ZONE ELECTROPHORESIS OF CRUDE PITUITARY EXTRACT

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While studying the nature of pituitary components involved in tumorigenesis in different species[1], the need arose to obtain these substances free from each other, and if possible, to preserve their native state.

The chemical fractionation procedures used so far, often resulted in the isolation of biologically highly active proteins. The native state of these hormones, however, remains doubtful. It has been demonstrated[2,3] for instance that even salt precipitation may change the isoelectric point of ICSH** and ACTH fractions; barium, calcium and lithium chlorides[4] and IO_3^- ions[5] alter the biological activity of gonadotrophins; changes in pH and temperature influence both the biological and physicochemical behaviour of various hormones[6–10]. Precautions have to be taken, lest proteolytic enzymes, present in aqueous pituitary extracts, destroy hormonal activity[11,2]. One of the most gentle techniques for the isolation of proteins is zone electrophoresis on a stabilizing medium, provided that (a) the supporting medium does not irreversibly adsorb the protein, (b) the temperature is kept constant at *ca.* 0°, (c) low ionic strength buffers and "inoffensive" ions are used, (d) a physiological pH value is chosen[12]. Starch block electrophoresis was found to be the best approach to the fulfilment of these conditions, and was therefore applied as the sole purification step in the studies described.

METHODS

Preparation of extract

Rats (strain R, Amsterdam, inbred AvL) were killed by decapitation after slight ether anaesthesia. The pituitaries were removed immediately, the anterior lobe dissected and transferred to ice-cold 0.005 *M* phosphate buffer of pH 7.4 (180 mg wet tissue/6 ml solution). Homogenization was carried out in an all glass homogenizer with a loose fitting pestle, while cooled in an ice bath. The homogenate was then centrifuged at 20,000 × *g* (60 min at 0°) in a PHYWE (Eispirouette) centrifuge. The supernatant was carefully pipetted and used for the electrophoretic experiments.

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** Abbreviations: STH = somatotrophic hormone (somatotrophin, growth hormone); TSH = thyroid stimulating hormone (thyrotrophin); ICSH = interstitial cell stimulating hormone (luteinizing hormone); FSH = follicle stimulating hormone; LTH = luteotrophic hormone, prolactin, mammotrophin; ACTH = adrenocorticotrophic hormone.

Electrophoresis

Vertical starch block electrophoresis at 6 V/cm, 6–8 mA, in phosphate buffer 0.025 μ, pH 7.4 for 16 to 22 hours was performed as described earlier[13]. The temperature of the block was kept between 0° and 3°, controlled at intervals during each experiment by measuring the electrical resistance of a thermistor inserted into the starch. The temperature of the room was —8°, hence the outer compartments of the electrode vessels had to be heated by an electrical element enclosed in glass, in order to maintain a constant temperature of 1° of the electrode buffer. After the electrophoretic run the block was sliced into 1 cm segments, using an improved cutting device[14]. Each segment was eluted with 6 ml ice-cold distilled water, the starch removed by brief centrifugation at 0°; the pH of the eluates remained between 7.1 and 7.7. The clear supernatant was used for estimation of protein content and for the bio-assays.

Protein estimation

Protein was determined by the modified Folin procedure according to LOWRY *et al.*[15]. The results were converted with the aid of a casein standard.

Bio-assays

All tests were performed in hypophysectomized immature male rats of the same strain (R, Amsterdam, inbred AvL) as the pituitary donors. Hypophysectomy was carried out by a method described by THOMAS[16] for mice, adapted for infantile rats, which gives a higher score of reliability[17] than the usual method of SMITH-FREUD.

0.2 ml aliquots of eluate from each segment to be assayed were injected into groups of 4 test animals at 24 hour intervals during 5 days. In addition each test animal received 5 μC ^{131}I ($Na^{131}I$, carrier-free, in sterile isotonic saline) concurrently with the last injection, 24 hours prior to killing.

For estimation of STH activity the tibia test[18] was used.

TSH recovery was determined by measuring the uptake of radioactive iodine by the thyroid gland[19].

The ventral prostate test[20] served as a measure for ICSH activity.

Increase in testicle weight[21], not accounted for by STH or ICSH, was considered to reflect FSH activity.

The degree of involution of the thymus gland[22] was used as indication for ACTH activity.

RESULTS

In a series of 20 experiments the protein pattern as well as the distribution of hormone activity obtained after each electrophoretic run were reproducible. In the first experiments all forty segments of the block were tested for their hormone activity, using only 2 to 3 test animals per segment. These experiments showed that all hormone activities were located in two regions of the starch block. Therefore in the subsequent experiments only fractions —10 to —4 and fractions +2 to +8 were tested for hormone activities. Fig. 1 shows such a typical distribution pattern. Obviously

maximal hormone activity eluted from the various segments does not coincide with the protein peaks, except for STH. This latter hormone could be entirely freed from all other hormones. A total dose of 50 μg in 5 days of this preparation caused a width of 235 μ of the tibia cartilage plate. Complete separation of TSH, ICSH, FSH

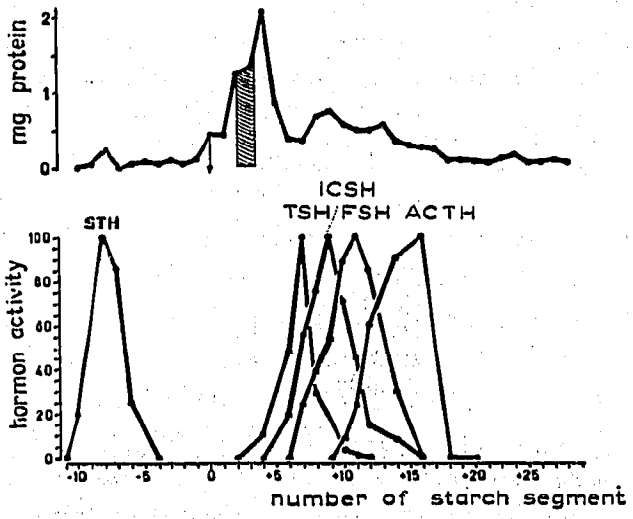

Fig. 1. Upper half: Protein distribution pattern after vertical starch block electrophoresis, 6 V/cm, 16 h in phosphate buffer 0.025 μ and pH 7.4. Segments 3 and 4 contain a haemoglobin band. The arrow indicates the starting point. Lower half: Hormone activity curve corresponding with the separated protein (upper half). The highest activity for each of the five hormones assayed in the respective segments was arbitrarily indicated as 100%. Absolute recovery values are given in Table I.

and ACTH could not be achieved by the single purification step applied. However, the fractions containing the highest activity of the four partially overlapping hormones showed a remarkable enrichment (*cf.* Table I); *e.g.* 40 % of the total TSH activity of the crude extract was recovered in fraction 5, which contained only 0.1 % FSH and 7.1 % ICSH. Neither STH nor ACTH activity could be detected in this fraction.

The recovery values were obtained for each segment by extrapolating the results of the bio-assay with the results obtained after injecting two dilutions of the crude extract (used in these particular experiments) into two groups of 4 test animals. For this purpose an amount of crude extract equal to that used for the electrophoretic experiment was mixed with starch and kept at 0° to 3° for the duration of the experi-

TABLE I

PERCENT RECOVERY IN THE FRACTIONS OF MAXIMAL ACTIVITY FOR THE FIVE HORMONES ASSAYED

Hormone activity assayed	*Segment number*				
	−8	*+5*	*+8*	*+11*	*+15*
STH	42	0	0	0	0
TSH	0	40	19	6.1	0
ICSH	0	7.1	37	12.2	0
FSH	0	0.1	11.4	30	10
ACTH	0	0	0	0.7	30

ment. It was then eluted with 6 ml of distilled water and centrifuged in the same way as for the segments. The supernatant of the crude extract, diluted with distilled water 1:1 and 1:9, was used for the injection into the two groups of test animals. The "peak fractions" (segments —8, +5, +11, +15) injected in two dilutions (undiluted and 1:4) showed exactly the same slopes as those obtained with the crude extract. This demonstrates that no alteration in biological behaviour was caused by the electrophoretic run.

TABLE II

TOTAL RECOVERY OF HORMONE ACTIVITY

These values were obtained by adding up the recovery percentages for each hormone in all fractions.

Hormone	*Recovery* %
STH	92
TSH	97
ICSH	120
FSH	115
ACTH*	—

* The thymus-involution test is not suitable for crude extracts (crude extracts contain STH, which antagonizes ACTH in this test).

Furthermore it was shown that mixing of the crude extract with starch did not result in detectable loss of hormonal activity.

The total recoveries for each hormone assayed are given in Table II. These values are the result of adding up the recovery percentages of each hormone in the various segments.

DISCUSSION

Zone electrophoresis on starch under the working conditions described, as the sole step in the fractionation of crude pituitary extracts has certain advantages in comparison with more complicated purification schemes, in which the separated end products are submitted to electrophoresis. All active factors of the original extract undergo exactly the same treatment, in no case is isolation achieved by the destruction of others. No loss or alteration of biological activity can be detected.

It appears from the results that the positions of the TSH fraction and of the STH fraction are not in accordance with the isoelectric points reported for these hormones (after their isolation by various purification steps) by other authors. HAYS AND STEELMAN[23] came to the conclusion that purified TSH had an isoelectric point between pH 8 and 8.5. Our experiments demonstrate that TSH should have an isoelectric point of less than 7.4. The reverse holds for the STH fraction. According to FØNSS-BECH AND LI[24] the isoelectric point of growth hormone obtained after the purification procedures described by these authors was 5.8, whereas the position of our STH activity suggests an isoelectric point higher than 7.4. This cannot be accounted

for by electroosmosis, since we corrected for this phenomenon by vertical arrangement of the block and by a difference in level of the buffer solution in the electrode vessels, so that electroosmosis was completely counterbalanced. Species specificity[25] may be an explanation for the differences found. However, parallel experiments with mouse and bovine pituitary tissue indicated that the position of the various hormonal activities is not specific for the rat pituitary[26]. In this connection it should be pointed out that POSTEL[27] using horizontal zone electrophoresis as the sole purification step for TSH, claimed to have found the hormone activity in quite a different position from that observed in our experiments. Further experiments are in progress to elucidate this question.

The position of ICSH and FSH are in agreement with the isoelectric point values reported by SQUIRE AND LI[2], LI *et al.*[28] and STEELMAN *et al.*[29].

With the biological assay employed no information on the localization of the LTH activity could be obtained. In one experiment LTH activity was therefore estimated by the method described by HADFIELD[30], using hypophysectomized mice of the A2G strain. In this experiment the LTH activity appeared to be localized in fractions 4,5 and 6 (Fig. 1), partially overlapping the TSH region. Since the method is cumbersome and appears not to be specific[31], it was not employed in further experiments. The information on the five other pituitary hormones was obtained by evaluating the response of the various target organs in the same test animals. We consider that in hypophysectomized test animals the criteria used for STH, TSH, ICSH and FSH are beyond reproach. The criteria for ACTH activity, however, are open to criticism and a better ACTH test for our purpose is being developed. Nevertheless, the bio-assay technique employed supplied a maximum of information with relatively *few* test animals (1700 rats!) and permitted us to test all segments containing hormone activity.

WALLACE AND FERGUSON[32] applied continuous electrophoresis as sole purification step to sheep pituitary extract without success. We ascribe their failure to the fact that these authors pooled their fractions according to the peaks of the protein distribution pattern. Our experiments show that this way of pooling fractions is quite an arbitrary one, since inactive proteins are responsible for most of the peaks as estimated with the Folin reagent.

SUMMARY

1. Starch block electrophoresis, under standardized conditions, of water-soluble protein hormones from rat anterior pituitary tissue is described. The recovery of four of the five hormones assayed proved to be 90–100 %.

2. A pure growth-promoting substance has been isolated by a single run.

3. The maxima of the separated hormone activities do not coincide with the protein peaks, with the exception of somatotrophic hormone (STH).

4. Follicle stimulating hormone (FSH), thyroid stimulating hormone (TSH) and interstitial cell stimulating hormone (ICSH) were partially separated. The peaks of both FSH and TSH contained only one major contaminant (ICSH).

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