the thyrotropic substance is quantitatively removed by pyridine extraction and that a considerable purification is thereby achieved.

(e) *Separation from gonadotropic substances and prolactin.* In view of the solubility of the gonadotropic substances in pyridine¹ it is improbable that the above-described solubility of thyrotropic substance in pyridine will be of any use in separating these different principles.

¹ The results on the immature guinea-pig receiving T.18.C and D are rather confusing as regards the partition of gonadotropic activity. The ovaries of those receiving the pyridine-insoluble averaged 61 mg., more than twice the weight of those receiving the pyridine-soluble fraction. The uteri weights were the reverse.

On the other hand, interesting results have been obtained with the lactation-stimulating substance. The pyridine-extracted residue (T. 14. A), which contained very small amounts only of thyrotropic activity, was tested on oestrin-sensitised immature rabbits, intact and ovariectomised and consistently gave a strong secretory proliferation of the mammary gland. A similar result was obtained with another pyridine-extracted residue tested on the mammary gland of intact or ovariectomised first oestrus rabbits. This result not only indicates that the thyrotropic and lactation-stimulating substances are distinct (as already shown by Riddle *et al.* [1933], but that they can be separated by pyridine extraction. Since Evans [1933] states that pyridine extraction usually leaves the growth hormone behind, it seems likely that a clean and simple separation of the growth hormone and prolactin from the thyrotropic and the gonadotropic substances may be effected by pyridine extraction.

(f) *Chemical properties.* In addition to what can be gathered of the chemical properties of the thyrotropic substance from the experiments described above, we are able to add qualitative data on certain other chemical properties. The substance is highly stable in the dry form. Ox pituitary tissue which had been desiccated more than a year was found to be active up to the usual standard. It is also stable to heat in the dry state. 7 mg. daily of a sample of T. 17. B which had been heated to 100° *in vacuo* produced an average thyroid weight of 68.4 mg., which, though rather lower than that given by the unheated material, is not sufficiently different to suggest appreciable destruction. Heated to 100° in aqueous solution a similar dose gave an average thyroid weight of only 31.4 mg., which indicates total destruction of the thyrotropic principle. This latter finding is in keeping with what is usually assumed, but is contrary to the work of Krichesky [1934]. The substance is readily soluble in water. The pyridine extract of T. 14 (see p. 1840) was extracted with 500 + 250 + 200 ml. of water. The insoluble fraction (T. 14. B), weighing 2.9 g., gave a negative assay, while the water-soluble fraction (T. 14. C), weighing 5.1 g., contained more than 500 units.

VI. *Simultaneous effects of total anterior lobe extracts on various organs.*

In addition to the weight of the thyroids, the weights of ovaries, uterus and adrenals were taken for all the animals described above. These records are of little interest here, except for those of the dose/response experiment (p. 1834). The average weight of the ovary at 200 g. body weight is about 30 mg. The highest average ovary weight for a group (82.5 mg.) was obtained from the urine of pregnancy experiment (p. 1836). Few of the anterior lobe extracts produced ovaries of more than 60 mg. The average uterus size for the group

Table VII. *Effect of various doses of anterior lobe extract on the organs of the guinea-pig.*

Daily dose mg.	Body weight g.	Thyroid weight mg.	Ovary weight mg.	Uterus weight mg.	Adrenal weight mg.
0	203	31.8	45.4	80.4	56.6
4	200	63.2	28.8	104.0	62.5
10	204	83.0	35.7	182.0	63.8
20	192	85.3	39.4	247.0	70.1
30	208	89.8	49.6	198.2	76.3
40	173	77.0	43.0	202.4	75.9

Five animals per group.

was extremely variable, ranging from 84 mg. to 202 mg. in 5-day experiments. Variation in adrenal size was comparatively slight though fairly regular and gave no suggestion that the normal immature guinea-pig would serve as a test animal for the adrenotropic hormone on gross size changes alone.

Fig. 6.

Table VII and Fig. 6 show the responses of the various organs to a wide range of dosage of T.17.B. The regularity of the responses of the thyroids and uterus is in marked contrast to the irregularity of the ovarian response.

VII. SUMMARY.

1. The thyrotropic activity of anterior pituitary extracts was tested by their capacity to increase the weight of the thyroids of immature female guinea-pigs. At 200 g. body weight, the normal thyroids weigh about 31 mg. and are growing at the rate of 1 mg. per 6 g. body weight.

2. The maximum increase produced by a constant daily dose of anterior lobe extract is attained in 8–10 days. The standard period of injection in these experiments was 5 days, the largest thyroids produced in this time having an average weight of 101 mg. in a group of five animals.

3. Thyroids caused to enlarge to 80 mg. return to normal size about 3 weeks after the last injection.

4. A curve for response/dose has been made on groups of ten animals for the purpose of standardising extracts, and, pending some international agreement, the unit adopted for the laboratory is the thyrotropic activity contained in an amount of extract which, given daily for 5 days, will cause the thyroids of the 200 g. guinea-pig to attain a weight of 60 mg., *i.e.* about double the normal.

5. The efficiency of some of the methods commonly used in the preparation of thyrotropic extracts is examined in the light of these quantitative observations.

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