CXI. THE PREPARATION OF A PURIFIED THYROTROPIC HORMONE BY CHEMICAL PRECIPITATION

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DURING the past few years an ever-increasing literature has developed regarding the thyrotropic action of the anterior lobe of the pituitary body, but so far no standardized unit of activity [cf. Junkmann & Schoeller, 1932; Loeser, 1932; Schoedel, 1933; Anderson & Collip, 1934; Rowlands & Parkes, 1934; Stimmel *et al.* 1936] or method of extraction of the gland has been generally adopted.

The hormone is soluble in water and potent extracts have been obtained by numerous workers [Loeb & Bassett, 1929; 1930; Loeser, 1932; Junkmann & Schoeller, 1932; Anderson & Collip, 1934; Rowlands & Parkes, 1934; Greep, 1935; see Junkmann, 1936], the initial stage of extraction involving the use of either acid, alkaline or neutral aqueous media. According to Mueller [1934], the thyrotropic hormone may best be extracted at pH 12.7, but he used only one extraction in his comparative experiments and a limited number of test animals. Junkmann & Schoeller [1932], on the other hand, comment on the instability of the hormone in alkaline solution. In our experience, the later purification of the hormone is facilitated by slightly acidic conditions, which render centrifuging easier, while contamination with soluble protein is less, and the recovery of hormone, after three such extractions, undiminished. Further purification has usually involved precipitation with alcohol or acetone, with or without preliminary removal of protein, followed by treatment with methanol [Loeser, 1932], or 50% pyridine [Rowlands & Parkes, 1934]. Anderson & Collip [1934], working with an evaporated extract, employed fractional precipitation with ammonium sulphate, alcohol and acetone; Junkmann & Schoeller stated, generally, that the hormone may be recovered from suitable precipitates (Junkmann [1936] favouring that obtained with picric acid), whilst Greep [1935] showed that it may be separated from the follicle-stimulating hormone by precipitation with benzoic acid.

In the method described in the present communication, concentration by evaporation has been eliminated, and the use of organic solvents reduced to a minimum. Most of the protein has been first removed by precipitation with salicylsulphonic acid, in dilute solution, and although this results in a loss of a proportion of the hormone, the residue may be rapidly and conveniently recovered in a concentrated form in the ensuing stages.

Method of preparation

Ox pituitary glands¹ were used throughout this series of experiments. Within half an hour of the death of the animals the heads were opened and the glands removed and placed in a refrigerator at about -11° . As soon as the

¹ We desire to express our thanks to the Metropolitan Meat Industry Commissioner, Sydney, and to Messrs Burroughs, Wellcome Co. (Aust.), Ltd., for their kindness in donating and facilitating, respectively, supplies of pituitary glands.

glands were frozen solid they were removed from the abattoirs and taken to the laboratory, where they were dissected and minced without delay, the glands being kept solid in the refrigerator until the moment of dissecting. After mincing, the material was dehydrated with acetone, using a total of 1300 ml. of acetone per 100 g. of mince, in three separate washings. The acetone was filtered off, final traces being removed in a vacuum desiccator and the dried material then powdered in a coffee-mill.

In a typical experiment, the dry milled powder (100 g.) was extracted at 0° with 1500, 500 and 400 ml. of acetic acid (0.5 %) for 24, 12 and 12 hr. respectively. The liquors were separated by centrifuging and totalled about 2 l. They were kept cool and then treated gradually with salicylsulphonic acid (20 %) until a test portion gave no further precipitate; 40–60 ml. of salicylsulphonic acid solution were usually sufficient. The precipitate of protein was centrifuged off and rejected. To the acid liquor, sodium tungstate (10 %)—*ca.* 30 ml.—was added slowly with shaking. The copious precipitate containing the active principle was centrifuged and washed with water (100 ml.). After an hour or so the main bulk of the liquor could be removed by siphoning before centrifuging. The precipitate, when dried, weighed about 4.5 g. Drying was usually omitted and the precipitate dissolved immediately in aqueous NaOH (0.1 %), enough being added to give a faint pink colour with phenolphthalein. Removal of tungstic acid was achieved by precipitation with aqueous BaCl₂ (*M*/20) and Ba(OH)₂ (*N*/2.5), the latter being added to preserve a slightly alkaline reaction. The centrifuged precipitate of barium tungstate was washed with water, which was added to the liquor, and the excess of Ba removed from the latter with Na₂SO₄ (*M*/20). Slow addition to the liquor of ethyl alcoholic benzoic acid (25 ml. of a solution containing 3 g. in 9 ml.), with vigorous stirring, then caused the formation of a heavy precipitate which was centrifuged and washed, first with water, then twice with acetone (200 and 100 ml.), to remove excess of benzoic acid and finally with alcohol and ether. A white powder was thus obtained weighing, when dry, about 2.5 g. (A). This, in doses of 1 mg. per day for 2 days caused marked hypertrophy and hyperplasia of the thyroid glands of guinea-pigs when injected in suspension intraperitoneally. At this stage, the precipitated hormone was insoluble in water, and it was further purified and rendered soluble as follows.

The substance was dissolved in water (25–30 ml.) containing 2*N* NH₄OH (2 ml.) and was centrifuged from a small quantity of insoluble material. The addition of acetone (10 vol.) caused only a faint turbidity, even after 12 hr. at 0°, and centrifuging gave only a few mg. of precipitate, which was discarded. The addition of alcoholic benzoic acid (2 ml.) to the aqueous acetone solution, however, brought down a copious precipitate, which was now almost completely soluble in distilled water (70 ml.). From this solution the hormone was thrown down with acetone (35 ml.) as a white amorphous precipitate, which after drying yielded a white powder (B), about 1.6 g. This powder was readily and completely soluble in water. A further purification by dissolving in water and precipitating with acetone does not appear to be necessary. It is interesting to note, however, that the hormone (B) now required, for complete precipitation, the addition of 10 vol. of acetone, yielding a white powder (C) weighing 1.4 g. when dry.

Properties

The final product (C) is very readily soluble in water and causes a definite change in the thyroids of 150–200 g. guinea-pigs in doses as low as 0.1 mg. spread over 2 days [cf. Junkmann & Schoeller, 1932]. It is free of gonadotropic activity as tested on four immature female rats in doses up to 1 mg. per day for 3 days

[*vide* Fevold & Meyer, 1934]. It gives a precipitate, in concentrated solutions, with salicylsulphonic acid.

The products (B) and (C) have been kept at 0° for 12 months without loss of activity or solubility. Various authors have commented on the insolubility of the hormone in concentrated organic solvents. However, if the above products (B) and (C) be triturated with acetone containing a trace of aqueous ammonia, centrifuged, and then dissolved in the minimum quantity of water, the addition of acetone up to 100 vol. does not cause a precipitate after 48 hours. The addition of a small quantity of alcoholic benzoic acid or a trace of an inorganic salt, however, brings about a complete precipitation.

The hormone (C) has been readily tolerated, under clinical conditions, when injected in doses up to 200 mg. (2000 units) per week.

Notes on method of preparation

(1) The salicylsulphonic acid precipitate (about 7 g.) contains a proportion of the hormone which was not recovered. Doses of 50 mg. for 2 days (injected intraperitoneally in suspension) gave a positive thyroid response. Probably at least 10% of the hormone is thus discarded, but the recovery of the residue appears to be quantitative.

(2) A second precipitation from the liquor with tungstic acid gave a product devoid of activity, even in large doses, so that the one treatment appears to be sufficient. Moreover, the liquors obtained after prior treatment with tungstic acid yielded inactive residues on evaporation *in vacuo*.

(3) Alkaline extraction (0.1% NaOH) yielded 0.44 g. from 15 g. of acetone-dried powder at stage (A). If the alkaline extract was first acidified with acetic acid a copious, highly coloured protein precipitate was obtained which was practically free of thyrotropic activity. Further precipitation with salicylsulphonic acid gave a liquor which could be treated, as outlined above, with sodium tungstate.

(4) Various workers have criticized the histological changes in the guinea-pig's thyroid as unreliable evidence of the thyrotropic activity of pituitary extract, but we agree with Junkmann & Schoeller [1932], Loeser [1932] and Greep [1935] that, with experience, an approximately quantitative expression of the activities of varying extracts may be obtained in comparative experiments. We have always used duplicate guinea-pigs, weighing 150–200 g., as test animals, these being injected intraperitoneally for 2 days and killed upon the third. The degree of response of the guinea-pig was graded according to:

- (i) character of epithelium, whether flattened, cubical, semi-columnar or columnar;
- (ii) presence of hyperplasia;
- (iii) absorption of colloid.

The first of these may be taken as the most reliable index, although some vacuolation of colloid was taken as indicative of slight activity before obvious changes in the epithelium occurred. Only when definite changes in the epithelium were observed was the reaction taken as positive.

Behaviour towards heat

It is usually considered that the thyrotropic principle is destroyed by heat when in aqueous solution. Krichesky [1934], however, using tadpoles as experimental animals, considers the hormone to be heat-stable.

In order to determine the behaviour of the thyrotropic hormone to heat, the following experiments were carried out. Extracts were made from fresh and dried glands, both by heating and in the cold, and the extracts were injected into guinea-pigs intraperitoneally. The histological appearance of the thyroid gland gave an index of the stability of the hormone to heat.

Extract I. To 10 g. of fresh minced pituitary gland was added acetic acid (0.5%, 40 ml.) and the mixture heated at 100° for 30 min. The extract was passed

through a Seitz filter, collected in ampoules and maintained at 0° until injection, as soon as possible after extraction.

Extract I *a*. To 10 g. of fresh minced gland was added acetic acid (0.5%, 40 ml.), and the mixture kept at 0° for 20 hr. with occasional shaking. The extract was passed through a Seitz filter as above.

Extract II. To 2 g. of acetone-dried powder was added acetic acid (0.5%, 40 ml.), and the mixture heated at 100° for 30 min. It was filtered and collected as above.

Extract II *a*. To 2 g. of acetone-dried gland powder was added acetic acid (0.5%, 40 ml.), and the mixture kept at 0° for 20 hr., with occasional shaking, and filtered and collected as above.

Extract III. To 7.5 mg. of purified hormone (C) was added acetic acid (0.5%, 7.5 ml.), and the mixture heated at 100° for 30 min. When cool, this was used for injection after adjusting to about pH 8.

Extract III *a*. 2.5 mg. of purified hormone (C) were dissolved in 2.5 ml. of isotonic saline and used for injection.

Extract III *b*. From extract III a precipitate formed on heating. This precipitate was suspended in 2 ml. of saline, and 1 ml. was used for the injection of each of two guinea-pigs.

In every case 1 ml. of the extract or suspension was used for injection of the guinea-pigs on each of two consecutive days; the animals were killed on the third day. Extracts III, III *a* and III *b* were made fresh on each occasion; the others were kept at 0° for the duration of the experiment.

<i>Results</i>			
Extract I	A (+)	B -	C +
Extract I <i>a</i>	D + + + (+)	E + + + (+)	
Extract II	F (+)	G + (+)	H + (+)
Extract II <i>a</i>	I + + + +	J (died)	
Extract III	K (+)	L (+)	M (+)
Extract III <i>a</i>	N + + + +	O + + +	
Extract III <i>b</i>	P +	Q + +	
Controls	R -	S -	

N.B. Each letter represents one guinea-pig. + sign indicates an approximate unit of activity of the extract; (+) sign indicates a doubtful unit; - sign denotes inactivity.

From these results it is concluded that under the above experimental conditions the hormone, either in the fresh or dried glands or, when purified, is almost entirely destroyed by heat. The question whether a separate metamorphic thermostable factor really exists [cf. Spaul, 1930; Uhlenhuth & Schwarzbach, 1935] as well as the mammalian principle, seems to render a series of comparative experiments on both guinea-pigs and tadpoles or axolotls highly desirable.

SUMMARY

1. A method is described for the rapid recovery of thyrotropic hormone from its aqueous extracts.
2. The behaviour of solutions of the hormone towards heat is examined.

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