EFFECT OF TRICHLORACETIC ACID ON BASOPHILS
AND ON CORTICOTROPHIN CONTENT OF
HUMAN PITUITARY GLANDS:
EXTRACTION OF LOW MOLECULAR WEIGHT
CORTICOTROPHIN

By

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ABSTRACT

Experiments are reported which show that 2.5\% trichloracetic acid (TCA) causes marked loss of periodic acid-Schiff staining of the basophil granules of the pituitary gland and that there is extraction of some ACTH into the TCA. There is, however, no direct correlation between the basophil changes and the extraction of the hormone from the gland. The vesiculate chromophobe, the colloid and the cytoplasmic granules of the acidophils are not apparently affected by TCA treatment.

Little is known about the chemical state of storage of ACTH in the human adenohypophysis. The amino acid structure and sequence of human ACTH has recently been proposed but there is as yet no chemical basis for a histochemical technique specific for ACTH.

The 2.5\% TCA-extractable ACTH, which represents about 30\% of the total ACTH activity of batches of 'old' pituitary glands, has been characterized by ultrafiltration, Sephadex column chromatography and ultracentrifugation as a single component of minimum molecular weight about 3200. This is a simple method for extracting low molecular weight ACTH-extraction in 2.5\% TCA (or water acidified to pH 5.0) for 1 hour at 4°C followed by ultrafiltration and Sephadex column chromatography.

About 68\% of the original activity in the TCA extract is recovered in the final product. Extraction was equally efficient whether 'pieces' or homogenates were used and in the presence or absence of posterior lobe tissue. Autolysis results in a decrease of both residual gland and TCA-extractable hormone.

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The nature of the hormone remaining in the residual gland after extraction is not known but it is presumed that it is protein or protein-bound and it is suggested that ACTH exists in the human pituitary gland in two chemical forms.

The possible physiological significance of the yield of low molecular weight ACTH in single 'fresh' pituitary glands is being studied.

Corticotrophin (ACTH) has been extracted from human pituitary glands (Lyons 1937; Hewett et al. 1954) and lyophilized extracts have been assayed for ACTH activity (Taylor et al. 1953). Recently Lee et al. (1961) have published a proposed amino acid sequence for the human hormone but little is known about its chemical state of storage in the pituitary gland. It is believed to be produced and secreted by the basophils (Currie & Symington 1955; Currie et al. 1958; McGarry et al. 1962) although it is not a glycoprotein as are other hormones produced and stored in these cells.

The present investigation began with a study of the effects of various solutions, precipitants or 'solvents' of animal proteins and known pituitary hormone extractants, on the staining properties of human pituitary cells. The aim was to find a solvent which selectively removed the acidophil or basophil granules, which did not otherwise interfere with the cell structure or with the staining methods used to demonstrate it, and which did not interfere with the biological activity of the hormones. None of them completely satisfied the criteria for the 'ideal' solvent, but treatment with trichloracetic acid (TCA) caused marked loss of the staining of basophil granules in sections stained by the periodic acid-Schiff (PAS) technique and did not apparently affect the cytoplasm of the acidophils. It did not interfere with the biological activity of ACTH, as measured by a bioassay method based on adrenal ascorbic acid depletion in cortisol-treated rats (Sayers et al. 1948; Hodges & Vernikos 1958; Dekanski & Harvie 1960).

The first part of this paper deals with the histological and histochemical changes produced in the mucoid cells by treatment with TCA and with the attempt to correlate these changes with extraction of ACTH from the glands. We found that only a proportion of the ACTH activity was extracted with TCA and in the second part of this communication we present the results of an investigation of the yields of TCA-extractable ACTH and of the residual gland hormone under varying experimental conditions. Thirdly we record our findings on the chemical nature of the TCA-extractable ACTH, and consider the chemical state of storage of the hormone in the gland.

**MATERIALS AND METHODS**

**Histological methods.** – The pituitary glands were cut in the horizontal plane and half or quarter was placed in TCA (0.1%o, 0.5%o, 1%o or 2.5%o) and the remainder in 10%o formol sublimate. The TCA-treated tissue was transferred about 24 hours later to the
same fixative. The tissue was thereafter dehydrated, cleared and paraffin-embedded. Sections cut at 5 μ were stained routinely by the periodic acid-Schiff-trichrome (PAS) technique and occasionally by other methods, including the performic acid-alcian blue-PAS technique (Adams & Swettenham 1958).

Extraction of ACTH. – Experiments involving extraction of single pituitary glands were done on complete glands collected less than 6 hours after death and stored at -20° C until required. Extractions of batches of pituitaries were always carried out on 'old' glands obtained more than 6 hours, and usually about 24 hours after death, and then stored at -20° C for intervals of up to several months.

After removal from store at -20° C the glands were kept on solid carbon dioxide before and after cutting. When single glands were extracted the pituitary gland was cut sagittally into two and then into a total of sixteen pieces. If two or three methods of extraction were to be compared a number of glands were used and care was taken to ensure that the batches were comparable with representative pieces of the glands in each batch. In one experiment homogenates were compared with pieces of glands extracted as already described: batches of 'sliced' pituitaries were homogenized in a pre-cooled Waring blender.

Twenty ml of solvent was used per gland. The solvents used were freshly prepared 2.5% TCA, freshly prepared 5% TCA, and distilled water acidified to pH 5.0 (1 litre distilled water acidified with 0.075 ml 0.01 n HCl). The mixture was stirred with an electric stirrer, vigorously but without frothing, for 24 hours or 1 hour at 4° C. It was centrifuged in tubes and trunnions pre-cooled in the deep freeze.

After removal of the residual glands by centrifugation, moistened De-acidite FF resin in the carbonate form was added stepwise to the extract in a beaker. Addition of resin was continued until the pH reached 2.7 in order to reduce the acidity of the extract for freeze drying and for injection into rats. (This pH is lower than the isoelectric point for ACTH.) The extract from a single gland was dried over P2O5 in a vacuum desiccator whereas that from a batch of glands was lyophilized.

ACTH extraction from residual gland. – Acetone-dried residual glands were extracted by three triturations with 75% acetone adjusted to pH 1.5-2.0. Three ml was used per trituration and the volume finally made up to 10 ml per gland. This was then poured into 90 ml acetone per gland, mixed well and allowed to stand overnight at 4° C. It was centrifuged and the precipitate finally all retained in one tube in which it was dried over P2O5 in a vacuum desiccator.

Ultrafiltration. – The lyophilized extract was dissolved in 2 n acetic acid giving pH of 2.0 (Curtis-Jones et al. 1950). Five ml acid was used per original pituitary gland. A fully soaked PT 300 cellophane membrane (pore size 3-4 mμ; impermeable to crystalline ribonuclease, molecular weight 13 700, and permeable to salmine, molecular weight 8000) was used under 6 kg/cm² nitrogen pressure at 4° C in a stainless steel apparatus supplied by Ultrafilter, Göttingen. The ultrafiltrate was lyophilized. The freeze-dried material was acid free. The film of material on the ultrafiltration membrane was dissolved in a total of 10 ml 0.9% NaCl containing 0.2 ml 0.01 n HCl added stepwise with 'rodding' after which the membrane was cut into fine shreds and left soaking in this saline overnight.

Sephadex column chromatography (Dr F. C. Greenwood). – A preliminary and a principal experiment were carried out using in both cases G-25 (about 30 g) and G-50 (about 15 g) Sephadex columns. To prepare and elute the columns 0.005 n HCl was used. The column parameters were determined by using crystalline bovine serum albumin and 0.07 m sodium barbitone. In each experiment a lyophilized ultrafiltrate from a 2.5% TCA extract from 27 'old' human pituitary glands was put on a G-25
column. In the preliminary experiment this was dissolved in 9 ml 0.005 N HCl but in the principal experiment, carried out at 4°C, when 0.5 ml of 0.005 N HCl was added to the lyophilized ultrafiltrate it gave a brown viscous solution. Addition of more HCl led to cloudiness and therefore 2 N acetic acid was added and the solution made up to a final volume of 10.2 ml. It was centrifuged at 2500 r. p. m. for 20 minutes to give an opalescent supernatant and a residue, which after digestion was found to account for only 0.155 mg N out of a total of 40.88 mg N in the ultrafiltrate used. Nine ml of the supernatant was put through a G-25 column. In both experiments Peak I was dissolved in 0.005 N HCl and re-run on a G-50 Sephadex column. In the principal experiment the weight of lyophilised Peak I was 13.5 mg and it dissolved easily in 3.0 ml 0.005 N HCl.

**Ultracentrifugation** (Dr P. A. Charlwood). — This was carried out on Peak Ib (Series 2) at a concentration of 10 mg/ml. Sedimentation velocity experiments were done at room temperature in a Spinco Model E machine in the usual way (Charlwood 1955). Owing to the low sedimentation rates to be measured, the cell used was of the synthetic boundary type (Klainer & Kegeles 1955) and the ultracentrifuge was operated at the highest permissible speed (59 780 r. p. m.). Since ordinary dialysis membranes were permeable to the solute, solutions were prepared by dissolving weighed amounts of sample in measured volumes of 0.1 m potassium chloride and the same potassium chloride solution was layered over the sample solution during the process of boundary formation. Sedimentation coefficients were corrected to water at 20°C in the usual way. The partial specific volume assumed in making the corrections (0.736) is not a critical factor in this experiment.

**Corticotrophin bioassay.** — The assay was a modification of the method of Dekanski & Harvie (1960) which in turn was developed from the method of Sayers et al. (1948). It is based on the decrease in adrenal ascobic acid caused by subcutaneous injections of ACTH in the cortisol-treated rat. Our modifications were to use a six- instead of a four-point assay, to employ different doses and injection times for the cortisol and to inject the ACTH at different hours.

Thirty male rats (140–170 g weight) were used per assay. These were obtained from Messrs Tuck, Rayleigh, Essex, or were the hybrid strain of the Imperial Cancer Research Fund, Mill Hill, London. Either pelleted diet (Bruce & Parke 1949) or later GR2 diet (Wyatt Ltd, Chard, Somerset) was used, food and water being given **ad libitum**. Intraperitoneal injections of cortisol acetate (11.3 mg/150 g rat) ("Cortel" acetate, Upjohn Ltd) were given on Day 1 at 3.15–3.30 p. m. and on Day 2 at 9.00 a. m. Doses of ACTH were diluted in 0.9% saline containing 0.2 ml 0.01 N HCl/10 ml. Standard doses of a total of 0.13, 0.40 and 1.2 International Units (IU) of crude acid-acetone preparations (Organon Ltd) were injected subcutaneously into the nape of the neck on Day 2 at 11.30 a.m., 12.15 and 1.30 p.m. The ratio between the dose levels of the 'unknown' preparation, injected at the same times, was also 3. The response was measured 1 hour after the last injection of ACTH when the adrenals were dissected, defatted, weighed to 0.1 mg and homogenized in 10 ml 2.5% metaphosphoric acid (MPA).

The ascobic acid was estimated spectrophotometrically by a phenol-indo-2:6 dichlorophenol method, modified from the reaction described in the XVI USP Pharmacopoeia, 1960. Our method followed the procedure of Dekanski & Harvie (1960) except that the sensitivity was improved by using 5 ml 4.53% w/v sodium acetate, hydrated, adjusted to pH 7.2 with 0.1 N acetic acid, by using 40 mm cells to increase the light path and by reading at 460 mu in a Unicam SP 600 spectrophotometer against distilled water. An aliquot of 8 ml of the homogenate was used if the adrenal weight was < 35 mg, and 5 ml aliquot plus 3 ml 2.5% MPA if > 35 mg.

By this assay method, it proved possible to identify correctly the strength of solu-
tions, ranging from 0.8 to 2.0 IU 2nd International Standard ACTH/ml, sent from another laboratory and tested as 'unknowns'. Discrimination was at least as good as 0.4 IU/ml. No tests for hormonal specificity were carried out in this laboratory but Sayers et al. (1943) have stated that adrenal ascorbic acid depletion is only caused by ACTH. The assays were analysed by Dr C. C. Spicer, using the method of Bliss & Marks (1939), and were statistically valid. Confidence limits of error calculated at the 5% level are given for the individual assays in the Tables: mean limits of error were 58.7–174.8. The indices of precision (\(\hat{z}\)) for four subcutaneous log. dose-response lines were 0.26, 0.27, 0.29 and 0.26 (mean 0.27).

RESULTS

Histology and histochemistry

After TCA extraction the intensity of PAS-staining of the granules of the basophils is much reduced (Fig. 1). This does not, however, apply to all PAS-

![Fig. 1.](image)

Human adenohypophysis. Periodic acid-Schiff-trichrome. \(\times 500\).

a. Control to show normal PAS-staining of basophil cells (dark cells) and of colloid (below). The majority of the other cells are acidophils.

b. Adenohypophysis (part of same gland as a) treated with 2.5% TCA. The majority of the cells are basophils but they show marked reduction in PAS-staining. Note the colloid (below) stains deeply.

c. Adenohypophysis treated with calcium hydroxide \(pH\) 11.0. There is marked distortion of the cells and it is difficult to distinguish one cell type from another. The colloid still stains deeply.
positive material since the vesiculate chromophobe and the colloid still stain brilliantly with the fuchsin. 'S' cells, that is cystine-rich basophils, are identifiable after TCA extraction (Currie & Sutton 1962), and the cytoplasm of the acidophils is apparently unaffected. There was no correlation of the cellular changes with the 'shift' of ACTH from tissue to solvent.

**Amount of 2.5% TCA-extractable and of residual ACTH**

Four batches of 'old' pituitary glands were extracted with 2.5% TCA for 24 hours. The hormonal activity of the extracts and of the extracted residual glands was determined, and it was shown that approximately one-third of the hormone was extracted by TCA (Table 1). The average total ACTH per gland in the four batches was about 15 International Units (IU).

We compared the efficiency of extraction using homogenates instead of 'pieces', and showed that the same yield of extractable hormone was obtained from both (Table 1, Batches A1, A2). No further activity was found on re-extraction or re-homogenization of these batches. The presence of the posterior pituitary lobe had no influence on the extraction results (Table 1, Batches B1, B2) and autolysis was associated with a marked decrease in the activity of both the residual gland and the extractable hormone.

Extraction with 2.5% TCA was compared with extraction using 5% TCA and water acidified to pH 5.0. This pH was chosen because the isoelectric point of crude ACTH preparations is pH 3.6 and for polypeptide ACTH (α-corticotrophin from sheep or corticotrophin A from pig) pH 6.8–8.0. There was approximately the same yield of hormone with the three solvents (Table 2, Batches D1, D2, D3).

When the time of extraction was reduced from 24 hours to 1 hour no difference was found in the hormonal activity of 2.5% TCA extracts (Table 2, Batches H1, H2). The hormone yield was the same when glands were extracted for 1 hour at 4°C with 2.5% TCA or distilled water acidified to pH 5.0 (Table 2, Batches K1, K2).

**Chemical nature of TCA-extractable hormone**

**Ultrafiltration.** – Three 24-hour 2.5% TCA extracts were ultrafiltered and the mean recovery of biological activity in the ultrafiltrates was 86% (Table 3). When the time of extraction was reduced from 24 hours to 1 hour the percentage of the original biological activity in the ultrafiltrate was 100% (Table 3, Batch K1). Batch K2 (Table 3) was extracted for 1 hour with water acidified to pH 5.0 and the percentage of the original biological activity in the ultrafiltrate was as high as that obtained with 2.5% TCA.

**Sephadex column chromatography** (Dr F. C. Greenwood). – The preliminary experiment with an ultrafiltrate from a 2.5% TCA extract of human pituitaries showed that on the G-25 Sephadex column, the first material to be eluted
Table 1.
ACTH content of 2.5% TCA extract and of residual tissue after TCA extraction of 'old' human pituitary glands.

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Description</th>
<th>Equivalent number of pituitaries</th>
<th>Wet weight (g)</th>
<th>2.5% TCA extract</th>
<th>Extracted residual glands</th>
<th>Total ACTH/extractable ACTH as % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Potency</td>
<td>/% Limits of error</td>
<td>ACTH content</td>
</tr>
<tr>
<td>Mean of 4</td>
<td>Stirred pieces</td>
<td>12.7</td>
<td>6.99</td>
<td>1.32</td>
<td>54.1-204.0</td>
<td>4.67</td>
</tr>
<tr>
<td>(A1, C1, D1, L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A1</td>
<td>Stirred pieces</td>
<td>10.0</td>
<td>5.64</td>
<td>0.78</td>
<td>55.8-242.0</td>
<td>3.72</td>
</tr>
<tr>
<td>A2</td>
<td>Homogenate</td>
<td>10.0</td>
<td>5.94</td>
<td>0.83</td>
<td>67.6-148.0</td>
<td>3.96</td>
</tr>
<tr>
<td>B1</td>
<td>Anterior lobes</td>
<td>10.0</td>
<td>4.38</td>
<td>1.97</td>
<td>64.4-164.2</td>
<td>7.85</td>
</tr>
<tr>
<td></td>
<td>Anterior lobes</td>
<td>10.0</td>
<td>4.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>posterior lobes</td>
<td>20.0</td>
<td>2.12</td>
<td>1.92</td>
<td>61.9-178.8</td>
<td>7.65</td>
</tr>
</tbody>
</table>

* Calculated at the 5% level.
† Aliquots of 1/3 total B1 and 1/6 total B2 were used for bioassay.
Table 2.
Amount of extractable and residual ACTH in 'old' human pituitary glands after treatment for 1 or 24 hours with several extractants: 2.5 % TCA, 5 % TCA, distilled water acidified to pH 5.0.

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Extractant</th>
<th>Equivalent number of pituitaries</th>
<th>Wet weight (g)</th>
<th>Time of extraction (h)</th>
<th>Extract</th>
<th>Extracted residual glands</th>
<th>Total ACTH/batch (IU)</th>
<th>Extractable ACTH as % of total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Potency</td>
<td>% Limits of error*</td>
<td>ACTH content (IU)</td>
<td>Potency</td>
</tr>
<tr>
<td>D1</td>
<td>2.5 % TCA</td>
<td>4.3</td>
<td>2.78</td>
<td>24</td>
<td>1.34†</td>
<td>52.5–190.0</td>
<td>23.8</td>
<td>2.57</td>
</tr>
<tr>
<td>D2</td>
<td>5.0 % TCA</td>
<td>4.3</td>
<td>2.87</td>
<td>24</td>
<td>1.15†</td>
<td>58.9–176.9</td>
<td>20.4</td>
<td>2.73</td>
</tr>
<tr>
<td>D3</td>
<td>Water pH 5.0</td>
<td>4.3</td>
<td>2.85</td>
<td>24</td>
<td>0.64†</td>
<td>64.6–147.0</td>
<td>20.2</td>
<td>2.33</td>
</tr>
<tr>
<td>H1</td>
<td>2.5 % TCA</td>
<td>6.0</td>
<td>3.68</td>
<td>1</td>
<td>1.49</td>
<td>62.2–173.0</td>
<td>23.8</td>
<td>2.33</td>
</tr>
<tr>
<td>H2</td>
<td>2.5 % TCA</td>
<td>6.0</td>
<td>3.78</td>
<td>24</td>
<td>1.49</td>
<td>45.0–176.5</td>
<td>23.8</td>
<td>2.33</td>
</tr>
<tr>
<td>K1</td>
<td>2.5 % TCA</td>
<td>30.0</td>
<td>16.33</td>
<td>1</td>
<td>0.75</td>
<td>55.8–170.0</td>
<td>120.0</td>
<td>2.33</td>
</tr>
<tr>
<td>K2</td>
<td>Water pH 5.0</td>
<td>30.0</td>
<td>15.95</td>
<td>1</td>
<td>0.74</td>
<td>59.8–158.0</td>
<td>118.5</td>
<td>2.33</td>
</tr>
</tbody>
</table>

* Calculated at the 5 % level.
† Aliquots of 9/10 of D1 and D2 and 1/2 of D3 were bioassayed.
Table 3.

Biological activity of ultrafiltrates of ACTH extracted from 'old' human pituitary glands using 2.5% TCA or distilled water acidified to $p_H$ 5.0.

<table>
<thead>
<tr>
<th>Batch No.</th>
<th>Extractant</th>
<th>Time of extraction (h)</th>
<th>Extract</th>
<th>Ultrafiltrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Potency</td>
<td>% Limits of error*</td>
</tr>
<tr>
<td>Mean of 3</td>
<td>2.5% TCA</td>
<td>24</td>
<td>0.86</td>
<td>59.2–161.2</td>
</tr>
<tr>
<td>(L, N, P)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K1</td>
<td>2.5% TCA</td>
<td>1</td>
<td>0.75</td>
<td>55.8–170.0</td>
</tr>
<tr>
<td>K2</td>
<td>Water $p_H$ 5.0</td>
<td>1</td>
<td>0.74</td>
<td>59.8–158.0</td>
</tr>
</tbody>
</table>

* Calculated at the 5% level.
† Similar aliquots of K1 and K2 were bioassayed.
(Peak I = 16 % total nitrogen of ultrafiltrate used) consisted of material with a high peptide content as shown by the Lowry reaction carried out both in the presence and absence of copper (Lowry et al. 1951). When this Peak was run on the G-50 column, it separated into Peaks Ia (5 % original nitrogen) and Ib (10 % original nitrogen), the latter being of low molecular weight (<8000–10 000).

The principal experiment was designed to repeat this and to obtain material from Peak Ib for biological testing and for an ultracentrifuge run. Fig. 2 shows that Peak I consisting of nitrogenous material both > and < 3500–4000 molecular weight was separated from Peak II which corresponds to the position of 0.07 M sodium barbitone ('salt peak'). As shown in Fig. 3, when Peak I was run on the G-50 column it gave two peaks, Ia (2.80 mg = 25.2 % by weight of Peak I) which appeared in the protein position, i.e. molecular weight > 8000–10 000 and Peak Ib (8.35 mg = 73.2 % by weight of Peak I) which was retained by the column, i.e. molecular weight < 8000–10 000. Seventy-two % of the hormonal activity originally used with the G-25 column was recovered from Peak Ib (Table 4).

**Fig. 2.**
G-25 Sephadex column run on ultrafiltrate from 2.5 % TCA extract of human pituitary glands. Peak I consisted of nitrogenous material both > and < 3500–4000. Peak I was run on a G-50 column (see Fig. 3).
G-50 Sephadex column run on Peak I material from G-25 column (see Fig. 2). It gave two peaks: la which appeared in the 'protein' position, i.e. molecular weight $> 8000-10000$, and which had no biological activity and lb which was retained by the column, i.e. molecular weight $< 8000-10000$, and in this Peak 72% of the hormonal activity originally used with the G-25 column was recovered.

Table 4.
Biological activity of an ultrafiltrate from a 2.5% TCA extract of human pituitary glands and of a component from the ultrafiltrate, Peak Ib, separated by Sephadex column chromatography (Fig. 3).

<table>
<thead>
<tr>
<th>Ultrafiltrate</th>
<th>Peak Ib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potency</td>
<td>% Limits of error*</td>
</tr>
<tr>
<td>1.05</td>
<td>55.6–182.5</td>
</tr>
</tbody>
</table>

* Calculated at the 5% level.

Ultracentrifugation (Dr P. A. Charlwood). – The ultracentrifuge run on Peak Ib indicated monodispersity (Fig. 4). From the sedimentation coefficient ($S_{20,w} = 0.75$), minimum molecular weight was calculated to be about 3200.

DISCUSSION

Histology and histochemistry
It was not possible to correlate the marked reduction in PAS staining of the basophil cells produced by TCA treatment with the 'shift' of ACTH from tissue to solvent. It is believed that the basophil is the cellular site of production

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Fig. 4.

Ultracentrifuge pattern obtained with 1% solution of Peak 1b (see Fig. 3) in 0.1 M potassium chloride after sedimentation (right to left) for 68 min at 21°C and 59780 r.p.m. in a synthetic boundary cell.

and storage of ACTH but this study does not throw any light on this point. Using a similar approach Barnett et al. (1956) claimed that follicle-stimulating hormone and thyroid-stimulating hormone were extracted from the rat pituitary gland by TCA extraction and, further, that these hormones were produced and stored by the basophil.

The PAS technique demonstrates 1,2-glycols and 1-hydroxy-2-primary amines whether the amino group is acylated or not. A number of biological materials, for example polysaccharides, glycolipids and adrenaline, can be identified using this technique but in practice a positive result is obtained most commonly in those structures containing a hexose component (Hale 1957). Purified human ACTH does not contain hexose, but since it has a terminal serine (Lee et al. 1961) it may stain with the PAS method (Hale 1957). Treatment with TCA removed other chemical components of the pituitary as well as some of the ACTH, and the TCA-treated gland, which shows such marked reduction of PAS staining of the basophil cells (see Fig. 1), still contains TCA-precipitated, presumably protein or protein-bound ACTH. McGarry et al. (1962) have shown with the fluorescent-antibody technique that ACTH is localized in the basophils of the pituitary gland in man but claim that it is not necessarily associated with the PAS-positive basophil granules. If this is the case it is not surprising that we have failed to show a direct correlation between the changes in PAS staining of the granules of the basophils and ACTH content. Since we do not know the chemical state of storage of the hormone in the cell – whether it is stored as ‘packaged’ polypeptide molecules, or whether it is
bound to a carrier protein – it would seem premature and indeed misleading at this stage to interpret much from a static histochemical picture. Adams & Pearse (1959) suggested that a type of basophil rich in cystine, the ‘S’ cell, was the corticotrophin cell. This claim was partly based on reports that ACTH contained cystine (Li et al. 1943; Sayers et al. 1943) or that it was associated with the cystine-rich protein (Sayers 1952) which Sayers later described as ‘white elephant’ protein (see Li 1962). Purified human ACTH does not contain cystine (Lee et al. 1961) but swine FSH is rich in cystine (Steelman & Segaloff 1959) and therefore could be expected to stain blue with the performic acid-alcian blue-PAS method used by Adams & Pearse (1959). Interestingly enough Pearse (1960) later suggested that the ‘S’ cell was the gonadotrophin cell. Clearly we do not yet know the function of the ‘S’ cell because our knowledge of the chemical structure and state of storage of all the human pituitary hormones is incomplete. Caution should also be exercised in interpreting the results of staining with fluorescent antibody (see Cruickshank & Currie 1958; Currie 1962): the picture here is dependent on the union of antibody with the antigenic groups of the molecule of the hormone that are free to unite with antibody. We do not know what these antigenic groups are, nor do we yet know if these are hormone-specific.

**TCA-extracted hormone – low molecular weight ACTH**

No reports have been found of the use of 2.5% TCA for the extraction of human pituitary ACTH nor has it been used in the first stage of ACTH extraction from animal glands. Li (1952) found semi-purified sheep ACTH (molecular weight 10,000) to be soluble in 5% though not in 25% TCA. ACTH obtained as polypeptide or small protein from either the unhydrolysed pig pituitary (corticotrophin A) or from the hydrolysed pig pituitary (corticotrophin B) is soluble in 2.5% TCA (Hewett C. L., personal communication).

The procedure of stirring pieces of human pituitary glands with 2.5% TCA at 4°C for 24 hours apparently completely removes all the ‘free’ low molecular weight ACTH. When this ‘standard procedure’ was modified by using homogenization, or 5% TCA or water acidified to pH 5.0 as the extractants, similar amounts of ACTH were obtained in the extracts. We should emphasize too that re-extraction or re-homogenization yielded no further hormonal activity and a 1-hour extraction period proved as efficient as the 24-hour extraction. Autolysis caused a decrease in the residual gland and in the TCA-extractable hormone.

The extracts with 2.5% TCA (and with water acidified to pH 5.0) were ultrafiltrable and after purification with G-25 Sephadex column chromatography a product was obtained which behaved on a G-50 Sephadex column and in the ultracentrifuge as a single component of minimum molecular weight about 3200. It is suggested that this three-stage method is a particularly easy
one for preparing low molecular weight ACTH. It may be contrasted with the seven stages used by Li's group (Li et al. 1954, 1955) to extract sheep α-corticotrophin: acid acetone extraction, sodium chloride fractionation, oxy-cellulose adsorption, dioxan fractionation, starch zone electrophoresis, IRC 50 chromatography and countercurrent distribution. However, if Peak Ib (see Fig. 3) consisted solely of ACTH, its activity would be about 10 IU/mg, which is much less than that of Li’s best preparations of α-corticotrophin (105–150 IU/mg). On the other hand, there is recovery of only about 14% of the biological activity of the starting extract in the case of the α-corticotrophin in contrast to about 68% with our method. Since we have used only Sephadex column chromatography and ultracentrifugation to investigate homogeneity we can not exclude the possibility that our material contained polypeptides of similar molecular weight but without corticotrophic activity. It is also perhaps relevant to recall the marked human adrenal response obtained by Symington & Davidson (1956) on injecting a crude acid-acetone preparation of human ACTH. The reaction was much greater than that obtained with similar doses of ACTH extracted from animal pituitary glands.

Lee et al. (1961) in their proposed amino acid sequence for human ACTH suggest that the polypeptide consists of 39 amino acid residues, the first 24 and the last 7 counting from the N terminus, being identical with those of homogeneous hormone preparations from pig, sheep, and beef, whilst the interspecies differences in composition and sequence from 25 to 32 are only slight (Li et al. 1955; Bell et al. 1956; Li et al. 1958). Molecular weights of 3500-4566 (analytical data) and 4500 (ultracentrifugation of the trichloracetate) for pig corticotrophin A and 4541 (analytical data) and 5363 (ultracentrifugation) for sheep α-corticotrophin have been reported (see Li 1956). There is general agreement that the presence of the first 23–24 amino acids is necessary to permit full hormonal activity as judged by adrenal ascorbic acid depletion in the rat and the steroidogenic effect in man. Hofmann et al. (1961) have synthesized a 23 amino acid compound of 100 IU/mg, a value similar to that of Li’s best preparations from sheep glands. A peptide of 19 amino acids has been found to be steroidogenically active in man and to cause adrenal ascorbic acid depletion in rats, but its potency is 7% less, whilst that of a 13 amino acid chain is only slight (Danowski et al. 1961). Our minimum molecular weight of about 3200 for human ACTH corresponds to the first 23 amino acid residues in the sequence.

**Two chemical forms of ACTH**

Whilst the ACTH remaining in the gland after TCA or water extraction has not been characterized chemically, from our experimental findings it would seem to be protein or protein-bound. We do not know whether TCA or water acidified to pH 5.0 extracts free low molecular weight hormone or if they
liberate this component from a high molecular weight precursor or carrier protein. The former seems more probable, since 2.5% TCA at 4°C or water acidified to pH 5.0 at the same temperature are unlikely to break peptide bonds or other covalent bonds in proteins. The observations that there is no difference in the amount of ACTH extracted with TCA or water acidified to pH 5.0, and that re-extraction does not yield more activity suggest that part of the ACTH, present in the gland in low molecular weight form, is simply being dissolved at an acid pH. Hence we suggest that ACTH is present in two chemical forms in the human pituitary gland.

ACTH in single human pituitary glands

The mean total yield of hormone extracted with TCA from single 'fresh' glands was about 21 IU per gland and this is similar to that reported by Hewett et al. (1954) using acid-acetone extraction. In batches of 'old' glands the mean total per gland was 15 IU (see Table 1) and about one-third of this was extractable with TCA or water acidified to pH 5.0. The completeness of the extraction with the procedures used has already been discussed.

It has still to be shown whether the amount of TCA-extractable hormone in single 'fresh' glands is of physiological significance. We are investigating this and in the small number so far studied it varies from gland to gland: the highest yield so far obtained was from the pituitary gland of a woman who had been bilaterally adrenalectomized for disseminated breast cancer 7 months before death whereas no activity was detected in the TCA extract of two pituitaries from patients who died within 2-3 days of the onset of acute stressing illnesses.

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