PREPARATION OF PITUITARY THYROTROPIC HORMONE*

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The existence of a thyroid-stimulating hormone in anterior pituitary gland tissue has been known for 30 years (1, 2). During this time, considerable work has been done on the physiological and the pharmacological effects of the thyrotropic hormone administered in the form of pituitary extracts which had been subjected to a varying degree of manipulation. Relatively little progress on the purification and chemical characterization of the thyrotropic hormone was made until 1931 (3). Since that time several methods of purification of the pituitary thyrotropic hormone have been described. Although some of these methods (4-9) effect significant concentration of the thyrotropic activity of pituitary tissue, the procedures either are not described in sufficient detail to allow ready repetition or result in poor yield of hormone. The most active preparation so far reported appears to be that of Fraenkel-Conrat, Fraenkel-Conrat, Simpson, and Evans (9), whose method yields material assaying 480 chick weight units per mg. of nitrogen. This represents a 100-fold increase in concentration of the hormone. Since their preparation contained 13 per cent N, it would assay about 65 chick weight units per mg. on a dry weight basis.

In no case has more than a crude attempt been made to characterize the chemical and physicochemical properties of the hormone. Available evidence indicates that the hormone is protein in nature.

It is the object of the present communication to present a description of a rather simple procedure which consistently yields significant amounts of a thyroid-stimulating product with a degree of physiological activity higher than any preparation previously described in the literature. A brief qualitative description of the thyrotropic preparation is given. At

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Some of the data in this paper are taken from a dissertation presented by Leon S. Ciereszko to the faculty of the Graduate School of Yale University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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a later time, the physicochemical properties of the product will be described.

In a previous publication from this laboratory, Bonsnes and White (10) described a procedure for the fractionation of saline extracts of fresh beef pituitary glands. By the use of isoelectric precipitation, followed by gradually increasing concentrations of acetone, it was possible to prepare a fraction with marked thyrotropic hormone activity. Essentially, this fraction represented material precipitated from a saline extract of pituitary tissue by 75 per cent acetone at pH 4.0 after the previous removal of precipitates at pH 5.4, 4.8, 4.0, and 4.0 with the addition of acetone to a concentration of 50 per cent.

The preparation of the thyrotropic fraction of Bonsnes and White has been simplified by omitting the isoelectric precipitations at pH 5.4 and 4.8. Thyrotropic hormone activity has been further concentrated by extraction of the 75 per cent acetone-insoluble precipitate with water, and treatment of this aqueous extract with lead acetate and with trichloroacetic acid. The product obtained from whole frozen beef pituitary glands by this procedure gives a positive histological response in the thyroids of white Leghorn chicks when given at a total dose of 1 γ. Growth, gonadotropic, and prolactin activities are absent from the preparations obtained from beef glands. Preliminary studies in the Tiselius electrophoresis apparatus and in the analytical ultracentrifuge indicate the homogeneity of the preparation.

**EXPERIMENTAL**

**Method of Assay**—The biological activities reported in this study are based on the histological changes in the thyroid of the 8 day-old male white Leghorn chick. Injections were started when the chicks were 3 days old. Single, subcutaneous doses were given daily for 5 days, a volume of 0.5 ml. being used for each injection. 24 hours after the last injection, the chicks were killed with illuminating gas and the thyroids carefully dissected out and weighed. The adrenals and gonads were also weighed at this time. The thyroids were then prepared for histological examination in the usual manner. The minimum effective dose, i.e. the minimum amount of the substance required to produce a definitely positive histological response, was determined for each preparation. The histological picture observed microscopically is similar to that in the photographs published recently by Jørgensen and Wade (11). Each dose level has been assayed on a minimum of eight chicks, and each assay was always accompanied by a group of eight uninjected control birds. The histology of the latter group served as a control for possible variations in chicks and in environmental conditions. White Leghorn chicks have been used
exclusively, and the birds were always purchased from the same hatchery. Environmental conditions were controlled by the use of constant temperature brooders. The non-stimulated histological appearance of the thyroids of the control chicks has been quite uniform and has made possible consistent assay results. Questionable stimulation has always been put in the negative group, and only definite, actual changes in the cells, with some resorption of the colloid and few resting interstitial cells, have been graded as a +1 or a minimum response.

A few comments are necessary regarding the chick assay, which is based on the work of Smelser (12). During the course of several years of investigation in this laboratory on the thyrotropic hormone, several thousand chick thyroids have been weighed and subjected to histological examination. At intervals 1, 2, 3, and 4 day-old chicks have been used for comparative assays. The 3 day-old chick was finally selected for two reasons: (1) a high mortality in the younger injected birds was generally encountered, and (2) a resting thyroid in the control non-injected chick was uniformly and consistently obtained when the chicks were permitted to adjust themselves to their new environment for a few days after arrival from the hatchery.

It may be added that little success attended efforts to use thyroid weight increase as a measure of the activity of thyrotropic hormone. Although careful dissection of the thyroids has been regularly conducted, it has not been possible to obtain a significant correlation between gland weight and histological response. It has been a very common experience to obtain definite histological alterations in some instances in which there were no changes in the weight of the thyroids. In other birds, thyroid weight increases were found without histological changes. Therefore, histological response alone was considered an accurate indication of thyrotropic hormone activity.

For purposes of convenience in the presentation of the data, the minimum amount of a preparation required to produce a definite histological response, under the conditions of the assay described above, is termed 1 unit.

Method of Preparation—In so far as possible the procedure is carried out at 3-5° with cold solvents and solutions. All pH measurements were made with the Beckman glass electrode.

1 kilo of frozen whole beef pituitary glands is ground finely in a motor-driven meat grinder. 5 liters of 2 per cent sodium chloride solution are added to the mash. The mixture is thoroughly agitated by means of mechanical stirring and the pH adjusted to 7.4 to 7.8 by the addition of about 50 ml. of 2 N NaOH. The extraction is conducted with continuous stirring for 3 to 4 hours. The tissue residues are removed by centrifuging.

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and the turbid supernatant acidified to pH 4.0 to 4.1 with 2 N HCl. The precipitate is then removed immediately by the use of the continuous Sharples supercentrifuge. To the supernatant (pH 4.0 to 4.1) an equal volume of acetone is added and the mixture allowed to stand overnight. The supernatant solution is siphoned off, clarified by filtration, and to it an equal volume of acetone is added. The precipitate which forms is allowed to settle out overnight. The supernatant acetone solution is siphoned off, and the precipitate is centrifuged and washed with a mixture of 3 parts of acetone and 1 part of water by dispersing the precipitate thoroughly in the aqueous acetone and centrifuging. The washing is repeated three times. The washed precipitate is then dried by triturating with acetone and centrifuging. The acetone washing is repeated three times. Finally, the precipitate is stirred up with ether, centrifuged, and put in a desiccator under a vacuum. After about 15 minutes, the precipitate is thoroughly broken up with a small spatula and replaced in the desiccator. The vacuum is turned on and released several times, the precipitate being broken up finely each time until the product is a fine dry powder. The yield is 5 to 6 gm. (Fraction CA).

An aqueous extract is then made by stirring the dry precipitate successively with one 100 ml. portion and three 50 ml. portions of distilled water in a centrifuge bottle for 5 to 10 minute periods. Each extraction mixture is centrifuged and the supernatant solutions are filtered and combined. The insoluble residue from the last extraction is discarded. The clear buff-colored extract obtained in this manner has a pH of approximately 7. A precipitate appears upon the addition of 1 N NaOH to pH 9. This precipitate is centrifuged off and the pH of the supernatant solution adjusted to 7.0. A solution of 5 per cent lead acetate (Pb(C₂H₃O₂)₂) is added until precipitation is complete. The amount of lead acetate solution required is determined on small measured samples of the water extract. After the addition of lead acetate, the solution is allowed to stand in the refrigerator until the precipitate settles out, leaving a clear supernatant. This takes less than an hour. After removal of the lead precipitate at the centrifuge, the pH of the supernatant varies from 5.0 to 5.4.

To the lead acetate supernatant, 20 per cent (1.25 M) trichloroacetic acid is added slowly and with stirring until a concentration of 8 per cent (0.5 M) trichloroacetic acid is obtained. The white precipitate which appears is allowed to flocculate by standing in the refrigerator for 1 hour and is then removed by centrifugation.

The clear and colorless supernatant, obtained after removal of the trichloroacetic acid-insoluble precipitate, has a pH of about 1.2. This solution is dialyzed in viscose tubing against running cold water until all
trichloroacetic acid has been removed. The dialyzed solution is then concentrated by pervaporation to a volume of 75 to 100 ml. and lyophilized. About 400 to 500 mg. of a white solid designated as Fraction SPbT are thus obtained from 1 kilo of frozen whole beef pituitaries.

The material obtained by the procedure described above produces histological changes in the thyroids of 3 day-old chicks injected over a period of 5 days with a total dose of 1 $\gamma$. It gives the usual protein color tests and is very soluble in water. The Molisch reaction is positive. Preliminary electrophoretic examination of the product, as well as ultracentrifuge studies, suggests that it contains but one protein component. The nitrogen content is 12.6 per cent; sulfur is 1.2 per cent. Tests for phosphorus are negative. Sulfosalicylic acid fails to precipitate the thyrotropically active protein; it is precipitated by phosphotungstic and picric acids, uranium acetate, and mercuric chloride. The lead used in the purification procedure is removed during the dialysis. Tests on the final product with hydrogen sulfide and dithizone indicate the absence of lead.

The thyrotropic hormone prepared by the method described above has been examined for other types of anterior pituitary activity. Growth-promoting action was tested in four hypophysectomized rats by injecting the substance into each animal in doses of 5 mg. daily for a 7 day period. There was no increase in body weight in any of the rats during the injection period.

Prolactin activity has been determined by the systemic method of Lyons (13), employing 6 week-old white Carneau pigeons. A total dose of 5 mg. produced no evidence of prolactin activity.

Gonadotropic activity has been evaluated throughout this study by determining the effect of various preparations on the weights of the testes of the same chicks employed in the thyrotropic assays. Although no effort has been made to determine variations in gonad weight as a measure of gonadotropic activity, certain conclusions are permissible from the many hundreds of chick gonads which have been weighed. The most striking and consistent observation has been the lack of evidence of definite gonadotropic potency in the beef thyrotropic preparation described above. Dr. W. U. Gardner of the Department of Anatomy has kindly examined the effect of the thyrotropic preparation on the gonads of the male hypophysectomized mouse, and reports no evidence of gonadotropic activity.

**DISCUSSION**

It was found that the fractionation procedure of Bonsnes and White (10) may be shortened without affecting the yield of the thyrotropic fraction. This was done by omitting the isoelectric precipitations at pH 5.4 and 4.8. The thyrotropic fraction (CA) represents less than 10 per cent
of the nitrogen originally present in the saline extract of the pituitary glands. The distribution of nitrogen in the course of the fractionation procedure is indicated in the accompanying flow chart.

1 kilo beef pituitary glands

2% saline extract at pH 7.4-7.8 (10 gm. N) Residue

M.e.d., * 0.10 mg.

Adjust to pH 4.1

Ppt. BA (4.5 gm. N) Supernatant

Acetone to 50%

Supernatant Ppt. C (2.0 gm. N)

Acetone to 75%

Supernatant (2.7 gm. N) Ppt. CA (5-6 gm.; 0.7 gm. N)

Extract with water

Residue Extract (0.53 gm. N)

Adjust pH to 9.0

Supernatant Ppt.

Adjust pH to 7.0

Add Pb(C₂H₂O₂)₂ in excess

Ppt. (0.2 gm. N) Supernatant (0.3 gm. N)

Add 1.25 M trichloroacetic acid to 0.5 M concentration

Supernatant Ppt. (0.17 gm. N)

Dialyze, concentrate, † lyophilize

Final product 0.4-0.5 gm. (0.05-0.06 gm. N)

(thyrotropic hormone) m.e.d., 0.001 mg.

* Minimum effective dose in chick assay.

† Solution concentrated in dialysis bag by pervaporation.

A constant fraction of the nitrogen in Precipitate CA is extracted by water. In four different experiments water extraction of various CA fractions yielded 92.4, 91.6, 92.5, and 89.0 mg. of nitrogen per gm. of
Fraction CA extracted. The water-extracted residue was inactive when assayed for thyrotropic activity.

Since thyrotropic activity began to appear in the precipitate at 30 per cent acetone from the water extracts of Fraction CA, the question arose as to whether significant loss of the hormone had occurred at the step just prior to removal of Fraction CA; that is, in the fraction of the saline extract insoluble at pH 4.1 in 50 per cent acetone (Fraction C'). Accordingly, the acetone-dried Fraction C was extracted with water, the extracted material precipitated by the addition of 3 volumes of acetone to the water extract, and the precipitate assayed. The material derived in this way from Precipitate C showed activity only at high dose levels in comparison with that from Fraction CA. In addition, it could be obtained only in small amounts, indicating that little thyrotropic activity was lost by the removal of Precipitate C in the fractionation of saline extracts.

Water extracts of Fraction CA, to which 5 per cent lead acetate solution has been added, still contain protein nitrogen after removal of the lead precipitate. Furthermore, it has been demonstrated that these supernatants from the lead precipitation retain the thyrotropic activity of Fraction CA, while the protein which can be recovered from the lead precipitate by extraction with dilute disodium hydrogen phosphate solution is thyrotropically inert.

Addition of trichloroacetic acid to the lead acetate supernatants precipitates about 55 per cent of the nitrogen present. The proportion of the nitrogen precipitated seems to be independent of the nitrogen concentration. For example, of 0.45, 0.90, and 1.23 mg. of N per ml. in lead acetate supernatants, 45, 45, and 46 per cent remained in solution after the addition of trichloroacetic acid to 0.5 M concentration. About half of the nitrogen in the trichloroacetic acid supernatant is removed by dialysis.

The yields of thyrotropic hormone obtained by the procedure here presented indicate that a good recovery of the hormone originally present in the saline extracts of pituitary tissue is obtained in the trichloroacetic acid-soluble fraction. The saline extract obtained from 1 kilo of frozen whole beef pituitaries contains about 62.5 gm. of protein material ($N \times 6.25$) and represents about 625,000 units of thyrotropic activity. The total yield of the thyrotropic protein fractions is about 500 mg. with a minimum effective dose of 1 $\gamma$. The yield of thyrotropic activity is thus about 500,000 units.

Thyrotropic preparations of equally high potency have been obtained from dissected sheep anterior lobes with the above procedure; however, these preparations always contain gonadotropin material. Beef glands contain relatively little gonadotropic hormone and yield a thyrotropic hormone preparation which is free from gonadotropic activity.
SUMMARY

Marked purification of the pituitary thyrotropic hormone has been achieved by a relatively simple procedure which yields good recoveries of thyroid-stimulating material from frozen whole beef pituitaries. 1 μg of the thyrotropic preparations obtained by this procedure produces definite histological changes in the thyroids of 3 day-old male white Leghorn chicks. The preparations do not have demonstrable prolactin, gonadotrophic, or growth hormone activity.

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