The Small-Scale Isolation and Characterization of Adrenocorticotrophin

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Several methods for isolating adrenocorticotrophin from small quantities of porcine and bovine pituitary tissue are compared. Initial extraction of the hormone by an acid-acetone technique was simpler and more efficient than one employing acetic acid extraction and ether precipitation. Subsequent purification procedures utilizing adsorption of the peptide on to oxycellulose realized the highest yields. CM-cellulose-column chromatography followed by Sephadex-gel filtration were suitable final steps for obtaining highly purified adrenocorticotrophin. The purity of the hormone was demonstrated by determining its amino acid composition, C-terminal analysis, polyacrylamide-gel electrophoresis, chymotrypsin digestion and paper electrophoresis and by radioimmunoassay and bioassay. Adrenocorticotrophin was found to be rapidly destroyed in intact and especially in homogenized glands kept at room temperature. At 4° the rate of destruction was less rapid and at -20° losses were minimal.

Smith (1927) first demonstrated that the adrenal atrophy which invariably followed total hypophysectomy in rats could be corrected by daily intramuscular implantation of fresh pituitary tissue. After this many attempts were made to extract and purify the active principle involved, ACTH.† Initial procedures using such classical protein techniques as aqueous extraction followed by isoelectric precipitation and salt or acetone fractionation (Collip, 1933; Chow, Greep & Van Dyke, 1939; Fevold, Lee, Hisaw & Cohn, 1940) proved relatively ineffective compared with those based on vigorous extractions (Bates, Riddle & Millar, 1940), which made use of the finding that ACTH was remarkably resistant to strong acids and to boiling (Collip, 1938; Li, Evans & Simpson, 1943). Thus Lyons (1937) described a method based on the use of concentrated hydrochloric acid and acetone, and Tyslowitz (1943) employed acetic acid. An acid-acetone procedure was used by Li *et al.* (1943) to obtain what they then considered was the pure peptide, and also for the

preparation of the Second International Standard, which has a biological activity of 1·14 i.u./mg.

Further advances in the extraction and purification of ACTH were made possible by the development of a sensitive and reliable bioassay by Sayers, Sayers & Woodbury (1948). Such work assumed practical as well as academic importance with the demonstration by Hench, Kendall, Slocumb & Polley (1949) of the dramatic therapeutic effects of ACTH in rheumatoid arthritis, since the material then available frequently caused allergic reactions and even death. Payne, Raben & Astwood (1950) made a major contribution to the subject when they obtained material having tenfold the biological activity by an adsorption technique with powdered cellulose. In the following year this group reported that oxycellulose was even more effective (Astwood, Raben, Payne & Grady, 1951). Subsequently workers in a number of laboratories adopted this technique (Lee & Lerner, 1956; Li, 1956; Rosenberg, Evans & Simpson, 1961) and a similar procedure was employed to prepare the Third International Standard, which has a biological activity of approx. 100 i.u./mg. when assayed by the Sayers subcutaneous technique (Bangham, Mussett & Stack-Dunne, 1962).

A number of techniques have been employed,

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† Abbreviations: ACTH, adrenocorticotrophin; α_p -, α_h - and α_b -ACTH refer to porcine, human and bovine ACTH respectively; MSH, melanocyte-stimulating hormone.

with or without prior oxycellulose treatment, for the final purification of ACTH. These include chromatography on cation-exchange resins (Dixon, Moore, Stacke-Dunne & Young, 1951; White & Fierce, 1953; Li *et al.* 1954; Dixon & Stack-Dunne, 1955; White & Peters, 1956; Li & Dixon, 1956), countercurrent distribution (Li *et al.* 1954; Li & Dixon, 1956; Shepherd *et al.* 1956), CM-cellulose and DEAE-cellulose chromatography (Farmer, 1959; Lee, Lerner & Buettner-Janusch, 1960; De Jager, Homan & de Wied, 1963; Pickering, Anderson, Lohmar, Birk & Li, 1963), SE-Sephadex chromatography (Island *et al.* 1965), Sephadex-gel filtration (Birk & Li, 1964), paper chromatography and paper electrophoresis (White, Fierce & Lesh, 1951; Jacobowitz, Marks & Vernikos-Danellis, 1963; Adiga, Uemura & Winnick, 1965). A recent technique (Papkoff & Li, 1966) has increased the customary yield from ovine pituitaries tenfold, to 100 mg. of ACTH/kg. of fresh glands.

The recent availability of synthetic peptides with corticotrophic activity means that the need for preparing purified natural ACTH for therapeutic purposes will diminish. There is, however, need for convenient small-scale isolation and purification procedures for biosynthetic studies. The present study arose from experiments concerned with the incorporation of radioactive amino acids into ACTH in surviving slices and cell-free ribosomal preparations derived from anterior pituitary tissue (Adiga *et al.* 1965; Adiga, Rao, Husa & Winnick, 1966). Its purpose was to compare several isolation procedures adapted to the scale of 1–20 g. quantities of pituitary tissue. Consideration was given to minimizing ACTH degradation by the pituitary before extraction, extracting crude ACTH in high yield, purifying the hormone and demonstrating its biological, immunological and physicochemical purity. The work was facilitated by the development of a rapid and sensitive radioimmunoassay.

MATERIALS

Samples of highly purified ACTH, α -MSH and β -MSH, all of porcine origin, were kindly provided by Dr A. B. Lerner, of synthetic α -ACTH by Ciba Laboratories, Basle, Switzerland, and of synthetic α -ACTH by Ferring Laboratories, Malmö, Sweden. Fresh bovine pituitary glands were obtained from the Hawaii Meat Co., Honolulu, Hawaii, and fresh porcine glands from T. Wall and Sons, London N.W.10.

Tennessee Eastman Co., Kingsport, Tenn., U.S.A., provided oxycellulose (10–12% carboxyl). The carboxylic resin Amberlite CG-50 (200–400 mesh) was obtained from Mallinckrodt Chemical Works, St Louis, Mo., U.S.A. Sephadex G-75 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, and the CM-cellulose (CM 23, fibrous, fines reduced) from Reeve Angel Co., London E.C.4. α -Chymotrypsin was a product of Sigma Chemical Co., St Louis, Mo., U.S.A. Peroxide-free diethyl ether and freshly distilled acetone were used throughout.

Sephadex gels and CM-cellulose were pretreated according to the manufacturer's suggestions. Amberlite CG-50 and oxycellulose were treated as described by Island *et al.* (1965) and Astwood *et al.* (1951) respectively.

METHODS

Isolation and partial purification of ACTH

Pituitary glands were removed within 30 min. of death and kept at 4° until their extraction after 2–4 hr. Whole porcine glands were used, unless otherwise stated. Bovine pituitaries were first decapsulated and dissected free of the neurohypophysis and pars intermedia and 20 g. amounts were then extracted and partially purified by one of the following procedures.

Acid-acetone extraction. Batches (from one to 15) of porcine pituitary glands were homogenized in HCl-acetone (2.5 vol. of conc. HCl + 97.5 vol. of acetone; 3 ml./g. wet wt.) as described by Lyons (1937). Cellular debris and protein were removed by centrifugation and the crude ACTH fraction was precipitated from the supernatant overnight at 4° by the addition of 3 vol. of acetone. This precipitate was collected by centrifugation, washed with acetone and dried.

Acid-acetone extraction and sodium chloride fractionation. The sequence of HCl-acetone extraction, acetone precipitation, NaCl fractionation, dialysis and freeze-drying was followed, as described by Papkoff & Li (1966).

Acetic acid extraction and purification with oxycellulose (acetic acid/oxycellulose procedure). Crude ACTH was prepared from batches, each of 15 porcine pituitaries, as described by Astwood *et al.* (1951). The glands were homogenized in acetic acid-acetone (2.5 ml. of acetone + 16 ml. of acetic acid/g. wet wt.) and heated for 30 min. at 70°. After centrifugation the residue was re-extracted, and further impurities were precipitated from the combined supernatants by the addition of 0.005 vol. of aq. 5 M-NaCl and 0.5 vol. of acetone. Crude ACTH was then precipitated overnight at 4° with 1 vol. of ether, washed with acetone and dried. The dried residue was extracted twice with 50 ml. of 0.1 M-acetic acid, and further fractionation accomplished by the batchwise addition of oxycellulose (50% of the weight of the crude ACTH) and shaking for 24 hr. followed by elution of the ACTH with three 50 ml. quantities of 0.1 M-HCl. The eluate was neutralized with NaOH and freeze-dried.

The bovine tissue was treated exactly as above except that 20 g. amounts were homogenized in 100 ml. of acetic acid before the heating, cellular debris and protein were precipitated by addition of 2 vol. of acetone and crude ACTH was precipitated with 3 vol. of ether.

Acetic acid extraction and purification with oxycellulose followed by cation-exchange chromatography (acetic acid/oxycellulose/CG-50 procedure). This was performed as above except that the neutralized eluate after the oxycellulose step was adsorbed on Amberlite CG-50 displaced in 50% acetic acid and freeze-dried (Dixon, 1959).

Acetic acid extraction and purification by cation-exchange chromatography (acetic acid/CG-50 procedure). The procedure was as above except that the ether precipitate was passed through an Amberlite CG-50 column as described by Island *et al.* (1965), the oxycellulose step thereby being omitted.

Acetic acid extraction and batchwise purification with CM-cellulose followed by cation-exchange chromatography (acetic

acid/CM-cellulose/CG-50 procedure). In this method CM-cellulose was employed batchwise in place of the oxycellulose used above (De Jager *et al.* 1963).

Extraction with hydrochloric acid and purification with oxycellulose followed by cation-exchange chromatography (hydrochloric acid/oxycellulose/CG-50 procedure). This method was that described by Rosenberg *et al.* (1961). It consisted of the extraction of homogenized pituitaries with 0.1M-HCl, neutralization of the extract to pH 5.6 with NaOH, heating at 85° to coagulate protein and filtration. The filtrate was then made 0.1M in acetic acid and further purification accomplished with oxycellulose followed by concentration on Amberlite CG-50.

Final purification of ACTH

CM-cellulose column chromatography. The method of Pickering *et al.* (1963) was employed. The crude hormone from the above methods was suspended in 1–2 ml. of 0.01M-ammonium acetate, pH 4.6, and applied to a 1 cm. × 15 cm. CM-cellulose column. A linear gradient was used from 0.01M-ammonium acetate, pH 4.6, to 0.2M-ammonium acetate, pH 6.7, with 50 ml. volumes in both the reservoir and mixing flasks. After the 100 ml. of buffer had passed through the column, 0.2M-ammonium acetate, pH 6.7, was applied until the ACTH region was eluted. Lastly, 0.1M-HCl was used to elute a residual peak, designated R (De Jager *et al.* 1963). Flow rates were from 1.0 to 2.5 ml./min. The E_{280} was monitored in an Instrument Specialties Co. (ISCO) flow-cell system. Fractions of volume 2–5 ml. were collected and those corresponding to each discrete peak or region were pooled separately and then freeze-dried several times to remove ammonium acetate completely.

Sephadex-gel filtration. This was performed as described by Andrews (1964) with a 2.5 cm. × 53 cm. column of Sephadex G-75, with 5% (v/v) acetic acid, pH 2.5, as the solvent. The flow rate was 40 ml./hr. The sample (1–20 mg. in 5% acetic acid containing 1–2% of sucrose) was layered on the bed of Sephadex. Fractions of volume 2–3 ml. were collected and the E_{278} was monitored. The yield of ACTH was made quantitative by plotting the E_{278} of the Sephadex fractions on graph paper, determining the area under the ACTH peak and comparing this value with the standard curve in Fig. 1.

Chemical characterization of ACTH

Chymotrypsin digestion was performed as described by Adiga *et al.* (1965). The carboxypeptidase procedure of Harris & Li (1955) was used to identify C-terminal amino acids. Polyacrylamide-gel electrophoresis and quantitative amino acid analysis were carried out as described by Rao, Robertson, Winnick & Winnick (1967).

Assay of ACTH

Biological activity. The hormone was assayed by the adrenal ascorbate-depletion technique of Sayers *et al.* (1948) and the steroidogenic method *in vitro* of Saffran & Schally (1955). These determinations were performed by the South Mountain Laboratories, Maplewood, N.J., U.S.A. In addition, the intrinsic MSH activity of various preparations was measured by the frog-skin assay (Lerner, 1966) at Yale

Fig. 1. Extinction and elution volume obtained with various quantities of highly purified α_p -ACTH with a 2.5 cm. × 53 cm. column of Sephadex G-75 with 5% acetic acid as buffer. (a) Reproducibility of the elution pattern; (b) standard curve obtained by plotting the area (expressed as weight of graph paper) under each peak versus the corresponding amount of ACTH employed.

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Immunological activity. A rapid radioimmunoassay was developed based on the use of an antibody directed against the amino acid sequence, composed of residues 16–24, common to all known species of ACTH. Synthetic α_p -ACTH was employed for standardization and radioiodination, the latter being performed as described by Landon, Livanou & Greenwood (1967). Curves were constructed by incubating various amounts of standard ACTH or unknown quantities of hormone with 500 pg. amounts of ^{125}I -labelled α_p -ACTH and antiserum (at a final dilution of 1:800) for 120 min. at room temperature. Under these conditions approx. 60% of labelled hormone was bound in the absence of unlabelled ACTH. The bound and free fractions of the labelled peptide were separated by a dextran-coated charcoal technique (Landon & Greenwood, 1968). The precision of the assay, as determined by 20 duplicate determinations, was $\pm 18\%$.

Assays based on this system can be used to determine all species of ACTH. Peptide fragments containing the whole antigenic determinant to the antibody used (the amino acid sequence 16–24) cause parallel inhibition slopes and are indistinguishable from the intact molecule. Fragments containing only part of this sequence may compete with the labelled hormone for antibody-binding sites. However, the inhibition slope is less steep (Landon, Girard & Greenwood, 1968).

RESULTS

Degradation of ACTH. Vigorous procedures, such as heating and treatment with acids, are used as a routine in the extraction of ACTH. Preliminary studies were performed to determine whether these measures caused degradation of the hormone as assessed by radioimmunoassay. Synthetic α_h -ACTH at a final concentration of 100 ng./ml. was

Table 1. *Content of ACTH of intact porcine pituitary glands kept at different temperatures*

Results are expressed as percentages of the control groups of pituitary glands.

Mean ACTH content immediately after removal ($\mu\text{g./pituitary}$)	Storage temp.	ACTH content (% of control)		
		2	6	21
Days after removal	...			
250	Room temp.	21	< 5	—
146	Room temp.	30	< 5	—
255	4°	87	53	—
174	4	80	31	—
180	-20	—	97	—
221	-20	—	117	110
230	-20	—	—	95

dissolved in 0.05M-sodium phosphate buffer, pH 7.5, in 0.1M-hydrochloric acid, in acetic acid and in 0.1M-sodium hydroxide. The tubes were kept in a boiling-water bath and samples removed at hourly intervals for 4 hr. After neutralization, the ACTH concentration of each sample was determined by immunoassay. In triplicate experiments no significant loss of ACTH occurred over the 4 hr. period in tubes containing phosphate buffer or hydrochloric acid. In tubes containing acetic acid approx. 50% of the ACTH had been destroyed at the end of the 4 hr. period at 100°, and in the tubes containing 0.1M-sodium hydroxide less than 10% of intact hormone was found after 1 hr.

Batches (each containing five fresh porcine pituitary glands) were homogenized in 0.25M-sucrose, pH 7.5. The ACTH concentration was determined by radioimmunoassay on samples removed immediately and 4, 24 and 48 hr. after homogenization. In three experiments in which the homogenates were kept at 4° the mean recovery was 91%, 60% and 30%. Less than 15% of immunologically active ACTH was found after 24 hr. in homogenates left at room temperature.

In a further series of studies batches, each of five intact fresh porcine pituitary glands, were kept at room temperature, at 4° or at -20°. After various intervals ACTH was extracted by the acid-acetone procedure and the amount present determined by radioimmunoassay. The results obtained were compared with those of batches of glands removed at the same time, from pigs of approximately the same size, weight and maturity, that had been extracted within 2 hr. of removal. The results are summarized in Table 1. Rapid losses were observed in batches of glands kept at room temperature, whereas there was no evidence of loss even after 3 weeks at -20°. At 4° about half the ACTH

Table 2. *Recovery of ACTH during the various stages of extraction with acetic acid*

Sequential procedures	ACTH content ($\mu\text{g./pituitary}$)	
	Expt. 1	Expt. 2
1 Glands homogenized in acetic acid, heated at 70° for 10 min.; centrifuged:		
Ppt.: re-extracted and extract assayed	6	8
Supernatant: assayed	365	212
2 NaCl and acetone added, left overnight:		
Ppt.: assayed	90	42
Supernatant: assayed	256	160
3 Ether added, left overnight:		
Ppt.: assayed	200	130
Supernatant: assayed	34	15

appeared to have been destroyed in intact glands after 6 days.

Isolation and partial purification of ACTH. The individual weights of the 175 fresh intact porcine pituitary glands used for studying the acid-acetone procedure were in the range 192–320 mg. with a mean value of 265 mg. The amount of material precipitated from the hydrochloric acid-acetone extract by addition of acetone ranged from 0.9 to 1.97% of the original weight of pituitary tissue used, with a mean value of 4.2 mg./gland. The ACTH content of the individual glands as determined by immunoassay of this precipitate, ranged from 136 to 504 $\mu\text{g.}$ with a mean value of 251 $\mu\text{g./pituitary.}$ The inhibition slope produced by this material was parallel with that of the standard curve. The amount of material left in the acetone supernatant, as determined after blowing off the acetone, was some three times that of the precipitate, with a mean value of 13 mg./original pituitary gland. Although large amounts of this material inhibited the binding of ^{125}I -labelled ACTH by antibody, the slope was much less steep than that produced by the standard, suggesting the presence of fragments of ACTH only.

A much lower yield of ACTH (96 $\mu\text{g./pituitary}$) was obtained from two batches, each of five glands, stored in acetone before the acid-acetone extraction, probably attributable to the difficulty experienced in homogenizing acetone-stored pituitaries. An improved yield (289 $\mu\text{g./gland}$) was obtained when fresh porcine pituitaries were homogenized in a minimum volume of 0.25M-sucrose immediately before the addition of hydrochloric acid-acetone. This may have related to the greater ease and efficiency of homogenizing the glands in sucrose than in acid-acetone. Decreasing the amount of

Fig. 2. Chromatography on 1 cm. x 15 cm. CM-cellulose columns of (a) 10-20 mg. of acetic acid-extracted oxycellulose-purified α_p -ACTH, (b) 10-20 mg. of acetic acid-extracted oxycellulose-purified α_b -ACTH, (c) 1 mg. of highly purified α_s -ACTH, (d) 5.5 mg. of β -MSH, (e) 3.8 mg. of α -MSH. A continuous ammonium acetate gradient (from 0.01 M, pH 4.6, to 0.2 M, pH 6.7) was employed. The first arrow indicates a buffer change, after completion of the gradient, to 0.2 M-ammonium acetate and the second arrow a change to 0.1 M-HCl. The tube numbers of the peaks were: I, 9-19; II, 31-35; II-III, 36-73; III, 74-85; R, 108-113.

concentrated hydrochloric acid in the acid-acetone from 2.5% to 1.0% or 0.75% did not significantly effect the yield of ACTH or the amount of material precipitated by the addition of acetone. Decreasing the concentration to 0.5% or 0.25%, however, caused the yield to fall to 63 and 23 μ g./pituitary respectively.

ACTH values, as determined by radioimmunoassay, were monitored throughout the processing of two batches each of 15 fresh porcine anterior pituitary lobes by the acetic acid procedure. The results are summarized in Table 2. The total amount of tissue used in these two experiments was 2820 and 2710 mg. and the total weight of crude ACTH (ether precipitate) obtained was 124 and 120 mg. respectively. Extraction was effective as evidenced by the observation that only a small quantity of ACTH could be obtained by re-extracting the residue. However, considerable losses occurred both in the sodium chloride-acetone precipitate and in the ether supernatant, and the final yields of ACTH (200 μ g. and 130 μ g./pituitary respectively) were lower than with the acid-acetone procedure. Two further batches were taken through these steps and then purified with oxycellulose. The two preparations of ACTH were still relatively impure, having an immunological activity of only 42% and 51% that of synthetic α_p -ACTH. Their respective biological activities were 40 and 56 i.u./mg. as com-

pared with 135 i.u./mg. for the synthetic hormone as assayed by the adrenal ascorbate-depletion technique.

Isolation of highly purified ACTH. The finding that the immunological and biological activity of the acetic acid/oxycellulose-prepared α_p -ACTH was less than half that of the synthetic hormone indicated that the material was impure. Therefore additional purification procedures were investigated with ACTH that had been partially purified by one of several methods.

(a) Chromatography on CM-cellulose. Acetic acid/oxycellulose-prepared α_p - and α_b -ACTH were resolved into several components by CM-cellulose-column chromatography and continuous gradient elution with ammonium acetate followed by 0.1 M-hydrochloric acid (see Fig. 2). The major peaks are designated as II, III and R. Standard preparations of α - and β -MSH were eluted from CM-cellulose in the same position as peak II and highly purified ACTH as a single peak in the III region. A much larger ACTH peak was obtained with the porcine than with the bovine material.

Fig. 3 shows the CM-cellulose profiles obtained with ACTH preparations derived from 20 g. amounts of fresh bovine anterior pituitary tissue subjected to a number of different isolation procedures. The yield of crude ACTH by these methods is given in Table 4. Various degrees of resolution of ACTH

Fig. 3. CM-cellulose chromatography, under conditions identical with those of Fig. 2, of crude α_b -ACTH isolated from 20g. amounts of fresh bovine anterior pituitary tissue (a) by acetic acid/oxycellulose/CG-50, (b) by acetic acid/CM-cellulose/CG-50, (c) by acetic acid/CG-50, (d) by HCl/oxycellulose/CG-50, (e) by acid-acetone extraction and NaCl fractionation.

(peak III) were observed. Most products, and especially that prepared by the acetic acid/CM-cellulose/CG-50 technique, were contaminated by MSH or some other material eluted in the peak II region. The most pure ACTH preparations, as evidenced by CM-cellulose chromatography, were those obtained by the acetic acid/oxycellulose/CG-50 method and by acid-acetone extraction and salt precipitation. The least satisfactory procedure for isolating ACTH was the acetic acid extraction combined only with cation-exchange chromatography. This procedure was handicapped, however, in that the quantity of material applied was excessive in relation to the size of the CM-cellulose column.

Peaks derived from CM-cellulose chromatography of acetic acid/oxycellulose-prepared α_p - or α_b -ACTH were compared in a series of studies with synthetic or highly purified natural porcine ACTH.

When analysed by polyacrylamide-gel electrophoresis, highly purified α_p -ACTH exhibited one major component near the origin and two minor ones nearer the anode, which may have represented deamidated forms of ACTH (Dixon & Stack-Dunne, 1955; Li, 1956). Peak III gave these bands together with an additional two bands still nearer the anode. Peak II gave a totally different band pattern (Fig. 4). The ninhydrin-staining pattern obtained after paper electrophoresis of chymotrypsin digests of the

Fig. 4. Polyacrylamide-gel electrophoresis of highly purified α_p -ACTH, peak II material, peak II-III material and peak III material after CM-cellulose chromatography of acetic acid/oxycellulose-isolated α_p -ACTH. Stippled, cross-hatched and solid areas indicate bands having an increasing intensity of staining.

peak III component also gave bands additional to those found with digests of the pure hormone. Although the porcine peak III material possessed approximately the same amino acid composition as synthetic α_p -ACTH, several of the amino acids values were not in close agreement. The apparent heterogeneity of peak III is probably due to its coming off the column shortly after a change of eluate. The immunological and biological activities of the major CM-cellulose peaks are summarized in Table 3. Peak II material from both sources showed none of the biological effects of ACTH and relatively little immunological activity. That from the whole porcine glands had much more melanocyte-stimulating activity than peak II material from bovine anterior tissue (as expected in view of the known abundance of MSH in the pars intermedia and neurohypophysis; Lerner, 1966). Peak III material from both sources displayed considerable potency in all three ACTH assays but not as great as that of the synthetic hormone. The frog-skin assays for porcine peak III gave a value somewhat higher than that expected for ACTH (2×10^8 i.u./g.; Lerner, 1966), probably reflecting contamination by MSH.

A residual peak (R) was apparent on CM-cellulose

Table 3. *Biological and immunological activities of various preparations of ACTH*

Method of assay	Activity (i.u./mg.)						Synthetic α_p -ACTH	
	With whole porcine pituitary material			With bovine anterior-lobe material				
	CM-cellulose peak ...	II	III	R	II	III		R
Adrenal ascorbate depletion		0	80	8	—	—	—	135
Adrenal steroidogenesis		—	—	—	0	47	2	—
Radioimmunoassay		12	112	3	6	55	3	140
Frog skin (MSH)		2×10^{11} *	4×10^9 *	3×10^7 *	5×10^7 *	—	3×10^6 *	—

* Units/g.

chromatography of all the crude ACTH preparations. It was largest with material obtained by using acetic acid for the initial extraction and had definite, though slight, MSH and ACTH activity (Table 3). Its amino acid composition (not shown) was totally different from that of ACTH and its hormonal activity was largely removed by gel filtration through a column of Sephadex G-75. Subsequent treatment with urea completely eliminated its ACTH-like properties (Hussa, 1968). When a sample of highly purified ACTH was manipulated through the steps of the acetic acid/oxy-cellulose/CG-50 procedure and then chromatographed on a CM-cellulose column, no R peak was obtained.

(b) Sephadex-gel filtration as the final step for ACTH purification. The findings summarized above indicate that none of the procedures used to isolate ACTH, even when combined with CM-cellulose chromatography, gave a pure product. Consequently, a further step was introduced, namely gel filtration on Sephadex G-75. In initial studies, the elution volume of ACTH was found to be highly reproducible and the hormone was well resolved both from larger molecules (such as growth hormone) and smaller peptides (including α - and β -MSH).

The contents of tubes 70–90 (peak III), from each CM-cellulose-purified preparation, were pooled, freeze-dried and then subjected to gel filtration. The profile obtained with 1.5 mg. of a peak III fraction from CM-cellulose chromatography of acetic acid/oxy-cellulose-prepared α_b -ACTH is shown in Fig. 5. Material in the central portion of this peak was freeze-dried before chemical and immunological analysis. The other profiles obtained are shown in Fig. 6 and the yields of purified ACTH isolated by each different method are summarized in Table 4. The most pure ACTH obtained after CM-cellulose chromatography, as evidenced by this technique, was that prepared by the acetic acid/oxy-cellulose/CG-50 procedure. The least pure was obtained by acetic acid extraction and cation-exchange chro-

Fig. 5. Gel filtration on 2.5 cm. $\times$ 53 cm. Sephadex G-75 columns with 5% acetic acid of 1.5 mg. of a peak III fraction from CM-cellulose chromatography of bovine ACTH previously isolated by the acetic acid/oxy-cellulose procedure. The material in the cross-hatched region was freeze-dried before chemical and immunological analysis.

matography only. The highest yield of ACTH (122 mg./kg. of bovine adenohypophysis) was provided by the hydrochloric acid/oxy-cellulose/CG-50 procedure. Batchwise treatment with CM-cellulose in place of oxy-cellulose and acid-acetone extraction followed by sodium chloride fractionation appeared to result in suboptimum recoveries of the hormone.

The immunological activity of the final product was similar to that of synthetic ACTH (see Table 4). It was homogeneous when analysed by polyacrylamide gel electrophoresis. It possessed the amino acid composition expected for α_b -ACTH (see Table 5), in that isoleucine, cystine and threonine were absent, as expected, and the slightly low serine value was explicable since no correction has been applied for destruction during hydrolysis. Endogenous peptidase activity may have caused the loss of some C-terminal phenylalanine before the isolation procedure was started. Phenylalanine and some glutamic acid were released after digestion with carboxypeptidase A for 1 hr. at 40°. After 18 hr. leucine was also detectable. These findings are in agreement with those of Li (1956) for ACTH.

Fig. 6. Gel filtration on Sephadex G-75 columns of the peak III fractions derived from CM-cellulose chromatography of the preparations as listed in Fig. 3.

Table 4. Yield and potency of ACTH isolated from fresh bovine adenohypophysis by several small-scale methods

All values are the means of duplicate experiments.

Initial fraction procedure	Yield of crude ACTH before CM-cellulose (g./kg. fresh wt. of tissue)	Purified hormone (after gel filtration)	
		Yield (mg./kg.)	Activity* (i.u./mg.)
Acetic acid/oxy-cellulose/CG-50	0.55	72	120-140
Acetic acid/CM-cellulose/CG-50	2.55	45	120-140
Acetic acid/CG-50	11.55	55	120-140
HCl/oxy-cellulose/CG-50	2.00	122	120-140
Acid-acetone and NaCl fractionation	3.85	45	60-80

* Based on radioimmunoassay, with synthetic ACTH (140 i.u./mg.) as reference standard.

Table 5. Amino acid analysis of bovine ACTH after final purification on Sephadex G-75

Amino acid	No. of residues	
	Theoretical	Experimental
Lys	4	3.9
His	1	0.9
Arg	3	2.9
Asp	2	2.2
Thr	0	0.0
Ser	3	2.4
Glu	5	5.1
Pro	4	4.0
Gly	3	3.1
Ala	3	3.1
Cys	0	0.0
Val	3	3.2
Met	1	0.9
Ile	0	0.0
Leu	1	1.0
Tyr	2	1.9
Phe	3	2.6

DISCUSSION

Procedures used to isolate ACTH should be as simple as possible, consistent with obtaining maximum yields of biologically active hormone and a final product uncontaminated by other peptides or proteins. The present investigation was designed to assess which of several procedures best fulfilled these criteria. The study was greatly facilitated by the development of a rapid, accurate and sensitive radioimmunoassay that could be applied to ACTH of all species. It must be noted, however, that such assays determine the number of molecules containing the antigenic determinant of the particular antibody used (Landon *et al.* 1968), in this case the amino acid sequence 16-24 of ACTH. Fragments containing this sequence will also be assayed and dissociation between results of immunoassay and of bioassay may occasionally be found, since the latter determines only molecules containing the *N*-terminal 24 amino acids.

Whether ACTH is isolated on a large scale for clinical purposes or on a small scale as in biosynthetic studies, it is essential to minimize its degradation before extraction. Substantial losses occurred within hours at room temperature, especially if the glands were homogenized and kept at neutral pH. The enzyme systems involved appear to be temperature-dependent, since there was no radioimmunological evidence of significant loss in intact glands kept at -20° for some weeks, or at $1-4^{\circ}$ for a few hours.

The first step, common to all isolation procedures, is the extraction of ACTH free from cellular debris,

proteins and large peptides. It is then necessary to separate it from other peptides of approximately the same size. In the present study initial extraction with hydrochloric acid-acetone proved better than that with acetic acid, because of greater simplicity and higher yield. The crude ACTH, so prepared, was stable for many months at 4° (J. Landon, unpublished work), indicating that both methods freed the hormone from contamination with proteolytic enzymes. Extraction with 0.1 M-hydrochloric acid, followed by heat coagulation of proteins, as described by Rosenberg *et al.* (1961) has also proved satisfactory. Such treatment, even in a boiling-water bath for 4 hr., did not destroy ACTH as evidenced by radioimmunoassay.

On the basis of the present results we now as a routine purify the crude ACTH by batchwise addition of oxycellulose followed by cation-exchange chromatography. Use of the optimum quantity of oxycellulose for obtaining pure ACTH (8% by wt. of the crude ACTH; Astwood *et al.* 1951) may result in incomplete recovery. To minimize any loss we use larger amounts of oxycellulose (50% by wt. of the crude ACTH) despite finding that, under these conditions, larger quantities of other peptides are also adsorbed. A further purification step is then introduced, namely CM-cellulose chromatography as described by Pickering *et al.* (1963).

The present experiments confirm the earlier observations of Farmer (1959), Lee *et al.* (1960), De Jager *et al.* (1963) and others, that crude ACTH can be resolved into several peaks on CM-cellulose. They indicate that peak II largely comprises MSH and peak III ACTH. A low activity of ACTH was also detected in the residual peak R, from which it could be removed by gel filtration or treatment with urea. To obtain a product immunologically and physicochemically identical with synthetic ACTH it was finally necessary to purify the peak III material further by gel filtration on Sephadex G-75. The yield (122 mg./kg. of bovine anterior pituitary tissue) obtained compares closely with that of Papkoff & Li (1966) of about 100 mg./kg. of whole sheep pituitary glands.

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