The Isolation and Characterization of Corticotropin from the Pituitary Gland of the Ostrich *Struthio camelus*

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1. Avian corticotropin (ACTH) was purified from both fresh and aged pituitary glands of the ostrich *Struthio camelus*. 2. The isolation of corticotropin in pure form involved acid/acetone extraction, NaCl fractionation, CM-cellulose chromatography and Sephadex G-50 chromatography. 3. The hormone preparations from fresh and aged glands behaved as single substances on polyacrylamide-gel electrophoresis, and both preparations were found to consist of 39 amino acid residues, in identical molar proportions for the different amino acids. 4. The isoelectric points of the two hormone preparations were estimated to be in the range pH 8.3–8.7, indicating possible differences in amide content, and the *N*-terminal amino acid of both preparations appeared to be serine. 5. The hormone preparations from fresh and aged glands exhibited similar biological potencies (73 and 77 i.u./mg respectively), as measured by steroidogenesis *in vitro*. 6. Apart from possible differences in amide content, the corticotropin preparations obtained from fresh and aged glands appear to be indistinguishable.

Of the various polypeptide and protein hormones produced by the pituitary gland, corticotropin (ACTH) was one of the first to be isolated in pure form from a variety of species, including pigs, sheep, cattle and man, and more recently from the dogfish *Squalus acanthias* (Oelofsen, 1975). All these corticotropin preparations show much similarity in terms of both chemical structure and biological activity. In mammalian species the difference is restricted only to one amino acid residue, but in the evolutionally very distant dogfish, the primary structure of corticotropin differs from that of the mammalian hormone with respect to 11 amino acid residues. The conformation of the molecule appears to be conserved among the various species, so it can be regarded as a characteristic property of corticotropin much like the active core and the binding site (Löw *et al.*, 1975).

To our knowledge no successful purification of corticotropin from avian species has so far been reported, which in part may be due to the difficulty of obtaining pituitary material in the required quantities from such species. The ostrich, being one of the tallest living birds which possesses a good-sized gland weighing approx. 0.4 g, offered us an opportunity to obtain sufficient amounts of adenohypophyseal material to attempt the purification of the hormone.

The present paper reports the isolation of the hormone in a state of high purity, allowing its further structural characterization and a comparison with

previously isolated hormones from non-avian sources. For practical reasons attention was also paid to the effect of post-mortem aging on the hormone recovered from the ostrich pituitary glands. Under the prevailing local circumstances it would greatly simplify the gland-collection procedure and lower the cost involved if moderately aged glands could be used in future isolation work. For this reason we report the results obtained with both 'fresh' and 'aged' glands.

Experimental

Biological material

Pituitary glands were collected immediately after death ('fresh' glands), or at most 3 h later ('aged' glands). Immediately after their removal the anterior and posterior lobes were separated and frozen separately in liquid N₂ (Oosthuizen *et al.*, 1976). The glands were stored under liquid N₂ during the usual 4-day collection period, and on arrival at the laboratory they were processed without delay. The average wet weight per single anterior lobe, as determined for 5677 lobes, was 0.225 g.

Starting material

The adenohypophyses were extracted by the acid/acetone procedure, and the resulting acid/acetone-dried powder was submitted to the NaCl-fractiona-

tion procedure to give the corticotropin Fraction D (Li *et al.*, 1951).

Fractionation on CM-cellulose

Fraction D was further purified by chromatography on a CM-cellulose (Cellex-CM; Bio-Rad, Richmond, CA, U.S.A.) column in a pH and ammonium acetate gradient (Birk & Li, 1964). In a typical run 1.3–2.1 g of Fraction D was dissolved in 45–60 ml of 0.01 M-ammonium acetate buffer, pH 4.6. The resultant slightly turbid solution was left in the cold-room for 1 h to allow a toffee-like material to settle fully and was then clarified by centrifugation at 43 500g for 1 h (Sorvall RC2-B centrifuge, SS-34 rotor at 4°C). The entire chromatography procedure was performed in a thermostatically controlled column at 25°C. Fractions (4 ml) were collected and monitored for A_{280} , pH and conductivity. The eluates corresponding to the various peaks were freeze-dried several times to remove ammonium acetate and were examined for biological activity.

Rechromatography on CM-cellulose

Peak-8 material (see the Results section) was rechromatographed on the same CM-cellulose column but by using a different gradient from that used for the initial chromatography. The pH and salt gradients were steepened by using half-volume quantities of the equilibration and gradient buffers used in the initial chromatography step.

Chromatography on Sephadex G-50

Material from peaks 8a and 8b (see the Results section) was submitted to gel filtration on a column (63 cm × 1.6 cm) of Sephadex G-50 (medium grade). Elution was performed with 0.1 M-acetic acid (Birk & Li, 1964).

Bioassay

The isolation of corticotropin activity was monitored by the adrenal steroidogenesis assay *in vitro* (Saffran & Schally, 1955). The Third International Working Standard of pig corticotropin was used as bioassay standard. This material was kindly supplied by the WHO International Laboratory for Biological Standards, London NW3 6RB, U.K.

Gel electrophoresis

Polyacrylamide-gel electrophoresis was used to demonstrate the homogeneity of the purified corticotropin fractions. Electrophoresis was performed at pH 4.5 (0.35 M- β -alanine/acetate buffer) for 3 h at 4 mA/tube in a Buchler analytical apparatus. The gels (7.5 cm × 0.5 cm) contained 15% (w/v) acrylamide and were maintained at 15°C during electrophoresis. Protein bands were made visible by staining with 0.5% (w/v) Amido Schwarz 10B in 7% (w/v) acetic acid, followed by electrophoretic destaining.

Isoelectric focusing

Electrofocusing was carried out on 4.6 mg of peak-8b' material (see the Results section) in the LKB 8101 (110 ml capacity) column, by using a linear 0–40% (w/v) sucrose gradient containing ampholyte carrier at a concentration of 1% (w/v) in the pH range 7–10. A potential of 500–600 V was applied for 94 h at a thermostatically controlled column temperature of 5°C. After completion of the run the sucrose gradient was pumped out of the column from the cathode end at the bottom and collected in fractions of 1.5 ml. For each fraction the pH was determined at 5°C, and the A_{280} was recorded.

Amino acid analysis

Samples from peaks 8a' and 8b' (see the Results section) were hydrolysed with 5.7 M-HCl in evacuated Pyrex tubes for 20 h at 110°C [in the presence of 10 μ l of 5% (w/v) phenol to prevent tyrosine loss], and the hydrolysates were examined with a Beckman 120B automatic analyser (Spackman *et al.*, 1958). The tyrosine-tryptophan content was analysed spectrophotometrically by the method of Bencze & Schmid (1957).

N-Terminal analysis

The N-terminal amino acid was identified by the dansyl (Dns, 5-dimethylaminonaphthalene-1-sulphonyl) procedure (Narita, 1970) against suitable Dns standards on silica gel G thin-layer plates. The unequivocal identification of the Dns derivative required a combination of two solvent systems: (a) chloroform/benzyl alcohol/acetic acid (70:30:3, by vol.) and (b) chloroform/2-methylpropan-2-ol/acetic acid (6:3:1, by vol.).

Results and Discussion

Isolation procedure

The elution pattern obtained from CM-cellulose chromatography of Fraction D is presented in Fig. 1. Although the pattern differs in some respects from that of Birk & Li (1964), it should be noted that the column used by us, as well as the pH and salt gradients, are not exactly comparable with those used by these previous workers. When comparing the CM-cellulose elution profiles and the subsequently freeze-dried components of Fraction D materials from fresh and aged glands, no distinct differences could be detected in terms of retention volumes, mass recoveries or biological activities. The biological potencies (i.u./mg) of peaks 1–9 from CM-cellulose chromatography (Fig. 1) were 0.2, 0.3, 1.5, 2.3, 1.8, 2.6, 9.6, 42.6 and 9.7 respectively. Peaks 4 and 6 also revealed lipolytic activity when assayed by the isolated-fat-cell procedure of Rodbell (1964).

Rechromatography of the material from peak 8 on a CM-cellulose column, by using a steeper gradient,

Fig. 1. *Chromatography of ostrich corticotropin concentrate on CM-cellulose*

Corticotropin Fraction D (2.13 g; dialysed and freeze-dried NaCl precipitate derived from the acid/acetone extract of ostrich pituitaries) was applied to a column (50cm×1.6cm) that had been previously equilibrated with 0.01M-ammonium acetate starting buffer, pH4.6. Gradient elution, with respect to pH and buffer concentration, was performed at 105 ml/h as described by Birk & Li (1964); preliminary elution was with 320 ml of starting buffer, followed by a gradient formed by introducing 0.1 M-ammonium acetate, pH6.7, through a 500 ml mixing flask containing the starting buffer. The gradient was later increased by substituting 0.2M- and 1M-ammonium acetate, pH6.7, as the solution flowing into the mixing flask. A_{280} (—), pH (○) and conductivity (●) of the eluted fractions were monitored. ↑, Effluents corresponding to the various peaks were pooled, freeze-dried and assayed for biological potency.

Fig. 2. *Rechromatography of an ostrich corticotropin-active fraction from CM-cellulose*

Peak-8 material (Fig. 1; 51 mg) was applied to a CM-cellulose column (50cm×1.6cm) under conditions exactly similar to those of Fig. 1, except for a different gradient procedure as indicated. ↑, Effluents corresponding to the various peaks were pooled, freeze-dried and assayed for biological potency.

yielded some peak-1 material carried over from the first run and two peaks, 8a and 8b (Fig. 2), exhibiting corticotropin activity. Peaks 8a and 8b from fresh and aged glands behaved similarly. When peak-8b

material, being the most active, was in turn re-chromatographed on CM-cellulose under the same re-run conditions as before, a single peak was obtained. This clearly demonstrated that peak 8a, obtained in the first re-run, was not merely a dissociation product of peak 8b but represented a discrete active component. Polyacrylamide-gel electrophoresis revealed one major band for peak-8b material, together with a minor apparently higher-molecular-weight contaminant, which had a decreased mobility towards the cathode.

Sephadex G-50 chromatography was originally introduced by Birk & Li (1964) to obtain good amino acid-analysis data on pure corticotropin samples, e.g. traces of threonine were removed in this manner. In similar experiments Birk & Li (1964) found that corticotropin emerged from the column directly after the void volume had been eluted. In the present study the corticotropin-active peaks, 8a' and 8b', only appeared after exactly 2 void-volumes had passed through the column, whereas an apparently higher-molecular-weight contaminant was eluted directly after the void volume. Peak 8b', representing the major active component, appeared homogeneous during polyacrylamide-gel electrophoresis, and no difference in mobility or in purity was evident, whether the preparation was derived from fresh or from aged glands. However, on polyacrylamide-gel electrophoresis

Table 1. *Purification of ostrich corticotropin from fresh and aged pituitary glands*

For each step (fraction) the upper value shown in each column refers to the fresh material and the lower value to aged material. Fiducial limits at $P = 0.05$ are shown in parentheses.

Step (fraction)	Mass recovery (mg)	Potency (i.u./mg)	Activity recovery (%)
Adenohypophyses	786000 1060000		
Acid/acetone extraction	30357 36630	1.40 (0.69–2.84) 1.13 (0.43–2.93)	100 100
Salt fractionation (Fraction D)	3809 4770	3.62 (1.71–7.68) 4.15 (2.04–8.47)	32.4 48.0
CM-cellulose column (peak 8)	51.0 69.5	43.03 (15.38–120.35) 42.62 (16.88–107.44)	5.2 7.2
CM-cellulose re-run column			
Peak 8a	9.8 10.7	8.65 (5.09–12.21) 10.40 (5.60–19.29)	0.20 0.27
Peak 8b	24.5 32.0	56.16 (19.87–157.74) 54.37 (19.46–151.88)	3.2 4.2
Sephadex G-50 column			
Peak 8a'	6.9 7.0	11.85 (8.82–15.93) 25.91 (11.90–56.25)	0.20 0.44
Peak 8b' (ostrich corticotropin)	19.7 26.3	73.48 (34.76–155.36) 76.95 (31.84–185.63)	3.4 4.9

peak 8a' still appeared to be contaminated with a component that had a greater mobility towards the cathode and which appeared to represent approx. 10% (w/w) of peak 8a'. Table 1 summarizes the results obtained during a typical isolation of corticotropin from fresh and aged ostrich pituitary glands.

Physical and chemical properties of corticotropin preparations

Electrofocusing of peak-8b' material from fresh and aged glands in the pH range 7–10 revealed three distinguishable fractions with pI values ranging from 8.3 to 8.7. Peak-8b' material from fresh glands revealed a major component of pI 8.7 and a minor component of pI 8.5. The same material from aged glands revealed two components in rather similar quantities at pI 8.3 and 8.5 respectively, and a minor component of pI 8.7. The different pI values observed could possibly be explained in terms of a variation in amide content between the hormone preparations from fresh and aged glands. Corticotropin preparations previously isolated from different species were found to contain at most two amide groups per molecule (Oelofsen, 1975). In the ostrich the most basic (pI 8.7) component, predominating in the preparation from fresh glands, could represent such a diamide variant. The less-basic components (pI 8.3 and 8.5) predominating in aged materials would seem to represent the totally and partly deamidated forms of the hormone, resulting from post-mortem amide-

Table 2. *Amino acid composition (molar proportions) of ostrich corticotropin and a related low-activity fraction*

For details see the text. Serine and threonine values are corrected for losses on hydrolysis. Values in parentheses are the nearest integers.

	Ostrich corticotropin (Peak 8b')		Low-activity fraction (Peak 8a')	
	Fresh glands	Aged glands	Fresh glands	Aged glands
Asp	1.1 (1)	1.2 (1)	0.9 (1)	0.9 (1)
Thr	1.1 (1)	1.1 (1)	0.9 (1)	0.8 (1)
Ser	2.6 (3)	2.6 (3)	2.2 (2)	2.4 (2)
Glu	5.8 (6)	6.2 (6)	1.9 (2)	1.8 (2)
Pro	4.1 (4)	3.7 (4)	0.9 (1)	0.7 (1)
Gly	4.3 (4)	4.0 (4)	3.3 (3)	3.0 (3)
Ala	0.3 (0)	0.4 (0)	2.4 (2)	2.2 (2)
Val	3.7 (4)	4.1 (4)	1.4 (1)	1.4 (1)
Met	0.9 (1)	1.0 (1)	0.8 (1)	0.7 (1)
Ile	0.1 (0)	0.1 (0)	0.5 (1)	0.5 (1)
Leu	1.1 (1)	1.2 (1)	1.6 (2)	1.6 (2)
Tyr	1.9 (2)	1.8 (2)	1.6 (2)	1.5 (2)
Phe	2.8 (3)	2.7 (3)	1.6 (2)	1.6 (2)
Lys	3.2 (3)	2.9 (3)	2.9 (3)	3.3 (3)
His	1.1 (1)	0.8 (1)	0.1 (0)	0.1 (0)
Arg	4.3 (4)	3.9 (4)	1.8 (2)	1.7 (2)
Trp	(1)	(1)	(0)	(0)
NH ₃	2.7 (0)	2.5 (0)	2.0 (0)	1.8 (0)
Total	39	39	26	26
Mol.wt. from formula	4610	4610	2946	2946

cleaving activity in the pituitary. This aspect could be further clarified by the results of sequencing studies. In any event, ostrich corticotropin seems to exhibit a more basic character than corticotropin from other species with pI values ranging from 7 to 8.

The N-terminal amino acid of peak-8b' material from both fresh and aged glands was identified as serine, which is in accordance with that from other species (Oelofsen, 1975). The results of the amino acid analysis of 20 h hydrolysates of each purified preparation are shown in Table 2. The values were obtained from two analyses in each case. The following points of interest emerge from these data. Firstly it is noted that, unlike all species so far investigated (pigs, ox, sheep, man and dogfish), avian corticotropin appears to be the only type containing a threonine residue in the molecule. It would be of considerable interest to know in which part of the sequence this unique residue occurs. Furthermore it appears that, as reported by Lowry *et al.* (1974) for the dogfish, alanine is also completely lacking from ostrich corticotropin, whereas all other species investigated have three alanine residues in the molecule. All in all it seems that for the six species so far investigated, all producing a 39-amino acid corticotropin molecule in the pituitary, these molecules only have an identical amino acid composition for four amino acid residues (Pro, 4; Tyr, 2; His, 1; Trp, 1). In the ostrich, a relatively low content of aspartic acid (one residue/molecule) coupled with a relatively high content of arginine (four residues/molecule) might explain the relative high basicity of the molecule reported in the present study.

Rechromatography of the corticotropin-active fraction 8 (Fig. 1) on CM-cellulose by using a steeper gradient consistently produced small quantities of material which, from its behaviour on the column, appeared to be slightly less basic than the major active peak 8b (Fig. 2) from which pure ostrich corticotropin was eventually recovered. This minor fraction (8a), furthermore possessed a low but significant adrenal-stimulating potency (Table 1). After further purification on Sephadex G-50 the resultant peak-8a' material still appeared on polyacrylamide-gel electrophoresis to be contaminated with a small amount (approx. 10%) of more basic material. No further purification of this fraction was attempted,

owing to the very low quantities available to us. Amino acid analysis of peak-8a' material showed it to differ considerably from the 39-amino acid ostrich corticotropin molecule (Table 2). Compared with the latter molecule, the low-activity fraction appears to be lacking with respect to Ser, Glu, Pro, Gly, Val, Phe, His, Arg and Trp, whereas it appears to have gained with respect to Ala, Ile and Leu. The possible relationship of this low-activity fraction to the major hormone or possibly to some other polypeptide or protein involved in the production of corticotropin in the gland can form the basis for interesting speculation. However, this aspect will have to await the accumulation and final purification of more of this material and the completion of sequence studies for ostrich corticotropin.

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